Hepatic triacylglycerol synthesizing activity during progression of alcoholic liver injury in the baboon

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Abstract To study the effects of alcoholic liver injury on the ability of ethanol to promote hepatic fat accumulation and hyperlipemia, baboons were pair-fed liquid diets containing 50% of energy either as ethanol or as additional carbohydrate (controls) for 1 to 7 years. Alcohol consumption produced triacylglycerol accumulation in the liver, hypertriacylglyceridemia, and various degrees of liver injury, including cirrhosis. At the early stages of fatty liver (with or without perivenular fibrosis), there was increased activity of microsomal diacylglycerol acyltransferase and of both microsomal and cytosolic phosphatidate phosphohydrolase, with no changes in glycerol-3-phosphate acyltransferase. With progression of the liver injury and development of septal fibrosis and/or cirrhosis, the rate of hepatic triacylglycerol accumulation and the magnitude of the hyperlipemia decreased, despite continuous ethanol intake. These changes were associated with disappearance of the increases in microsomal diacylglycerol acyltransferase and cytosolic phosphatidate phosphohydrolase activities, whereas those of microsomal phosphatidate phosphohydrolase remained elevated and glycerol-3-phosphate acyltransferase was unaffected.▪ Thus, changes in the activity of two enzymes of the triacylglycerol-synthesizing pathway, namely the microsomal diacylglycerol acyltransferase and the cytosolic phosphatidate phosphohydrolase, may contribute to the differences in the rate of hepatic triacylglycerol accumulation and the degree of hyperlipemia during progression of the alcoholic liver damage.—**Savolainen, M. J., E. Baraona, P. Pikkarainen, and C. S. Lieber.** Hepatic triacylglycerol synthesizing activity during progression of alcoholic liver injury in the baboon. *J. Lipid Res.* 1984. **25:** 813–820.

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One of the earliest and most striking manifestations of alcoholic liver damage is the hepatic accumulation of triacylglycerols. However, despite continuous ethanol intake, the accumulation of triacylglycerols in the liver stabilizes and sometimes decreases during progression of alcoholic liver damage (1). Also, alcoholic hyperlipemia is more marked in alcoholics with mild liver damage than in those with advanced lesions (2, 3). The exact mechanism of the hyperlipemia is not fully clarified, but the evidence gathered hitherto indicates that the hyperlipemia is due in part to enhanced release of triacylglycerol-rich lipoproteins from the liver (4–6). Thus, both effects may reflect changes in the capacity of the liver to synthesize triacylglycerols during progression of the liver injury. This could be due, at least in part, to the attenuation of the redox shift induced by ethanol after prolonged consumption (1). By promoting better oxidation of the fatty acids and a lesser production of *sn*-glycerol-3-phosphate, the attenuation of the redox shift may decrease the supply of these precursors for the synthesis of both triacylglycerols and phospholipids. However, one must consider the possibility that the activities of the enzymes involved in the synthesis of triacylglycerols may also play a regulatory role. Therefore, this study was undertaken to investigate whether changes in the activity of these enzymes may contribute to the attenuation in the rate of hepatic fat accumulation and in the degree of hyperlipemia during progression of the alcoholic liver damage.

MATERIALS AND METHODS

Materials

sn-[^{14}C(U)]Glycerol-3-phosphate (144 Ci/mol), disodium salt, and Liquifluor^R were purchased from New England Nuclear Corp. (Boston, MA); [9,10(n)-^{3}H]oleic acid (5.04 Ci/mol) was from Radiochemical Center (Amersham, Bucks, U.K.).

Animal procedures

Female baboons (*Papio hamadryas*) were matched according to body weight and approximate age and pair-fed nutritionally adequate liquid diets containing 50% of total energy either as ethanol or as additional carbohydrate for 1–7 years. The feeding techniques and dietary constituents have been previously reported in detail (7, 8). In twelve pairs of animals we had multiple mea-

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surements of liver triacylglycerol concentrations and they were used for assessment of the rate of hepatic accumulation at the fatty liver and fibrotic stages of alcoholic liver injury. The studies on liver lipid composition, microsomal triacylglycerol synthesizing enzymes and serum lipids were done only once in animals previously selected on the basis of histological findings. In all experiments, the ethanol-fed baboons and their pair-fed controls were studied the same day. The ethanol-containing diet was removed at 4:00 PM the day before surgery, and both the ethanol-fed animals and their controls were given control diet equivalent to one-third of the usual intake for an additional 8 hr, after which complete fasting was established. On the next day, at 9:00 AM, the animals were anesthesized with ketamine, blood samples were obtained from the femoral artery for the measurement of serum triacylglycerols (9) and cholesterol (10), and a piece of liver was surgically removed.

