Chronic alcohol feeding impairs hepatic translation initiation by modulating eIF2 and eIF4E


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Abstract

Chronic alcohol feeding impairs hepatic translation initiation by modulating eIF2 and eIF4E. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E805-E814, 1999.—The present study examined potential cellular mechanisms responsible for the inhibition of protein synthesis in liver after chronic alcohol consumption. Rats were maintained on an alcohol-containing diet for 14 wk; control animals were fed isocalorically. Hepatic ATP content was not different in alcohol-fed and control animals. No alcohol-induced reduction in total hepatic RNA content (an estimate of ribosomal RNA) was detected, suggesting that alcohol decreased translational efficiency. Alcohol feeding increased the proportion of 40S and 60S ribosomal subunits in the nonpolysome-associated fraction by 30%. To identify mechanisms responsible for the impairment in initiation, several eukaryotic initiation factors (eIF) were analyzed. Alcohol feeding decreased hepatic eIF2β activity by 36%. This reduction was associated with a 20% decrease in eIF2β content and a 90% increase in eIF2α phosphorylation. Alcohol also dramatically influenced the distribution of eIF4E. Compared with pair-fed control values, alcohol feeding increased the amount of eIF4E present in the inactive 4E-binding protein 1 (4E-BP1)-eIF4E complex by 80% and decreased binding of eIF4G to eIF4E by 70%. However, the phosphorylation status of 4E-BP1 and eIF4E was not altered by alcohol. Although the plasma concentrations of threonine, proline, and citrulline were mildly decreased, the circulating amount of total amino acids was not altered by alcohol feeding. In summary, these data suggest that chronic alcohol consumption impairs translation initiation in liver by altering eIF2β activity as well as eIF4E function via changes in eIF4E availability.

Keywords:
eukaryotic initiation factor 2; eukaryotic initiation factor 4E; ribosomal subunits; amino acids; liver; rats

The liver plays a central role in regulating circulating amino acid and protein concentrations, which are necessary for maintenance of whole body metabolic homeostasis and host defense. Alcohol is known to have diverse effects on hepatic protein turnover (35). The large majority of in vitro studies indicate that addition of ethanol to isolated hepatocytes inhibits protein synthesis (6, 23, 46). It also appears that, for at least physiologically relevant concentrations, the ability of ethanol to inhibit protein synthesis is attributable to its metabolites (23). Furthermore, ethanol also decreases protein (i.e., albumin) synthesis in isolated perfused rabbit liver (27). Recent studies indicate that both acute alcohol intoxication (31, 42) and chronic (>6 wk) feeding of an alcohol-containing diet (32, 41) significantly decrease hepatic protein synthesis in vivo.

The mechanism by which alcohol reduces the synthesis of hepatic protein remains largely unexamined. Early studies indicated that chronic alcohol consumption in rats decreases both protein synthesis and total RNA in liver (32). Because the large majority (>80%) of total liver RNA is ribosomal, these data were interpreted to mean that the alcohol-induced impairment in protein synthesis occurred secondary to a reduced number of ribosomes. However, the decrease in protein synthesis in this early study was proportionately greater than the decrease in RNA. Hence, an impairment in translational efficiency must likely exist as well. Translational efficiency reflects how well the existing protein synthetic machinery is functioning. The translational phase of protein synthesis can be categorized into three phases: peptide-chain initiation, elongation, and termination (2). Indirect evidence based on the abundance of polysomes suggests that alcohol impairs translational efficiency by decreasing peptide-chain initiation (24). Translation initiation is regulated by a large number of protein factors, termed eukaryotic initiation factors (eIFs). One of these initiation factors, eIF2, mediates the first step in initiation, which involves the attachment of the initiator methionyl-tRNA (met-tRNA\textsuperscript{methyl}) to the 40S ribosomal subunit to form the 43S preinitiation complex (2). A second regulatory step in initiation involves the binding of mRNA to the 43S preinitiation complex, which is mediated by eIF4F (34). Both of these regulatory steps are altered in other conditions that are associated with decreases in protein synthesis (2, 34, 48). However, there is no information on the effects of chronic alcohol on either of these two steps involved in peptide-chain initiation. Therefore, the purpose of the present study was to determine whether chronic alcohol consumption in rats impairs regulation of the initiation process by modulating eIF2 and/or eIF4F in liver.

