Undernutrition enhances alcohol-induced hepatocyte proliferation in the liver of rats fed via total enteral nutrition

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Baumgardner JN, Shankar K, Korourian S, Badger TM, Ronis MJ. Undernutrition enhances alcohol-induced hepatocyte proliferation in the liver of rats fed via total enteral nutrition. Am J Physiol Gastrointest Liver Physiol 293: G355-G364, 2007. First published May 17, 2007; doi:10.1152/ajpgi.00038.2007.—To assess the relative contributions of undernutrition and ethanol (EtOH) exposure to alcohol-induced hepatotoxicity, female Sprague-Dawley rats were intragastrically infused liquid diets containing 187 or 154 kcal·kg⁻³/₄·day⁻¹ with or without 11 g·kg⁻¹·day⁻¹ EtOH. EtOH clearance was impaired in the 154 kcal·kg⁻³/₄·day⁻¹ EtOH group (P ≤ 0.05). A combination of undernutrition and EtOH also increased the induction of hepatic cytochrome P-450 (CYP)2E1 and CYP4A1 mRNA, apoprotein, and activities (P ≤ 0.05). This was accompanied by increased oxidative stress (P ≤ 0.05). The severity of liver steatosis, macrophage infiltration, and focal necrosis was comparable in both EtOH groups. Alanine aminotransferase levels were elevated (P ≤ 0.05) but did not significantly differ between the two EtOH groups. TUNEL analysis also demonstrated a comparable increase in apoptosis in the two EtOH groups (P ≥ 0.05). The development of alcohol-induced liver pathology was accompanied by little change in fatty acid (FA) synthesis or degradation at 187 kcal·kg⁻³/₄·day⁻¹ but at 154 kcal·kg⁻³/₄·day⁻¹ was accompanied by decreased expression of FA synthesis genes and increased expression of peroxisome proliferator-activated receptor-α (PPAR-α)-regulated FA degradation pathways (P ≤ 0.05). In addition, 154 kcal·kg⁻³/₄·day⁻¹ EtOH group livers exhibited greater hepatocyte proliferation (P ≤ 0.05). We conclude that undernutrition does not exacerbate alcoholic steatohepatitis despite additional oxidative stress produced by an increased induction of CYP2E1 and CYP4A1. However, enhanced ethanol-induced cellular proliferation, perhaps as a result of enhanced PPAR-α signaling, may contribute to an increased risk of hepatocellular carcinoma in undernourished alcoholics.

ethanol; liver injury; cell proliferation; peroxisome-proliferator activated receptor-α

DECREASED CALORIC INTAKE AND FRANK UNDERRUTRITION are often observed in alcoholic patients (14, 23, 25). Long-term caloric restriction in humans and animal experiments has been shown to decrease the incidence of diseases, increase lifespan, and present overall benefit to the organism (3). Reduced caloric intake has also been shown to increase the regenerative capacity of the liver (3). However, animal studies have not been conducted to systematically study the interaction of reduced calories and ethanol (EtOH) on the development of liver pathology. This is largely due to the lack of suitable animal models. High rates of EtOH clearance result in little or no blood EtOH or liver pathology in rodents fed EtOH in drinking water ad libitum, while the aversion to the taste of EtOH in liquid diets results in significantly reduced caloric intake and a necessity for pair feeding. In addition, the various rodent models of alcohol-induced liver damage (ALD) differ significantly with regard to species, animal age, growth rate, and the development of endotoxemia preceding pathology (28, 33, 46, 47).

We employed a rat total enteral nutrition (TEN) model in which EtOH-containing liquid diets are infused intragastrically (5–7, 38). The infusion of diets occurs over a 14-h period (overnight from 18:00 to 8:00 hours) when the animals are normally awake. TEN overcomes the problem of the aversion of rodents to EtOH, and it allows complete control over caloric intake, diet composition, and EtOH dose. Overnight infusion also better mimics human drinking patterns without compromising normal sleep or eating cycles. In this model, the development of ALD (steatohepatitis) above and beyond simple steatosis is dependent on a low dietary carbohydrate-to-fat ratio and dietary polyunsaturated dietary fatty acids (FAs) (21). Moreover, in this model, ALD develops without significant elevations in endotoxin (41). Data from some models of ALD, including the TEN model, have suggested that liver pathology results from a process involving EtOH-induced oxidative stress and free radical production. Increased lipid peroxidation, impaired antioxidant enzyme defenses, and the appearance of free radical adducts derived from cytochrome P-450 (CYP)2E1-dependent EtOH metabolism to the 1-hydroxyethyl radical, FA breakdown, and uncoupling of mitochondrial respiration have all been demonstrated to precede the development of liver pathology (1, 34, 37, 42, 55).