The liver size was estimated by adaptation to the baboon (11) of a roentgenographic procedure used in man. The coefficient of variation of the index obtained by dividing the product of 3 radiographic diameters by the actual liver volume of autopsy was 11% in seven animals that died shortly after radiography.

Preparation and subcellular fraction of the liver tissue

The specific gravity was assessed by displacement of saline by a weighed piece of tissue, and was used for conversion of the liver size into liver weight. Part of the sample (approximately 0.5 g) was homogenized in chloroform–methanol 2:1 (v/v) and the lipids were extracted (12) and measured (13). Liver triacylglycerols were separated by thin-layer chromatography (13), eluted with ether, and measured by their ester bonds (14). Phospholipids were measured in the lipid extracts by phosphorus assay (15), and hepatic cholesterol was measured by a modification (16) of the method of Searcy and Bergquist (17).

Another piece of liver (5–7 g) was chopped and washed in 0.25 M sucrose in order to remove excess blood interfering with the determination of cytochrome P-450 in the homogenates, used as a marker for the microsomal recovery. The liver tissue was then homogenized in 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4, and 1 mM ethylene diamine tetracetic acid (EDTA). After sedimentation of nuclei and mitochondrial fraction at 9,000 g for 30 min, the microsomes were sedimented by centrifugation of the supernatants at 105,000 g for 60 min. The supernatant of this second centrifugation is referred to as soluble fraction or cytosol. The recovery of the microsomes was assessed by comparison of the content of cytochrome P-450 in the homogenates and in the microsomal fractions (18).

Protein was measured by the method of Lowry et al. (19) and DNA by the method of Burton (20).

Triacylglycerol-synthesizing enzymes

Since the livers of the alcohol-fed animals differed from those of controls, not only in weight but also in protein and DNA contents (usual parameters of reference for enzyme activities), all the activities were expressed not only per mg of microsomal protein, but also per g of liver and per total liver per kg body weight, since in both ethanol-fed and control animals the liver weight correlated with the body weight (11).

sn-Glycerol-3-phosphate acyltransferase (EC 2.3.1.15)

The activity of this first enzyme in the glycerolipid synthesizing pathway was assayed in the liver microsomes essentially as described by Lamb and Fallon (21), measuring the incorporation of labeled glycerol-3-phosphate into total glycerolipids. Microsomes (150–200 μg of protein) were incubated for 5 min (22) at 37°C in a medium containing 7.5 μmol Tris-HCl, pH 7.4, 0.825 μmol MgCl$_2$, 6.25 nmol Coenzyme A, 0.4 μmol ATP, 0.175 μmol sn-[^{14}C(U)]glycerol-3-phosphate (0.32 Ci/mol), 0.15 μmol ammonium palmitate, and 1.25 mg of fatty acid-free bovine serum albumin in a volume of 0.25 ml. The reaction was stopped by extracting the glycerolipids by a modification (23) of the Folch, Lees, and Sloane Stanley (12) procedure, designed to have a better extraction of lysophosphatidic acid, one of the products of this reaction. To this effect, 3 ml of chloroform–methanol 1:1 (v/v) containing 0.1 N HCl was added to the incubation flasks. After shaking for 10 min at room temperature and transferring into graduated centrifuge tubes, 1 ml of chloroform and 2 ml of 2 M KCl were added, mixed, and the tubes were centrifuged at low speed to separate the phases. The upper phase was aspirated by suction and discarded. The chloroform phase was washed twice with 2 ml of chloroform–methanol–water 1:48:47 (by vol) and transferred into scintillation vials. After drying the solvent under nitrogen, the glycerolipids were dissolved in toluene-containing Liquifluor® and their radioactivity was measured in a Beckman scintillation counter. The reaction was linear for at least 15 min and in a range of 140 to 850 μg of microsomal protein.

