STIMULATION OF HEPATIC PHOSPHATIDATE PHOSPHOHYDROLASE ACTIVITY BY A SINGLE DOSE OF ETHANOL

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SUMMARY

An acute ethanol load (5 g per kg body wt) given by gastric intubation to fasted rats caused a significant increase in phosphatidate phosphohydrolase activity in the soluble fraction of the liver. The activity was two-fold at 8 hours and three-fold at 16 hours after the ethanol administration and decreased to the control level a few hours after the disappearance of ethanol from the blood. Results from in vivo experiments with intraportally injected [^{3}H]glycerol showed an ethanol-induced cross-over point between glycerol incorporation into phosphatidic acid and neutral glycerolipids. This cross-over could be observed only when the phosphatidate phosphohydrolase activity was increased.

The mechanisms by which ethanol administration enhances hepatic triacylglycerol synthesis (1-3) are not clear (4). It has been suggested that cytoplasmic phosphatidate phosphohydrolase (EC 3.1.3.4) is a rate limiting enzyme in the hepatic glycerolipid biosynthesis (5). The present study investigates the role of phosphatidate phosphohydrolase in the development of an acute ethanol-induced fatty liver.

MATERIALS AND METHODS

Reagents. Phosphatidic acid (prepared from egg phosphatidylcholine) was obtained from Koch-Light, the other biochemicals and lipid standards from Sigma, [1-^{14}C]palmitic acid (56 Ci/mol) and [1,3-^{3}H]glycerol (2.8 Ci/mmol) from Amersham, and t.l.c. plates and solvents from Merck. 1-Acyl-sn-3-phosphate was synthesized from 1-acyl-sn-glycero-3-phosphorylcholine (6).

Treatment of animals. Male Long-Evans rats weighing 175-220 g and which had fasted for 24 hours before the experiment were used. Ethanol was administered through a stomach tube at a dose of 5 g per kg body weight as a 20 % (w/v) solution in water. The control animals received the same amount of water and a third group received an isocaloric dose of glucose (8.7 g per kg body weight). The rats were anaesthetized with diethyl ether 2, 8, 16, 24 or 36 hours after gastric intubation or without intubation.

Preparation of the tissue samples. The liver was homogenized in 4 volumes of 0.225 M sucrose containing 1 mM EDTA and 50 mM Tris-HCl, pH 7.5, with a glass homogenizer fitted with a teflon

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pestle (all procedures at 2°). The homogenate was then centrifuged at 15 000 g for 20 min, and the supernatant further centrifuged at 105 000 g for 60 min. To the supernatant 0.23 g of $(NH_4)_2SO_4$ per ml was added. After standing for 15 min, and centrifuging at 10 000 g for 20 min, the pellet surface was washed gently three times with 1 ml of 0.25 M sucrose containing 0.5 mM dithiothreitol and 20 mM Tris-HCl, pH 7.4, and the pellet was dissolved in this buffer. The enzyme was assayed either immediately or stored at -20° until assayed.

Assay of phosphatidate phosphohydrolase activity. In the standard method, an aqueous dispersion of phosphatidic acid was used as substrate (7). The incubation medium contained, in a volume of 0.5 ml, 20 µmol Tris-HCl pH 7.4, 1 µmol $MgCl_2$, 0.8 µmol phosphatidylcholine, 0.5 µmol dithiothreitol, 0.2 µmol phosphatidic acid (disodium salt), and the enzyme (either 105 000 g supernatant or $(NH_4)_2SO_4$ fraction). After a 10-min incubation at 37°, 1 ml of 10 % trichloroacetic acid was added, and after centrifugation, the supernatant was extracted with H_2O-saturated diethyl ether. The amount of inorganic phosphate in the water phase was determined (8). The values obtained were corrected for blank values observed in the control tubes incubated without phosphatidic acid.

Phosphatidate phosphohydrolase activity was also determined using membrane-bound [^{14}C]phosphatidic acid as a substrate (9). The substrate was synthesized on microsomes from [^{14}C]palmitic acid (5). A sample of the soluble supernatant (1.1 - 1.2 mg protein) was incubated at 37°C for 15 min in the presence of 5 µmol potassium phosphate, pH 6.8, 2.5 mg fatty acid poor albumin and microsome-bound [^{14}C]phosphatidic acid. The diacylglycerol which formed was separated from the substrate by t.l.c. and the radioactivity in the diacylglycerol fraction was determined as described below.

