Oral Leucine Enhances Myocardial Protein Synthesis in Rats Acutely Administered Ethanol\textsuperscript{1,2}

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Abstract

Acute alcohol ingestion induces an inhibition of myocardial protein synthesis by impairing mRNA translation initiation. Elevating plasma leucine (Leu) concentrations via oral gavage stimulates mRNA translation initiation in several tissues, although the effect in heart has not been well defined. The experiments described herein were designed to test the effects of a gavage solution containing Leu on protein synthesis and potential mechanisms important in accelerating mRNA translation initiation in cardiac muscle of rats given ethanol acutely to mimic "binge" drinking. Gavage with Leu stimulated protein synthesis and enhanced the assembly of the active eukaryotic initiation factor (eIF)4G-eIF4E complex. Increased assembly of the active eIF4G-eIF4E complex was associated with a 130\% rise in phosphorylation of eIF4G (Ser\textsuperscript{1106}) and a decreased assembly (~30\%) of inactive eIF4E-binding protein1 (4EBP1)-eIF4E complex in rats-administered ethanol. The reduced assembly of the 4EBP1-eIF4E complex was associated with an increase in phosphorylation of 4EBP1 in the hyperphosphorylated γ-form following Leu gavage. Phosphorylation of mammalian target of rapamycin on Ser\textsuperscript{2448}, an upstream regulator of phosphorylation of 4EBP1, was elevated following Leu gavage. Neither the phosphorylation of 70-kDa ribosomal protein S6 kinase on Thr\textsuperscript{389} nor eIF4E phosphorylation was increased following Leu gavage under any condition. Leu gavage accelerates myocardial protein synthesis following acute ethanol intoxication by enhancing eIF4G-eIF4E complex assembly through increased phosphorylation of eIF4G and decreased association of 4EBP1 with eIF4E. J. Nutr. 139: 1439–1444, 2009.

Introduction

Alcoholism remains a major health problem in the United States, affecting as much as \(~8\%\) of the population. Heavy, chronic alcohol consumption (>80 g/d ethanol for >10 y) can cause a syndrome termed alcoholic heart muscle disease. Alcoholic heart muscle disease represents an important source of the morbidity and mortality associated with alcoholism, because the disease pathogenesis can lead to development of a dilated cardiomyopathy (1,2). The metabolic basis of alcoholic heart muscle disease is probably multifactorial, with defects in myocardial protein metabolism contributing to the precipitation and progression of the disease (3,4). The dynamic balance of proteins in the myocardium is dependent upon both protein synthesis and degradation. We and others have provided evidence that acute ethanol intoxication (binge drinking) inhibits rates of protein synthesis in cardiac muscle at the processes involved in mRNA translation initiation (3,5–9).

Regulation of myocardial protein synthesis in response to acute or chronic alcohol consumption (4) involves 7-methyl guanine triphosphate cap-dependent translation factors and their regulatory proteins [eukaryotic translation initiation factor (eIF)\textsuperscript{3} 4E-binding protein1 (4EBP1)] such as changes in their phosphorylation state [eIF4E, eIF4G, mammalian target of rapamycin (mTOR), PKB, 4EBP1] and availability (eIF4E, eIF4G) to associate and form functional complexes (eIF4F) capable of enhancing rates of peptide-chain initiation. Alcohol limits assembly of the active eIF4G-eIF4E complex, thereby limiting mRNA translation initiation by inhibiting the binding of mRNA to the 43S preinitiation complex (4,6,9–11). Formation of the eIF4G-eIF4E complex appears to be regulated in part by the proline kinase mTOR. Indeed, mTOR controls protein synthesis through a stunning number of downstream effector proteins that impinge on the protein synthesis apparatus. mTOR can be regulated via changes in its content and/or activity. In this regard, mTOR activity appears to be dependent upon multi-site phosphorylation (12). Both acute, binge ethanol intoxication (8) and chronic alcohol feeding (13) impair mTOR(Ser\textsuperscript{2448}) phosphorylation in hearts from fed rats without affecting the myocardial mTOR content (6).