Methods and Materials

Experimental protocol. Specific pathogen-free, male Sprague-Dawley rats (Charles River Breeding Laboratories, Cambridge, MA) were maintained on an ethanol-containing agar block diet for 14 wk (1, 20). Initially, all rats were provided agar without alcohol for 2 days. Thereafter, one-half of the animals were fed agar containing 10% alcohol, and the others were provided agar containing an equal caloric amount of dextrin-maltose. The ethanol content of the agar was increased to 20% and then finally to 30% at 1-wk intervals.

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Rats were maintained on the 30% ethanol-agar block for the remainder of the experimental protocol. Calculated ethanol intake during the final 10 wk of the experimental protocol averaged 16 ± 3 g·kg⁻¹·day⁻¹. Control animals were provided the same amount of agar, but isocaloric dextrin-maltose was substituted for the ethanol.

The nutrient intake for both groups of animals was supplied by consumption of standard solid rodent chow (Harlan Teklad no. 8604; Madison, WI) because the agar block, although providing a source of water and/or ethanol, has nominal nutritional value. The alcohol-fed rats were permitted solid chow ad libitum. Control animals were provided the same amount of solid chow as that consumed by the alcohol-fed group. Over the final 10 wk, alcohol-fed rats had an average total energy consumption (food + ethanol) of 274 ± 19 kcal·kg⁻¹·day⁻¹, which is sufficient to meet the caloric requirements of growing rats (25) and did not differ from the average total energy consumption (food) of 254 ± 19 kcal·kg⁻¹·day⁻¹. The nutrient intake of both groups was also essentially identical, because the sole source of nutritional support was derived from solid chow (24% protein, 5% fat, and 71% carbohydrate).

On the morning of the experiment, rats were anesthetized with pentobarbital sodium (60 mg/kg), and total body electrical conductivity was measured noninvasively to estimate body composition (EM-SCAN, Springfield, IL). This device estimates lean body mass based on the property that the strength of the electromagnetic field is proportional to the amount of fat free mass, which is more electrically conductive than adipose tissue. Strong linear correlations between electrical conductivity and total potassium, total body water, and lean body mass have been reported in subjects with varying body weights and adiposity (3). Thereafter, a laparotomy was performed. The entire left lateral lobe of the liver was excised and weighed. The tip of the lobe was homogenized directly for analysis of total RNA, ribosomal subunits, and eIF2B activity. Another portion of the lobe was weighed and dried at 100°C for 48 h to determine total water content. The remainder of the lobe was frozen in liquid nitrogen-cooled clamps. Frozen liver samples were powdered under liquid nitrogen and stored at −70°C. The remainder of the liver was excised, dissected free of connective tissue, blotted dry, and weighed. Total liver weight was calculated as the sum of the weights of the left lateral lobe and the remaining liver tissue. This protocol was approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University, College of Medicine.

Determination of total RNA. Total RNA was measured from homogenates of liver samples (44). Briefly, 0.3 g of fresh liver was homogenized in five volumes of ice-cold 10% trichloroacetic acid. The homogenate was centrifuged at 9,000 g for 11 min at 4°C. The supernatant was discarded, and 2.5 ml of 6% perchloric acid (PCA) was added to the remaining pellet. The sample was centrifuged at 9,000 g for 6 min, the supernatant was discarded, and the procedure was repeated. Next, 1.5 ml of 0.3 N KOH was added to the pellet, and the samples were heated for 1 h at 50°C. Samples were then mixed with 5 ml of 0.5 N PCA and centrifuged at 9,000 g for 11 min. The concentration of RNA in the supernatant was determined by absorbance at 260 nm corrected by the absorbance at 232 nm. Total RNA was expressed as milligram RNA per gram tissue.

Isolation of ribosomal subunits. Fresh liver was used to isolate 40S and 60S ribosomal subunits by sucrose density gradient centrifugation, as described previously (9). Briefly, liver was homogenized in a Dounce homogenizer in seven volumes of homogenization buffer [in mM: 25 HEPES (pH 7.5), 2 magnesium acetate, 250 KCl, 0.5 dithiothreitol (DTT), 0.08 EDTA, and 250 sucrose]. The homogenate was centrifuged at 10,000 g for 15 min, and the supernatant was recovered. Aliquots of the samples (0.5 ml), to which 0.1 vol of 10% (wt/vol) Triton X-100 and deoxycholate solution had been added, were then layered onto 0.44–2.0 M sucrose gradients. The samples were centrifuged at 167,000 g in a SW41 rotor (Beckman Instruments) for 2 h to resolve the 40S and 60S ribosomal subunits. The absorbance of the gradients was monitored at 254 nm, and fractions were collected using a density gradient fractionator (Instrumentation Specialties, Lincoln, NE).