Incorporation of [^{3}H]glycerol into intermediates of hepatic glycerolipid synthesis in vivo. The rats were anaesthetized with diethyl ether 0.5 hours or 16 hours after ethanol administration (Fig. 3), and 0.1 ml of a solution containing 0.1 mCi of [1,3-^{3}H]-glycerol (2.8 Ci/mmol) in 0.15 M NaCl was injected into the portal vein (10). After the time intervals indicated in Fig. 3 a liver sample was taken by the freeze-clamp technique (11). For the extraction of liver lipids, 500 mg of the frozen tissue was dropped into 10 ml of chloroform-methanol (2:1, v/v) and homogenized immediately with an Ultra-Turrax homogenizer. The homogenate was broken into two phases by adding 2.5 ml of 0.15 M NaCl containing 0.11 M glycerol. The chloroform phase was washed twice with a synthetic upper phase (chloroform-methanol-water, 1:48:47, by vol.), dried under a stream of nitrogen at 40° and dissolved in 1 ml of chloroform.

Lipid analysis. The radioactivity in the total lipids was determined directly by scintillation counting and the lipid classes were separated on Kieselgel 60 precoated t.l.c. plates. The plates were developed for about 60 % of their length with chloroform-acetic acid-acetone-methanol-water (10:2:4:2:1, by vol.) dried at room temperature, and then developed in the same direction for their full length with hexane-diethyl ether-acetic acid (70:20:1, by vol.) in order to separate neutral lipids from phospholipids (12). 1-Acyl-sn-glycerol-3-phosphate and 3-sn-phosphatidylcholine chromatographed together. No attempt was made to separate these compounds from each other, since 1-acyl-sn-glycerol-3-phosphate is not quantitatively extracted by the Folch procedure (13). The phospholipids were detected with a molybdate reagent (14), and the neutral lipids after heating for 3 min at 120°. The lipid

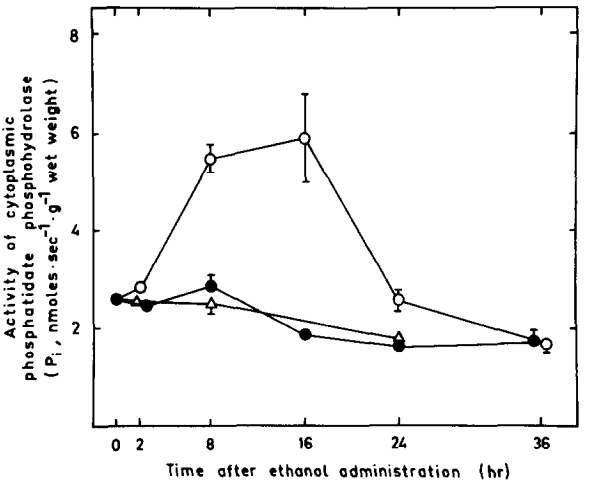

Figure 1. Effect of a single ethanol load on the activity of soluble phosphatidate phosphohydrolase in rat liver. Phosphatidate phosphohydrolase activity was measured using an aqueous dispersion of phosphatidic acid as substrate and the results were expressed as nmoles of P_i released per sec per g wet weight of liver. The results are the means ± S.E.M. of 4-6 separate experiments. At zero time one group of animals received ethanol (5 g/kg body weight), a second group an isoenergetic dose of glucose (8.7 g/kg body weight), and a third group an equal amount of water (25 ml/kg body weight). Symbols: ethanol group -o-o-o-; glucose group -Δ-Δ-Δ-; water control group -●-●-●-.

spots were scraped into counting vials and their radioactivity was determined in a Wallac liquid scintillation spectrometer. Disintegrations per second were obtained by quenching correction through the use of an automatic external standardization procedure.

RESULTS

Ethanol caused a rapid increase in the phosphatidate phosphohydrolase activity of the soluble fraction of rat liver (Fig. 1). This was already statistically significant 2 hours after ethanol administration (14 %; $P<0.05$), and the activity was two- and threefold at 8 and 16 hours respectively. At 24 hours, i.e. about two hours after the disappearance of ethanol from the blood, the phosphatidate phosphohydrolase activity was still 50 % higher than in the controls ($P<0.0025$), whereas at 36 hours there was no difference between the groups. Glucose administration caused no significant changes in the cytoplasmic phosphatidate phosphohydrolase activity as compared to the water control group.

