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Ethanol stimulates lipid biosynthesis in the rat reticulocyte by activating glycerol kinase

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In the liver, the ability of ethanol to stimulate glycerolipid biosynthesis has been attributed mainly to its oxidation by alcohol dehydrogenase and to the subsequent enhanced production of glycerol 3-phosphate via the dihydroxyacetone phosphate pathway. To explore alternative pathways, rat reticulocytes, which are devoid of alcohol deshydrogenase, have been exposed to ethanol and their glycerolipid metabolism has been investigated using glycerol as a lipid precursor. The results indicate that short-term (<30 min) cell exposure to ethanol (50–500 mM) stimulated the incorporation of [^{14}C]glycerol into glycerol 3-phosphate, and thereby enhanced glycerolipid biosynthesis. The involvment of glycerol kinase in the stimulatory effect was confirmed by the stimulation by ethanol of glycerol kinase activity directly assayed in reticulocyte lysates. This ethanol effect may be important under physiological or pathophysiological conditions associated with increased glycerol to glucose ratio in blood. (J. Nutr. Biochem. 8:312–315, 1997) © *Elsevier Science Inc. 1997*

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Introduction

In liver, the accumulation of triacylglycerol (TAG) after chronic alcohol consumption reflects enhanced lipid biosynthesis, a metabolic response currently explained by ethanol-dependent increases in the two main precursor molecules, glycerol 3-phosphate (G3P) and fatty acid (for review, see Ref. 1). The dependency of the ethanol-induced increase in TAG synthesis on G3P has been documented: (1) the ethanol-induced stimulation of TAG synthesis from palmitate in hepatocytes isolated from fasted rats is associated with increased G3P level,[2] and (2) rapid rises in G3P concentration have been measured either in rat liver after ingestion of a single dose of ethanol,[3] in hepatocytes incubated with ethanol[2,4] or in rat liver perfused with ethanol.[5] The increased formation of G3P is ascribed mainly to higher levels of reduction of dihydroxyacetone phosphate (DHAP) associated with a high NADH/NAD ratio resulting from alcohol oxidation by alcohol and acetaldehyde dehydrogenases. Beside the DHAP pathway, G3P may also be generated in most cells via the phosphorylation of glycerol by glycerol kinase (GK; EC 2.7.1.30). Under basal conditions, this pathway seems to be of little importance, because of the low level (<0.5 mM) of glycerol in blood, compared with glucose and the glycolytic pathway. However, there are examples that, on increase in the glycerol to glucose ratio in the extracellular medium, the cell uptake and utilization of glycerol for lipid synthesis seem to be effective, and at least partly regulated by GK.[6] Alternatively, GK deficiency in humans results in increased glycerol concentration in blood.[7]

Reticulocytes are relatively easy to obtain with essentially no membrane damage. In addition, this cell model, used shortly after blood withdrawal, displays readily measurable lipogenic activity, in contrast to more mature red blood cells.[8] Using rat reticulocytes, we have shown previously that the utilization of glycerol is enhanced markedly by ethanol, leading to increased glycerolipid biosynthesis.[9] In this study, we present additional data on the kinetics and mechanism of this lipogenic effect. The results indicate that

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the phosphorylation of glycerol by GK is implicated directly in the stimulatory effect of ethanol on lipid biosynthesis.

Methods and materials

U,L-[^{14}C]glycerol-3-phosphate ([^{14}C]G3P; 156 mCi/mmol) was purchased from Amersham International (UK) and U,L-[^{14}C]glycerol (150 mCi/mmol) from Isotopchim (Peyruis-France). Phenylhydrazine hydrochloride and 4-methylpyrazole were obtained from Sigma (St. Louis, MO, USA). Defatted bovine serum albumine (BSA) was from Calbiochem-Behring (La Jolla, CA, USA). DEAE-cellulose disks (DE 81) were from Whatman (Maidstone, Great Britain).