The second enzyme in the glycerolipid synthesis, namely the 1-acyl-sn-glycerol-3-phosphate acyltransferase, was not measured because it is known to have very high activity (21, 22) and therefore it is not likely to play a regulatory role in the synthetic pathway.

Phosphatidate phosphohydrolase (EC 3.1.3.4)

The activity of this third enzyme in the triacylglycerol-synthesizing pathway was determined both in the microsomes as described by Lamb and Fallon (21) and in the

cytosol, as previously reported (24–26), by measuring the release of inorganic phosphate from an aqueous dispersion of sodium phosphatidate. In the microsomes, this method slightly overestimates the activity in the microsomes because of some deacylation of the phosphatidate and hydrolysis of the glycerol-3-phosphate by nonspecific lipases and phosphatases (27). But the alternative approach, namely the measurement of diacylglycerols formed, slightly underestimates the activity because of deacylation of the diacylglycerols (27). In the cytosol, the formation of diacylglycerols fully accounts for the release of phosphate (27). The dispersion of sodium phosphatidate was obtained by sonication of phosphatidic acid in 0.25 M sucrose adjusted to pH 9–10 with NaOH in a Branson W 140 sonifier for 1 min. The pH was brought to neutrality before use.

For the cytosolic enzyme, the incubation medium contained, in a volume of 0.5 ml, 20 μmol of Tris-HCl, pH 7.4, 1 μmol of $MgCl_2$, 0.8 μmol of phosphatidylcholine (this cofactor was dispersed by 1-min sonication in 0.25 M sucrose), 0.5 μmol of dithiothreitol, 100 nmol of ethylene glycol bis(β-aminoethyl ether) N,N'-tetracetic acid (EGTA), 0.2 μmol of sodium phosphatidate, and approximately 200 μg of protein of the enzyme preparation. To avoid the high blank values caused by inorganic phosphate of the cytosol, the enzyme was precipitated from the cytosol by adding an equal volume of 3.5 M $(NH_4)_2SO_4$. After standing for 15 min at 4°C and centrifugation at 10,000 g for 20 min, the pellet was redissolved to the initial volume of cytosol with 0.25 M sucrose containing 0.5 mM dithiothreitol and 20 mM Tris-HCl, pH 7.4 (25). The enzyme was either assayed immediately or stored at −20°C for up to 3 months. After a 10-min incubation with the substrate at 37°C, the reaction was stopped with 1 ml of 10% trichloroacetic acid. After centrifugation of the precipitates at 9,000 g for 10 min, the inorganic phosphorus was measured in the supernatants (28).

Acyl-CoA:1,2-diacylglycerol-O-acyltransferase (EC 2.3.1.20)

The activity of this fourth enzyme, which determines the branching of the pathway toward the synthesis of triacylglycerol (rather than phospholipids), was assessed by the conversion of diacylglycerol to triacylglycerol by the liver microsomes. This method is similar to that reported by Fallon et al. (29), except that the membrane-bound diacylglycerol was prepared from labeled phosphatidic acid previously extracted and partially purified rather than from membrane-bound phosphatidic acid, in order to increase the concentration of microsomal diacylglycerol to a level (88.8 ± 23 nmol/mg of microsomal protein in alchol fed animals and 54.9 ± 12.2 in controls; n = 6 pairs; N.S.) at which the transferase activity is independent of the substrate concentration (29). The labeled phosphatidic acid was prepared by incubation of rat liver microsomes with [9,10(n)-^{3}H]oleic acid, extracted and purified by thin-layer chromatography, as previously described (26). The membrane-bound diacylglycerol was then prepared by incubating 1 mg of baboon microsomal protein with 0.4 μmol of the [^{3}H]phosphatidic acid (11.0 Ci/mol) in a medium containing 24 μmol of Tris-HCl, pH 7.4, 12 μmol of $MgCl_2$, 7 μmol of dithiothreitol, and 2.7 mg of bovine serum albumin in a final volume of 2 ml. After preincubation for 30 min at 37°C, the reaction mixture was centrifuged at 100,000 g at 4°C for 60 min, and rehomogenized in a buffer containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), and 1 mM dithiothreitol. A 30-min rather than a 5-min preincubation was used because we found no difference in enzyme activity within these periods and we achieved better equilibration of the label in the membranes. The diacylglycerol specific activities achieved were similar in the ethanol-fed animals and in the controls (8125 ± 1931 dpm/nmol vs. 7703 ± 2153, in controls; n = 6 pairs; N.S.).