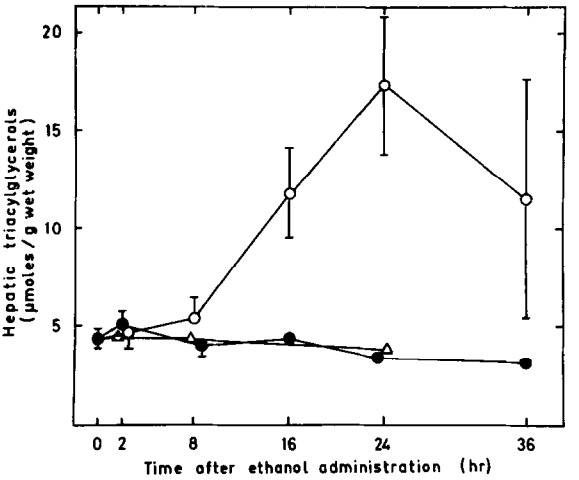

Figure 2. Effect of a single ethanol load on hepatic triacylglycerol concentration. The results are the means ± S.E.M. of 4-6 separate experiments. Symbols and experimental conditions as in Fig. 1.

The increase in the phosphatidate phosphohydrolase activity in the ethanol-treated rats was followed by an accumulation of neutral glycerolipids in the liver (Fig. 2), and a decline in the activity to control level resulted in a subsequent decline in the hepatic triacylglycerol concentrations.

In some experiments, the cytoplasmic phosphatidate phosphohydrolase was also assayed using ^{14}C-labelled microsome-bound phosphatidic acid as substrate. The activity was significantly higher in the livers from the ethanol-treated rats as compared with the control group (Table 1).

To evaluate the significance of the ethanol-induced increase in the phosphatidate phosphohydrolase activity *in vivo*, the incorporation of radioactivity from [^{3}H]glycerol into intermediates of hepatic glycerolipid synthesis was followed (Fig. 3). The measurements were made 0.5 and 16 hours after the administration of ethanol. These time points were chosen because they allow a comparison of the pattern of incorporation during ethanol oxidation in the absence of, and presence of, an increase in the soluble phosphatidate phosphohydrolase activity. In the control rats, phosphatidic acid attained maximal radioactivity at about 10 seconds after the injection and diacylglycerol at 20-30 seconds,

Table 1. Effect of ethanol on the activity of cytoplasmic phosphatidate phosphohydrolase with microsome-bound [^{14}C]phosphatidic acid as substrate

	Phosphatidate phosphohydrolase activity (nmol of diacylglycerol formed/s per g wet weight of liver)	
Control	1.71 ± 0.35	
		(P<0.001)
Ethanol	5.28 ± 0.16	

The results are the means ± S.E.M. of six different experiments. Liver samples were taken 16 hours after the administration of ethanol or water. The soluble supernatant was incubated with microsome-bound [^{14}C]phosphatidic acid at 37° for 15 min and the increase in the radioactivity in the diacylglycerol fraction was determined.

whereas the labeling of triacylglycerol proceeded continuously throughout the experimental period (10-60 seconds). At 0.5 hours after the ethanol administration, the labeling of neutral glycerolipids was somewhat retarded in the ethanol-treated rats, although there was no significant difference in the total incorporation of [^{3}H]glycerol into hepatic lipids. At 16 hours the pattern of incorporation of [^{3}H]glycerol was significantly different in the ethanol-treated rats. The pattern of incorporation of radioactivity into intermediates of hepatic glycerolipid synthesis showed an ethanol-induced cross-over point between phosphatidic acid and diacylglycerol, i.e. in the step catalyzed by phosphatidate phosphohydrolase.

