Alcohol impairs leucine-mediated phosphorylation of 4E-BP1, S6K1, eIF4G, and mTOR in skeletal muscle

Charles H. Lang, Robert A. Frost, Nobuko Deshpande, Vinayshree Kumar, Thomas C. Vary, Leonard S. Jefferson, and Scot R. Kimball. Alcohol impairs leucine-mediated phosphorylation of 4E-BP1, S6K1, eIF4G, and mTOR in skeletal muscle. Am J Physiol Endocrinol Metab 285: E1205–E1215, 2003.—Acute alcohol (EtOH) intoxication impairs skeletal muscle protein synthesis. Although this impairment is not associated with a decrease in the total plasma amino acid concentration, EtOH may blunt the anabolic response to amino acids. To examine this hypothesis, rats were administered EtOH or saline (Sal) and 2.5 h thereafter were orally administered either leucine (Leu) or Sal. The gastrocnemius was removed 20 min later to assess protein synthesis and signaling components important in translational control of protein synthesis. Oral Leu increased muscle protein synthesis by the same magnitude in Sal- and EtOH-treated rats. However, the increase in the latter group was insufficient to overcome the suppressive effect of EtOH, and the rate of synthesis remained lower than that observed in rats from the Sal-Sal group. Leu markedly increased phosphorylation of Thr residues 36, 47, and 70 on 4E-binding protein (BP)1 in muscle from rats not receiving EtOH, and this response was associated with a redistribution of eukaryotic initiation factor (eIF) 4E from the inactive eIF4E·4E-BP1 to the active eIF4E·eIF4G complex. In EtOH-treated rats, the Leu-induced phosphorylation of 4E-BP1 and changes in eIF4E availability were partially abrogated. EtOH also prevented the Leu-induced increase in phosphorylation of eIF4G, the serine/threonine protein kinase S6K1, and the ribosomal protein S6. Moreover, EtOH attenuated the Leu-induced phosphorylation of the mammalian target of rapamycin (mTOR). The ability of EtOH to blunt the anabolic effects of Leu could not be attributed to differences in the plasma concentrations of insulin, insulin-like growth factor I, or Leu. Finally, although EtOH increased the plasma corticosterone concentration, inhibition of glucocorticoid action by RU-486 was unable to prevent EtOH-induced deficits in the ability of Leu to stimulate 4E-BP1, S6K1, and mTOR phosphorylation. Hence, ethanol produces a leucine resistance in skeletal muscle, as evidenced by the impaired phosphorylation of 4E-BP1, eIF4G, S6K1, and mTOR, that is independent of elevations in endogenous glucocorticoids.

AMINO ACIDS, acting as nutritional signals, are of central importance in the regulation of many cellular processes, including protein synthesis. Extensive data in the literature indicate that amino acid feeding after brief starvation increases the synthesis of mixed proteins in skeletal muscle. Furthermore, elevated amino acids also increase the synthesis of both myofibrillar and sarcoplasmic proteins. Amino acids not only provide substrate for protein synthesis but are also now recognized to stimulate a number of cell-signaling pathways important in the regulation of mRNA translation. Although the recognition site(s) by which amino acid availability is sensed is a matter of controversy, the anabolic effects of amino acids are known to involve signal transduction pathways leading to the stimulation of translational efficiency. Translation of mRNA constitutes a multistep reaction catalyzed by a large number of factors that regulate the processes of initiation, elongation, and termination. Under in vivo conditions and in the isolated perfused muscle preparation, amino acids stimulate protein synthesis by increasing the rate of translation initiation. Moreover, the ability of a full complement of amino acids to increase the protein synthetic rate is largely, if not exclusively, dependent on the presence of the branched-chain amino acid leucine.

