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SHORT COMMUNICATION

Incorporation of Exogenous Precursors into Neutral Lipids and Phospholipids in Rat Hepatocytes: Effect of Ethanol *In Vitro*

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ABSTRACT. We studied the incorporation of different radioactively labeled exogenous substrates into the lipids of rat hepatocytes previously incubated with ethanol. Glycerol, oleate, and serine were all incorporated into neutral lipids to a significantly greater degree in the presence of ethanol, the increase in radioactivity in the triacylglycerol fraction being quite substantial. A similar ethanol-induced increase was found in the incorporation of these substrates into the various phospholipids. This lipogenic activity did not occur when the metabolism of ethanol was blocked by 4-methylpyrazole, an inhibitor of hepatic ADH (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) activity, thus demonstrating that one of the initial effects of ethanol on lipid biosynthesis was mediated by some products of its metabolism in the liver. The only alteration that persisted in the presence of 4-methylpyrazole was an inhibitory effect on the esterification of free cholesterol from oleate, suggesting that ethanol specifically inhibits hepatic ACAT (acyl CoA:cholesterol *O*-acyltransferase, EC 2.3.1.26) activity. BIOCHEM PHARMACOL **56**;12:1639–1644, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. rat hepatocytes; ethanol; neutral lipids; phospholipids

The effects of prolonged ethanol administration have been widely studied, ethanol being well known to cause marked alterations to lipid metabolism in both hepatic [1] and extrahepatic tissues [2]. Chronic ethanol administration increases fat mobilization from adipose tissue, increases the synthesis of fatty acids in the liver, enhances the esterification of fatty acids into TAG† and decreases the egress of TAG from the liver. Furthermore, ethanol ingestion impairs the mitochondrial oxidation of fatty acids in rats and decreases the activity of the tricarboxylic acid cycle, probably as a consequence of alterations in the redox state [reviewed in Refs. 3 and 4]. All these effects of ethanol on lipid metabolism may well be responsible for the accumulation of fat in the liver, hyperlipemia, and macrovacuolar steatosis which are characteristic of alcoholics.

In spite of substantial experimental evidence concerning the effects of ethanol after prolonged ingestion, data on the early mechanisms which it exerts on the different metabolic pathways are scarce and inconclusive. Studies *in vitro* allow us to analyze the effects of ethanol under controled conditions by exposing the cells to constant levels of ethanol for a short period of time. We have recently demonstrated that incubating hepatocytes for 3 hr with 100 mM ethanol inhibits the incorporation of choline and ethanolamine by the cytidine diphosphate-derivative pathway [5]. This alteration seems to be caused directly by ethanol itself, since it occurs when its metabolism is blocked by an inhibitor of ADH (EC 1.1.1.1) activity. A similar reduction in the incorporation of choline into phosphatidylcholine is also observed after chronic ethanol ingestion [6].

To obtain further information about the mechanisms behind the effects produced by alcohol, we made a comparative analysis in rat hepatocytes of the influence of ethanol *in vitro* on the biosynthetic pathways of neutral lipids and phospholipids using labeled glycerol, oleate, and serine as exogenous precursors. Bearing in mind that ethanol may exert its pharmacological effects either by direct interaction with different structural components of biological membranes or via metabolites produced during its oxidation in the liver, we analyzed the alterations induced by alcohol on lipid metabolism, both in the presence and absence of 4-methylpyrazole, a specific inhibitor of ADH activity and thus of ethanol metabolism in the liver.

MATERIALS AND METHODS

Materials

[1(3)^{3}H]Glycerol, [9,10(n)-^{3}H]oleic acid, and L-[3-^{3}H]serine were bought from Amersham International. Fat-

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† *Abbreviations:* ACAT, acyl CoA:cholesterol *O*-acyltransferase; ADH, alcohol:NAD^+ oxidoreductase; DAG, diacylglycerols; and TAG, triacylglycerols.

