Effect of Ethanol on Polyamine Synthesis during Liver Regeneration in Rats

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Abstract

Ethanol consumption retards the hepatic regenerative response to injury. This may contribute to the pathogenesis of liver injury in alcoholic individuals. The mechanisms responsible for ethanol-associated inhibition of liver regeneration are poorly understood. To determine if the antiregenerative effects of ethanol involve modulation of polyamine metabolism, parameters of polyamine synthesis were compared before and during surgically induced liver regeneration in ethanol-fed rats and isocalorically maintained controls. After partial hepatectomy, induction of the activity of ornithine decarboxylase (ODC), the rate limiting enzyme for polyamine synthesis, was delayed in rats that had been fed ethanol. This was correlated with reduced levels of putrescine, ODC’s immediate product. Increases in hepatic spermidine and spermine were also inhibited. Differences in ODC activity between ethanol-fed and control rats could not be explained by differences in the expression of ODC mRNA or by differences in ODC apoenzyme concentrations, suggesting that chronic ethanol intake inactivates ODC posttranslationally. Supplemental putrescine, administered at partial hepatectomy and 4 and 8 h thereafter, increased hepatic putrescine concentrations and markedly improved DNA synthesis and liver regeneration in ethanol-fed rats. These data suggest that altered polyamine metabolism may contribute to the inhibition of liver regeneration that occurs after chronic exposure to ethanol. (J. Clin. Invest. 1990. 85:385–390.)

Introduction

There is evidence that ethanol inhibits hepatic DNA synthesis and liver regeneration (1–7). Since liver regeneration is important for recovery from many forms of liver injury (8–11), the antiregenerative effects of ethanol may contribute to the pathogenesis and progression of liver disease in alcoholic individuals. The mechanisms by which ethanol inhibits liver regeneration are unknown but may be important to understand in order to design successful treatments for alcoholic liver disease.

Regulation of regenerative growth is, in general, poorly understood. However, it is known that polyamine synthesis is required for regeneration in a number of tissues, including the liver (12–14). Polyamines have been implicated as regulators of both DNA (15, 16) and protein (17, 18) synthesis and may also affect the expression of genes which regulate cell division (19, 20). Biosynthesis of the polyamines (putrescine, spermidine, and spermine) is controlled by the activity of ornithine decarboxylase (ODC), the first and rate limiting enzyme of the pathway, as well as by the availability of ornithine, the substrate of ODC (14).

The antiproliferative effects of ethanol may be mediated, at least partly, by inhibition of polyamine synthesis. In support of this belief are data that acute exposure to ethanol inhibits ODC activity (21, 22), as well as the availability of ornithine (23). In opposition to it are observations that ODC activity is apparently unaffected by chronic ethanol consumption (24, 25).

It is known that chronic consumption of ethanol inhibits DNA synthesis and liver regeneration 24 h after partial hepatectomy (2–5, 25). One purpose of the present study was to determine if polyamine synthesis is also inhibited under identical conditions. To answer this question, several parameters of polyamine metabolism were compared in ethanol-fed rats and isocalorically maintained controls before and at various times during the first 24 h after partial hepatectomy. Secondly, in order to determine if ethanol-associated alterations in polyamine synthesis contribute to ethanol’s inhibition of liver regeneration, the ability of supplemental putrescine to improve regeneration in ethanol rats was tested.

Methods

Materials. The 1982 formulations of the Lieber de Carli ethanol and control diets were purchased from Bio-Serv (Frenchtown, NJ). In each liter of control diet, fat contributes 350 kcal, protein 180 kcal, and carbohydrate 470 kcal. The ethanol diet is identical except ethanol is substituted for 355 of the carbohydrate calories. All chemicals used in HPLC, DNA, and enzyme assays were from Sigma Chemical Co. with the exception of [1-14C]ornithine, [3H]thymidine, and [3H]difluoromethylornithine (DFMO) which were purchased from DuPont-New England Nuclear (Boston, MA). Reagents for determination of hepatic protein content were purchased from Bio-Rad Laboratories (Richmond, CA). The prepacked 300 × 3.9 mm i.d. μBondapak C18 column used for HPLC separation of polyamines was from Waters Associates (Milford, MA).

