Effects of chronic alcohol consumption on regulation of myocardial protein synthesis

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Vary, Thomas C., Christopher J. Lynch, and Charles H. Lang. Effects of chronic alcohol consumption on regulation of myocardial protein synthesis. Am J Physiol Heart Circ Physiol 281: H1242–H1251, 2001.—Heart disease represents an important etiology of mortality in chronic alcoholics. The purpose of the present study was to examine potential mechanisms for the inhibitory effect of chronic alcohol exposure (16 wk) on the regulation of myocardial protein metabolism. Chronic alcohol feeding resulted in a lower heart weight and 25% loss of cardiac protein per heart compared with pair-fed controls. The loss of protein mass resulted in part from a diminished (30%) rate of protein synthesis. Ethanol exerted its inhibition of protein synthesis through diminished translational efficiency rather than lower RNA content. Chronic ethanol administration decreased the abundance of eukaryotic initiation factor (eIF)4E associated with eIF4E in the myocardium by 36% and increased the abundance of the translation response protein (4E-BP1) associated with eIF4E. In addition, chronic alcohol feeding significantly reduced the extent of p70S6 kinase (p70S6K) phosphorylation. The decreases in the phosphorylation of 4E-BP1 and p70S6K did not result from a reduced abundance of mammalian target of rapamycin (mTOR). These data suggest that a chronic alcohol-induced impairment in myocardial protein synthesis results in part from inhibition in peptide chain initiation secondary to marked changes in eIF4E availability and p70S6K phosphorylation.

Cardiomyopathy; peptide chain initiation; eukaryotic initiation factor 4E; 4E-BP1; eukaryotic initiation factor 4G; heart; translational efficiency; p70S6K; mTOR

Heart disease as well as cirrhosis represent an important etiology of mortality in chronic alcoholics. Excessive ethanol consumption can result in a syndrome referred to as alcoholic heart muscle disease. Alcoholic heart muscle disease is rarely produced by short-term ethanol administration and thus necessitates a more prolonged exposure to alcohol. It occurs in those patients for whom the sole causative agent is excessive and prolonged alcohol consumption (>80 g of ethanol/day for >10 yr). The clinical feature of this syndrome is a defect in myocardial contractility as assessed by a reduction in ejection fraction, which is usually detected by echocardiogram. The degree of cardiac dysfunction is proportional to the duration and severity of alcohol consumption (43).

The major pathological features revealed through biopsy or postmortem examination include generalized enlargement of the heart (especially of both ventricles), thinning of the ventricular wall with fibrosis and endocardial fibroelastic thickening, interstitial edema, and focal areas of necrosis within the ventricular wall (7, 13, 14). Microscopic examination of biopsy specimens obtained from humans reveals a thinning of the ventricular wall, myocyte degeneration, loss of striations, and myofilament dissolution, which is consistent with alterations in structural and myofibrillar proteins (2, 3, 7, 13, 14). An in vitro study using myocytes has shown that ethanol reduces the number and uniformity of the myofibrils (1). It therefore appears that in alcoholic heart disease muscle, the mechanical performance of the heart as a pump is seriously compromised by loss of contractile elements or functional impairment of these elements by fragmentation and disarrangement.

Numerous hypotheses have been put forth to explain the development of alcoholic heart muscle disease, which suggest that the metabolic basis for this disease is probably multifactorial. As alcoholic heart muscle disease is associated with reduced contractility and derangements in myofibrillar architecture, it follows that one explanation for these changes is that the integrity of cellular proteins may be compromised by ethanol toxicity. Indeed, one of the metabolic hallmarks of chronic alcohol abuse is the negative nitrogen balance resulting from the net catabolism of muscle protein (29–31, 33–35). When prolonged, this imbalance in protein metabolism leads to the erosion of lean body mass and the myopathy commonly observed in alcoholics. These effects most likely result from ethanol per se. Early work (39) indicated that chronic ethanol consumption led to decreased association of actin and myosin in vitro, and it was suggested that persistent changes in some proteins may have occurred and may have been related to an inhibition of protein synthesis. It is therefore of major importance to evaluate protein turnover after prolonged and sustained ethanol exposure.
The dynamic balance of proteins in the myocardium is dependent on both protein synthesis and degradation. Preedy and co-workers (31) failed to demonstrate an effect of ethanol on cardiac protein synthesis in rats fed alcohol for 6 wk. This led to the apparent paradox that acute (23, 32) but not chronic alcohol ingestion modulated protein synthesis in cardiac muscle. Because of the long time required for evolution of changes in myocytes in humans, we reasoned that 6 wk might not be sufficiently long to induce changes in protein metabolism. Therefore, we placed rats on an alcohol-containing diet for 16 wk (21, 22, 25). The purpose of the present set of experiments was to ascertain whether protein synthesis is inhibited in hearts from rats chronically fed alcohol for 16 wk and, if so, to investigate potential mechanisms.