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ty-acid-free BSA, collagenase (Type A from *Clostridium histolyticum*) and trypsin inhibitor were supplied by Boehringer Mannheim. Glycerol, oleic acid, L-serine, and L-methionine were purchased from Sigma. TLC plates came from Sigma and Percoll from Pharmacia. All other reagents used were of analytical grade.

Isolation of Hepatocytes

Male Sprague–Dawley rats (200–250 g) were fed *ad lib.* on a standard chow diet in a chamber with a light cycle from 9 a.m. to 7 p.m. and a controled temperature of 25°. Hepatocytes were isolated by a modification of Seglen's perfusion technique [7] using collagenase. According to this method, the rats were kept fasted the night before the hepatocytes were to be isolated. They were anaesthetized by an intraperitoneal injection of sodium ketamine (222 mg/kg of body weight). The livers were perfused initially in Ca^{2+}-free Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 0.08 mM of EGTA and 10 mM of HEPES. Once the liver had been cleared of blood, the perfusion medium was changed for a Krebs–Henseleit bicarbonate medium containing 5 mM of $CaCl_2$, collagenase A, and tripsin inhibitor (8.52 mg/100 mL of perfusion medium). The temperature was maintained at 37° and the perfusion was continued for 15 min. All solutions were thoroughly gassed with a mixture of O_2/CO_2 (95%:5%). After perfusion with collagenase, the liver was transferred to a Petri dish and gently dispersed. The resulting suspension was filtered through a nylon mesh and the hepatocytes were placed on Percoll (1.08 mg/mL) in a centrifuge tube. After a 30-min centrifugation at 30,000 *g*, the viable cells were collected and washed twice at 50 *g* for 2 min before being resuspended in Krebs–Henseleit buffer containing 1.5% BSA and 10 mM of glucose. Aliquots of the suspension were tested for viability by trypan blue exclusion. The viability of the cells was higher than 90% in all the preparations used and this level was maintained throughout all our experiments, both in control and ethanol-treated cells.

Incubation of Hepatocytes

Cells (5×10^6) were incubated both in the presence and absence of 100 mM of ethanol at 37° in a shaken water bath (80 oscillations/min) in 2.5 mL of Krebs–Henseleit, pH 7.4, containing 1.5% BSA (w/v) and 10 mM of glucose, under an atmosphere of carbogen (95% CO_2; 5% O_2). The reactions were begun after a 150-min incubation by adding either 50 μM of methionine and [1(3)-^{3}H]glycerol (50 μM; 200 dpm/pmol) or [9,10(n)-^{3}H]oleate (100 μM, 26.5 dpm/pmol), prepared as described by Stremmel and Berk [8], or L-[3-^{3}H]serine (50 μM, 150 dpm/pmol). The reactions were continued for 30 min at 37° and stopped by the addition of 7.5 mL of ice-cold Krebs–Henseleit. The cells were washed twice in Krebs–Henseleit medium at 50 *g* for 5 min and the pellet was collected and used for the analysis of neutral lipids and phospholipids. Incubations with 4-methylpyrazole proceeded as described above, except that the Krebs–Henseleit medium also contained 3 mM of 4-methylpyrazole.

Extraction and Analysis of Neutral Lipids and Phospholipids

Lipids were extracted from the cell pellet according to the procedure of Folch *et al.* [9]. The chloroform layer was dried by evaporation under a stream of nitrogen. The lipid residue was dissolved in chloroform, and the different neutral lipids were separated on silica gel 60 G TLC plates using a mixture of n-hexane/ethyl ether/acetic acid (70:30:1) as solvent. The different phospholipids were separated on silica gel 60 G TLC plates as described by Higgins [10], using a mixture of chloroform/methanol/acetic acid/water (60:50:1:4) as solvent. The spots corresponding to each compound were visualized by exposing the plates to iodine vapors, then scraped and transferred to scintillation vials for radioactivity measurements in a Beckman 6000-TA liquid scintillation counter.