Relative amounts of eIF2 and eIF2B. The relative amounts of the α-subunit of eIF2 (eIF2α) and the ε-subunit of eIF2B (eIF2Bε) in liver were estimated by protein immunoblot analysis, as described previously (10, 14, 45). eIF2 and eIF2B were chosen because changes in these initiation factors correlate with alterations in protein synthesis (2). eIF2 consists of three subunits, of which the α-subunit appears important in regulating protein synthesis (48). Previous studies have established that the expression of the α-subunit is representative of the other subunits of eIF2 (47). Likewise, eIF2B is a multimeric protein consisting of five subunits, with the ε-subunit being the catalytic subunit (47). Therefore, the relative abundance of eIF2Bε was taken as representative of the eIF2B holoenzyme. Briefly, liver was homogenized in 7 vol of buffer composed of (in mM) 20 Tris (pH 7.4), 250 sucrose, 100 KCl, 0.2 EDTA, 1 DTT, 50 NaF, 50 β-glycerophosphate, 1 phenylmethylsulfonyl fluoride (PMSF), 1 benzamidine, and 0.5 sodium vanadate. The samples were mixed with 2× Laemmli sodium dodecyl sulfate (SDS) buffer, boiled for 3 min, and centrifuged. Equal amounts of protein (−160 µg) from liver homogenates were electrophoresed at 60 mA in a 12.5% polyacrylamide gel. After electrophoresis, proteins in the gel were transferred to nitrocellulose. After blocking for 30 min with nonfat milk (5% wt/vol) in 25 mM Tris (pH 7.6)-0.9% saline containing 0.01% Tween 20 (Tris-NaCl-Tween), the membranes were washed extensively in Tris-NaCl-Tween. The nitrocellulose was incubated for 1 h at room temperature with an antibody specific for either eIF2α or eIF2Bε (47). Antibodies were visualized using an enhanced chemiluminescence (ECL) procedure, with the secondary antibody linked to horseradish peroxidase (Amersham). The blots were exposed to X-ray film in a cassette equipped with a Du Pont Lightning Plus intensifying screen. After development, the X-ray film was scanned (Microtek ScanMaker IV) and quantitated using NIH Image 1.6 software.

Measurement of phosphorylation state of eIF2α. The relative amount of eIF2α present in the phosphorylated form, eIF2α(P), was estimated by immunologic visualization of proteins after separation by use of slab-gel isoelectric focusing (IEF) (43). Livers were homogenized in the same buffer as described above for eIF2. A 75-µl aliquot of the homogenate was mixed with 42.9 mg of urea and 300 µl of 1E buffer (9.5 M urea, 2% Nonidet P-40, ampholytes (BHD Resolyte 4–8), and 0.7 M β-mercaptoethanol). The samples were electrophoresed and then transferred electrophoretically onto polyvinylidene fluoride membranes. The membranes were subsequently incubated with an eIF2α monoclonal antibody, and eIF2α was visualized as described above. The proportion of eIF2α present in the phosphorylated state was measured by densitometric scanning of the membranes and is expressed as a percentage of the total eIF2α content (i.e., phosphorylated + unphosphorylated). Previous studies have demonstrated that eIF2α(P) can be determined on frozen tissue samples (43).

Determination of eIF2B activity. Hepatic eIF2B activity was measured in postmitochondrial supernatants by use of a
then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was assayed immediately for eIF2B activity, as described previously (10, 14). Briefly, aliquots of the reaction mixture were analyzed for eIF2B activity by measuring the decrease in $[^3H]GDP$ bound to eIF2. The rate of exchange was linear over the time points measured (data not shown). Under these conditions, ~50% (0.3 pmol) of the $[^3H]GDP$ was released from the eIF2-$[^3H]GDP$ complex during the 1.5-min assay. eIF2B activity was expressed as picomoles of GDP exchanged per minute per milligram protein.