The reaction was started by the addition of 50 μmol of palmitoyl-CoA to 0.1 mg of microsomal protein of the preparation containing the labeled diacylglycerol and the enzyme in a final volume of 0.25 ml. After 5 and 10 min of incubation at 37°C, the reaction was stopped by adding 1.5 ml of heptane–isopropanol–0.5 M H_2SO_4 10:40:1 (by vol). This solvent mixture was added to the blank tubes immediately after addition of the palmitoyl-CoA. After standing at room temperature for 30–60 min, 1 ml of 0.1 M $KHCO_3$ and 1 ml of heptane containing 1 mg of glyceroltrioleate were added. After slow-speed centrifugation, the upper phase was transferred into another tube and the lower phase was washed with 1 ml of heptane. The heptane phases were combined and dried under nitrogen. Triacylglycerols and diacylglycerols were separated by thin-layer chromatography, eluted with ethyl ether, and counted. Aliquots of the diacylglycerol fractions were hydrolyzed with ethanolic KOH at 60°C and the free glycerol was measured, after neutralization, with glycerol kinase (9) in order to determine the specific activity of the diacylglycerol substrate and to calculate the nmoles of triacylglycerols formed per minute.

Statistics

All values are expressed by their mean ± standard error of the mean (SE). Values obtained in the alcohol-fed baboons were compared to those obtained in their pair-fed controls and the mean of the individual differences was tested by the Student's t-test (paired comparisons) (30). The significance of the effect of the degree of liver injury was assessed by one-way analysis of the variance (30).

TABLE 1. Effects of ethanol feeding and degree of alcoholic liver damage on liver size and composition[a]

	Alcohol-fed Baboons with Fatty Liver	Pair-fed Controls	P[b]	Alcohol-fed Baboons with Liver Fibrosis	Pair-fed Controls	P[b]	ANOVA[c]
Liver weight / Body weight	0.0375 ± 0.0036	0.0270 ± 0.0016	<0.01	0.0309 ± 0.0021	0.0237 ± 0.0014	<0.01	<0.002
Dry weight / Wet weight	0.417 ± 0.018	0.313 ± 0.030	<0.02	0.355 ± 0.018	0.302 ± 0.009	N.S.	<0.001
Proteins							
mg/g of liver	122.1 ± 6.6	138.7 ± 3.9	<0.02	122.9 ± 7.4	136.0 ± 7.2	N.S.	N.S.
g/kg body weight	4.19 ± 0.56	3.56 ± 0.23	N.S.	3.80 ± 0.20	2.95 ± 0.23	<0.02	<0.001
Lipids							
mg/g of liver	171 ± 26	58 ± 10	<0.01	168 ± 14	73 ± 10	<0.01	<0.001
g/kg body weight	5.59 ± 1.09	1.62 ± 0.31	<0.01	4.90 ± 1.08	1.73 ± 0.24	<0.01	<0.001
DNA							
mg/g of liver	1.88 ± 0.10	1.86 ± 0.12	N.S.	1.53 ± 0.07	1.52 ± 0.12	N.S.	<0.005
g/kg body weight	0.0599 ± 0.0006	0.0436 ± 0.0051	<0.05	0.0446 ± 0.0030	0.0365 ± 0.0046	N.S.	<0.001

[a] Mean ± SE of ten alcohol-fed babons with fatty liver (with or without perivenular fibrosis) and their pair-fed controls, and of six alcohol-fed baboons with septal fibrosis or cirrhosis and their pair-fed controls.
[b] Paired comparisons.
[c] ANOVA, analysis of the variance, one-way.