Animals. 108 adult male Wistar-Furth rats (mean initial weight = 150 g) were divided into two groups. One group (ethanol fed, n = 54) was fed a liquid chow diet in which ethanol contributed 36% of the total calories. A second group (pair fed, n = 54) was fed isocaloric quantities of the same diet but dextrin-maltose was substituted for ethanol (26). Although such “pair feeding” restricts the caloric intake of the control group, previous studies have demonstrated no significant differences in ODC activity or DNA synthesis before or 24 h after partial hepatectomy in pair-fed rats and rats allowed ad lib. access to

1. Abbreviations used in this paper: ASGP, asialoglycoprotein; DFMO, [3H]difluoromethylornithine; ODC, ornithine decarboxylase; PH, partial hepatectomy; PLP, pyridoxal 5’-phosphate.
chow (25). Therefore, ad lib.-fed controls were not included in the present experiments. The animals were housed in individual cages with a 12-h light and 12-h dark cycle and fed their respective diets for 6 wk. During that period of time dietary intake and body weight were monitored. Previous studies in our laboratory have shown that under such conditions (a) ethanol and control rats grow comparably; (b) mid-morning blood ethanol levels range from 75 to 150 mg/dl and (c) the ethanol diet produces hepatic steatosis without significant necroinflammation or fibrosis (25). These results are similar to those of others utilizing this dietary protocol (26).

After 6 wk on the respective diets, the animals underwent a 70% partial hepatectomy (PH) with light ether anesthesia between 9 and 11 a.m. (27). Partial hepatectomy has been shown to substantially increase the proportion of hepatocytes in S phase and is therefore a standard technique used to assess the response to various effectors of regeneration (28). Sham-operated controls were not included in these experiments since a previous study has demonstrated that the effects of partial hepatectomy on DNA synthesis and ODC activity are not reproduced by sham laparotomy (25).

At various times (0.5, 1, 3, 6, 12, or 24 h) postoperatively, the liver remnants were resected and the rats were killed by exsanguination under light ether anesthesia. The livers were immediately weighed and freeze clamped in liquid nitrogen. The tissues were stored at −70°C for up to 2 wk, until they were analyzed for ODC activity, polyamine levels, ODC mRNA expression, ODC apoenzyme concentration, and total protein content.

In separate experiments 60 similar rats were fed either ethanol (n = 45) or control (n = 15) diets for 6 wk as described above. At the time of partial hepatectomy and 4 and 8 h thereafter, these rats received intraperitoneal injections of either normal saline or putrescine (1 or 4 mg/kg body wt). 1 h before death they were injected intraperitoneally with [3H]thyminde (10 μCi/200 g rat). Rats were killed 24 h after partial hepatectomy and livers were immediately weighed, frozen with liquid nitrogen, and stored at −70°C subsequent analysis of [3H]thyminde incorporation by scintillation counting and autoradiography. DNA and protein content and polyamine concentrations.

**Determination of ODC activity.** ODC activity was measured in liver homogenates by the quantitation of [3H]CO liberated from [14C]-ornithine using a modification (25) of the method described by Luk and Baylin (29). Briefly, the livers were homogenized 1:10 (wt/vol) in 100 mM sodium phosphate buffer, pH 7.2, containing 5 mM DTT and centrifuged at 100,000 g for 20 min. The final reaction mixture (500 μl) contained 100 μM unlabeled ornithine, 1 μCi [1-14C]ornithine hydrochloride (20 μCi/mmol, New England Nuclear), 100 μM pyridoxal phosphate and 300 μl of supernatant. The reaction mixture contained abundant pyridoxal phosphate to minimize underestimation of the amount of apoenzyme present due to its variable cofactor deficiency. Both reagent blanks and heated homogenate blanks were routinely included in the assay to control for ODC-independent, nonspecific deaminoplation of [14C]ornithine. The reactions were run in capped, 25-ml vials incubated at 37°C in a shaking water bath for 30 min. The reaction was terminated with 10% TCA. The [3H]CO trapped on a filter impregnated with 2 M hyamine hydroxide was assessed. Results are expressed as nanomoles of CO released/hour per milligram protein.