An important control point in translation initiation involves the recruitment of the 43S preinitiation complex to the mRNA that is mediated by the eukaryotic initiation factor (eIF)-4F complex. This complex is heterotrimeric, being composed of eIF4E, -4G, and -4A, with each subunit having discrete functions. Of these subunits, eIF4E is least abundant in muscle and under many conditions is considered to be rate limiting in the binding of mRNA to ribosomes. eIF4E binds to the mRNA cap structure present at the 5' end of all nuclear transcribed mRNAs to form an eIF4E·mRNA complex. During translation initiation, the eIF4E·mRNA complex binds to the scaffold protein eIF4G and the RNA helicase eIF4A to form the active eIF4F complex, thereby allowing translation to proceed. The binding of eIF4E to eIF4G is controlled in part by a family of...
cap-dependent translational repressors, referred to as eIF4E-binding proteins (BP). In skeletal muscle the most prominent member of this family is 4E-BP1. This binding protein acts as a molecular mimic of eIF4G and obstructs the interaction of eIF4G with eIF4E, limiting assembly of the active eIF4F complex (17). Hyperphosphorylation of 4E-BP1 liberates it from eIF4E and thereby facilitates binding of eIF4E with eIF4G. Modulating eIF4F complex assembly by regulating the phosphorylation status of 4E-BP1 has been observed in cultured L6 myoblasts (20) as well as in various tissues in vivo, including skeletal muscle (3, 31), in response to leucine.

Whereas a wealth of evidence supports the theory that activation of protein kinase B (PKB) is required for insulin and other growth factors to stimulate protein synthesis (11), amino acids appear to stimulate 4E-BP1 phosphorylation and protein synthesis via a PKB-independent pathway (15, 17, 20). The mammalian homolog of the yeast target of rapamycin protein kinase (mTOR) appears to represent a site of convergence for the anabolic effects of insulin and amino acids, and it also appears responsible for the phosphorylation of several downstream signaling molecules, including 4E-BP1. Inhibition of mTOR activity by the immunosuppressant drug rapamycin decreases the extent of 4E-BP1 phosphorylation induced by amino acids (3, 14). However, whether mTOR increases 4E-BP1 phosphorylation directly by functioning as a protein kinase or indirectly by inhibiting the activity of a protein phosphatase that dephosphorylates 4E-BP1 is unresolved.

The phosphorylation and activation of the serine (Ser)/threonine (Thr) protein kinase S6K1 are also markedly increased by either a complete mixture of amino acids or leucine alone (3, 9, 16). Full and complete activation of S6K1 requires hierarchical multisite phosphorylation of sites in the linker region. Of these phosphorylation sites, Thr389 is of critical importance in this regard (49). Moreover, the leucine-induced phosphorylation of Thr389 is also inhibited in a rapamycin-dependent manner (3), implicating mTOR in the activation of this downstream kinase. A physiologically relevant substrate for S6K1 is the ribosomal protein (rp) S6, which is important in the translational regulation of the mRNA family that encodes proteins containing a terminal oligopyrimidine tract (TOP) downstream of their transcription initiation site (6). Leucine causes a rapid phosphorylation of rpS6 in skeletal muscle (3) and in other tissues (e.g., liver; see Ref. 4) and stimulates translation initiation by enhancing the affinity of the ribosome for binding TOP-containing mRNAs. As expected, pretreatment with rapamycin completely prevents the leucine-induced phosphorylation of S6 (3). Therefore, collectively, these data suggest that mTOR either directly or indirectly controls the phosphorylation of both 4E-BP1 and S6K1 and, furthermore, represents a likely bifurcation point for distal parallel signaling pathways (43).

Acute alcohol intoxication decreases muscle protein synthesis, and this response results from an impairment in translation initiation (21, 23–25, 38). Specifically, under basal conditions, alcohol impairs the phosphorylation of both 4E-BP1 and S6K1 in the gastrocnemius (21). This impairment cannot be ascribed to decreases in the prevailing plasma concentrations of insulin, insulin-like growth factor (IGF)-I, or leucine (21–24, 26). However, the ability of exogenous insulin and IGF-I to stimulate phosphorylation of S6K1, and to a lesser extent 4E-BP1, in muscle is markedly decreased in alcohol-treated rats (21). Therefore, the purpose of the present study was to determine whether acute alcohol intoxication prevents or attenuates the ability of leucine to stimulate the phosphorylation of S6K1 and/or 4E-BP1 in skeletal muscle. Moreover, we also determined whether alcohol impaired basal and leucine-stimulated phosphorylation of mTOR, because this kinase appears to be a common intermediate and represents a point of convergence for the stimulatory action of growth factors and amino acids on protein synthesis. Additionally, because acute alcohol intoxication also produces a rapid and sustained increase in the plasma glucocorticoid concentration, the role of endogenous corticosterone in mediating the alcohol-induced changes in leucine-stimulated changes in the phosphorylation status of mTOR, 4E-BP1, and S6K1 was also assessed by use of the glucocorticoid receptor antagonist RU-486.