RESULTS

The consumption of ethanol-containing diets for 1–7 years resulted in a variety of liver lesions. All alcohol-fed baboons had visible accumulation of fat in the liver (steatosis). Sequential morphological observations, previously reported in these animals (31), allowed us to distinguish at least four stages in the progression of the liver injury on the basis of the development of fibrosis: a) fatty liver without fibrosis (four animals); b) fatty liver with deposition of a small amount of fibrous tissue around the terminal hepatic venules (six animals); c) fatty liver with development of fibrous septa, generally spreading into the parenchyma from the perivenular area (four animals); and d) fatty liver with micronodular cirrhosis (two animals). We did not detect differences in the measurements reported in this study between animals with fatty liver with or without perivenular fibrous tissue deposition or between animals with septal fibrosis or cirrhosis. Therefore, except in Fig. 1, we pooled the animals into two groups, hereinafter referred to as fatty liver and fibrosis, for the purpose of comparing the effects of mild and severe liver injury. The intake of ethanol remained unchanged during progression of the liver damage: 4.34 ± 0.28 g/kg per day at the fatty liver stage and 4.41 ± 0.19, at the fibrosis stage.

Effects of alcohol feeding and degree of liver injury on liver size, composition, and hepatic lipids

Feeding alcohol to the baboons resulted in enlargement of the liver and a striking accumulation of triacylglycerols. In every pair, the liver/body weight ratio was higher in the alcohol-fed than in the control baboons (Table 1).

The liver protein was slightly decreased when expressed per gram of liver, probably as a consequence of the severe steatosis and decreased water space in the alcohol-fed animals, but increased when expressed per total liver. DNA per total liver increased at early stages of alcoholic liver injury probably reflecting mesenchymal cell proliferation and increased number of inflammatory cells (31), but the difference with the controls disappeared at more advanced stages.
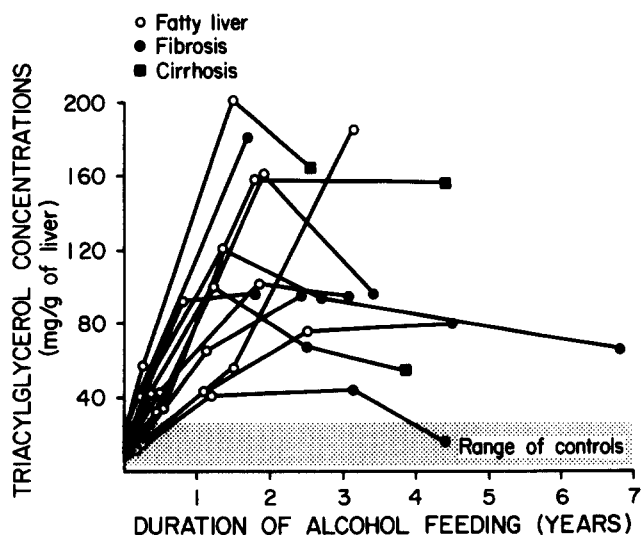

Fig. 1. Sequential determinations of hepatic triacylglycerol concentrations during development of alcoholic liver injury in the baboons. The rapid accumulation that occurs at early stages of liver damage (fatty liver with or without perivenular fibrosis) slows down or disappears with the development of the more advanced stages (septal fibrosis or cirrhosis), despite continuous alcohol consumption. There is marked individual variation in the duration of alcohol intake required for this transition.

TABLE 2. Effects of ethanol feeding and degree of alcoholic liver damage on liver lipids[a]

	Alcohol-fed Baboons with Fatty Liver	Pair-fed Controls	P[b]	Alcohol-fed Baboons with Liver Fibrosis	Pair-fed Controls	P[b]	ANOVA[c]
Triacylglycerols							
mg/g of liver	151 ± 24	26 ± 6	<0.001	120 ± 15	23 ± 10	<0.01	<0.001
g/kg body weight	5.4 ± 0.8	0.6 ± 0.2	<0.001	3.9 ± 0.6	0.8 ± 0.4	<0.02	<0.001
Phospholipids							
mg/g of liver	33.3 ± 3.0	41.0 ± 2.6	N.S.	37.1 ± 1.9	40.1 ± 4.0	N.S.	N.S.
g/kg body weight	1.2 ± 0.1	1.1 ± 0.1	N.S.	1.2 ± 0.1	0.9 ± 0.1	N.S.	N.S.
Cholesterol							
mg/g of liver	2.63 ± 0.53	3.23 ± 0.49	N.S.	3.51 ± 0.84	3.75 ± 0.68	N.S.	N.S.
g/kg body weight	0.09 ± 0.02	0.09 ± 0.01	N.S.	0.12 ± 0.01	0.12 ± 0.02	N.S.	N.S.