Lipid biosynthesis in intact reticulocytes was estimated from the incorporation of [^{14}C]glycerol as described previously.[9] Incubations were carried out in 0.4 mL of medium A made of (in mM): 140 NaCl, 5 KCl, 1 $MgSO_4$, 10 Tris-HCl, pH 7.4, 5 glucose, containing variable (0.1 to 50 μM; 10^4 to 10^6 dpm) concentrations of [^{14}C]glycerol and 0.6 mg BSA. Incorporations were started by addition of ~7 × 10^8 cells. After incubation, lipids were extracted with chloroform/methanol (1:1, by vol). Total lipid biosynthesis was estimated by measuring the amount of [^{14}C]glycerol converted into chloroform-soluble material. Triplicate incorporation assays agreed within 2.8 ± 1.3% (n = 12) of [^{14}C]glycerol incorporated. Cell viability, as determined from the extent of hemolysis,[10] was >98% after 60 min of incubation both in the absence or presence of ethanol (up to 750 mM).

GK activity was assayed radiochemically by measuring the conversion of [^{14}C]glycerol into [^{14}C]G3P.[11] In the standard assay (final volume, 0.4 mL), 10 μM [^{14}C]glycerol (1.3 × 10^6 dpm), 3 mM ATP, 3 mM $MgCl_2$, 0.16% (wt/vol) BSA in medium A were incubated for 10 min at 37°C. The reaction was initiated by adding 0.1 mL cell lysate (~2 × 10^8 cells) and stopped with 0.5 mL cold ethanol. For blank values, ethanol was added before the enzyme. After incubation, the mixture was kept for 30 min in ice, then centrifuged at 1600 g for 10 min. To quantitate [^{14}C]G3P, a 30 μL aliquot of the clear supernatant was applied on DEAE-cellulose disks, which adsorb G3P;[11] after drying at 20°C, the disks were washed three times with 20 mL H_2O, then dropped into vials containing 1 mL 0.5 M NaCl; scintillator fluid was added and the radioactivity was counted. Over 95% of total [^{14}C]G3P was recovered in the disk after washing. Less than 2% of added [^{14}C]glycerol was consumed during incubation and no ^{14}C-labeled glycerolipids were detected in the assay medium. The formation of [^{14}C]G3P was proportional to time and amounts of lysate.

Reticulocyte-enriched blood cell suspensions were obtained from female Sprague-Dawley rats injected with phenylhydrazine hydrochloride (30 mg/kg/day) for 4 days, as described previously.[12] Suspensions contain an average of 75 ± 9% (n = 20) reticulocytes; no attempt was made to remove mature cells, which do not synthesize lipids. [^{14}C]G3P produced in cells from [^{14}C]glycerol was quantitated as described for the GK assay. G3P and fatty acid levels in cells were measured according to Michal and Lang[13] and Ho,[14] respectively.

Results and discussion

When cells were incubated for 15 min with increasing (0.5 to 50 μM) concentration of [^{14}C]glycerol, the labeling of G3P and glycerolipids markedly increased up to 5 μM, and then slowed down (*Figure 1*). In incubations performed in 50 μM [^{14}C]glycerol as a function of time, a rapid (<10 min) increased in [^{14}C]G3P led after 20 min to a nearly steady state, whereas the labeling of glycerolipids was initially low and then increased, supporting a precursor-to-

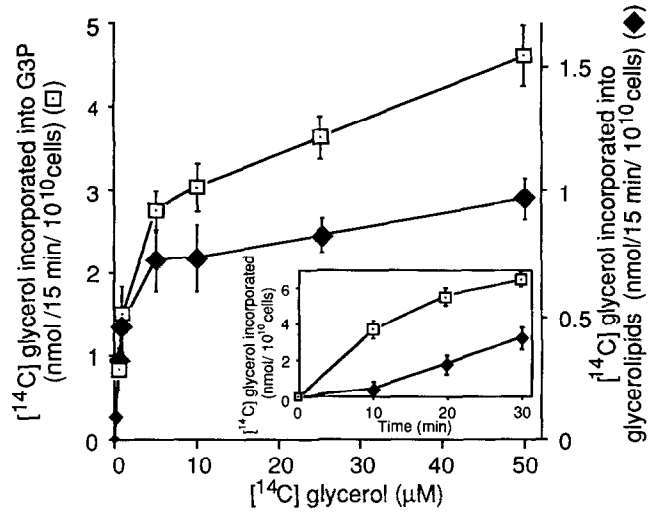

Figure 1 Effects of the addition of [^{14}C]glycerol (0.1 to 50 μM) to reticulocyte suspension on the labeling at 15 min of G3P and glycerolipids. Values are the mean (±SD) of four experiments with assays in triplicate. Note the different ordinate scales for the labeling of G3P and glycerolipids. *Inset*, Time course of the labeling of G3P (□) and glycerolipids (◆) in reticulocytes continuously labeled with [^{14}C]glycerol (50 μM). Each time point represents the mean value of triplicate samples from a representative experiment which was repeated four times.