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\textsuperscript{3} Abbreviations used: eIF, eukaryotic initiation factor; 4EBP1, eIF4E-binding protein1; IP, intraperitoneal; mTOR, mammalian target of rapamycin; PKB, protein kinase B; PRAS40, proline rich AKT substrate of 40 kDa; PVDF, polyvinylidene fluoride; S6K1, 70-kDa ribosomal protein S6 kinase.
Amino acids, leucine (Leu) in particular, have the ability to regulate protein synthesis (14,15). Emerging evidence indicates that amino acids acting as nutrient signals themselves modulate cellular processes leading to an acceleration of protein synthesis through augmented translation initiation (9). For example, formation of the active elf4E-elf4G complex is dependent upon the amino acids in the postprandial state (16). Meal feeding or oral administration of Leu enhances protein synthesis through changes in the regulation of proposed effectors of mRNA translation initiation, as evidenced by increased phosphorylation of the translational repressor, 4EBP1, the association of elf4E with elf4E, and the phosphorylation of the 70-kDa ribosomal protein S6 kinase (S6K1) in striated muscle (9,17,18).

The purpose of the present set of experiments was to test the hypothesis that Leu stimulates formation of the active elf4E-elf4G complex in hearts from rats administered ethanol and in doing so accelerates rates of protein synthesis.

Materials and Methods

Rats and experimental design. In these experiments, the ability of Leu to stimulate protein synthesis was assessed in hearts from pathogen-free, male Sprague-Dawley rats (Charles River Breeding Laboratories) injected once with ethanol. On the day before the experiment, food was removed from the cages. The food-deprived rats were divided into 2 groups: an alcohol-treated [intraperitoneal (IP) injection 75 mmol/kg body weight] group and a control group (IP injection of an equal volume of saline) at T 0. After 1.5 h, Leu (1.35 g/kg body weight) or water was administered via oral gavage as described previously (19–21). After 2.25 h, the rats were anesthetized (50 mg/kg nembutal) and L-[3H]phenylalanine injected via the jugular vein for measurement of protein synthesis in vivo as described previously (22,23). Blood samples were taken from a catheter placed in the carotid artery at 2, 6, and 10 min following in vivo as described previously (22,23). Blood samples were taken from a catheter placed in the carotid artery at 2, 6, and 10 min following injection of L-[3H]phenylalanine for determination of radioactivity of the plasma phenylalanine as the precursor pool. The specific radioactivity of L-[3H]phenylalanine was measured by HPLC analysis of supernatant from the injection of L-[3H]phenylalanine for determination of radioactive specific activity of L-[3H]phenylalanine (24) and ethanol in the plasma. Plasma ethanol concentrations increased from 8 ± 2 mg/dl to 300 ± 6 mg/dl (P < 0.0001). After the last blood sample, the heart was excised and frozen between aluminum clamps cooled to the temperature of liquid nitrogen for subsequent measurement of protein synthesis rates in vivo and analysis of the phosphorylation states of mTOR, S6K1, 4EBP1, elf4E, and elf4G, as well as the association of elf4E with elf4G and 4EBP1. The dose of Leu given in gavage solution is equivalent to the amount of Leu consumed by rats of this age and strain during a 24-h period (19,20,25). This protocol raises the plasma Leu concentration in the blood from 0.175 to ~3 mmol/L within 30 min (26). In control rats, the rise in plasma concentrations and its stimulation of protein synthesis we have previously published a dose response and time dependency of the blood from 0.175 to ~3 mmol/L within 30 min (26). In control rats, meal feeding or oral administration of Leu enhances protein synthesis through changes in the regulation of proposed effectors of mRNA translation initiation, as evidenced by increased phosphorylation of the translational repressor, 4EBP1, the association of elf4E with elf4E, and the phosphorylation of the 70-kDa ribosomal protein S6 kinase (S6K1) in striated muscle (9,17,18).

The purpose of the present set of experiments was to test the hypothesis that Leu stimulates formation of the active elf4E-elf4G complex in hearts from rats administered ethanol and in doing so accelerates rates of protein synthesis.