Analysis of Radioactivity in the DAG Moiety of Phospholipids

The amount of radioactivity from L-[3-^{3}H]serine associated with the head group and DAG moiety of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine molecules was determined by phospholipase C (EC 3.1.4.3) digestion according to the method of Kuksis *et al.* [11] with minor modifications. Phospholipids isolated by TLC were dried under nitrogen. Each phospholipid was dissolved in 1 mL of diethyl ether and 5.5 mL of Tris–HCl buffer (13.3 mM, pH 7.4) containing 0.24% $CaCl_2$ and 1 unit of phospholipase C from *Bacillus cereus* was added to the sample. The tubes were incubated at 30° for 3 hr in a shaken bath (250 oscillations/min). Digestion was stopped by the addition of 15 mL of chloroform/methanol (2:1) and 0.2 mL of 0.1 M HCl. The radioactivity associated with DAG was determined after its isolation by TLC.

Statistical Analysis

The results are expressed as means ± SEM, as indicated in the legends to the tables. Data from ethanol-treated and control hepatocytes were compared by Student's *t*-test, taking a value of $P < 0.05$ as the criterion for significance.

RESULTS AND DISCUSSION

In the present study, we have analyzed the effects of ethanol *in vitro* on the biosynthesis of neutral lipids and phospholipids in isolated rat hepatocytes. To explore the possible influence of ethanol upon the different steps of the lipid biosynthetic pathways, we used glycerol, oleate, and serine as exogenous precursors, which were incubated in the presence or absence of ethanol as described in the Materials

TABLE 1. Effect of 100 mM ethanol on the incorporation of glycerol, oleate, and serine into neutral lipids in rat hepatocytes

	Glycerol		Oleate		Serine	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
DAG	0.25 ± 0.01	0.47 ± 0.01†	3.08 ± 0.06	8.91 ± 0.40†	0.19 ± 0.02	0.38 ± 0.02*
TAG	6.05 ± 0.33	16.31 ± 0.31†	126.09 ± 4.59	308.47 ± 4.34†	0.10 ± 0.01	0.64 ± 0.02†
Cholesterol ester	ND	ND	9.48 ± 0.33	6.90 ± 0.33*	ND	0.12 ± 0.02†

Data are expressed as pmol of radioactive label incorporated/min per 10^6 cells. Values are expressed as means ± SEM for three experiments. Statistical significance of differences from the controls are indicated by: *$P \leq 0.05$; †$P \leq 0.0001$; ND, not detected.

and Methods section. In order to analyze the effects of ethanol itself with no interference on the part of its various metabolites, we also investigated the effects of ethanol on the incorporation of these precursors in the presence of 4-methylpyrazole, a specific inhibitor of ADH activity.

[1(3)^{3}H]Glycerol can be phosphorylated into glycerol 3-phosphate, which is then acylated to yield phosphatidate, a common intermediate in the synthesis of phospholipids and TAG. Thus, by using labeled glycerol as a metabolic precursor, we have the advantage of being able to analyze the activity of both biosynthetic pathways simultaneously. The results of the analysis of the incorporation of [1(3)^{3}H]glycerol into different neutral lipids are set out in Table 1, where it can be seen that in the control cells there was a substantial uptake of this substrate into TAG, whilst DAG were labeled to a much lesser degree, radioactivity levels in TAG being nearly 25 times higher than those found in DAG. This indicates that in liver cells DAG produced by *de novo* synthesis is actively acylated by DAG acyltransferase (acyl CoA:diacylglycerol *O*-acyltransferase, EC 2.3.1.20) to yield TAG. When labeled oleate was used as lipid precursor in the control hepatocytes with no ethanol, TAG also exhibited a significantly higher level of radioactive label compared to DAG. Interestingly, the TAG/DAG ratio, which can be used as an indicator of DAG acyltransferase activity, was clearly higher when the exogenous substrate was oleate rather than glycerol (Fig. 1). Since some previous reports have indicated that DAG acyltransferase activity in rat liver microsomes is stimulated in the presence of oleic or palmitic acid [12], the high uptake rate of oleate into TAG observed in our study may be due both to the availability of intracellular fatty acids as substrates for the acylation process and the high activity of the enzyme involved in the synthesis of TAG; however, it is possible that the increase in the TAG/DAG ratio might in part be due to the fact that some TAG molecules contain two labeled oleates, thus increasing the amount of radioactivity per TAG molecule.