Quantification of 4E-BP1-eIF4E and eIF4G·eIF4E complexes. The association of eIF4E with 4E-binding protein 1 (4E-BP1) and eIF4G was determined as previously described (5, 15). Briefly, liver was homogenized in seven volumes of buffer A (20 mM HEPES, pH 7.4, 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM DTT, 50 mM NaF, 50 mM o-glyceroephosphate, 0.1 mM PMSF, 1 mM benzamidine, 0.5 mM sodium vanadate, and 1 µM microcystin LR) with a Polytron homogenizer. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. eIF2 and eIF2B·GDP complexes were immunoprecipitated from aliquots of 10,000 g supernatants by use of an anti-eIF4E monoclonal antibody. The antibody-antigen complex was collected by incubation for 1 h with BioMag goat anti-mouse IgG beads (Perseptive Biosystems, Framingham, MA). Before use, the beads were washed in 1% nonfat dry milk in buffer B (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% o-mercaptoethanol, 0.5% Triton X-100, 50 mM NaF, 50 mM o-glyceroephosphate, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM sodium vanadate). The beads were captured using a magnetic sample rack and were washed twice with buffer B and once with buffer B containing 500 mM, rather than 150 mM, NaCl. Proteins remaining bound were eluted by resuspending the beads in SDS-sample buffer, followed by boiling for 5 min. The beads were collected by centrifugation, and the supernatants were subjected to electrophoresis either on a 7.5% polyacrylamide gel, as previously described (15). 4E-BP1 was immunoprecipitated as described in the previous section for immunoprecipitation of eIF4E. The immunoprecipitates were solubilized with SDS sample buffer. The various phosphorylated forms of 4E-BP1 were separated by electrophoresis and quantitated by protein immunoblot analysis, as described previously (5, 13). For detection of p70 S6 kinase, an aliquot of cell homogenate was combined with an equal volume of SDS sample buffer, and the diluted samples were subjected to electrophoresis on a 7.5% polyacrylamide gel, as previously described (13). The samples were then analyzed by protein immunoblot analysis by use of a rabbit anti-rat p70 S6 kinase antibody.

Tissue ATP content. An aliquot of powdered liver was extracted in cold PCA, neutralized, and used for the determination of adenosine triphosphate (ATP) by standard fluorometric methods.

Liver enzyme and plasma alcohol and amino acid levels. Aspartate aminotransferase (AST; EN 2.6.1.1) activity in plasma was determined using a standard enzymatic assay (Sigma). The alcohol concentration in plasma samples was determined using a rapid alcohol analyzer (model GL5; Analox Instruments, Lunenburg, MA). Plasma was deproteinized with sulfoalicylic acid and the supernatant used for amino acid analysis by ion-exchange HPLC (model 6300, Beckman Instruments, Fullerton, CA). Absorbance was measured at 440 and 570 nm after postcolumn Ninhydrin treatment. (S)-2-aminoethyl-L-cysteine was used as an internal standard, and data acquisition and management were performed by Beckman System Gold 8.10.

Statistics. Values are presented as means ± SE. The number of rats per group is indicated in the legends to the figures and tables. Data were analyzed by Student's t-test to determine treatment effect. Statistical significance was set at P < 0.05.

RESULTS

Whole body composition and liver weight. The body weight was 16% lower in animals fed the alcohol-containing diet for 14 wk, compared with pair-fed control animals (427 ± 8 vs. 517 ± 12 g, respectively; P < 0.05). Despite the same caloric intake, the average weight gain in control animals was 4.4 g/day, compared with 3.5 g/day for the alcohol-fed group (P < 0.05). Estimates of body composition indicated that alcohol feeding decreased the amount of lean body mass (312 ± 12 vs. 399 ± 9 g; P < 0.05) without significantly altering the amount of body fat (115 ± 6 vs. 118 ± 5 g; P < 0.05).

However, estimates of liver body mass based on electrical conductance and related methodologies may be influenced by perturbations in the hydration state of the subject (3, 8). The effect of chronic alcohol consumption on the volume and ionic composition of various body fluid compartments was not directly measured in the current study. However, there are several lines of evidence that suggest our alcohol-fed rats do not have major changes in fluid compartments. First, tissue water content was essentially identical between control and alcohol-fed rats for both skeletal muscle (control, 747 ± 0.004 ml/g; alcohol, 745 ± 0.006 ml/g) and liver (see Table 1). Second, there was no significant difference in hematocrit between the two groups (control, 43 ± 2%; alcohol, 42 ± 2%), indicating that non-red blood cell volume was most likely unaffected by alcohol feeding.