Rates of protein synthesis. Rates of protein synthesis (nmol phenylalanine incorporated/h/mg protein) were determined by the incorporation of radioactive phenylalanine into muscle proteins (23) and were calculated as described earlier using the mean specific radioactivity of the plasma phenylalanine as the precursor pool. The specific radioactivity of the plasma phenylalanine was measured by HPLC analysis of supernatants from trichloroacetic acid extracts of plasma via HPLC (24). The specific radioactivities from the 3 time points were averaged. The assumption in using this technique to measure rates of protein synthesis in vivo is that the intracellular concentration (>1 mmol/L) of phenylalanine is elevated to such an extent that any dilution effect of nonradioactive phenylalanine derived from the proteolysis on the specific radioactivity is negligible. Under the condition of elevated plasma phenylalanine concentrations, the specific radioactivity of the intracellular phenylalanine equals the specific radioactivity of the tRNA-bound phenylalanine (27–29).

Preparation of heart lysates. Frozen, powder tissue was homogenized in 7 volumes of buffer A (20 mmol/L HEPES, pH 7.4, 100 mmol/L potassium chloride, 0.2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L diithiothreitol, 50 mmol/L sodium fluoride, 50 mmol/L β-glycerophosphate, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 0.5 mmol/L sodium vanadate, and 1 mmol/L microcystin LR) using a Polytron PT 10 homogenizer set at 60% of maximum power. The homogenate was centrifuged at 10,000 × g; 10 min at 4°C and the pellet was discarded. An aliquot of the 10,000 × g supernatant was mixed with an equal volume of 2× Laemmli SDS sample buffer (65°C) and then subjected to protein immunoblot analysis. Another aliquot of the homogenate was used to measure the protein concentration by the Biuret method with crystalline bovine serum albumin serving as a standard.

Determination of phosphorylation state of elf4G or mTOR. To measure the relative extent of phosphorylation of elf4G and mTOR, proteins in the homogenate were separated by using 5% criterion gel electrophoresis (PAGE) (16,30–32). Following electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Biotrace, PALL), which were then incubated with an antibody that recognizes the phosphorylated form of elf4G (Ser1108), mTOR(Ser2448), or mTOR(Ser2481) (Cell Signaling Technology). The blots were then developed using an Enhanced Chemiluminescence Plus (ECLPlus) Western blotting kit as per the manufacturer’s instructions (GE Heathcare). Films were scanned using a Epson scanner equipped with a transparent media adaptor connected to a Macintosh computer. Densitometric analysis of the images obtained were quantitated using Image J software. Following development of the immunoblot, the membranes were treated with a solution containing 62.5 mmol/L Tris-HCl (pH 6.7), 100 mmol/L β-mercaptoethanol, and 2% (wt:v) SDS to remove antibodies as per the manufacturer’s instructions. The membranes were then immunoblotted with antibodies that recognize elf4G or mTOR independently of its phosphorylation state (Bethyl Laboratories). The blots were developed using ECLPlus (GE Heathcare) and the autoradiographs were scanned and analyzed as described above. The phosphorylated elf4G or mTOR signal densities were normalized to the respective total elf4G or mTOR signal to reflect the relative ratio of phosphorylated elf4G or mTOR to total elf4G or mTOR, respectively.

Quantification of elf4G elf4E and elf4E 4EBP1 complexes. The association of elf4G with elf4E and elf4E with 4EBP1 was determined in cardiac muscle using immunoblot techniques as previously described in our laboratory (18,21,30,33). elf4G-elf4E and 4EBP1-elf4E complexes were immunoprecipitated from aliquots of 10,000 × g supernatants using an anti-elf4G monoclonal antibody (gift of Dr. Leonard Jefferson, Penn State University College of Medicine, Hershey, PA). Samples were subjected to immunoblot analysis with a monoclonal antibody elf4G or polyclonal antibody 4EBP1. The abundance of 4EBP1 was normalized to the amount of elf4E in the immunoprecipitate.