In the present study, we examined the effects of EtOH with or without undernutrition on EtOH metabolism, the development of oxidative stress, and liver pathology in the rat TEN model. We found that the severity of early stage steatohepatitis following EtOH treatment was not increased due to caloric restriction; however, an increase in hepatocyte proliferation in the face of EtOH and undernutrition was observed. This is perhaps due to alterations in peroxisome proliferator-activated receptor-α (PPAR-α) signaling and may be associated with an increased long-term risk of liver cancer in undernourished compared with well-fed alcoholics.

MATERIALS AND METHODS

Reagents. EDTA, PMFS, and glycerol were purchased from Sigma-Aldrich (St. Louis, MO). Potassium chloride, potassium phos-
cytochrome for 50 days with either non-EtOH-containing diets (control) or EtOH
ment. Animals were randomly assigned to groups and were infused
14 days to recover before the diet infusion as described previously (5–7,
had an intragastric cannula surgically inserted and were allowed 7
animals were approved by the Institutional Animal Care and Use Committee. Rats
house in an Association for Assessment and Accred-
chemiluminescent detection in Western blot analysis was from Am-
accorded by the National Research Council. Twenty-four-hour urine ethanol con-
during the period of infusion. All
by the Ethical guidelines for animal research established and
children were from Invitrogen Life Technologies (Rockville, MD). Reagents
for the assessment of RNA quality using the Agilent Bioanalyzer were
from Agilent Technologies (Foster City, CA). ECL for
phate, and potassium ferricyanide were purchased from Fisher Scien-
tific (Hampton, NH). TRIzol LS used for RNA extraction was ob-
tained from Invitrogen Life Technologies (Rockville, MD). Reagents
for the assessment of RNA quality using the Agilent Bioanalyzer were
needed for death. Serum and livers were collected and stored at −20 and −70°C,
respectively.
Biochemical analysis. Plasma alanine aminotransferase (ALT) lev-
were measured at death using Infinity ALT liquid stable reagent
by Thermco Electron, Waltham, MA) according to the manufacturer’s
protocols. Liver microsomes were prepared by differential centrifug-
and stored at −70°C until analysis. Protein concentrations of
mics were determined by the Bradford method using the
by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Microsomal carbon
tetrachloride-dependent lipid peroxidation was assessed according to
against Johansson and Ingelman-Sundberg (18). p-Nitrophenol hydroxylation
was measured spectrophotometrically as described by Koop et al.
(20). Liver lipid peroxidation was assessed as a measure of oxidative
stress as described by Ohkawa et al. (35). Western immunoblot
analysis of apoprotein expression for CYP2E1 and CYP4A1 was
as conducted as previously described (37) except that cross-reactive
proteins were detected by ECL using horseradish peroxidase-linked
goat antibody to rabbit IgG or rabbit antibody to sheep IgG in the case of
CYP4A1. CYP2E1 was a gift from the laboratory of Dr. Magnus
Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden) (19).
CYP4A1 was detected using a polyclonal sheep antibody to rat
cycle. Serum and livers were collected and stored at −20 and −70°C,
cycle. Serum and livers were collected and stored at −20 and −70°C,
respectively.