METHODS AND MATERIALS

Chronic alcohol feeding. Pathogen-free male Sprague-Dawley rats (Charles River Breeding Laboratories; Cambridge, MA) were maintained for 16 wk on an ethanol-containing diet in which alcohol was provided in both drinking water and previously described agar blocks (4, 22, 25). Initially, all rats were provided the agar block without ethanol for 2 days. Thereafter, the animals were randomly assigned to either an alcohol or a control group. Animals in the alcohol group were given free access to ethanol-containing agar blocks. The concentration of ethanol in the agar blocks was increased in 10% increments from 10% to 30% over the first 2 wk (21, 22, 25). Ethanol-fed rats remained on the 30% ethanol agar-block diet for the remainder of the experimental protocol. Control agar blocks contained an equal caloric amount of dextrin-maltose. Nutrient intake in both groups was furnished by standard rat chow (no. 8604; Harlan Teklad; Madison, MI). Control rats were provided the same amount of solid food as was consumed by the alcohol-fed group (21, 22, 25).

Measurement of protein synthesis in vivo. Rates of protein synthesis in vivo were estimated using the flooding-dose method previously described in our laboratory (21, 23, 24, 46–48). An incision was made in the neck of animals anesthetized with Nembutal (50 mg/kg body wt) and polyethylene (PE) catheters (PE-50 tubing) were inserted into the carotid artery and jugular vein. A bolus infusion of L-[3H]phenylalanine (0.2 μCi·ml−1·μmol−1, 30 μCi/100 g body wt, 1 ml/100 g body wt) was delivered over a 10- to 15-s interval via the catheter in the jugular vein (46–48). Ten minutes after injection of the radioisotope, an arterial blood sample (3 ml) was withdrawn for measurement of phenylalanine concentrations and radioactivity. The concentration of phenylalanine was measured by HPLC analysis of supernatants from myocardial high-energy phosphate content. For determination of ATP, ADP, AMP, creatine phosphate, and creatine, an aliquot of the frozen heart powder was homogenized with a porcelain mortar and pestle at 4°C in 3 ml of 6% (wt/vol) perchloric acid. After centrifugation, the supernatant was neutralized with KOH. The potassium perchlorate precipitate was removed by centrifugation, and the neutralized supernatant was used to measure high-energy phosphate metabolites via standard spectrophotometric techniques (44, 49).

Analysis of 4E-BP1-eIF4E and eIF4G-eIF4E complexes. The association of eIF4E with 4E-BP1 or eIF4G was determined using immunoblot techniques as previously described in our laboratory (21, 24). Heart muscle was homogenized in seven volumes of buffer A [in mM: 20 HEPES (pH 7.4), 100 KCl, 0.2 EDTA, 2 EGTA, 1 dithiothreitol, 50 NaF, 50 β-glycerolphosphate, 0.1 phenylmethylsulfonyl fluoride (PMSF), 1 benzamidine, and 0.5 sodium vanadate and 1 μM microcystin LR] using a Polytron homogenizer. The homogenate was either used directly or centrifuged at 10,000 g for 10 min at 4°C, and the pellet was discarded. The supernatant was used to evaluate the regulation of the eukaryotic initiation factor (eIF)4E system. eIF4E as well as the 4E-BP1-eIF4E and eIF4G-eIF4E complexes were immunoprecipitated from aliquots of 10,000-g supernatants using 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 [in mM: 50 Tris-HCl (pH 7.4), 150 NaCl, 5 EDTA, 50 NaF, 50 β-glycerolphosphate, 0.1 PMSF, 1 benzamidine, and 0.5 sodium vanadate plus 0.1% β-mercaptoethanol and 0.5% Triton X-100]. The beads were captured using a magnetic sample rack and washed twice with buffer B and once with buffer B.
containing 500 mM rather than 150 mM NaCl. Resuspending
the beads in SDS sample buffer and boiling for 5 min eluted
protein bound to the beads. The beads were collected by
centrifugation and the supernatants were subjected to elec-
trophoresis either on a 7.5% polyacrylamide gel for quanti-
tation of eIF4G or on a 15% polyacrylamide gel for quantita-
tion of 4E-BP1 and eIF4E. Proteins were then electro-
phoretically transferred to a nitrocellulose membrane. The
membranes were incubated with a mouse anti-human eIF4E
antibody, a rabbit anti-rat 4E-BP1 antibody, or a rabbit
anti-eIF4G antibody for 1 h at room temperature. The blots
were then developed using enhanced chemiluminescence
(ECL; Amersham Pharmacia Biotech; Piscataway, NJ). The
blots were exposed to X-ray film in a cassette equipped with
DuPont Lightning Plus intensifying screens. After develop-
ment, the film was scanned (Microtek ScanMaker IV) and
quantitated using NIH Image 1.6 software.