Determination of phosphorylation state of elf4E, 4EBP1, PRAS 40, PKB, and S6K1. The various phosphorylated forms of 4EBP1 (designated α, β, and γ) were separated by SDS-PAGE electrophoresis and quantitated by protein immunoblot analysis as described previously (18,21,30,33). To examine the phosphorylation of proline rich AKT substrate of 40 kDa (PRAS40), PKB, S6K1, and elf4E, homogenates of cardiac muscle were mixed with 2× Laemmli SDS sample buffer and subjected to electrophoresis on 12.5% SDS-PAGE criterion gels (Bio-Rad) (16,31–33). Separate samples were electrophoresed for each individual protein assayed. The membranes were probed with phospho-specific antibodies that recognize phospho-PRAS40(Thr246) (Biosource), phospho-PKB(Thr308), phospho-PKB(Ser473), phospho-S6K1(Thr389), and phospho-elf4E(Ser209) (Cell Signaling Technology). After quantification of the relative intensity of the signal for phosphorylation, the phospho-specific antibodies were removed from PVDF membranes as described above for elf4G. The blots were then probed with an antibody that recognizes both phosphorylated and unphosphorylated (total) forms of PRAS40 (Biosource), PKB, S6K1 (Santa Cruz Biotechnology), and elf4E. Results are presented as the ratio of the densitometric analysis of blot for phosphorylated PRAS40(Thr246), PKB(Thr308), PKB(Ser473), elf4E(Ser209), 4EBP1, PRAS40, and S6K1.
S6K1 (Thr\textsuperscript{389}), or eIF4E (Ser\textsuperscript{209}) divided by their respective total abundance of PRAS40, PKB, S6K1, or eIF4E measured using the same PVDF membrane.

**Statistical analysis.** Values are presented as means ± SEM of densitometric analyses of extracts from individual hearts for each group. Data were executed using 2-way ANOVA with Leu (Leu) gavage (saline + Leu vs. ethanol + Leu) and condition (saline + water vs. ethanol + water) were accomplished with the Prizm Statistical software package (GraphPad Prizm4 Software). A Sidak post hoc test for multiple comparisons was performed to determine significant differences between means for all significant interactions and main effects. For all analyses, differences were considered significant at \( P \leq 0.05 \). Linear regression analyses were performed using the InStat statistical software package. This program also tests whether the slope is significantly different from zero, with \( P < 0.05 \) considered significant.

**Results**

**Rates of protein synthesis.** Acute gavage with a solution containing Leu stimulates protein synthesis in various tissues, including cardiac muscle. In the present set of experiments, Leu gavage accelerated myocardial rates of protein synthesis by 35\% in control rats. Acute alcohol intoxication depressed rates of myocardial protein synthesis by 40\% (Table 1) compared with hearts from time-matched control rats injected with saline, consistent with our previous reports (10,34). Gavage with a solution composed of Leu increased protein synthesis by 130\% in rats injected with ethanol to values similar to those in controls gavaged with Leu (Table 1).

**Assembly of a active eIF4G-eIF4E complex.** The mechanistic interactions between alcohol and Leu were investigated through scrutiny of known regulatory steps controlling protein synthesis. In this regard, the rate of protein synthesis may depend upon the formation of an active eIF4G-eIF4E complex. Consistent with this supposition, injection of ethanol was reduced by 40\% in rats treated with ethanol alone (Table 2). The abundance of the eIF4G-eIF4E complex was significantly increased by 70\% in hearts from control rats gavaged with Leu. The abundance of eIF4G associated with eIF4E was 120\% greater following gavage with a solution containing Leu in rats injected with ethanol (Table 2). The changes in eIF4G-eIF4E did not result in significant differences in the abundance of eIF4E in the immunoprecipitate in hearts from control and ethanol-treated rats in the presence or absence of Leu gavage (data not shown).

We have previously posited that translation initiation is limited through a reduced assembly of an active eIF4G-eIF4E complex during acute ethanol intoxication (4,8,11). Consistent with this hypothesis, the abundance of the eIF4G-eIF4E complex was significantly reduced by 40\% following ethanol administration. Acute Leu administration resulted in an increased abundance of the eIF4G-eIF4E complex in rats given saline (70\%) or ethanol (100\%). Moreover, a positive linear relationship with a slope different than zero \( (r^2 = 0.98; P < 0.01, F\text{-test} = 84.65) \) between rates of protein synthesis and association of eIF4G with eIF4E was observed in the present study. Although the correlation between rates of protein synthesis and association of eIF4G with eIF4E does not prove cause and effect, the relationship is consistent with the proposed role of the eIF4G-eIF4E complex in the overall regulation of eukaryotic protein synthesis similar to results obtained in skeletal muscle in response to insulin.