Table 2. Effects of EtOH and undernutrition on growth, UEC, and ADH activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g (at death)</th>
<th>Body Weight Gain, g/day</th>
<th>Liver Weight, g (at death)</th>
<th>Liver Weight/BODY Weight, %</th>
<th>Mean UEC, mg/dl</th>
<th>ADH Activity, nmol·mg⁻¹·min⁻¹</th>
</tr>
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<tbody>
<tr>
<td>187 kcal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>375.6±16.0</td>
<td>2.4±0.3</td>
<td>10.8±0.6</td>
<td>2.9±0.1</td>
<td>0</td>
<td>2.8±0.4</td>
</tr>
<tr>
<td>EtOH</td>
<td>363.6±7.9</td>
<td>2.4±0.1</td>
<td>12.9±0.5</td>
<td>3.5±0.2</td>
<td>114±9.8</td>
<td>3.9±0.4</td>
</tr>
<tr>
<td>154 kcal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>290.3±9.6</td>
<td>1.3±0.1</td>
<td>9.0±0.3</td>
<td>3.1±0.1</td>
<td>0</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td>EtOH</td>
<td>292.9±15.3</td>
<td>1.2±0.1</td>
<td>11.0±0.7</td>
<td>3.8±0.2</td>
<td>169±16.6</td>
<td>4.0±0.5</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 8–10 animals/group. Both ethanol (EtOH)-treated groups received equivalent EtOH doses (10.6 ± 0.1 g·kg⁻¹·day⁻¹) throughout the study. The 187 and 154 kcal groups received 187 kcal and 154 kcal·EtOH⁻¹·day⁻¹ diets, respectively. UEC, urine EtOH concentration. Statistical significance was determined by two-way ANOVA followed by Student-Newman-Keuls post hoc analysis. P ≤ 0.05 as follows: *187 kcal + EtOH vs. 187 kcal control; b154 kcal + EtOH vs. 154 kcal control; c154 kcal + EtOH vs. 187 kcal + EtOH; d154 kcal control vs. 187 kcal control; and etOH groups vs. control groups.
Real-time RT-PCR. Total RNA was extracted from livers using RNeasy mini-columns (Qiagen, Valencia, CA). Total RNA (1 μg) was reverse transcribed using the iScript Reverse Transcription Kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. Reverse-transcribed cDNA (10 ng) was utilized for real-time PCR using 2× SYBR green master mix and monitored on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Gene-specific probes were designed using Primer Express Software (Applied Biosystems, Foster City, CA; Table 1), and the relative amounts of gene expression were quantitated using a standard curve according to the manufacturer’s instructions.

Pathological evaluation. Liver pathology was assessed in hematoxylin-eosin-stained liver sections and scored using blinded samples by a board-certified pathologist (S. Korourian). For statistical comparisons, level of steatosis (macro- and microvesicular), inflammation, and necrosis were scored on a scale of 1–5, where 1 = no pathology and 5 = maximal pathology, and total pathology was defined as the sum of steatosis, inflammation, and necrosis scores (21). Apoptosis was assessed by in situ end labeling of free 3' hydroxyl ends generated during apoptosis (TUNEL) using a commercial kit (Frag-EL DNA Fragmentation Detection Kit, Fisher Scientific, Hampton, NH). Sections were counterstained with Gill’s hematoxylin. Apoptotic bodies and cells appeared brown. At least 2,000 cells were counted from each liver section.

Immunohistochemistry. Proliferating cell nuclear antigen (PCNA) immunohistochemical analysis was conducted as described by Greenwell et al. (16). Briefly, liver sections mounted on glass slides were first blocked with casein (0.5%) for 20 min and then reacted with monoclonal antibody (1:5,000) to PCNA (PC.10, Dako, Carpentaria, CA) for 60 min. Antigen retrieval was performed by heating slides in 1% zinc sulfate solution for 6.5 min. The antibody was then linked with biotinylated goat anti-mouse IgG antibody (1:500 for 20 min, Jackson Immunoresearch, West Grove, PA). Brown color was developed by exposing the peroxidase to diaminobenzidine (one tablet in 10 ml PBS, filtered, and 3% hydrogen peroxide) for 10 min. Sections were counterstained with Gill’s hematoxylin. The nuclei of G0-phase cells were blue, G1-phase cells had light brown nuclei, and S-phase nuclei stained dark brown. G2-phase cells showed brown cytoplasmic staining with or without brown speckling of the nucleus. M-phase cells were identified by mitotic bodies. For histomorphometric analysis, each section was scored for cells in different phases of the cell cycle in six high-powered fields as reported previously by Wang et al. (49).