Hepatic weight was decreased 25% in alcohol-fed rats compared with values from control rats (Table 1). Liver weight was still decreased by 10% in alcohol-consuming rats when hepatic weight was normalized for the difference in body weight. There was no difference in the amount of protein per gram of liver between the two groups. As a result of these changes, there was a significant decrease in the total liver protein content of alcohol-fed rats compared with control values.
Total RNA content and ribosomal subunits. A decreased rate of protein synthesis may result from alterations in either the number of ribosomes or the efficiency of translation. Ribosomal RNA accounts for as much as 80% of the total RNA in liver. Hence, changes in total tissue RNA content presumably reflect changes in ribosomal RNA. There was no detectable difference in the total RNA content of liver between control and alcohol-fed rats (Fig. 1A).

We examined whether alcohol-fed rats had changes in peptide-chain initiation relative to elongation by determining changes in the distribution of 40S and 60S ribosomal subunits between polysome-associated and nonpolysome (i.e., free) pools. Figure 1B illustrates that the number of free 40S and 60S subunits isolated from liver was increased 30% by alcohol feeding, indicating that alcohol decreased the rate of initiation relative to elongation/termination.

Relative amounts of eIF2 and eIF2B, and eIF2B activity. One possible mechanism for the alcohol-induced decrease in translation is via alterations in the amount and/or activity of specific eIF proteins. There was no significant difference in the amount of total eIF2 in liver from control and alcohol-fed rats (data not shown). However, the percentage of eIF2 in the phosphorylated form was increased almost twofold in alcohol-consuming rats (Fig. 2).

The effect of chronic alcohol feeding on eIF2B activity was measured in postmitochondrial supernatants of liver from control and experimental rats. Hepatic eIF2B activity was significantly decreased 36% in alcohol-fed rats (Fig. 3). A portion of the alcohol-induced decrease in eIF2B activity may be the result of the concomitant reduction (20%) in the amount of eIF2B protein determined by Western blot analysis in liver from alcohol-consuming rats compared with controls (Fig. 4).

Regulation of eIF4E. Another potential mechanism for decreasing hepatic protein synthesis involves altered regulation of eIF4E. Binding of the translational repressor 4E-BP1 to eIF4E forms an inactive complex. This is visualized on an immunoblot as an increase in

<table>
<thead>
<tr>
<th>Liver Wt, g</th>
<th>Liver Wt/Body Wt, g/kg</th>
<th>Liver Protein Concentration, mg protein/g wet weight</th>
<th>Total Liver Protein Content, g protein/whole liver</th>
<th>Tissue Water Content, ml/g wet wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.8 ± 0.5</td>
<td>30.7 ± 1.1</td>
<td>210 ± 6</td>
<td>3.35 ± 0.21</td>
</tr>
<tr>
<td>Alcohol</td>
<td>11.7 ± 0.5*</td>
<td>27.4 ± 0.9*</td>
<td>207 ± 3</td>
<td>2.53 ± 0.25*</td>
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Values are means ± SE; n = 10 rats per group. Total liver protein content was calculated by multiplying mg protein/g wet weight times total liver weight. *P < 0.05 compared with control values.

Fig. 1. A: total RNA content of liver from control (open bar) and alcohol-consuming (solid bar) rats. B: effect of chronic alcohol consumption on the content of free ribosomal subunits in liver. Liver was obtained from pair-fed control animals or alcohol-fed rats. For both groups, values are means ± SE; n = 10 rats per group. *P < 0.05 compared with control values.

Fig. 2. A: effect of alcohol feeding on phosphorylation state of hepatic eukaryotic initiation factor 2α (eIF2α). Liver homogenates were subjected to isoelectric focusing (IEF) slab gel electrophoresis, transferred to nitrocellulose, and visualized with eIF2α-specific monoclonal antibody. B: proportion of eIF2α in the phosphorylated (P) state. Values were quantitated by densitometric analysis of immunoblots of IEF gels. For both groups, values are means ± SE; n = 10 rats per group. *P < 0.05 compared with control values.
the amount of 4E-BP1 present in an eIF4E immunoprecipitate. Figure 5A illustrates that alcohol feeding increased the amount of both the α- and β-forms of 4E-BP1 in the immunoprecipitate. Densitometric analysis of both bands indicates an 80% increase in the total amount of 4E-BP1 associated with eIF4E in liver from alcohol-fed rats (Fig. 5B).