Determination of phosphorylation state of 4E-BP1. The
phosphorylated forms of 4E-BP1 were measured in heart
extracts. In brief, an aliquot (200 µl) of the myocardial
homogenate was immediately boiled for 5 min. The boiled
homogenate was centrifuged in a microcentrifuge at room
temperature and the supernatant was decanted. An equal
volume of 2x Laemmli SDS buffer (at 60°C) was then added
and boiled for 5 min. The boiled homogenate was then
subjected to SDS-PAGE Criterion gels (Bio-Rad; Hercules,
CA). Proteins were then electro-
phoretically transferred onto polyvinylidene fluoride (PVDF;
Cruz Biotechnology; Santa Cruz, CA) that recognizes total
p70S6K (i.e., both phosphorylated and unphosphorylated
forms). Results are presented as the ratio of the densitomet-
ric analysis of blot for phosphorylated Thr389 divided by total
p70S6K performed on the same gel.

Determination of phosphorylation state of p70 S6K. Avruch
and co-workers (51) have provided evidence that after phos-
phorylation, p70 activity in vivo is most closely related to the
activation of the kinase. After analysis of the relative
phosphorylation of residue Thr389. To examine the phosphor-
ylation of p70S6K, we performed phosphorylation
\[ \text{Thr}^{389} \]
activity in vivo is most closely related to the
activation of the kinase. After analysis of the relative
of Thr389. To examine the phosphor-
ylation of p70S6K in the cell, the blood alcohol concentration averaged 21
mM. This corresponds to a blood alcohol content of
21% in intoxicated humans

RESULTS

Total caloric consumption was not significantly dif-
ferent between control (289 ± 5 kcal·kg body
wt\(^{-1}\)·day\(^{-1}\)) and alcohol-fed rats (298 ± 11 kcal·kg
body wt\(^{-1}\)·day\(^{-1}\)). Thus control and alcohol-fed rats
consumed the same amount of calories over the course of
the study. During the final 6 wk of the experimental
protocol, rats consumed alcohol at the rate of 18 ± 1
mg·kg body wt\(^{-1}\)·day\(^{-1}\). Although blood alcohol concen-
trations normally fluctuate throughout the feeding cycle,
the blood alcohol concentration averaged 21 ± 5
mM. This corresponds to a blood alcohol content of
0.1%. The blood alcohol content in these rats was
comparable to that observed in intoxicated humans
displaying impaired mental and motor skills (52).

Beginning during the second week of alcohol feeding,
rats were weighed on a weekly basis. Figure 1 shows
the average weights of the pair-fed control and alcohol
fed rats. Both antibodies were incubated with PVDF membranes for 1 h at room temperature and then overnight at 4°C. The first was a rabbit anti-
FRAP polyclonal antibody (anti-FRAP) from StressGen Biotechnologies (Victoria, Canada). This antibody was directed against a keyhole limpet hemocyanin (KHL)-linked peptide, QVELLIKQATSHENL-(COOH), which represents residues 2,524–2,538 of FRAP (the human form of mTOR), and was used at a dilution of 1:1250 per the manufacturer’s recommenda-
tion. The second antibody, MBAT5 (produced in our lab-
atory), was directed against a KHL-linked peptide, (COOH)-
QREPKEMQKQPYWRHTFEE, representing a more NH\(_2\)-
terminal sequence (residues 221–240 of RAFT, a rat form of
mTOR) and was used at a dilution of 1:10,000. An anti-FLAG
monoclonal antibody ( Sigma; St. Louis, MO) was used to
detect the recombinant protein according to the supplier’s
protocol. Antibody immunoreactivity was detected using
ECL reagents (Amersham-Pharmacia) as described (see Analysis of 4E-BP1-eIF4E and eIF4G-eIF4E complexes).

Statistics. Values are presented as means ± SE for the number of rats per group as indicated in the figures and
tables. The data were analyzed using Student’s t-test to
determine treatment effects among the means. Differences
were considered significant when \( P < 0.05 \).
animals over the 16-wk course of the experiment. Except for week 3 and a period between weeks 6 and 9, there were no significant differences in the weight of the animals between the two groups.