**ODC apoenzyme quantitation.** In separate assays, ODC apoenzyme was quantitated in the aforementioned 100,000 g supernatants of liver homogenates by measuring the binding of tritium-labeled DMFO ([3H]DMFO) (30). As above, both reagent and boiled blanks were included in the assay. Separate controls incubated with the same amount of cold DMFO were assessed for residual ODC activity to insure that sufficient [3H]DMFO had been added to completely inactivate the ODC present. The final reaction mixture (110 μl) contained 25 μl of supernatant, 100 μM pyridoxal 5′-phosphate (PLP), and 1 μCi of [3H]DMFO (20 Ci/mmol, New England Nuclear). Binding was allowed to proceed in a shaking water bath at 37°C for 2 h before the reaction was terminated by the addition of 12% perchloric acid. Subsequently, the acid precipitates were filtered and the filters washed sequentially with 6 vol of 12% perchloric acid, a mixture of ethanol/chloroform/ether (2:1:1) and ether. Filters were then air dried and solubilized with soluene. Radioactivity of the solubilized protein was determined. Results are expressed as disintegrations per milligram protein.

**Polyamine determination.** A prepacked 300 × 3.9 mm i.d., 10-μm particle sized Bondapak C18 column was employed to chromatograph the dansylated polyamines. The mobile phase consisted of 0.02 M PIC B-7 reagent (1-heptanesulfonyl acid) pumped in tandem with acetonitrile. Two high-pressure pumps were used to deliver the mobile phase. A concave gradient was used to elute the various polyamines from the column. Gradient parameters were 50% acetonitrile and 50% of the 0.02 M 1-heptanesulfonic acid at zero time. Upon injection, the acetonitrile was increased from 50% to 80% within a 20-min period. Total analysis time was 30 min. Flow rate for the dual pumping system was 2 ml/min. Column pressures ranged between 75 and 85 bar. All separations were performed at room temperature. 1.6-Diaminohexane was used as an internal standard. Each specimen was run in triplicate to ensure reproducibility. Peak areas were measured by an on-line computing integrator. The detection limit of the method was 1 pmol on column with a signal-to-noise ratio of 3:1. A fluorometer (Amicon, Rockville, MD), equipped with a 365-nm excitation and 485-nm emission filter was employed for fluorometric detection (31). Results are expressed as picomoles of di- or polyamine/mg protein. Protein content was determined by the method of Bradford (32) using BSA as standard.

**DNA probes.** A 1,600-bp cDNA clone (clone pOD48) of mouse ornithine decarboxylase was supplied by Dr. P. Coffino, University of California, San Francisco (33). A 1,169 bp cDNA clone (clone 22) of the rat liver asialoglycoprotein receptor (ASGP) was supplied by K. Drickamer, Columbia University (34).

**RNA isolation and Northern blotting.** RNA isolation, poly(A+)RNA enrichment and electrophoresis on horizontal denaturing formaldehyde-agarose gels with subsequent transfer to nitrocellulose membranes was performed as previously described (35). After prehybridization in the hybridization buffer for 5 h at 42°C, nylon membranes were hybridized to 32P-nick-translated DNA probes (specific activity 1 × 108 cpm/μg DNA) overnight at 42°C in 50% deionized formamide, 5 × SSC (1× SSC = 0.15 M NaCl/0.015 M trisodium citrate), 5 × E buffer (1 × E buffer = 10 mM phosphate buffer, pH 7.5), 3 × PM (1 × PM = 0.2% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA) and 0.1% SDS with 5 mg/ml yeast tRNA carrier. Blots were washed at moderate stringency (2× SSC/0.1% SDS at room temperature for 30 min with a total of three changes and 0.1× SSC/0.1% SDS at 45°C for 20 min with a total of two changes), and exposed to Kodak XAR-5 film with intensifying screens. After exposure to film, Northern blot was washed (boiled for 5 min in water) and reprobed with albumin, ASPG, and GAPDH cDNA probes to confirm that all lanes had equal amounts of RNA.