4E-BP1 has at least five potential phosphorylation sites, and the various phosphorylated forms of the protein are resolved into three bands by SDS-PAGE (5, 15). The total amount of all three phosphorylated forms (i.e., α-, β-, and γ-forms) did not differ between control and alcohol-fed rats [601 ± 49 vs. 513 ± 53 arbitrary units (AU), respectively], indicating that the total amount of 4E-BP1 in liver was not altered by alcohol. Hyperphosphorylation of 4E-BP1 in response to hormones, such as insulin, results in a shift in distribution to the γ-form with a resulting release of eIF4E from the inactive 4E-BP1·eIF4E complex, and an increase in translation (41). However, in the current study, the amount of 4E-BP1 in the γ-form was not significantly different between the two groups (Control = 23.1 ± 2.7% vs. Alcohol = 25.3 ± 2.3%). In addition, there was no detectable change in the electrophoretic mobility of p70 S6 kinase in liver from alcohol-fed rats (data not shown), suggesting that the phosphorylation state of this protein was also not altered by alcohol feeding.

Similarly, eIF4E immunoprecipitates were used to measure the association of eIF4E with eIF4G. The liver from alcohol-fed rats demonstrated a 71% decrease in the amount of eIF4G that immunoprecipitated with eIF4E (Fig. 6). This decrease was not the result of a reduction in the amount of eIF4E in the immunoprecipitate between control and alcohol-fed rats (Fig. 7).

To further define the mechanism through which alcohol inhibits translation in this tissue, the phosphorylation of eIF4E was examined. In liver from control...
animals, 66% of the total eIF4E was in the phosphorylated state. However, chronic alcohol consumption did not significantly alter the phosphorylation status of eIF4E (Fig. 8).

Plasma amino acid concentrations. Table 2 presents the plasma amino acid concentrations in control and alcohol-fed rats. There was no significant difference between the two groups for the various gluconeogenic, branched-chain, or aromatic amino acids. Alcohol feeding decreased the concentration of threonine (26%), proline (18%), and citrulline (39%), as well as increasing the concentration of taurine (114%), 3-methylhistidine (45%), and α-amino-n-butyric acid (5-fold). However, the plasma concentration of total amino acids was essentially identical between control and alcohol-fed rats.

**DISCUSSION**

Recent work has demonstrated that chronic alcohol consumption decreases the in vivo rate of hepatic protein synthesis (32, 40, 42). However, there is a paucity of data related to the cellular mechanism responsible for this impairment. Inhibition of protein synthesis can result from a decreased number of ribosomes and/or a reduction in translational efficiency. Because 80–90% of the cellular RNA is ribosomal, changes in liver RNA content primarily reflect changes in ribosomal content. In the present study, we were unable to confirm an alcohol-induced decrease in the total hepatic RNA content described previously by Preedy and Peters (32). The reason for this difference is
unclear. However, an impairment in translational efficiency is a commonality of both studies. The impaired translational efficiency may result from a reduction in either peptide-chain initiation or elongation/termination (2). Analysis of the distribution of ribosomal subunits between free subunits (i.e., non-polyosomal associated) and polyosomes was used to estimate the rate of initiation relative to elongation. In general, the amount of RNA in free 40S and 60S ribosomal subunits is indicative of a balance between the two processes. That is, when the rate of initiation is decreased relative to elongation/termination, free ribosomal subunits are binding to mRNA at a slower rate (initiation) than they are moving along (elongation) and exiting (termination) mRNA. The net result of this defect is an increase in the abundance of free ribosomal subunits. Our analysis of subunits indicated a moderate increase in the accumulation of free 40S and 60S ribosomal subunits in liver from alcohol-fed rats. This finding is consistent with the previous demonstration that alcohol decreases hepatic polysome aggregation (27). Collectively, this ribosomal redistribution is characteristic of a decrease in the rate of initiation of peptide chains relative to the rate of elongation/termination (4).

eIF2 (a heterotrimer consisting of α-, β-, and γ-sub-units) represents a major regulatory control point for initiation (34). The first step in initiation is the formation of a ternary complex consisting of eIF2, GTP, and met-tRNA<sub>met</sub>. eIF2 mediates the binding of met-tRNA<sub>met</sub> to the 40S ribosomal subunit to form the 43S preinitiation complex. A reduction in the tissue content of eIF2 protein, therefore, could lead to a decrease in initiation. In several nonmuscle tissues (including liver), the cellular eIF2 content is linearly related to rates of protein synthesis (17, 43). However, in the present study, there was no detectable difference in eIF2 content (as assessed by Western blot analysis of eIF2<sub>x</sub>) of liver between control and alcohol-fed rats. Thus the defect in translation initiation is not mediated by reduced hepatic eIF2 content.