[a] Mean ± SE of ten alcohol-fed baboons with fatty liver (with or without perivenular fibrosis) and their pair-fed controls, and of six alcohol-fed baboons with septal fibrosis or cirrhosis and their pair-fed controls.
[b] Paired comparisons.
[c] ANOVA, analysis of the variance, one-way.

The major changes occurred in the lipid fraction. Serial determinations of liver lipids in some of these baboons indicated that, during the fatty liver stage, there was a high rate of triacylglycerol accumulation (**Fig. 1**). By contrast, during the development of fibrosis, the hepatic concentration of triacylglycerols generally remained stable or decreased. There were no significant changes with time between the pair-fed controls.

In contrast to the marked changes in triacylglycerols (**Table 2**), the hepatic concentration of phospholipids and cholesterol did not change. There was only a slight increase when expressed per total liver due to the hepatomegaly of the alcohol-fed animals.

Effect of alcohol feeding and degree of liver injury on serum lipids

Alcohol feeding increased serum triacylglycerols and cholesterol even in the fasting state (**Table 3**). However, these changes greatly diminished with progression of the liver injury.

Effects of alcohol feeding and degree of liver injury on hepatic triacylglycerol-synthesizing enzymes

Regardless of the severity of the liver damage, the recovery of the microsomal marker, cytochrome P-450,

was similar in alcohol-fed and control baboons (43.2 ± 3.8% vs. 51.5 ± 6.0, in controls; N.S.). Corrected for the recovery of the marker, the amount of microsomal protein per gram of liver was also similar (23.9 ± 3.3 mg/g of liver vs. 25.7 ± 2.4, in the controls; N.S.), although it tended to be higher in alcohol-fed animals when expressed per kg body weight (799 ± 28 mg vs. 691 ± 89, in controls, N.S.)

Chronic ethanol administration did not change the activity of glycerophosphate acyltransferase of liver microsomes at any of the stages of alcoholic liver injury (**Table 4**). By contrast, the phosphatidate phosphohydrolase activity in both soluble and microsomal fractions increased by 50 and 47%, respectively. However, these activities differed from each other at later stages of alcoholic liver damage: the activity of the soluble enzyme was only 20% higher than that of the control (a statistically nonsignificant difference), whereas the activity of the microsomal enzyme remained significantly elevated at 34% above that of controls.

The only enzyme specific for the synthesis of triacylglycerols, the diacylglycerol acyltransferase of liver microsomes, underwent changes during the progression of liver damage, similar to those of the soluble phosphatidate phosphohydrolase. At the fatty liver stage, the activity

TABLE 3. Effects of ethanol feeding and degree of alcoholic liver damage on fasting serum lipids[a]

	Alcohol-fed Baboons with Fatty Liver	Pair-fed Controls	P[b]	Alcohol-fed Baboons with Liver Fibrosis	Pair-fed Controls	P[b]	ANOVA[c]
Triacylglycerols							
mmol/liter	1.009 ± 0.141	0.412 ± 0.049	<0.001	0.684 ± 0.075	0.392 ± 0.062	<0.01	<0.001
Cholesterol							
mmol/liter	3.24 ± 0.27	2.30 ± 0.11	<0.01	2.75 ± 0.40	2.48 ± 0.19	N.S.	<0.05

[a] Mean ± SE of six (cholesterol) to twelve (triacylglycerols) alcohol-fed baboons with fatty liver (with or without perivenular fibrosis) and their pair-fed controls, and six alcohol-fed baboons with septal fibrosis or cirrhosis and their pair-fed controls.
[b] Paired comparisons.
[c] ANOVA, analysis of the variance, one-way.