Chronic ingestion of alcohol significantly reduced the heart weight (left and right ventricles) by 10% (see Table 1) compared with pair-fed controls. There was no significant difference in the wet weight-to-dry weight ratio between the two groups (data not shown). Instead, the loss of heart weight appeared to result from a 25% drop in the amount of protein in the heart ($P < 0.05$), such that the total protein per heart was significantly decreased in alcohol-treated rats compared with controls (see Table 1). These results indicate that alcohol feeding reduces heart weight secondary to a loss of myocardial proteins.

**Rates of myocardial protein synthesis.** To better understand the processes responsible for the loss of myocardial proteins, protein synthesis was estimated by measuring the rate of incorporation of radioactive phenylalanine into muscle proteins. Initially, pilot experiments were performed to establish that the feeding regimen per se did not induce changes in myocardial protein synthesis in controls. To do this, we compared rates of myocardial protein synthesis in rats fed rat chow ad libitum and control animals pair-fed to consume the same amount of calories as alcohol-fed rats (see Table 2). There were no significant differences in rates of protein synthesis in hearts from pair-fed control rats compared with ad libitum-fed animals. Thus the feeding regimen per se did not adversely affect rates of protein synthesis compared with control rats fed ad libitum.

In marked contrast, the rate of myocardial protein synthesis was decreased ~30% ($P < 0.01$) in animals consuming the alcohol-containing diet compared with the rates observed in pair-fed controls. This reduction in protein synthesis occurred despite equal caloric and nitrogen intakes between the pair-fed control and alcohol-fed groups. Hence, the derangements in protein synthesis over the course of the 16-wk feeding regime were independent of the nutritional status of the alcohol-fed rats. Next, we examined whether the inhibition of protein synthesis was a generalized phenomenon observed in muscles composed of slow-twitch fibers such as the soleus. Unlike the heart, no decrease in protein synthesis was observed in the soleus between control and alcohol-fed groups. Therefore, the defect in protein synthesis appears somewhat specific for cardiac muscle rather than a general effect of ethanol feeding on muscles composed of slow-twitch fibers.

The number of ribosomes and/or the translational efficiency can regulate protein synthesis in cardiac muscle (for a review, see Refs. 9 and 20). Changes in total RNA content presumably reflect alterations in rRNA and hence the number of ribosomes. Total tissue RNA was not altered in hearts from alcohol-treated rats ($1.2 \pm 0.1$ mg of RNA/g) compared with pair-fed control ($1.1 \pm 0.1$ mg of RNA/g). Hence, the decreased rate of protein synthesis was not the result of decreased numbers of ribosomes in hearts of alcohol-fed rats. Diminished rates of protein synthesis can also result from a reduced translational efficiency. The translational efficiency, which is estimated by dividing the rate of protein synthesis in ventricular muscle by the myocardial RNA content, is a measure of the ability of the protein synthetic machinery to translate mRNA into protein. Chronic alcohol ingestion reduced the translational efficiency by 38% [control, $12 \pm 1$% day$^{-1}$, (mg RNA/g)$^{-1}$ vs. alcohol, $7.5 \pm 0.5$% day$^{-1}$ (mg RNA/g)$^{-1}$, $P < 0.005$]. Thus chronic alcohol ingestion induces specific defects in myocardial translational efficiency that result in reduced rates of protein synthesis.

**Myocardial high-energy phosphate concentrations.** An "energy deficit" represents one possible mechanism to account for the observed reduction in translational efficiency after chronic alcohol consumption. Reduced ATP, creatine phosphate, and ATP/ADP ratios have been shown to correlate with reduced rates of protein synthesis in hearts. However, there were no significant differences in the concentrations of ATP, ADP, AMP, creatine phosphate, or creatine in alcohol-treated rats compared with pair-fed controls (see Table 3). Therefore, it is doubtful that a reduction in high-energy phosphates was responsible for the reduced rates of myocardial protein synthesis observed in rats that were chronically fed alcohol.

### Table 1. Effect of chronic alcohol feeding on ventricular weight and protein content

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ventricular Weight, g</th>
<th>Protein Content, mg protein/heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.25 ± 0.02</td>
<td>312 ± 36</td>
</tr>
<tr>
<td>Alcohol fed</td>
<td>1.10 ± 0.03*</td>
<td>209 ± 23*</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 8$ hearts in each group. *$P < 0.05$ vs. control.