**In vitro transcription and hybridization.** Isolation of nuclei and transcription analysis were performed according to the method of J. A. Silverman and J. D. Yager (manuscript in preparation). In short, at specific times after PH, samples of tissue were excised, frozen in liquid nitrogen and stored at −70°C until analyzed. To obtain nuclei, 0.5 g of frozen liver fragments were pulverized in liquid nitrogen and Dounce homogenized in buffer containing 0.32 M sucrose, 3 mM MgCl2 and 1 mM Hepes, pH 6.8 and then centrifuged for 10 min at 1,600 g for 1 h at 4°C. The crude nuclear pellet was layered onto a 2.1 M sucrose, 1 mM Hepes, pH 6.8, and 2 mM MgCl2 cushion and centrifuged at 70,000 g for 1 h at 4°C. The nuclear pellet was gently resuspended and washed twice in 0.25 M sucrose, 1 mM Hepes, pH 6.8, and 1 mM MgCl2. The nuclei were then resuspended in transcription buffer containing 0.2 M Tris-HCl, pH 8.0, 20% glycerol, 140 mM KC1, 5 mM MgCl2, 1 mM MnCl2, 14 mM β-mercaptoethanol and 1 mM each of ATP, CTP, and GTP. An aliquot of 100 μCi of [3H]UTP (3,000 Ci/ mmol, New England Nuclear) was added to the suspension which was then incubated at 30°C for 25 min with periodic mixing. After recovering the nuclei by centrifugation at 1,000 g for 10 min at 4°C, the nuclei were lysed in buffer containing 0.5 M MgCl2, 0.5 M NaCl, 0.01 M
Tris-HCl, pH 7.4 and 100 μg/ml DNAse (RNAse free; Worthington Biochemical, Malvern, PA). RNA was extracted with phenol/chloroform and precipitated with ethanol. 2 × 10^6 cpm of labeled RNA was hybridized to nylon filters (MSI, Westboro, MA) to which 1.0 μg of the indicated probe fragment was bound by slot blot. The filters were hybridized for 4 d at 42°C, washed according to standard techniques, and autoradiographed.

[3H]Thymidine incorporation. DNA synthesis was estimated by [3H]thymidine incorporation over the 1-h period before death. 0.5-g pieces of liver were homogenized in 0.25 M sucrose with 1 mM MgCl₂ and then precipitated with 5% TCA. The tritium radioactivity of the precipitates was measured and expressed as disintegrations per minute per milligram DNA, per milligram protein and per gram of liver weight (12, 25). DNA content in the liver homogenates was estimated fluorometrically as described by Labarca and Paigen (36). The incorporation of [3H]thymidine into DNA has been validated as an excellent measure of DNA synthesis in liver regeneration (37). However, autoradiography was done for additional confirmation. Coded histologic sections from each rat were reviewed to determine the percentage of labeled nuclei. At least 500 nuclei were counted per section and a labeling index was derived by dividing the number of labeled nuclei by the total number of nuclei counted and then multiplying by 100 (25, 38).

Calculation of liver mass restoration. The weight of the liver segment resected at partial hepatectomy was taken as 70% of the total prehepatectomy liver weight. Thus, the total prehepatectomy liver weight was calculated as (100/70) × resected liver weight. The expected weight of the un-resected liver remnant was therefore (30/100) × the prehepatectomy liver weight. At the time of death, the liver remnant was weighed. The percentage of the prehepatectomy liver weight that was regained 24 h after surgery was calculated as: [actual weight of liver remnant – expected weight of liver remnant] + prehepatectomy liver weight × 100 (25).

Statistical analysis. Data were analyzed by analysis of variance, Student's t test, and linear regression analysis (39).