EMSA. Nuclear extracts were isolated from livers frozen at −70°C using a nuclear extraction kit from Sigma. The protein concentration of the nuclear extracts was determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories). EMSAs were performed as previously described (17). In brief, double-stranded oligonucleotides coding for the acyl CoA oxidase-peroxisome proliferator response element (PPRE), 5′-gatcCTCCCGGAAGTGCCTT-TGTCCTGGTG-3′, were prepared by combining and heating equimolar amounts of complementary single-stranded DNA to 95°C for 5 min in distilled H2O and cooling to room temperature. Annealed oligonucleotides were diluted to a concentration of 40 μM and stored at −20°C. EMSAs were carried out in 15-μl volumes containing 50 mM KCl, 12 mM HEPES, 1 mM EDTA, 1.0 mM DTT, 15% glycerol, and 1 μg of poly(dl-dc) (Roche Molecular Biochemicals). Nuclear
Fig. 2. Representative Western blots. Representative Western blots show the effect of diets with or without 11 g EtOH·kg\(^{-1}\)·day\(^{-1}\) with either 187 or 154 kcal·kg\(^{-3/4}\)·day\(^{-1}\) on hepatic CYP2E1 and CYP4A1 expression. Each lane represents liver microsomal protein from individual rats.

**CYP2E1**

- **A**
  - 187 kcal/kg\(^{3/4}\)/day
  - 154 kcal/kg\(^{3/4}\)/day
  - **Control**
  - **EtOH**

- **B**
  - 187 kcal/kg\(^{3/4}\)/day
  - 154 kcal/kg\(^{3/4}\)/day
  - **Control**
  - **EtOH**

- **C**
  - 187 kcal/kg\(^{3/4}\)/day
  - 154 kcal/kg\(^{3/4}\)/day
  - **Control**
  - **EtOH**

**CYP4A1**

- **A**
  - 187 kcal/kg\(^{3/4}\)/day
  - 154 kcal/kg\(^{3/4}\)/day
  - **Control**
  - **EtOH**

- **B**
  - 187 kcal/kg\(^{3/4}\)/day
  - 154 kcal/kg\(^{3/4}\)/day
  - **Control**
  - **EtOH**

- **C**
  - 187 kcal/kg\(^{3/4}\)/day
  - 154 kcal/kg\(^{3/4}\)/day
  - **Control**
  - **EtOH**

- **Statistical analysis.** Data are expressed as means ± SE. Quantitation of Western blot autoradiograms was performed using Quantity One software (Bio-Rad Laboratories). SigmaStat software package version 3.0 (SPSS, Chicago, IL) was used to perform all statistical tests. In all experiments, statistical significances between control and TEN-EtOH diets at the same caloric intake were analyzed by Student’s t-test. Data were tested using Levene’s test for equality of variance. Pearson product moment correlation was performed using SigmaStat software. Group differences were evaluated via two-way ANOVA followed by Student-Newman-Keuls post hoc comparisons test unless otherwise stated. Grubbs’test, also called the extreme Studentized deviate method, was used to determine whether values were significant outliers from the rest. P values of ≤0.05 were considered as statistically significant.

**RESULTS**

**Effects of alcohol and caloric intake on body and liver weights.** As in previous studies with the TEN model, infusion of diets at 187 kcal·kg\(^{-3/4}\)·day\(^{-1}\) resulted in weight gains similar to those in ad libitum chow-fed rats. Undernutrition as the result of infusion of 154 kcal·kg\(^{-3/4}\)·day\(^{-1}\) resulted in substantial reductions in weight gain (P ≤ 0.05; Table 2). However, there was no significant loss of weight gain following isocaloric infusion of EtOH-containing TEN diets at either level of caloric intake (Table 2). Comparable increases in liver weight were observed in both EtOH-treated groups (P ≤ 0.05; Table 2).

**Effects of undernutrition on EtOH metabolism.** Since 24-h UECs accurately estimate blood EtOH concentration (BECs) (5, 6, 42), we monitored UECs in animals receiving EtOH diets (11 g·kg\(^{-1}\)·day\(^{-1}\)) at two levels of caloric intake (187 and 154 kcal groups, respectively). Mean UECs were significantly lower in the animals receiving EtOH in the 187 kcal group.