### Table 2. Effect of chronic alcohol consumption on protein synthesis in the heart and soleus

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Ad Libitum Control</th>
<th>Pair-Fed Control</th>
<th>Alcohol Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>15 ± 1</td>
<td>13 ± 1</td>
<td>9 ± 0.5*</td>
</tr>
<tr>
<td>Soleus</td>
<td>18 ± 2</td>
<td>16 ± 1</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 8$ per group. *$P < 0.05$ vs. both ad libitum control and pair-fed control animals.

### Table 3. Comparison of high-energy phosphate content in hearts from alcohol-fed and control rats

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>Alcohol Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>24.38 ± 0.88</td>
<td>23.01 ± 0.92</td>
</tr>
<tr>
<td>ADP</td>
<td>4.66 ± 0.44</td>
<td>4.33 ± 0.54</td>
</tr>
<tr>
<td>AMP</td>
<td>0.55 ± 0.29</td>
<td>0.77 ± 0.31</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>27.55 ± 3.01</td>
<td>29.03 ± 2.06</td>
</tr>
<tr>
<td>Creatine</td>
<td>28.91 ± 2.04</td>
<td>31.07 ± 4.11</td>
</tr>
<tr>
<td>Energy charge</td>
<td>0.90 ± 0.01</td>
<td>0.89 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 8$ hearts in each group. Units for metabolites are μmol/g dry weight except for energy charge, which was calculated from the high-energy phosphate content of cardiac muscle as follows: (ATP + 1/2ADP)/(ATP + ADP + AMP).
Regulation of eIF4E availability. Based on the distribution of ribosomal subunits in hearts from alcohol-fed rats, we proposed that both peptide chain initiation and elongation were affected by chronic alcohol ingestion (21). With regard to peptide chain initiation, we previously reported that the eIF2B system in cardiac muscle did not appear to be affected by chronic ingestion of alcohol (21). Because the eIF4E system has also been implicated in regulating global rates of protein synthesis, we examined the possible role of eIF4E in limiting protein synthesis during chronic alcohol feeding. One mechanism for modulating eIF4E involves its relative distribution between active (eIF4G•eIF4E) and inactive (4E-BP1•eIF4E) complexes. To investigate the effects of alcohol feeding on the association of eIF4G with eIF4E, eIF4E immunoprecipitates were used to measure the amount of eIF4G that coprecipitates with eIF4E. eIF4E immunoprecipitates demonstrated a 36% reduction in the amount of eIF4E bound to eIF4G (see Fig. 2).

The decreased amount of eIF4E•eIF4G complex could result from changes in the amount of eIF4E in the immunoprecipitate. However, the amount of eIF4E was not significantly reduced in the immunoprecipitate between control (450 ± 40 arbitrary units/mg protein) and alcohol-treated rats (465 ± 35 arbitrary units/mg protein). We also examined the possibility that the reduced abundance of eIF4E•eIF4G complex resulted from a decreased amount of eIF4G in the myocardium of rats fed alcohol. The myocardial content of eIF4G was not significantly reduced between control (69 ± 6 arbitrary units/mg protein) and alcohol-treated rats (70 ± 5 arbitrary units/mg protein). Moreover, there was no evidence that chronic alcohol feeding increased the amount of eIF4G proteolytic fragments (data not shown).

It has been postulated that the availability of eIF4E for binding to eIF4G can be modulated by its association with the translation repressor 4E-BP1. The binding of 4E-BP1 to eIF4E forms an inactive complex, thereby limiting translation initiation. The interaction between 4E-BP1 and eIF4E is regulated by the extent of 4E-BP1 phosphorylation. 4E-BP1 possesses multiple phosphorylation states, and the various phosphorylated forms of the protein are resolved into three bands (α-, β-, and γ-bands) using SDS-PAGE (26, 28). The most highly phosphorylated form of the protein, the γ-form, does not bind eIF4E (26, 28). In contrast, the hypophosphorylated α- and β-forms of 4E-BP1 do bind to eIF4E (26, 28). The total myocardial content of 4E-BP1 (the sum of all three phosphorylated forms) did not differ significantly between control (11,539 ± 1,393 arbitrary units/mg protein) and alcohol-treated (12,028 ± 1,206 arbitrary units/mg protein) rats. In contrast, the amount of 4E-BP1 in the γ-form in cardiac muscle was decreased ~25% after chronic alcohol consumption (see Fig. 3). Furthermore, the decrease in the hyperphosphorylated γ-form was associated with a corresponding increase in the prominence of the α- and β-bands.