**MATERIALS AND METHODS**

**Experimental protocol.** Pathogen-free male Sprague-Dawley rats (200 ± 10 g, Charles River Breeding Laboratories, Cambridge, MA) were used in all studies. The rats were housed in a controlled environment and provided commercial laboratory food and water ad libitum for ≥1 wk before the start of the study. On the day of the experiment, overnightfasted rats were divided randomly into two groups (–16–18 animals/group); the alcohol-treated group was injected intraperitoneally with ethanol (75 mmol/kg body wt; 20% wt/vol in saline), and the control group was injected intraperitoneally with an equal volume of 0.9% saline. This dose of ethanol was selected because it significantly decreases muscle protein synthesis (23, 38). Both saline- and alcohol-treated rats were then administered either saline (0.155 mol/l) or 1.35 g/kg body wt leucine (prepared as 54.0 g/l of l-amino acid in distilled water) by oral gavage 2.5 h after administration of alcohol (1–3). The amount of leucine administered approximates that consumed in a 24-h period when rats of this age and strain are provided free access to food (1). Therefore, four groups of rats were used in these studies: saline-saline (also referred to as the “control” group), saline-leucine, alcohol-saline, and alcohol-leucine.

In the first study, rats in all four experimental groups were injected intravenously with 1-[3,4,5,6-3H]phenylalanine (Phe; 150 mM, 30 μCi/ml; 1 ml/100 g body wt) 10 min after the oral administration of leucine or saline. After an additional 10-min period, trunk blood was collected in heparinized tubes (23, 48). The gastrocnemius was rapidly excised and frozen between aluminum blocks precooled to the temperature of liquid nitrogen. The frozen tissues were powdered under liquid nitrogen with a mortar and pestle. Blood samples were centrifuged (13,000 g for 1 min at 4°C), and plasma was collected. Tissue and plasma samples were stored at 70°C until analyzed. A portion of the powdered tissue was homogenized in ice-cold perchloric acid, and the supernatant
was used to estimate the rate of incorporation of [3H]Phe into protein (23, 48). The specific radioactivity was calculated by dividing the amount of radioactivity in the peak corresponding to Phe by the concentration of the amino acid in the same fraction. Rates of protein synthesis were calculated as previously described (23, 48) by using the mean plasma Phe specific activity as the precursor pool.

In the second study, rats were prepared as described for the first study except that Phe was not injected. Rats were anesthetized intraperitoneally with pentobarbital sodium, and tissues were removed 20 min after oral administration of alcohol or saline and used to determine signaling components central to the regulation of peptide-chain initiation.

Previous studies demonstrate that acute alcohol intoxication increases the circulating corticosterone concentration severalfold (21) and, in addition, that elevated glucocorticoids impair translation initiation in rats (44). Therefore, a third study was performed to assess the role of glucocorticoids in mediating the alcohol-induced changes in leucine-stimulated signal transduction. Rats were pretreated with the glucocorticoid receptor antagonist RU-486 (Mifepristone; Sigma, St. Louis, MO) or saline. RU-486 was given intraperitoneally in a 1:1 ratio of ice-cold homogenization buffer (in mM: 20 HEPES, pH 7.4, 2 EGTA, 50 NaF, 100 KCl, 0.2 Na2HPO4, 0.1 MgCl2, 0.1 CaCl2, and 0.5 sodium vanadate) with a Polytron homogenizer in phosphate-buffered saline including 0.1% Tween 20) and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit) at room temperature for 2 h. The blots were developed with enhanced chemiluminescence (ECL) Western blotting reagents by following the manufacturer’s (Amersham) instructions. The blots were exposed to X-ray film and quantified using a BioRad UltraMatic Plus intensifying screen. After development, the film was scanned (Microtek ScanMaker IV) and analyzed using National Institutes of Health Image 1.6 software.