**Fig. 3.** Effects of EtOH and undernutrition on CYP4A1. A: lauric acid 12-hydroxylase activity from rats fed diets with or without 11 g EtOH·kg\(^{-1}\)·day\(^{-1}\) at either 187 or 154 kcal·kg\(^{-3/4}\)·day\(^{-1}\). Data represent means ± SE; n = 8–10. *P ≤ 0.05 vs. the 187 kcal control group; *P ≤ 0.05 vs. the 154 kcal control group. B: hepatic CYP4A1 mRNA activities from rats fed diets with or without 11 g EtOH·kg\(^{-1}\)·day\(^{-1}\) at either 187 or 154 kcal·kg\(^{-3/4}\)·day\(^{-1}\). Data represent means ± SE; n = 8–10. *P ≤ 0.05 vs. the 154 kcal control group; *P ≤ 0.05 vs. the 187 kcal + EtOH group. C: densitometric quantitation of CYP4A1 protein levels from rats fed diets with or without 11 g EtOH·kg\(^{-1}\)·day\(^{-1}\) at either 187 or 154 kcal·kg\(^{-3/4}\)·day\(^{-1}\). Data represent means ± SE; n = 8–10. *P ≤ 0.05 vs. the 187 kcal control group; *P ≤ 0.05 vs. the 187 kcal + EtOH group.
compared with the 154 kcal group (P ≤ 0.05; Table 2), suggesting impaired EtOH clearance in undernourished animals.

Nutritional status effect on hepatic ADH class I activity and mRNA. To understand the underlying mechanisms whereby undernutrition impairs EtOH clearance, we examined the major hepatic EtOH metabolizing enzyme ADH I. Hepatic ADH I activity was increased by EtOH treatment but did not differ significantly between the 187 and 154 kcal + EtOH groups (Table 2).

Effects of EtOH and undernutrition on CYP2E1. Hepatic microsomal CYP2E1 apoprotein expression was increased twofold by ethanol treatment in the 187 kcal group (P ≤ 0.05; Fig. 1C), but activity, mRNA expression, and apoprotein levels were all increased in the undernourished EtOH-fed animals compared with the 187 kcal + EtOH group (P ≤ 0.05; Fig. 1, A–C).

FA ω-hydroxylation and CYP4A1 expression. EtOH treatment in the 187 kcal group increased CYP4A1 apoprotein expression and activity (P ≤ 0.05), whereas a combination of EtOH and undernutrition further increased CYP4A1 apoprotein expression and increased CYP4A1 mRNA expression in the 154 kcal group (P ≤ 0.05; Figs. 2 and 3).

Oxidative stress. Increased (P ≤ 0.05) lipid peroxidation (thiobarbituric acid-reactive substances) was observed in the 154 kcal + EtOH group compared with the 187 kcal + EtOH group, indicative of greater oxidative stress following EtOH combined with undernutrition (Table 3).

Liver histopathology. Liver pathology revealed comparable steatosis, inflammation, and necrosis in both EtOH groups (Table 3 and Fig. 4). Serum ALT activity levels were elevated (P ≤ 0.05) but were also not significantly different between the EtOH groups (Table 3). TUNEL analysis demonstrated an increase in apoptotic cell death following EtOH treatment (P ≤ 0.05; Table 3 and Fig. 5), but this did not differ as a function of caloric intake.

FA homeostasis. We examined the steady-state mRNA expression of genes that regulate FA synthesis and FA degradation and found no effects other than an increase in carnitine palmitoyl transferase (CPT-1) gene in the 187 kcal + EtOH group (P ≤ 0.05). The FA synthesis genes acyl CoA carboxylase-1 (ACC-1) and FA synthase (FAS) were decreased (P ≤ 0.05), whereas CPT-1 and a gene involved in FA β-oxidation by mitochondria, hydroxacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (HADHA), were increased in the 187 kcal + EtOH group (P ≤ 0.05; Table 4). EMSA showed an increase in PPAR-α binding to its PPRE in the 154 kcal + EtOH group (P ≤ 0.05; Table 4 and Fig. 6).

Cellular proliferation. Hepatocyte proliferation was examined by PCNA analysis (Fig. 7). Most hepatocytes were found in the quiescent G0 phase; however, both undernutrition alone and EtOH treatment increased proliferation (P ≤ 0.05). The 154 kcal + EtOH group livers exhibited more progression through the cell division cycle than 154 kcal + EtOH group livers (P ≤ 0.05; Table 5).

DISCUSSION

 Dietary factors and nutritional status are well known to influence the hepatotoxic effects of EtOH and other drugs (21, 25, 37); however, in the present study, the severity of alcoholic steatohepatitis appeared to be similar in well-nourished and undernourished rats. The lack of effect of undernutrition occurred despite significantly increased oxidative stress associated with increased expression of CYP2E1 and CYP4A1. These data suggest that oxidative stress and CYP2E1-dependent metabolism of EtOH may be less important factors in the early stages of ALD than previously thought. The mechanisms by which oxidative stress contributes to alcohol toxicity are still not completely understood. The available evidence indicates that, by favoring mitochondrial permeability transition, oxidative stress promotes hepatocyte necrosis and/or apoptosis and is implicated in the alcohol-induced sensitization of hepatocytes to the proapoptotic action of TNF-α.