As serine is also metabolized to pyruvate, which may then be converted either into glycerol or acetyl CoA, we determined the incorporation of radioactivity from serine into different neutral lipids and phospholipids. As far as the radioactivity found in neutral lipids is concerned (Table 1), there was quite a low degree of incorporation into DAG or TAG while esterified cholesterol showed no radioactive label. Thus, the low radioactivity found in neutral lipids under our experimental conditions suggests that in our control hepatocytes serine was hardly used to synthesize these lipids.

The effects of 100 mM of ethanol upon the incorporation of labeled oleate, glycerol, or serine into neutral lipids are also shown in Table 1. The incubation of hepatocytes with ethanol for 3 hr resulted in a significant increase in the uptake of all the exogenous substrates into both DAG and TAG compared to the control hepatocytes. The rise in the radioactive content of these lipids induced by ethanol may well explain the higher levels of TAG that we found in hepatocytes after incubation with alcohol (24.27 ± 1.78 μg of TAG/10^6 cells in control hepatocytes vs 33.38 ± 2.66 μg of TAG/10^6 cells in ethanol-treated hepatocytes). Other authors have also shown that ethanol causes an increase in TAG levels in primary cultures of hepatocytes [13], although longer periods of exposure were required, probably due to the lower concentrations of ethanol used. Castro *et al.* [14] and Dich *et al.* [15] have shown that incubations of rat hepatocytes with ethanol cause a marked increase in the concentration of glycerol 3-phosphate, and recently it has also been demonstrated that NADH produced during ethanol oxidation is used in the formation of a pool of glycerol 3-phosphate, which gives rise to TAG and possibly

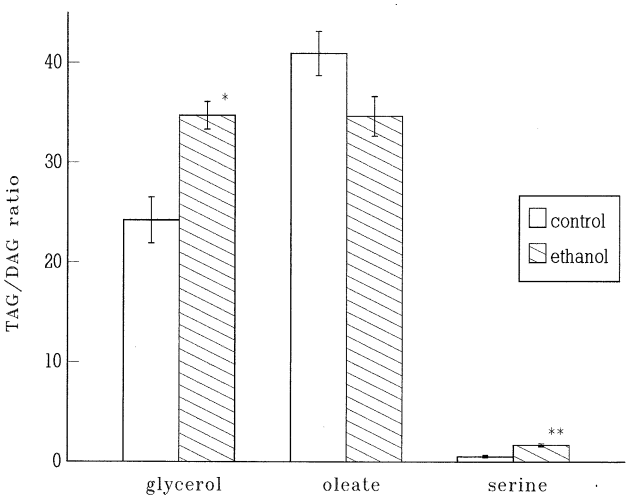

FIG. 1. Effect of 100 mM ethanol on the TAG/DAG radioactivity ratio from glycerol, oleate, or serine in rat hepatocytes. Values are expressed as means ± SEM for three experiments. Statistical significance of differences from the controls are indicated by: *P ≤ 0.02; **P ≤ 0.003.

TABLE 2. Effect of 100 mM ethanol on the incorporation of glycerol, oleate, and serine into phospholipids in rat hepatocytes

	Glycerol		Oleate		Serine	
	Control	Ethanol	Control	Ethanol	Control	Ethanol
Phosphatidylcholine	3.66 ± 0.13	5.52 ± 0.17†	18.54 ± 0.82	29.88 ± 1.68*	0.29 ± 0.03	0.59 ± 0.04*
Phosphatidylethanolamine	1.10 ± 0.02	1.81 ± 0.05†	5.67 ± 0.21	9.74 ± 0.06†	0.25 ± 0.02	0.36 ± 0.02*
Phosphatidylinositol	0.14 ± 0.01	0.27 ± 0.01†	1.81 ± 0.03	3.52 ± 0.12†	ND	ND
Phosphatidylserine	ND	ND	0.40 ± 0.04	0.71 ± 0.06*	0.07 ± 0.01	0.09 ± 0.01