Results

As shown in Fig. 1, hepatic ODC activities were virtually identical in ethanol-fed rats and pair-fed controls immediately before PH. In control rats, ODC activity peaked biphasically after PH. In contrast, induction of ODC activity after PH was blunted in ethanol-fed rats. In this group, the early peak in ODC activity did not occur. Instead, ODC activity gradually increased until it reached control levels by 24 h after PH. ODC activity in ethanol fed rats was significantly less than control at 1, 3, and 6 h. Putrescine levels were measured in order to determine if ethanol-associated inhibition of ODC activity was also associated with reduced hepatic levels of polyamines. As shown in Fig. 2, putrescine levels were similar in ethanol and control rats before and for the first hour after PH. However, by 3 h putrescine levels in ethanol-fed rats were significantly less than those of control animals. They remained below control values until 12 h after PH. In both ethanol and control rats, ODC activity was significantly correlated with putrescine levels (r = 0.8-0.9, P < 0.01).

Because putrescine is the substrate for spermidine and spermine synthesis, levels of these polyamines were also measured. Spermidine concentration tended to be greater in ethanol-fed than control rats before PH. In marked contrast to the significant increase in spermidine levels that occurred in control rats over the first 12 h after PH, spermidine levels remained virtually unchanged in ethanol-treated animals (Fig. 3). Changes in spermine concentration after partial hepatectomy paralleled those of spermidine (Fig. 4).

These data suggest that chronic ethanol consumption inhibits both ODC activity and the synthesis of polyamines. To determine if ethanol-induced inhibition of ODC activity was due to reduced levels of ODC apoenzyme, ODC mRNA expression and levels of ODC apoenzyme were measured. Expression of ODC mRNA before and after partial hepatectomy is illustrated in Fig. 5. The major hybridizing band at 2.1 kb is consistent with published results (40, 41). Our probe also identified a minor band at 2.4 kb. Although ODC mRNA expression markedly increased after PH in both control and ethanol fed groups at 12 and 24 h, there were no significant differences between the two groups. These data suggest that ethanol does not inhibit the transcription of ODC mRNA at the times indicated. Interestingly, although there was a significant increase in ODC mRNA levels at 12 and 24 h post-PH,
nuclear run-offs showed no significant change in in vitro transcription of ODC at 0, 6, 12, and 24 h in either group (data not shown).

Since tissue content of ODC may not always reflect ODC mRNA expression, levels of ODC apoenzyme were measured. By using an established technique to measure DFMO binding, the equivalent amount of ODC apoenzyme was determined (30). The amount of ODC apoenzyme in ethanol rats was similar to that of control rats both before and after PH (Table 1). These data suggest that ethanol inhibition of ODC activity cannot be explained by reduced synthesis or levels of ODC apoenzyme and hence, may result from posttranslational inactivation of the enzyme.

As shown in Fig. 6, ethanol significantly inhibited DNA synthesis and liver regeneration 24 h after partial hepatectomy. Supplemental putrescine significantly increased hepatic putrescine levels during the initial 6 h after PH in both ethanol and pair fed rats (data not shown). The effect of putrescine therapy on liver regeneration in controls was comparable to that of normal saline treatment. However, in ethanol-fed rats, supplemental putrescine (4 mg/kg body wt) significantly increased [3H]thymidine incorporation/mg hepatic DNA and the nuclear labeling index. Restoration of liver mass was also improved.

Discussion

Alcoholic liver injury is a major cause of chronic liver disease in industrialized societies (42). Even in subjects without obvious liver disease, habitual alcohol consumption is ubiquitous in many of these populations (43). Since regeneration is essential for recovery from many forms of liver injury, the effect of chronic ethanol consumption on the regenerative capacity of the hepatocyte has many clinically relevant implications.

This study was devised to define potential mechanisms responsible for the anti-regenerative effects of chronic ethanol consumption. Partial hepatic resection was used to trigger liver regeneration because it is highly reproducible and has been extensively studied, permitting clear definition of the normal sequence of regenerative events (28). Despite previous data, which suggested that chronic exposure to ethanol does not adversely affect polyamine synthesis (24, 25), this process was carefully scrutinized because it is known to be essential for hepatic regeneration (12–14) and is acutely inhibited by ethanol (21–23).