Undernutrition significantly increased UECs. This was associated with impairment of EtOH clearance and was apparently independent of changes in hepatic expression of either ADH-I, which was unchanged, or CYP2E1, which was increased in the 154 versus 187 kcal + EtOH groups. It has been suggested that there may be a linear relationship between basal metabolic rate and the rate of EtOH metabolism (24, 25, 26, 29). It is therefore possible that decreased mitochondrial respiration may be the rate-limiting step in EtOH metabolism in these animals. The urine volume remained relatively constant.

Table 3. Effect of EtOH and undernutrition on oxidative stress, liver pathology, and hepatic cell death

<table>
<thead>
<tr>
<th>Group</th>
<th>TBARS, nmol Reactive TBA Product/mg Protein</th>
<th>Steatosis</th>
<th>Inflammation</th>
<th>Necrosis</th>
<th>Total</th>
<th>Serum ALT, SFU/l</th>
<th>TUNEL, % Apoptosis</th>
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<tbody>
<tr>
<td>187 kcal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.22±0.03</td>
<td>1.1±0.1</td>
<td>1.6±0.1</td>
<td>1.3±0.3</td>
<td>4.0±0.6</td>
<td>44.7±3.2</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.27±0.03</td>
<td>3.6±0.3</td>
<td>2.0±0.2</td>
<td>1.3±0.3</td>
<td>6.9±0.8</td>
<td>142.9±16.9</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>154 kcal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.25±0.03</td>
<td>1</td>
<td>1.7±0.2</td>
<td>1</td>
<td>3.7±0.2</td>
<td>49.2±3.6</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.36±0.03</td>
<td>3.0±0.5</td>
<td>1.8±0.3</td>
<td>1.1±0.1</td>
<td>5.8±0.8</td>
<td>153.4±34.2</td>
<td>1.2±0.4</td>
</tr>
</tbody>
</table>

Statistical significance as indicated by superscripts a–c (P ≤ 0.05) was determined by two-way ANOVA followed by Student’s Newman-Keuls post hoc analysis, except for the pathology scores where statistical significance was determined by two-way ANOVA of Ranks followed by Dunn’s test. a is 187 kcal EtOH vs. 187 kcal control group; b is 154 kcal EtOH vs. 154 kcal control group, and c is 154 kcal EtOH vs. 187 kcal EtOH group.

Data are means ± SE; n = 8–10 animals/group. TBARS, thiobarbituric acid (TBA)-reactive substances; ALT, alanine aminotransferase. Steatosis, inflammation, and necrosis were scored as in MATERIALS AND METHODS, where 0 = normal pathology; the total score was also determined as in MATERIALS AND METHODS, where 3 = normal pathology. All apoptotic cells were identified by morphology (TUNEL) and are expressed as the percentage of the total numbers of hepatocytes per 10 high-powered fields (×200 magnification). Statistical significance was determined by two-way ANOVA followed by Student-Newman-Keuls post hoc analysis except for the pathology scores, which used two-way ANOVA of ranks followed by Dunn’s test. P ≤ 0.05 as follows: *187 kcal + EtOH vs. 187 kcal control; +154 kcal + EtOH vs. 154 kcal control; and +154 kcal + EtOH vs. 187 kcal + EtOH.
throughout the experiment for all animals/groups; moreover, changes in UECs were not the result of increases or decreases in urine output. Alcohol equilibrates with body water, the major route of excretion for the EtOH is the urine (previous studies have carefully demonstrated that there is essentially no loss or no significant loss of EtOH in feces or expired air of rats fed by TEN at the doses we used in the present study), and we have previously demonstrated that UECs accurately track BECs in this model (5–7). Body weight was significantly decreased in the 154 kcal group, suggesting an overall decrease in total body water and volume of distribution; this could be a cause of the increased UECs in that group.