Data are expressed as pmol of radioactive label incorporated/min per 10^6 cells. Values are expressed as means ± SEM for three experiments. Statistical significance of differences from the controls are indicated by: *$P \leq 0.05$; †$P \leq 0.001$; ND, not detected.

to fatty liver [16]. The availability of glycerol 3-phosphate may be important for the rate of TAG synthesis in the liver, especially when acyl CoA is provided in excess, as occurs during ethanol metabolism [17]. Thus, it is likely that when the hepatocytes are incubated with ethanol, the concentration of fatty acids may be sufficient to support the esterification reaction of glycerol 3-phosphate to supply the TAG-synthesizing pathway.

It is interesting to note that the TAG/DAG ratio increased when the substrate was serine or glycerol (Fig. 1), which suggests that ethanol stimulates DAG acyltransferase activity, probably as a consequence of the increase in levels of fatty acids synthesized from ethanol via acetate. Ethanol did not increase this ratio with oleate as the lipid precursor because in the control hepatocytes incubated with oleate, the activity of DAG acyltransferase was already high and was thus not increased any further by the fatty acids synthesized *de novo* from ethanol.

To clarify whether the changes in lipid metabolism caused by ethanol *in vitro* were associated with alcohol metabolism, we incubated hepatocytes in the presence of 4-methylpyrazol, a specific inhibitor of ADH activity. The incubation of control hepatocytes with 4-methylpyrazole did not significantly alter the incorporation of the different labeled substrates into the lipid fraction. When the cells were incubated in the presence of ethanol together with 4-methylpyrazole, none of the above-mentioned changes to the metabolism of DAG or TAG occurred (data not shown). Taken jointly, these results indicate that the increase caused by ethanol in the uptake of glycerol, serine, and oleate into lipids results exclusively from the metabolism of alcohol in the liver via ADH activity.

Rat hepatocytes also incorporate oleate into cholesterol esters. This esterification is catalyzed by ACAT (EC 2.3.1.26), an enzyme located in the rough endoplasmic reticulum. Our results demonstrate that oleate was incorporated into the cholesterol ester fraction to a much less significant degree when the hepatocytes were incubated with alcohol (Table 1), suggesting that ethanol inhibits ACAT activity. This inhibition of cholesterol esterification clearly contrasts with the increase in hepatic ACAT activity previously described by us [18] and other authors [19] in different experimental animals after chronic ethanol ingestion, one related to an increase in free-cholesterol levels. This mechanism can be excluded in our experimental situation since after 3 hr of exposure to ethanol the cholesterol levels in hepatocytes remained unaltered. This inhibition was also observed in the presence of 4-methylpyrazole (6.90 ± 0.33 pmol/min per 10^6 cells in ethanol-treated hepatocytes vs 6.87 ± 0.48 pmol/min per 10^6 cells in hepatocytes incubated with ethanol and 4-methylpyrazole), indicating that the inhibitory effect may reflect a direct action of ethanol on the enzyme and/or an effect on the enzyme lipid microenvironment being produced by ethanol per se rather than any of its metabolite. This suggestion is supported by other studies which have shown that the activity of membrane-embedded enzymes may well be influenced by alterations to the physico-chemical properties of the membrane itself, induced either by ethanol [20] or other organic solvents [reviewed in Ref. 21].