Both the extent of eIF4G phosphorylation and availability of eIF4E can modulate the assembly of the active eIF4G-eIF4E complex. In the present experiments, the extent of eIF4G phosphorylation was reduced by ~50\% by ethanol intoxication (Table 2). The extent of eIF4G phosphorylation increased by almost 130\% following Leu gavage in ethanol-injected rats. In contrast, gavage with Leu did not increase the phosphorylation state of eIF4G in controls. Changes in eIF4G phosphorylation were not a result of Leu- or ethanol-induced change in the content of total eIF4G (data not shown).

**Binding of eIF4E with 4EBP1.** Availability of eIF4E for assembly of the active eIF4G-eIF4E complex is posited to be related to the formation of an inactive eIF4E-4EBP1 complex. Alcohol intoxication increased the association of eIF4E with 4EBP1 compared with rats injected with saline. Leu orally administered to rats given ethanol markedly decreased the amount of the inactive eIF4E-4EBP1 complex in rats gavaged with Leu compared with their corresponding water-gavaged groups (Table 2). The eIF4E-4EBP1 complex did not differ in hearts from rats gavaged with Leu.

**Phosphorylation of 4EBP1.** The association of eIF4E with 4EBP1 is inversely related to the phosphorylation state of 4EBP1, with decreased phosphorylation associated with increased formation of an inactive eIF4E-4EBP1 complex. When phosphorylated, 4EBP1 resolves into distinct electrophoretic forms \((\alpha, \beta, \text{and } \gamma)\), with the \( \gamma \)-form representing the highest

| TABLE 1 | Effect of oral Leu gavage on protein synthesis rates in cardiac muscle following acute alcohol intoxication in rats\(^1\) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| IP injection + gavage | Saline + water | Saline + Leu | Ethanol + water | Ethanol + Leu |
| Protein synthesis, nmol | 270 ± 15 | 367 ± 24 | 192 ± 32\(^a\) | 290 ± 25 |
| Phe p慰 vet \( \times \) | 10 | 10 | 10 | 10 |

\(^1\) Values are means ± SEM; \( n = 6 \). Gavage with Leu affected protein synthesis, \( P < 0.001 \). \(^a\) Different from saline + water, \( P < 0.0005 \).

| Table 2 | Effect of oral Leu gavage on eIF4G-eIF4E association, eIF4E-4EBP1 association, eIF4E phosphorylation, and eIF4G phosphorylation in cardiac muscle following acute alcohol intoxication in rats\(^1\) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| IP injection + gavage | Saline + water | Saline + Leu | Ethanol + water | Ethanol + Leu |
| eIF4G-eIF4E association, eIF4G/eIF4E | 10 ± 1 | 17 ± 2 | 6 ± 1\(^a\) | 13 ± 1 |
| eIF4G phosphorylation, phospho-eIF4G(Ser\textsuperscript{112})/total eIF4G | 19 ± 2 | 21 ± 2 | 10 ± 2\(^b\) | 23 ± 3 |
| eIF4E-4EBP1 association, 4EBP1/eIF4E | 10 ± 1 | 8 ± 1 | 14 ± 0.7\(^b\) | 10 ± 1 |
| eIF4E phosphorylation, phospho-eIF4E(Ser\textsuperscript{209})/total eIF4G | 16 ± 3 | 9 ± 1 | 18 ± 3 | 10 ± 2 |

\(^1\) Values are means ± SEM; \( n = 6–13 \). Gavage with Leu affected all variables, \( P < 0.005 \). Letters indicate different from saline + water: \( ^a P < 0.05 \), \( ^b P < 0.001 \).
Table 3: Effect of oral Leu gavage on mTOR phosphorylation, 4EBP1 phosphorylation and S6K1 phosphorylation in cardiac muscle following acute alcohol intoxication in rats