Compensatory tissue repair is known to influence the final outcome of hepatotoxicity (8, 31, 44), and it is known that nutritional factors may modulate the tissue repair response in addition to altering the metabolic activation of hepatotoxicants (32). Timely onset of cell division and sustained continuation of the cell proliferative response are of pivotal importance for

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Fig. 4. Representative hematoxylin-eosin (H&E)-stained liver sections. A–D: representative H&E-stained liver sections of the 187 kcal control (A), 187 kcal + EtOH (B), 154 kcal control (C), and 154 kcal + EtOH (D) groups.

Fig. 5. Representative liver sections stained with the TUNEL assay. A–D: representative liver sections stained with the TUNEL assay of the 187 kcal control (A), 187 kcal + EtOH (B), 154 kcal control (C), and 154 kcal + EtOH (D) groups. All TUNEL-positive cells showed a very distinct nuclear staining.
survival in the face of liver injury; however, such a sustained increase in the hepatocyte proliferative rate will also increase the risk of carcinogenesis (15). Therefore, although the increase in hepatocyte proliferation in response to a combination of EtOH and undernutrition reported in the present study may prevent an increase in severity of steatohepatitis, undernutrition in alcoholics may significantly increase the long-term risk of liver cancer. There are little data in the literature that examine undernutrition as a predisposing component of liver cancer development after chronic alcohol consumption; however, epidemiological data have suggested that poor nutritional status is an important risk factor for esophageal cancer in alcoholics (27). Hepatocytes are normally highly differentiated, metabolically active cells existing in the resting G0 state. The exact status of hepatocyte proliferation and liver regeneration following EtOH consumption has been the subject of many contradictory reports (4, 9, 12–14, 22, 36, 52–54). While some reports have suggested impaired liver regeneration following partial hepatectomy or chemically induced acute liver injury in EtOH-treated rodents, others [including recent studies using the Lieber DeCarli rat model (4, 10)] have suggested enhanced hepatocyte proliferation following chronic EtOH consumption per se. As far as we are aware, this is the first report showing that undernutrition significantly enhances hepatocyte proliferation in response to chronic EtOH treatment; however, it has previously been shown that dietary restriction can protect against acute hepatotoxicity from xenobiotics such as thioacetamide by stimulation of promitogenic signaling (3).

The molecular mechanisms underlying the effects of EtOH on hepatocyte proliferation are as yet poorly understood. It has been suggested that hepatic retinoic acid depletion may play a role (10). Chronic EtOH treatment has been shown to reduce hepatic vitamin A content and increase expression of c-Jun protein (23, 47). Vitamin A controls cell proliferation by delaying the progression of cells into the S phase, while c-Jun, a component of activator protein-1, is required for progression through the G1 phase by a mechanism involving direct transcriptional control of the cyclin D1 gene (50). Studies demonstrating that increased expression of c-Jun in the rat liver following EtOH treatment can be reversed by retinoic acid supplementation have implied a causal relationship between EtOH-induced vitamin A depletion and hepatocyte proliferation via regulation of c-Jun expression (10). It is unlikely, however, that effects on hepatic retinoic acid content could explain the significantly increased proliferation associated with a combination of EtOH and undernutrition since the vitamin A

Table 4. Effect of EtOH and undernutrition on peroxisome proliferator-activated receptor-regulated genes

<table>
<thead>
<tr>
<th>Group</th>
<th>ACC-1 mRNA</th>
<th>FAS mRNA</th>
<th>ACO mRNA</th>
<th>HADHA mRNA</th>
<th>CPT-1 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>187 kcal Control</td>
<td>1.1±0.1</td>
<td>1.0±0.2</td>
<td>1.8±0.2</td>
<td>1.2±0.1</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>EtOH</td>
<td>1.1±0.1</td>
<td>1.1±0.2</td>
<td>2.3±0.3</td>
<td>1.5±0.1</td>
<td>3.8±0.5</td>
</tr>
<tr>
<td>154 kcal Control</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>2.1±0.1</td>
<td>1.1±0.1</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.6±0.1abc</td>
<td>0.5±0.1bce</td>
<td>2.3±0.3</td>
<td>1.7±0.1b</td>
<td>4.6±0.3b</td>
</tr>
</tbody>
</table>

Data are means ± SE (as relative expression levels); n = 8–10 animals/group. Statistical significance was determined by two-way ANOVA followed by Student-Newman-Keuls post hoc analysis. P ≤ 0.05 as follows: *187 kcal + EtOH vs. 187 kcal control; †154 kcal + EtOH vs. 154 kcal control; ‡154 kcal + EtOH vs. 187 kcal + EtOH.