Alternatively, a decrease in the activity of another eIF, eIF2B, can also decrease the ability of eIF2 to form the ternary complex. eIF2 is bound to GDP as an inactive complex when it is released from the ribosome, and this GDP must be exchanged for GTP before binding another molecule of met-tRNA<sub>met</sub>. The replacement of GDP for GTP on eIF2 is catalyzed by eIF2B, a guanine nucleotide exchange factor, under physiological conditions. This initiation factor is important in the recycling and activation of eIF2. Hence, a decrease in eIF2B activity would ultimately limit translation initiation by reducing the amount of eIF2-GTP that is available to bind to tRNA<sub>met</sub>. Livers from alcohol-fed rats clearly demonstrated a decrease in eIF2B activity (Fig. 9). These data are consistent with previous studies in other conditions that have reported a proportional decrease in both eIF2B activity and protein synthesis in liver (17, 43). Therefore, a decrease in eIF2B-mediated guanine nucleotide exchange appears to be at least partially responsible for the alcohol-induced decrease in initiation.

Several mechanisms are known to regulate eIF2B activity (48). A major mechanism involves phosphorylation of the α-subunit of eIF2, which increases the affinity of eIF2 for eIF2B (18). Phosphorylation of eIF2<sub>x</sub> effectively converts eIF2 from a substrate into a competitive inhibitor, thereby limiting the recycling of the eIF2-GDP complex. Previous work by Kimball et al. (16) demonstrated that the eIF2B-to-eIF2 ratio in liver is ~0.6. Thus the formation of the highly stable eIF2<sub>x</sub>(P)·eIF2B complex would be expected to sequester available eIF2B. The net result of this sequestration is a reduction in guanine nucleotide exchange activity and impairment of initiation. In selected in vitro systems, the extent of eIF2<sub>x</sub> phosphorylation has been demonstrated to be inversely proportional to the rate of protein synthesis (21, 36). In the present study, chronic alcohol consumption almost doubled the amount of phosphorylated eIF2<sub>x</sub>. Theoretically the alcohol-induced increase in eIF2<sub>x</sub>(P) could result from an increase in protein kinase or a...
E812 ALCOHOL IMPAIRS TRANSLATION INITIATION

...and an increase in the extent of eIF2 activity results from both a decrease in eIF2B content that the alcohol-induced decrease in hepatic eIF2B be an important regulator of hepatic eIF2B activity in our experimental model. Therefore, our data indicate that the alcohol-induced decrease in hepatic eIF2B activity results from both a decrease in eIF2B content and an increase in the extent of eIF2α phosphorylation.

A second regulatory step controlling peptide-chain initiation involves the binding of mRNA to the 43S preinitiation complex, which is mediated by eIF4F (34). One of the components of the eIF4F complex, eIF4E, binds directly to the m7GTP cap structure present at the 5′-end of all eukaryotic mRNAs and stimulates mRNA binding to the small ribosomal subunit. During translation initiation, the eIF4E·mRNA complex binds with eIF4G and eIF4A to form the functional eIF4F cap-binding complex. One mechanism for modulating the formation of the eIF4F complex is by regulating the relative distribution of eIF4E between inactive and active protein complexes with other proteins. 4E-BP1 (also called PHAS-I) binds to amino acid residues in eIF4E that also bind eIF4G to form the active eIF4F complex. Thus 4E-BP1 functions as a repressor of translation initiation. In the current study, the amount of 4E-BP1 bound to eIF4E was markedly increased in liver from alcohol-fed rats (Fig. 9). We also observed a concomitant decrease in the amount of eIF4E bound to eIF4G in these animals. These data strongly suggest that alcohol feeding decreases initiation, at least in part, by an impairment in eIF4F function secondary to a decrease in eIF4E binding to eIF4G.