<table>
<thead>
<tr>
<th>IP injection + gavage</th>
<th>Saline + water</th>
<th>Saline + Leu</th>
<th>Ethanol + water</th>
<th>Ethanol + Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTOR phosphorylation, phospho-mTOR(Ser2448)/total mTOR</td>
<td>215 ± 12</td>
<td>317 ± 23</td>
<td>155 ± 32</td>
<td>336 ± 51</td>
</tr>
<tr>
<td>mTOR phosphorylation, phospho-mTOR(Ser2448)/total mTOR</td>
<td>74 ± 3</td>
<td>110 ± 11</td>
<td>114 ± 14</td>
<td>138 ± 35</td>
</tr>
<tr>
<td>PRAS40 phosphorylation, phospho-mTOR(Ser2448)/total PRAS40</td>
<td>14 ± 2</td>
<td>13 ± 2</td>
<td>13 ± 2</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>PKB phosphorylation, phospho-PKB(Thr308)/total PKB</td>
<td>52 ± 8</td>
<td>51 ± 13</td>
<td>54 ± 7</td>
<td>68 ± 10</td>
</tr>
<tr>
<td>PKB phosphorylation, phospho-PKB(Thr308)/total PKB</td>
<td>15 ± 3</td>
<td>21 ± 6</td>
<td>14 ± 2</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>4EBP1 phosphorylation, γ-form/total 4EBP1</td>
<td>32 ± 2</td>
<td>40 ± 1</td>
<td>25 ± 2</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>S6K1 phosphorylation, phospho-S6K1(Thr389)/total S6K1</td>
<td>15 ± 2</td>
<td>17 ± 3</td>
<td>17 ± 1</td>
<td>14 ± 3</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 6–22. Gavage with Leu affected all variables for mTOR(Ser2448) and 4EBP1 phosphorylation, P < 0.005. *Different from saline + water, P < 0.05.

Discussion

Acutely elevating the blood ethanol concentration (>70 mmol/L) results in an inhibition of myocardial protein synthesis in rats. In the present set of experiments, acute ethanol injection caused a 50% reduction in the rate of protein synthesis in vivo. More importantly, the present study indicates that raising the plasma Leu concentration through oral gavage with a solution containing Leu in food-deprived animals reverses the inhibition of protein synthesis in hearts from rats following the equivalent of a binge-drinking episode. This observation differs significantly from the effects of Leu on protein synthesis in skeletal muscle, where acute ethanol intoxication induces Leu resistance (38). Hence, fundamental distinctions in the response to Leu following ethanol intoxication exist between muscles composed of fast-twitch fibers (gastrocnemius) and cardiomyocytes.

To understand these divergences in the response of protein synthesis to Leu, potential mechanistic interactions between alcohol intoxication and Leu administration in cardiac muscle were investigated by analysis of regulatory steps that modulate mRNA translation initiation. We have previously posited that translation initiation is limited by a reduced assembly of an active eIF4G-eIF4E complex (36). Therefore, we examined phosphorylation of the Thr389 residue as a measure of S6K1. Oral Leu administration did not alter phosphorylation of S6K1 in hearts under any condition (Table 3).

Phosphorylation of mTOR. mTOR, a proline-directed serine-threonine protein kinase, is posited as a common upstream kinase in a signaling cascade leading to phosphorylation of 4EBP1 and S6K1 (37). mTOR’s activity is regulated in part by phosphorylation. Ethanol intoxication did not significantly affect the extent of mTOR phosphorylated on Ser2448 or Ser2481 (Table 3). However, Leu gavage significantly increased the extent of phosphorylation of mTOR(Ser2448). None of the treatments altered the total amount of mTOR in cardiac muscle (data not shown).

PRAS40 (proline-rich Akt/PKB substrate 40 kDa) is an mTOR-binding protein that mediates PKB signaling to mTOR. PRAS40 silencing uncouples the response of mTOR to PKB signals. Furthermore, PRAS40 phosphorylation by PKB and association with 14–3–3, a cytosolic anchor protein, appear crucial for growth hormone-stimulated mTOR activity. Therefore, we examined the possible role of ethanol intoxication and Leu gavage on phosphorylation of PRAS40. Neither ethanol intoxication nor Leu gavage significantly affected the extent of PRAS40 phosphorylated on Thr246 (Table 3). None of the treatments altered the total amount of PRAS40 in cardiac muscle (data not shown).