Fig. 6. Proliferating cell nuclear antigen (PCNA) immunohistochemical analysis. A–D: PCNA assay in liver sections of the 187 kcal control (A), 187 kcal + EtOH (B), 154 kcal control (C), and 154 kcal + EtOH (D) groups. G0-phase cells show blue nuclear staining; G1-phase cells show light brown nuclear staining; S-phase cells show dark brown nuclear staining; and G2-phase cells show cytoplasmic staining and with or without a speckled nuclear staining.

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levels in both EtOH TEN diets were identical even though the macronutrient content was reduced.

Our data point to a possible role for enhanced PPAR-α signaling as a potential mediator of the synergistic increases in hepatocyte proliferation associated with a combination of EtOH and undernutrition. In contrast to some reports (11, 51) in EtOH-fed mice, where impaired PPAR-α signaling and reduced FA degradation have been suggested to play a role in the development of steatosis, we and others (30, 40) have previously reported an induction of the PPAR-α-dependent CYP4A1 gene following EtOH treatment in rats. In the present study, although PPAR-α binding to its response element was unaffected in EMSA assays from EtOH-treated rat livers in rats fed 187 kcal·kg\(^{-3/4}\)·day\(^{-1}\), an induction of CYP4A1 was observed and was accompanied by similar effects on expression of another PPAR-α target gene, CPT-1. EtOH-induced steatosis in the undernourished rat appeared to be accompanied by homeostatic effects on hepatic FA homeostasis with reduced expression of genes (such as FAS and ACC-1) involved in de novo FA synthesis and stimulation of FA β- and ω-oxidation. The combination of EtOH and undernutrition further increased expression of CYP4A1, CPT-1, and another PPAR-α-dependent gene involved in mitochondrial β-oxidation, HADHA. In addition, significantly increased binding of PPAR-α to its response element was observed in EMSA assays (Fig. 4 and Table 4). It has been shown that peroxisomal proliferators, which activate PPAR-α signaling in the rodent liver, are mitogenic and that PPAR-α-null mice have impaired liver regeneration (2). This suggests a positive role for PPAR-α in the regulation of hepatocyte proliferation. Studies in diabetic mice have demonstrated that increased PPAR-α activation provide protection against acute hepatotoxicity from acetaminophen as the result of increased hepatocyte proliferation in response to toxic challenge associated with upregulation of cyclin D\(_1\) (43). This protection was abolished in diabetic PPAR-α knockout mice (43). A similar mechanism may underlie the increased hepatocyte proliferation observed with EtOH treatment in underfed rats in the present study.

In conclusion, undernutrition does not increase the severity of early stage steatohepatitis following EtOH treatment. However, a highly significant synergistic interaction between undernutrition and EtOH results in increased hepatocyte proliferation, possibly as a result of alterations in PPAR-α signaling. This may be associated with increased long-term risk of liver cancer in undernourished compared with well-fed alcoholics.

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Table 5. Proliferating cell nuclear antigen immunohistochemical analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>(G_0) Phase</th>
<th>(G_1) Phase</th>
<th>S Phase</th>
<th>(G_2) Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>187 kcal</td>
<td>Control</td>
<td>98.1±0.5</td>
<td>1.4±0.5</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>EtOH</td>
<td>90.3±2.5(^a)</td>
<td>5.0±1.9(^b)</td>
<td>3.8±1.4(^c)</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>154 kcal</td>
<td>Control</td>
<td>93.3±0.9</td>
<td>5.1±1.1</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>EtOH</td>
<td>65.4±6.9(^b)</td>
<td>17.4±3.2(^b)</td>
<td>17.1±4.5(^b)</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>

Data are means ± SE (in %); \(n = 8–10\) animals/group. Percentage of cells in different gap phases were calculated from a total of 1,000 counted hepatocytes/slide. Statistical significance was determined by two-way ANOVA followed by Student-Newman-Keuls post hoc analysis. \(P \leq 0.05\) as follows: \(^a\)187 kcal + EtOH vs. 187 kcal control; \(^b\)154 kcal EtOH vs. 154 kcal control; \(^c\)154 kcal + EtOH vs. 187 kcal + EtOH.
REFERENCES