Table 1. ODC Apoenzyme Concentrations after Partial Hepatectomy

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>ODC apoenzyme concentration (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>31.8±9.6</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0</td>
<td>29.8±7.4</td>
</tr>
<tr>
<td>Control</td>
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<td>29.4±5.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1</td>
<td>40.7±8.6</td>
</tr>
<tr>
<td>Control</td>
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<td>65±4.0</td>
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<tr>
<td>Ethanol</td>
<td>3</td>
<td>68±6.1</td>
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<tr>
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<td>6</td>
<td>58.5±7.5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6</td>
<td>72.0±10.0</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>33.0±10.6</td>
</tr>
<tr>
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<td>12</td>
<td>14.3±8.7</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>31.8±9.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>24</td>
<td>33.0±4.1</td>
</tr>
</tbody>
</table>

ODC apoenzyme concentrations were determined in liver homogenates by measuring the binding of tritium-labeled difluoromethylornithine, a specific, irreversible inhibitor of ODC. Values at each time represent the mean±SD of at least nine rats from each group.
In the model used, chronic ethanol consumption clearly delays the induction of ODC activity that normally occurs after PH. Previous studies, which failed to document any inhibitory effect of chronic ethanol exposure on ODC activity, probably overlooked this inhibition because tissues were studied either before or long after the regenerative stimulus was applied. In ethanol-fed rats, as in many other models in which ODC activity is inhibited, the inhibition of enzyme activity is highly correlated with reduced polyamine levels, impaired DNA synthesis and cellular proliferation (12, 13, 44, 45). Even the transient delay in polyamine synthesis associated with ethanol consumption appears to be pathophysiological significant. This is demonstrated by the finding that supplemental polyamines dramatically improve DNA synthesis in polyamine-deficient, ethanol-fed rats.

Despite significant differences in ODC activity between ethanol-fed and control animals during the initial 6 h after PH, expression of ODC mRNA and the concentration of ODC apoenzyme were similar in both groups at all time points assessed. Since [3H]DFMO may bind to non-ODC proteins under certain assay conditions, the present apoenzyme data must be confirmed using immunoprecipitation techniques. If substantiated, these observations indicate that ethanol inhibition of ODC activity occurs at neither transcriptional nor translational levels. This suggests that chronic ethanol consumption affects posttranslational inhibition of ODC activity.

It is unclear how this posttranslational inactivation occurs. Because chronic ethanol consumption reduces plasma and tissue concentrations of PLP (46), a factor necessary for ODC activity (47), it is tempting to speculate that posttranslational inhibition of ODC activity is due to PLP deficiency. However, while PLP deficiency may contribute to ethanol-associated inhibition of polyamine synthesis in vivo, it cannot explain the inhibition of ODC activity noted in the present study. In these experiments, ODC activity was determined in vitro using standard assay conditions that optimize PLP concentrations (48). Hence, the reduced activity of ODC noted in tissues of chronically ethanol-fed rats cannot be explained by PLP deficiency.

ODC can be posttranslationally modified by various mechanisms including noncovalent binding to an inhibitory “antizyme,” microsomal oxidation, transglutamination and phosphorylation (49, 50). Each of these processes is induced by elevated tissue levels of particular polyamines. Basal levels of spermidine and spermine tended to be elevated in ethanol-treated animals. These two polyamines have been shown to induce a kinase that inactivates ODC by phosphorylation (51). Some workers have suggested that the phosphorylated ODC and the polyamine-dependent kinase are also regulatory proteins that can affect the expression of genes involved in cellular proliferation (20).

The identification of an ethanol-associated defect in polyamine synthesis, a vital step in hepatic regeneration, will help delineate the effects of chronic ethanol consumption on cellular proliferation. Such knowledge may suggest therapeutic approaches to improve the hepatic regenerative capacity of alcoholic individuals. These efforts, in turn, may improve the morbidity and mortality of alcoholic liver disease.

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References

Ethanol Effects on Polyamine Synthesis during Liver Regeneration 389