TABLE 4. Effects of ethanol feeding and degree of alcoholic liver damage on the activity of triacylglycerol-synthesizing enzymes in the liver[a]

	Alcohol-fed Baboons with Fatty Liver	Pair-fed Controls	Alcohol-fed Baboons with Liver Fibrosis	Pair-fed Controls	ANOVA
sn-Glycerol-3-phosphate acyltransferase nmol glycerolipid-glycerol/min					
per mg of microsomal protein	9.18 ± 0.87 (4)	9.57 ± 0.97 (4)	9.78 ± 1.45 (2)	12.62 ± 0.44 (2)	N.S.
per g of wet liver	220.8 ± 32.3	277.3 ± 68.7	236.6 ± 28.5	274.4 ± 9.2	
per kg body weight	7890 ± 608	7728 ± 2240	7612 ± 1668	7519 ± 857	
Microsomal phosphatidate phosphohydrolase nmol phosphorus released/min					
per mg of microsomal protein	9.67 ± 0.74^b (10)	6.60 ± 0.43 (10)	10.03 ± 0.98^c (6)	7.47 ± 0.93 (6)	<0.01
per g of wet liver	242 ± 24^d	192 ± 14	251 ± 35^c	175 ± 30	
per kg body weight	8449 ± 766^b	5037 ± 368	7070 ± 859^c	4077 ± 678	
Cytosolic phosphatidate phosphohydrolase nmol phosphorus released/min					
per mg cytosolic protein	5.70 ± 0.65^b (5)	3.80 ± 0.25 (5)	4.52 ± 0.88 (4)	3.75 ± 0.19 (4)	<0.01
per g of wet liver	298 ± 28^d	225 ± 37	242 ± 54	199 ± 16	
per kg body weight	10959 ± 1537^d	6068 ± 988	5937 ± 1630	4866 ± 687	
Acyl-CoA:1,2-diacylglycerol acyltransferase nmol triacylglycerol formed/min					
per mg of microsomal protein	3.02 ± 0.29^b (4)	1.57 ± 0.33 (4)	2.17 ± 0.38 (2)	2.16 ± 0.36 (2)	<0.05
per g of wet liver	69.5 ± 5.6	48.89 ± 15.2	42.6 ± 17.6	46.5 ± 4.5	
per kg body weight	2570 ± 147^d	1318 ± 400	1657 ± 419	1300 ± 308	

[a] Mean $\pm$ SE of enzyme activities. Numbers in parentheses are the number of paired comparisons. ANOVA, analysis of the variance, one-way.
[b] $P < 0.02$.
[c] $P < 0.01$.
[d] $P < 0.05$.

was 92% higher in the alcohol-fed animals than in the controls, but this activity decreased to within the normal range in the alcohol-fed animals with more advanced liver injury.

DISCUSSION

Our findings indicate that the progression of the liver damage induced by chronic administration of ethanol is associated with a decreased rate of triacylglycerol accumulation in the liver, with attenuation of alcoholic hyperlipemia and with disappearance of the increased activities of some triacylglycerol-synthesizing enzymes.

It is almost universally accepted that the primary alteration leading to the development of alcoholic fatty liver is the defective oxidation of fatty acids, the major normal fuel for the liver, due to the redox shift produced by acute ethanol administration and the mitochondrial damage that follows its chronic administration (32). The fatty acids are diverted to the synthesis of glycerolipids. This is associated with a rapid increase in the activities of enzymes involved in this pathway. The sn-glycerol-3-phosphate-dependent release of coenzyme A from acyl-CoA was found to be increased by microsomes of alcohol-fed rats (33). Initially, this was attributed to the activity

of glycerol-3-phosphate acyltransferase, but now it appears that, in addition to this enzyme, the release of CoA depends on the activity of at least two other enzymes in the pathway, the 1-acylglycerol-3-phosphate acyltransferase and the diacylglycerol acyltransferase. Thus, the enzyme activity playing a regulatory role in the pathway remains unknown. An assay more specifically designed to measure the glycerol-3-phosphate acyltransferase, the first enzyme of the pathway, did not reveal significant changes of this activity in the alcohol-fed baboons. It must be pointed out that this activity is sensitive to changes in dietary lipids (34) and the liquid diets given to the baboon are poorer in fat content than those given to rats (8). The second enzyme in the synthesis of glycerolipids, namely the 1-acyl-glycerol-3-phosphate acyltransferase, which catalyses the acylation of lysophosphatidic to phosphatidic acid, is known to have very high activity (21, 22) and is not likely to represent a limiting step.