Because reductions in the amount of 4E-BP1 in the γ-form in hearts from alcohol-fed rats would be expected to affect the binding of eIF4E to 4E-BP1, we examined the abundance of eIF4E bound to 4E-BP1. To investigate this possibility, eIF4E immunoprecipitates were analyzed for 4E-BP1 content (see Fig. 4). The immunoblot in Fig. 4 illustrates that only the α- and β-forms of 4E-BP1 were detected in the immunoprecipitates. Chronic alcohol feeding caused a 70% increase in the total amount of 4E-BP1 associated with eIF4E compared with pair-fed control rats.

Phosphorylation of p70S6K. We also examined the extent of phosphorylation of p70S6K to determine whether this was an important regulatory mechanism in cardiac muscle after chronic alcohol intoxication. To
alcohol intoxication significantly \( (P < 0.005) \) reduced the prominence of the phospho-Thr^{389} immunoreactivity in the myocardium (see Fig. 5). The change in phosphorylation of Thr^{389} occurred without any significant alteration in total p70 S6K and indicates a net decrease in the phosphorylation state of the protein.

One potential mechanism to account for a decreased phosphorylation of p70^{S6K} would be a diminished cellular content of mTOR, the kinase responsible for the phosphorylation of p70^{S6K}. However, heretofore no measurements of the abundance of mTOR in cardiac muscle have been reported. Therefore, we established the methodology to measure mTOR in cardiac muscle. Both the commercial anti-FRAP antibody from StressGen Biotechnologies (data not shown) and the mTAB5 antibody detected a ~240-kDa band from baclovirus-infected Sf-9 insect cell lysates expressing FLAG-FRAP (see Fig. 6, top). FLAG-FRAP is a recombinant form of human mTOR containing an amino terminal peptide, MDYKDDDDK, as an epitope (FLAG) tag. The FLAG-FRAP band from infected Sf-9 cells also reacted with anti-FLAG monoclonal antibody. Myosin became separated from mTOR (data not shown). This allowed for the detection of mTOR using the antibodies described. Figure 6 (top and middle) shows the densitometric analysis of the anti-FRAP immunoreactivity in hearts from control and alcohol-treated rats. These results show that chronic alcohol treatment had no significant effect on the amount of mTOR in the heart (see Fig. 6, bottom).

**DISCUSSION**

Chronic alcohol feeding resulted in a lower heart weight compared with pair-fed controls. Moreover, there was a 25% loss in cardiac protein per heart from animals consuming alcohol for 16 wk. The loss of protein mass resulted in part from a diminished (30%) rate of protein synthesis. The effect of chronic alcohol administration to inhibit myocardial protein synthesis in vivo reported herein is consistent with reports examining protein synthesis in hearts from guinea pigs chronically fed ethanol for 40 wk and then perfused in

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**Fig. 4.** Effect of chronic alcohol feeding on the amount of 4E-BP1 bound to eIF4E. To determine the amount of 4E-BP1 bound to eIF4E, eIF4E was immunoprecipitated from 10,000-g extracts of cardiac muscle homogenates (as described in Fig. 2) by use of an anti-eIF4E monoclonal antibody. Immunoprecipitates were subsequently subjected to protein immunoblot analysis using anti-4E-BP1 antibodies. Top: representative immunoblots of 4E-BP1 in eIF4E immunoprecipitates from control and alcohol-treated rats. Bottom: summary of densitometric analysis of all immunoblots. Results represent means ± SE for 8–12 rats/group. *\( P < 0.05 \) vs. control.

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**Fig. 5.** Effect of chronic alcohol feeding on phosphorylation of p70^{S6K} kinase (p70^{S6K}) at Thr^{389}. Proteins (200 \( \mu \)g of protein/lane) from cardiac muscle homogenates were separated on 7.5% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane at 100 V for 30 min in 10% 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11) and 10% methanol transfer buffer. Top: representative lanes of 4E-BP1 in eIF4E immunoprecipitates from control and alcohol-treated rats. Membranes were probed with antibody that only recognizes p70^{S6K} phosphorylated on the site required for activation of the kinase, Thr^{389} (Thr-389-P). Middle: membranes were stripped and reprobed with antibody that only recognizes total p70^{S6K}. In this gel system, there are not significant shifts in mobility with increased p70^{S6K} phosphorylation. Bottom: phospho-specific and total p70^{S6K} blots were analyzed by densitometry, and the ratio of phospho-specific (Thr^{389}) to total p70^{S6K} immunoreactivity was determined. Results are means ± SE for \( n = 8–10 \) rats/group. *\( P < 0.005 \) vs. control.
The rate of protein synthesis is known to be sensitive to the changes in the myocardial content of ATP (8, 16). However, it is unlikely that the decreased rate of protein synthesis in cardiac muscle of rats fed alcohol for 16 wk was caused by a diminished high-energy phosphate content because the myocardial ATP and creatine phosphate content were not reduced compared with controls. Likewise, amino acids are required as a substrate for the synthesis of proteins. Indeed, nutrient deprivation reduces cardiac protein synthesis (50). We have previously established (25) that chronic alcohol ingestion produced minor alterations in a few individual amino acids (threonine, proline, and taurine) but did not significantly change the total concentration of amino acids in the blood. Therefore, it also appears unlikely that an alcohol-induced depletion of amino acids was responsible for the inhibition of protein synthesis in cardiac muscle.