Because PKB is involved in the phosphorylation of PRAS40, we also examined the extent of phosphorylation of PKB. Neither ethanol intoxication nor Leu gavage significantly affected the extent of PKB phosphorylated on either Thr308 or Ser473 (Table 3). Hence, we do not think changes in PRAS40 phosphorylation influence myocardial mTOR phosphorylation following acute ethanol administration.

eIF4E phosphorylation. To further investigate the potential mechanisms responsible for the ethanol-induced inhibition of protein synthesis, we examined the effect of Leu on phosphorylation of eIF4E (Table 1). Neither ethanol intoxication nor Leu gavage significantly affected the extent of phosphorylated eIF4E in cardiac muscle. Therefore, it is unlikely that the ability of Leu to augment rates of protein synthesis occurred as a result of increased phosphorylation state of eIF4E.
isoforms of 4E-BP bind to eIF4E with high affinity, preventing assembly of a translationally active eIF4F complex (43,44). The binding affinity of 4E-BP is controlled through phosphorylation. Phosphorylation of 4E-BP reduces the binding affinity for eIF4E and thereby relieves the translational repression. Acute ethanol administration increased the sequestration of 4EBP1 in the inactive eIF4E-4EBP1 complex. Gavage with a solution containing Leu reduced the binding of the eIF4E to 4EBP1 in cardiac muscle from rats injected with ethanol. Hence, reduced binding of eIF4E with 4EBP1 would be expected to enhance availability for eIF4E to associate with eIF4G. Taken together, the results are consistent with a combined role of eIF4G phosphorylation and increased availability of eIF4E as a means to increase the assembly of an active eIF4G-eIF4E complex following oral gavage with Leu in hearts from rats given ethanol.

The biochemical mechanism enabling Leu to stimulate eIF4G-eIF4E assembly and hence protein synthesis has not been fully illuminated. The pathways activated by mTOR have been implicated in controlling protein synthesis. In the present study, the extent of mTOR phosphorylation on both Ser2448 and Ser2481 did not significantly differ in animals receiving water in their gavage. In contrast, gavage with a solution containing Leu raised the extent of phosphorylation of mTOR on Ser2448, phosphorylation on Ser2481, however, was not affected. Likewise, mTOR Ser2481 phosphorylation in the myocardium is not increased in rats provided a meal. Hence, activation of the mTOR Ser2481 pathway is not required for the stimulation of eIF4G-eIF4E complex formation in heart. Leu did not affect the phosphorylation of Thr389 of S6K1. Similarly, rats provided with a meal did not enhance S6K1 phosphorylation in cardiac muscle (18). Furthermore, studies in cells in culture indicate outputs from mTOR to modify phosphorylation of 4EBP1 and S6K1 are distinct (45). The results of the present study are consistent with these reports.

In summary, Leu gavage stimulated protein synthesis in the myocardium from rats given ethanol acutely. The mechanism may involve an enhanced formation of the active eIF4G-eIF4E complex in cardiac muscle following acute ethanol intoxication. A positive linear relationship with a slope significantly different than zero between rates of protein synthesis and association of eIF4G with eIF4E was observed in the present study, similar to previous conclusions obtained in skeletal muscle in response to insulin (32). The mechanism for the increased formation of the eIF4G-eIF4E complex appeared to differ in control and ethanol-treated rats. In rats injected with saline, we could not detect any significant differences in potential mechanisms responsible for this change. Leu gavage did not significantly increase the eIF4G phosphorylation state or decrease the association of eIF4E with 4EBP1 in control rats. This result is in contrast to previous reports in heart, where the increase in the eIF4G-eIF4E complex following meal feeding was associated with an augmented eIF4G phosphorylation state and reduced association of eIF4E with 4EBP1. The reason for this apparent discrepancy is unknown. However, the effect of meal feeding on these parameters is no longer sustained after 3 h of cessation of feeding. In the present study, the eIF4G phosphorylation state and association of eIF4E with 4EBP1 were measured 2.5 h after administration of Leu and therefore may be at a time when the effects of Leu on these signaling pathways are waning. In contrast, Leu gavage enhanced phosphorylation of eIF4G and decreased the abundance of eIF4E associated with 4EBP1 in cardiac muscle from rats acutely given ethanol. The effect of Leu gavage to enhance the abundance of myocardial eIF4E-eIF4G could be related to the same pathway activated in control rats and/or the eIF4G phosphorylation state and association of eIF4E with 4EBP1 in rats treated with ethanol.

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