product relationship (*Figure 1, inset*). At 15 min, approximately 2% of [^{14}C]glycerol had entered the cells, approximately 80% of which was water-soluble and identified as G3P; the chloroform-soluble fraction contained phospholipids (≈65%) and neutral lipids (mainly DAG). Thus, the GK-dependent production of G3P behaved as a limiting factor for glycerolipid biosynthesis beyond 10 μM added [^{14}C]glycerol, a value compatible with the apparent Km range (10 to 100 μM) for glycerol uptake by mammalian cells.[15] Fatty acids are not limiting in the synthetic process: reticulocytes contain, on average, 70 ± 15 μM (n = 8) fatty acids, i.e., a concentration far more higher than that of G3P (5 ± 1 μM, n = 6) attained in cells 15 min after incubation with 50 μM [^{14}C]glycerol, as calculated from the specific activity of [^{14}C]glycerol. In the absence of added [^{14}C]glycerol, no G3P could be detected in cells at any time of incubation, suggesting that [^{14}C]G3P produced from [^{14}C]glycerol during incubation was not significantly diluted by endogenous G3P, and should therefore approach the actual intracellular concentration.

In reticulocytes exposed to ethanol, the incorporation of [^{14}C]glycerol into G3P and glycerolipids increased markedly between 0.5 and 10 μM and culminated above the control (no ethanol) value at approximately 80% for G3P and 40% for glycerolipids (*Figure 2*), confirming previous results.[16] In 10 μM glycerol, ethanol stimulated in a dose-dependent manner the formation of G3P up to 500 mM, and that of glycerolipids up to 200 mM (*Figure 2, inset*). The addition of 4-methylpyrazole (1 mM), an inhibitor of ethanol oxidation, did not modify the stimulatory effects. Taking into account that: (1) glycerol is not used as glucose precursor in the reticulocyte,[8] and (2) GK activity (*Figure 1*) and the ethanol-stimulated production of G3P (*Figure 2*) similarly depend on glycerol concentration, we

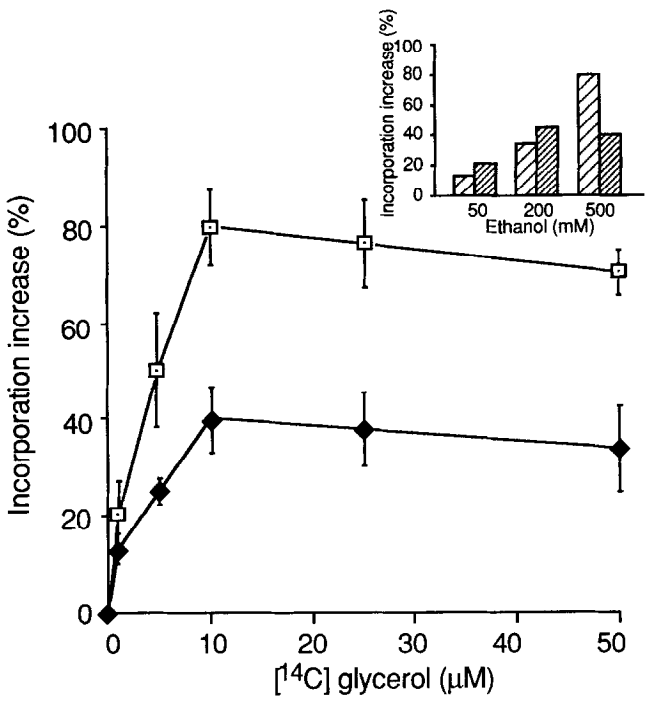

Figure 2 Effects of ethanol (500 mM) as a function of glycerol concentration on the incorporation of [^{14}C]glycerol into G3P (□) and glycerolipids (◆) in reticulocytes incubated for 15 min. Data points are expressed relative to control samples incubated without ethanol. Values are the mean (±SD) from four experiments with assays in triplicate. *Inset*, dose-response of the ethanol effect for G3P (▨) and glycerolipids (▨) with 10 μM [^{14}C]glycerol.