There was no decrease in the protein synthetic apparatus as estimated by total tissue RNA. Instead, ethanol exerted its inhibitory effect on protein synthesis through a diminished translational efficiency (an estimate of how well the existing synthetic machinery is functioning) as indicated by the reduction in the amount of protein synthesized per unit of RNA. This response may be due to ethanol exerting its effect on one or more of the many steps whereby amino acids are incorporated into functional cardiac muscle proteins.

The inhibition in cardiac muscle protein synthesis results from a diminished translation efficiency secondary to blocks in both peptide chain initiation and elongation (21). In the present set of experiments, we examined the effect of chronic alcohol feeding on peptide chain initiation. The process of peptide chain initiation involves essentially four major steps (for reviews, see Refs. 9 and 20): 1) dissociation of the 80S
ribosome into 40S and 60Sribosomal subunits, 2) formation of the 43Spreinitiation complex with binding of the initiator met-tRNA\textsuperscript{met} to the 40S subunit, 3) binding of mRNA to the 43Spreinitiation complex, and 4) association of the 60S ribosomal subunit to form an active 80S ribosome.

Two of the steps involved in peptide chain initiation appear important as major regulatory points in the overall control of protein synthesis in vivo. The first step controlling peptide chain initiation is the binding of met-tRNA\textsuperscript{met} to the 40S ribosomal subunit to form the 43Spreinitiation complex. This reaction is mediated by eIF2 and is regulated by the activity of another initiation factor, eIF2B. We have previously established (21) that altered regulation of eIF2 is probably not responsible for the reduced translation initiation during chronic alcohol intoxication.

The second regulatory step involves the binding of mRNA to the 43Spreinitiation complex, which is mediated by eIF4F, a complex of several subunits. One of the subunits, eIF4E, binds the 7-methylguanosine 5'-triphosphate (m\textsuperscript{7}GTP) cap structure present at the 5'end of most eukaryotic mRNAs to form an eIF4E·mRNA complex (37). During translation initiation, the eIF4E·mRNA complex binds to eIF4G and eIF4A to form the active eIF4F complex (37, 38, 42). The active eIF4E·eIF4G complex allows binding of mRNA to the 43Spreinitiation complex, and elongation then proceeds. Because translation of mRNAs in eukaryotic cells relies heavily on a cap-dependent process involving eIF4E, it might be expected that modulation of eIF4E would also contribute to the inhibition of myocardial protein synthesis during chronic alcohol feeding. Indeed, changes in the distribution of eIF4E between the active eIF4E·eIF4G complex and the inactive eIF4E·eIF4B·BP1 complex have been suggested as the mechanism through which hormones such as insulin or insulin-like growth factor I regulate global rates of protein synthesis (17–19, 45) and acute alcohol administration modulates myocardial protein synthesis (23).

eIF4E is regulated by several mechanisms. During translation initiation, mRNA binds either directly to eIF4E already associated with 40S ribosomes or to free eIF4E with subsequent binding of the mRNA·eIF4E·eIF4G complex to the ribosome (37, 38, 42). With either scenario, a decreased amount of eIF4E associated with eIF4G would diminish the association of mRNA with ribosomes and hence limit translation initiation. In the present set of experiments, chronic ethanol administration diminished the abundance of eIF4E associated with eIF4E in the myocardium by 36%. The diminished binding of eIF4G to eIF4E did not result from a diminished cellular content of either of these two eukaryotic initiation factors.