Phosphatidate phosphohydrolase, the third enzyme in the pathway, which catalyses the hydrolysis of the phosphatidic acid intermediate, has a lower activity and could be a rate-limiting step in the synthesis of glycerolipids. Acute (24, 35, 36) and chronic (37) administration of ethanol to rodents increased the activity of this enzyme both in the microsomes (26, 35, 36) and the cytosol (24, 36). The increase in this microsomal activity correlated

well with the enhanced triacylglycerol synthesis by microsomes of alcohol-fed hamsters (36). A stimulation at the phosphatidate phosphohydrolase locus would facilitate the synthesis of the two main types of glycerolipids, namely phospholipids and triacylglycerols. Indeed, a moderate increase in hepatic phospholipids has been observed in alcohol-fed rats (37). Moreover, the rate of synthesis of some phospholipids (38, 39) and the activities of phosphatidyl ethanolamine methyltransferase and choline phosphotransferase (40) are increased in ethanol-fed rats. Proportionally, however, the accumulation of triacylglycerols is much greater than that of phospholipids. In our present study, the administration of greater amounts of ethanol and for a much longer time to the baboon not only exaggerated the accumulation of hepatic triacylglycerols, but produced no changes in other lipids including phospholipids. This suggested the existence of another regulatory site more specific for the synthesis of triacylglycerols.

The branching of the glycerolipid synthetic pathway toward the formation of triacylglycerols is determined by the activity of the diacylglycerol acyltransferase. The administration of ethanol to the baboon markedly increased this activity. Previously, this activity had been reported to the unaltered after acute ethanol administration to the rat (35). More recently, however, when the assay included the cytosolic fraction in addition to the microsomes it has been found to be threefold higher in the liver of rats after an acute ethanol dose (41). The cytosolic factor is most likely an enzyme activity which governs the phosphorylation-dephosphorylation state of the microsomal enzyme protein (42). In this study, it was observed that chronic alcohol feeding to the baboon increased diacylglycerol acyltransferase activity two-fold even in the absence of the cytosolic activator. The possible role of the cytosolic factors in our model remains to be determined.

Previous observations (1–3) suggested that the enhanced rate of triacylglycerol synthesis could decrease with progression of alcoholic liver damage. This provided us with the opportunity to assess the possible regulatory role of these enzyme activities. Indeed, sequential determinations indicated that active accumulation of hepatic triacylglycerols takes place during the initial stages of the alcohol-induced liver damage of the baboon, whereas this accumulation stopped or the concentration of triacylglycerols even decreased at more advanced stages of the liver injury despite the continuous ingestion of alcohol. These differences in concentration were not due to differences in the degree of hepatomegaly between these two groups. Also, hyperlipemia decreased in advanced liver damage. These effects were associated with a change in two enzyme activities, the cytosolic fraction of the phosphatidate phosphohydrolase and the microsomal diacylglycerol acyltransferase, whereas the glycerol-3-phosphate acyltransferase remained unaffected and the microsomal fraction of the phosphatidate phosphohydrolase remained increased. Although the exact role of the soluble fraction of the phosphatidate phosphohydrolase has not been clarified, it differs from the membrane-bound fraction in its response to a variety of dietary and pharmacologic changes (25, 26), suggesting a regulatory role for the entire pathway. The present findings tend to support this concept, but it must be pointed out that quantitative dissociations between the activity of the soluble enzyme and the rate of triacylglycerol synthesis have also been reported (23).

Another site for these effects could be the activity of diacylglycerol acyltransferase, which may play a role in both hepatic triacylglycerol synthesis and the production of very low density lipoproteins (43). In keeping with this possibility, the increase in diacylglycerol acyltransferase activity found in baboons with moderate alcoholic liver injury disappeared with progression of the hepatic lesions. It remains to be determined whether the decrease in this activity is due to a change in the microsomal enzyme, in the cytosolic factors that are believed to regulate its activity (42), or in the availability of fatty acids, upon which this activity is also dependent (43). Nevertheless, the hepatic diacylglycerol acyltransferase represents the most likely site for modulation of triacylglycerol synthesis and secretion in the course of alcoholic liver injury.

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