DISCUSSION

The *in vivo* incorporation of fatty acids into hepatic glycerolipids is increased after an acute intake of ethanol (1-3). Enhanced esterification *in vitro* has also been found with microsomal preparations from fasted rats given a single large dose of ethanol, whereas addition of ethanol to the medium does not alter the incorporation of palmitic acid into triacylglycerols by liver homogenates or microsomes from either ethanol-fed rats or glucose-fed controls (3). This difference suggests that ethanol or its metabolites do not simply activate some enzymes involved in the

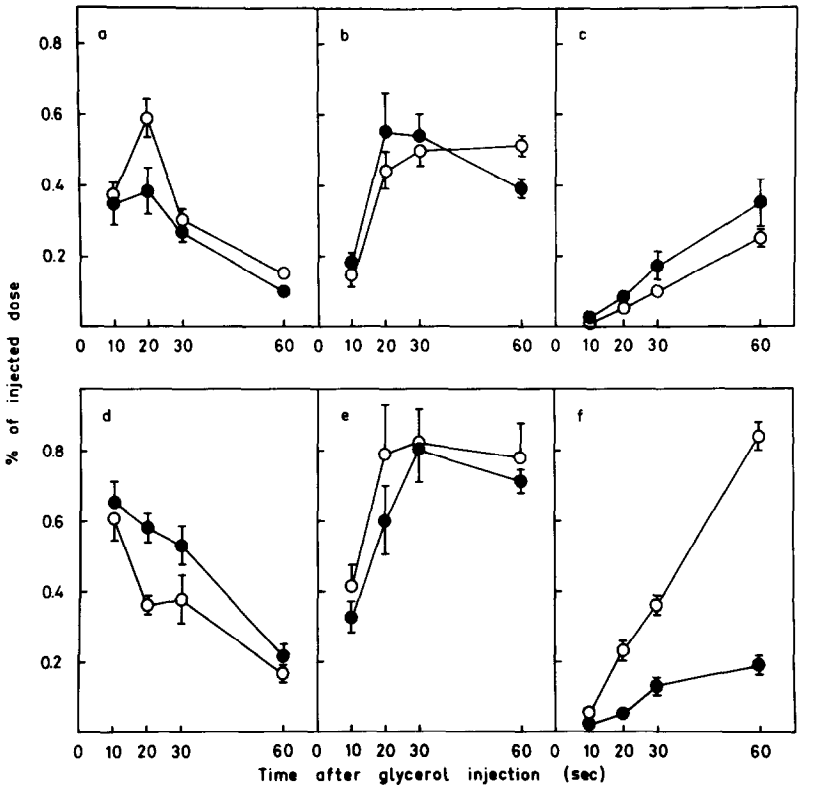

Figure 3. Effect of a single ethanol load on the initial incorporation of glycerol into intermediates of hepatic glycerolipid synthesis: phosphatidic acid (a,d), diacylglycerol (b,e) and triacylglycerol (c,f). 0.1 mCi of [1,3-^{3}H]glycerol was injected into the portal vein 0.5 h (a-c) or 16 h (d-f) after the administration of ethanol (5g/kg body wt.) or water (25 ml/kg body wt.). Liver samples were taken 10, 20, 30 or 60 seconds after the injection and the radioactivity in the lipid classes were determined. Open circles represent the ethanol group and closed circles the control group. The results are the means ± S.E.M. of 4-6 separate experiments.

glycerolipid synthesis, but act by inducing the synthesis of rate-limiting enzymes.

Cytoplasmic phosphatidate phosphohydrolase has been reported as a rate-limiting enzyme in neutral glycerolipid synthesis in the liver (5). The activity of this enzyme is elevated after partial hepatectomy (15), fasting (16) and a carbohydrate-rich diet (17). In the present study, a three-fold increase in the activity of the soluble phosphatidate phosphohydrolase was found in the livers of ethanol-treated rats. The temporal pattern of the

changes in the activity of phosphatidate phosphohydrolase and the time course of the accumulation of neutral lipids in the liver suggest that a causal relationship exists between the increase in the phosphatidate phosphohydrolase activity and the triacylglycerol accumulation.

The results of the incorporation experiments with ^{3}H-labelled glycerol also demonstrated that under these experimental conditions the activity of phosphatidate phosphohydrolase is rate-limiting, as has been previously suggested by the activity determination of the enzymes of the sequence of glycerolipid synthesis under various dietary and experimental conditions (5,17). These results can also be interpreted as showing that the regulation of the enzyme activities may be a more important regulator than substrate availability, e.g. the concentration of sn-glycerol-3-phosphate, which has usually been held as being the main regulator of glycerolipid synthesis in the liver at least during the metabolism of ethanol (for a review, see 4).

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