The different exogenous substrates used in our study can also be incorporated into phospholipids. Since serine can be incorporated both into the polar head group of phospholipids and into their DAG moieties [5, 22], we quantified the distribution of radioactivity in both the polar and apolar moieties of phospholipids as described in the Materials and Methods section, and the data for serine are expressed as the radioactivity found in the DAG moiety. As can be seen in Table 2, the highest levels of radioactivity are found in phosphatidylcholine whatever the precursor used in the control hepatocytes, although phosphatidyethanolamine also shows significant labeling. Interestingly, when hepatocytes were incubated for 3 hr in the presence of 100 mM of ethanol, significantly greater quantities of all the substrates were incorporated into the different phospholipids studied.

Several studies with animals have shown that the greater capacity of the esterification pathway associated with alcohol intake is due to increased phosphatidate phosphohydrolase (3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) activity [17, 23]. It has been clearly demonstrated that the activity of this enzyme increases rapidly in homogenates of hepatocyte monolayers incubated with oleate, probably due to the translocation of cytosolic phosphohydrolase to membranes [24]. Bearing in mind that ethanol produces an increase in endogenous free fatty acids and a concomitant activation of phosphatidate phosphohydrolase, it may well contribute to the stimulated incorporation of radioactivity from exogenous precursors into TAG and phospholipids

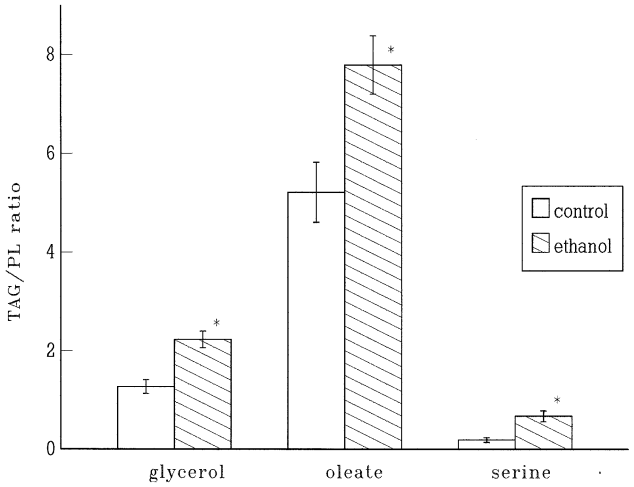

FIG. 2. Effect of 100 mM ethanol on the TAG/PL radioactivity ratio from glycerol, oleate or serine in rat hepatocytes. Values are expressed as means ± SEM for three experiments. Statistical significance of differences from the controls are indicated by: *P ≤ 0.04.

that we found when hepatocytes were incubated with ethanol.

We must also take into account that the ethanol-mediated increase in DAG biosynthesis should result in a corresponding rise in both TAG and phospholipid synthesis, since DAG is used in the final step in the synthesis of both TAG and phosphatidylcholine or phosphatidylethanolamine. Thus, by calculating the ratio of radioactivity incorporated into TAG and phosphatidylcholine plus phosphatidylethanolamine (TAG/PL), we can determine whether ethanol acts on the DAG "branch-point" and preferentially stimulates the shunt of DAG into TAG or phospholipids. As can be seen in Fig. 2, ethanol increased the TAG/PL ratio markedly when oleate, glycerol or serine was used as exogenous substrate. From the values of this ratio, it is evident that after exposure to ethanol DAG is preferentially used for TAG synthesis.

Finally, when the cells were incubated in the presence of ethanol and 4-methylpyrazole, thus inhibiting ethanol metabolism, no detectable change in the biosynthesis of the different phospholipids was apparent. This would suggest that the increase induced by ethanol on the uptake of exogenous precursors into phospholipids results from its metabolism, as occurs in the TAG and DAG biosynthetic pathways (see above).

Consequently, our results taken together show that ethanol exerts some of its early effects by altering the metabolic pathways of phospholipids and neutral lipids. Thus, in the presence of ethanol, rat hepatocytes show a higher tendency to synthesize lipids, which could well account for the accumulation of lipids observed in cases of chronic alcoholism. All these effects appear to be related to metabolites of ethanol produced in the liver and are controlable *in vitro* by specific inhibitors of hepatic ADH activity.

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