Instead, recent evidence suggests that the availability of eIF4E for binding to eIF4G is controlled through binding to small acid- and heat-labile proteins [termed 4E-BP1 (PHAS-I), 4E-BP2 (PHAS-II), and 4E-BP3] and forming an inactive 4E-BP1·eIF4E complex (26, 28). In rat muscle, the predominant form of these translation repressor proteins is 4E-BP1. Unphosphorylated 4E-BP1 binds to eIF4E to form an inactive 4E-BP1·eIF4E complex. When eIF4E is bound to 4E-BP1, eIF4E binds to mRNA but cannot form an active eIF4E·eIF4G complex. Thus formation of the 4E-BP1·eIF4E complex prevents binding of mRNA to the ribosome. 4E-BP1 binding to eIF4E essentially limits cap-dependent mRNA translation by physically sequestering eIF4E into an inactive complex. In the present set of experiments, chronic ethanol administration increased the abundance of 4E-BP1 associated with eIF4E in the myocardium consistent with the decrease in amount of eIF4E·eIF4G.

The interaction between 4E-BP1 and eIF4E is regulated by the extent of 4E-BP1 phosphorylation. Hypophosphorylation of 4E-BP1 binds to eIF4E to form an inactive 4E-BP1·eIF4E complex, which prevents binding of eIF4G to eIF4E. Conversely, hyperphosphorylation of 4E-BP1 does not bind to eIF4E, which thereby releases eIF4E from the 4E-BP1·eIF4E complex. In this simplistic model, the release of eIF4E from the 4E-BP1·eIF4E complex after hyperphosphorylation of 4E-BP1 allows eIF4E to bind to eIF4G and form an active eIF4E·eIF4G complex (26, 28). In the present set of experiments, chronic alcohol feeding reduced the phosphorylation of 4E-BP1. Thus we observed a reciprocal relationship between eIF4E found in the inactive 4E-BP1·eIF4E complex and 4E-BP1 phosphorylation after chronic alcohol feeding.

Like mTOR, p70\textsuperscript{S6K} is a serine/threonine kinase. The enzyme phosphorylates the S6 ribosomal protein (for a review, see Ref. 36) and has been implicated in stimulating skeletal muscle protein synthesis after insulin treatment (10). Phosphorylation of ribosomal S6 protein enhances translation of mRNA into protein (for reviews, see Refs. 12 and 36). Ribosomal S6 protein is uniquely positioned to regulate mRNA translation by its location at the rRNA binding site on the 40S ribosome. Ribosomal S6 protein is phosphorylated by a family of 70-kDa protein kinases referred to as p70\textsuperscript{S6K} (12, 36). Phosphorylation of ribosomal S6 protein appears to control the translation of a set of mRNAs that possesses 5'-terminal tracts of pyrimidines (5'-TOP mRNAs) (15), and some encode for ribosomal proteins and elongation factors. Furthermore, reduced p70\textsuperscript{S6K} activity is associated with diminished rates of protein synthesis (10). In the present studies, chronic alcohol feeding significantly reduced the extent of phosphorylation of the p70\textsuperscript{S6K}. This was evidenced by the decrease in phosphorylation of Thr\textsuperscript{389}, which has been proposed to be the phosphorylation site most closely associated with p70\textsuperscript{S6K} activity (51). Thus inactivation of p70\textsuperscript{S6K} by dephosphorylation may also play a role in limiting myocardial protein synthesis after chronic alcohol administration.

It is currently thought that phosphorylation of p70\textsuperscript{S6K}, like 4E-BP1, lies distal to mTOR in the signaling pathway. Hence, stimuli that affect the activity of mTOR should influence the extent of phosphorylation of both p70\textsuperscript{S6K} and 4E-BP1. One mechanism to account
for the changes in phosphorylation of p70S6K would be a diminished cellular content of mTOR in animals fed alcohol. We found no significant differences in myocardial content of mTOR between control and alcohol-fed rats. Alternatively, chronic alcohol feeding could lower mTOR activity through a yet-to-be-determined pathway and/or stimulate phosphatase activity.

In summary, the results of the present experiments provide evidence that chronic ethanol administration inhibits protein synthesis in cardiac muscle of rats. Ethanol exerted its inhibitory effect on protein synthesis through a diminished translational efficiency rather than a reduction in the RNA content. Chronic ethanol administration diminished the abundance of eIF4E associated with eIF4E in the myocardium and increased the abundance of 4E-BP1 associated with eIF4E. In addition, chronic alcohol feeding significantly reduced the extent of p70S6K phosphorylation. The alterations in myocardial eIF4E and p70S6K phosphorylation in the present study were similar to the changes previously reported to occur after acute alcohol intoxication (29). These data suggest that chronic alcohol-induced impairments in myocardial protein synthesis result in part from an inhibition of translation initiation secondary to marked changes in eIF4E availability and p70S6K phosphorylation.

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