4E-BP1 activity can be regulated by phosphorylation of either 4E-BP1 or eIF4E (15). Phosphorylation of 4E-BP1 releases eIF4E from the 4E-BP1·eIF4E complex, thereby permitting the eIF4E·mRNA complex to bind first to eIF4G and then ultimately to the small ribosomal subunit. Hence, stimuli that limit translation initiation and protein synthesis are associated with a decreased percentage of 4E-BP1 in the phosphorylated γ-form (15). However, there was no detectable alteration in the phosphorylation state of 4E-BP1 in liver from alcohol-fed rats. We cannot exclude the possibility that the analytical methods used lacked sufficient sensitivity to detect a change in phosphorylation of 4E-BP1. However, we also failed to detect a shift in the electrophoretic mobility of p70 S6 kinase in alcohol-fed rats. Because both 4E-BP1 and p70 S6 kinase are phosphorylated and activated by a rapamycin-sensitive pathway involving the upstream regulator mTOR (13, 29), these data suggest that alcohol-induced changes in eIF4E are not mediated by a change in the phosphorylation state of 4E-BP1.

Changes in the phosphorylation state of eIF4E can also influence eIF4E binding to mRNA. Although both phosphorylated and nonphosphorylated eIF4E bind to the mRNA cap structure, phosphorylation of eIF4E enhances the affinity of the factor for the m7GTP cap severalfold (22). Moreover, in vitro studies have demonstrated that increases in phosphorylation are proportional to increases in translation (40). However, again, chronic alcohol consumption failed to produce a detectable alteration in the phosphorylation state of eIF4E.

Alterations in hepatic protein balance can be induced by changes in either the rate of protein synthesis or the rate of proteolysis. Although our results indicate that alcohol impairs peptide-chain initiation (and likely protein synthesis), we cannot exclude the possibility that a portion of the alcohol-induced decrease in liver weight results from an increase in hepatic protein degradation. However, this scenario appears unlikely. Ethanol has previously been shown to have either no effect on protein degradation in isolated hepatocytes (46) or to actually inhibit proteolysis when determined in the isolated perfused rat liver or under in vivo conditions (30).

Amino acids can regulate translation initiation and protein synthesis by modulating various signaling pathways involving eIF2 and eIF4E (13, 29). However, chronic alcohol ingestion produced only mild changes in a small number of individual amino acids and did not change the total concentration of amino acids in the blood. Therefore, it appears unlikely that an alcohol-induced decrease in plasma amino acids was responsible for any of the observed changes in initiation. Although acute administration of ethanol can dramatically decrease a large number of amino acids (7), our data are consistent with those of a previous study that failed to detect major changes in plasma amino acids in rats fed a nutritionally adequate liquid diet containing alcohol for 8–10 wk (39). There were changes in two amino acids, however, that are noteworthy. First, alcohol-fed rats demonstrated a fivefold elevation in α-amino-n-butyric acid compared with control animals. A similar finding has been previously reported (37) and appears to result from an alcohol-induced stimulation of hepatic production of this amino acid (38). In contrast, the plasma concentration of α-amino-n-butyric acid (and that of the branched-chain amino acids) is decreased in animals and humans by dietary protein deficiency (37). These data further support the conclusion that dietary protein intake was adequate in alcohol-fed rats and that a difference in nutrient consumption was not a primary mediator of the observed changes in hepatic translation initiation. Second, alcohol-fed rats also demonstrated a 45% increase in the plasma concentration of 3-methylhistidine. Concentrations of this...
amino acid in blood or urine have been used as an indirect estimate of myofibrillar protein degradation. In rodents, the primary sites of production for this amino acid appear to be the intestine and skeletal muscle (26). Hence, elevated circulating levels of 3-methylhistidine suggest an enhanced rate of proteolysis in these tissues and are consistent with the decreased lean body mass in alcohol-fed rats.

In summary, our data suggest that chronic alcohol consumption in rats results in an imbalance in hepatic protein metabolism mediated, at least in part, by a reduction in peptide-chain initiation. The alcohol-induced inhibition of translation initiation appears to result from a decrease in eIF2B activity. This diminished activity is associated with a moderate reduction in eIF2Be and a larger increase in eIF2α phosphorylation. In addition, alcohol-fed rats also had an impairment in eIF4F function as evidenced by the increase in 4E-BP1 bound to eIF4E and the corresponding decrease in eIF2E bound to eIF4G. This redistribution of eIF4E could not be explained by changes in the phosphorylation status of either 4E-BP1 or eIF4E. As eIF4E could not be explained by changes in the association of eIF-4E and PHAS-I in rat skeletal muscle. Am. J. Physiol. 270 (Cell Physiol. 39): C705–C709, 1996.