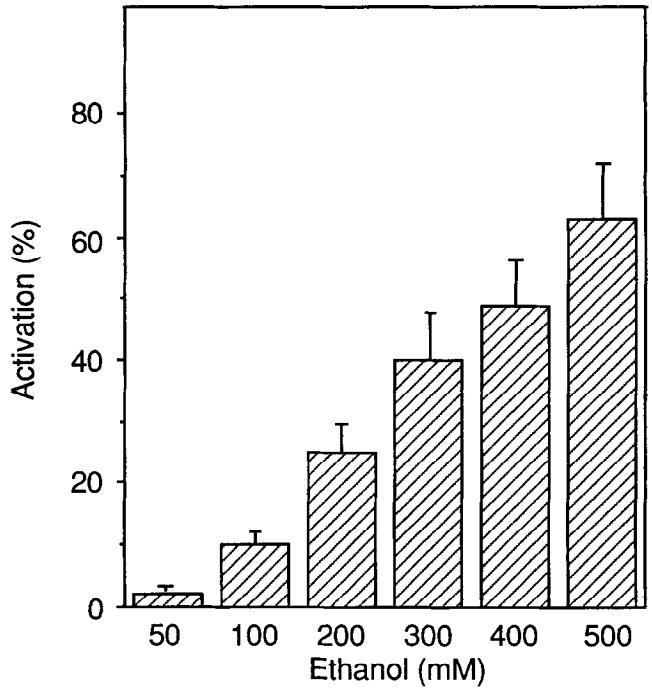

Figure 3 Effect of ethanol (50 to 500 mM) on glycerol kinase activity assayed in reticulocyte lysates. Data points are the mean (±SD) of four experiments with assays in triplicate. Glycerol kinase activity was measured at 10 min with 10 μM [^{14}C]glycerol, as the substrate; basal values are 0.5 ± 0.04 nmol G3P/min/10^{10} cells, equivalent to 1.7 pmol/min/mg protein.

inferred that ethanol stimulated glycerolipid biosynthesis from glycerol by increasing GK activity. This was confirmed by showing that ethanol directly activated GK activity in reticulocyte lysates (*Figure 3*), according to a dose-dependent (50 to 500 mM) pattern mimicking that shown for G3P formation in intact cells (*Figure 2, inset*). The particulate fraction obtained by centrifugation (100,000g, 60 min, 4°C) of lysate contains >80% of total cellular GK activity and gave similar results. GK activity measured in the soluble fraction was unchanged by ethanol, suggesting that some features of the native molecular environment of the enzyme in its membrane site is required for the stimulatory effect; the disordering action of ethanol on the membrane structure has been described long ago.[17] The ethanol-dependent increase in GK was reversible: activity returned to control values as soon as cells were transferred to an ethanol-free medium (not shown). No free [^{14}C]glycerol could be detected in cells, whether ethanol was present or not in the medium, suggesting that in reticulocytes, the GK-dependent phosphorylation of glycerol is rate-limiting for glycerol uptake, as is the case in procaryotes.[18,19]

In conclusion, we show that: (1) in the presence of a physiological concentration of glucose, rat reticulocytes are able to use glycerol for glycerolipid biosynthesis via the GK pathway and (2) ethanol enhances glycerolipid biosynthesis from glycerol at least in part by stimulating GK activity. Earlier results have been conflicting regarding the effects of ethanol on GK. A 6-hour exposure to ethanol (1 to 50 mM) of rat hepatocyte monolayers produced an increase in GK activity.[20,21] In contrast, an inhibitory effect was reported on GK in rat liver homogenates[22] and in the liver of genetically-obese rats given an ethanol diet.[23] Only the bovine liver enzyme, used in the crystalline state, was activated by ethanol (870 mM), whereas GK from guinea pig and rabbit were not.[22]

The liver is the chief source of GK in the body and accounts for at least three-fourths of the total body capacity for the utilization of glycerol.[24] On the other hand, basal concentrations (0.04 to 0.4 mM) of glycerol in blood may increase severalfold by starvation, exercise, exposure to cold and ingestion of some dietary (including glycerol-rich) products and such increases should produce GK-dependent increments in lipid biosynthesis. Interestingly, alcoholic drinks, which may contain, besides ethanol, large amounts of glycerol (up to 30 g/L), display the two specific features for enhancing lipid biosynthesis via the GK pathway.

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