The 4E-BP1/eIF4E and eIF4G/eIF4E complexes were quantified as described previously (23, 24, 43). Briefly, eIF4E was immunoprecipitated from aliquots of 10,000 g supernatants with an anti-eIF4E monoclonal antibody (Drs. Jefferson and Kimball; Hershey, PA). Antibody-antigen complexes were collected using magnetic beads, as described above, and subjected to electrophoresis using a 7.5 or 15% polyacrylamide gel. Proteins were then electrophoretically transferred to a nitrocellulose membrane. The blots were incubated with a mouse anti-human eIF4E antibody, a rabbit anti-rat 4E-BP1 antibody, or a goat anti-eIF4F antibody for 12 h at 4°C. The phosphorylated forms of 4E-BP1 were measured after immunoprecipitation of 4E-BP1 from the tissue homogenates after centrifugation at 10,000 g. The various phosphorylated forms of 4E-BP1 were separated by SDS-PAGE and analyzed by protein immunoblotting. The blots were then developed with ECL, and the autoradiographs were scanned for analysis as we have described.

Statistical analysis. Experimental data for each condition are summarized as means ± SE, where the number of animals was 8–9 for each treatment group. Statistical evaluation of the data was performed using ANOVA followed by the Student-Newman-Keuls test to determine treatment effect. Differences between the groups were considered significant when P < 0.05.

RESULTS

Alcohol- and leucine-induced changes in muscle protein synthesis. Figure 1 illustrates that acute alcohol intoxication markedly decreased protein synthesis in gastrocnemius by ~70%. Oral leucine increased the protein synthetic rate an equivalent amount in both groups (e.g., ~0.3 nmol Phe·mg protein−1·h−1). However, the rate of muscle protein synthesis in the alcohol + leucine group was still significantly lower than that observed in control (e.g., saline + saline) animals.

Alterations in eIF4E distribution and eIF4G phosphorylation. The mechanistic interactions between alcohol and leucine were investigated by analyzing known regulatory steps in the control of translation
initiation. In this regard, neither alcohol nor leucine significantly altered the total amount of eIF4E in gastrocnemius (Fig. 2A). In contrast, acute alcohol intoxication increased the amount of eIF4E bound to 4E-BP1 (73%; Fig. 2B and C) and decreased the amount of eIF4E bound to eIF4G (64%; Fig. 2D and E) under basal (e.g., no leucine) conditions. Orally administered leucine markedly decreased the amount of the inactive eIF4E-4E-BP1 complex (60%) and increased the amount of the active eIF4E-eIF4G complex (64%) in rats not receiving alcohol. These changes are consistent with the known anabolic nature of the leucine stimulus (2, 3). Leucine administration produced quantitatively similar changes in eIF4E distribution in muscle from alcohol-treated rats. However, the leucine-induced changes in alcohol-treated rats only returned values to those observed in the saline-saline group.

The interaction between eIF4E and eIF4G can also be regulated, at least in part, by the phosphorylation of eIF4G. The phosphorylation of this component of the eIF4F complex is enhanced by mitogen and serum stimulation (40) and inhibited by rapamycin (39). In saline-saline rats, there was a low constitutive phosphorylation of eIF4G (Fig. 3, top) in skeletal muscle. Alcohol produced a small, albeit statistically significant, decrease in the content of phosphorylated eIF4G (Fig. 3). The extent of eIF4G phosphorylation in muscle was increased more than fivefold in control rats administered leucine compared with values from the salinesaline group. In contrast, there was no significant leucine-induced increase in eIF4G phosphorylation in rats administered alcohol. Finally, the abovementioned changes in eIF4G phosphorylation were not due to a significant leucine- or alcohol-induced change in the content of total eIF4G in muscle (Fig. 3, top).

Phosphorylation of 4E-BP1. To further define the mechanism through which alcohol and amino acids interact to modulate eIF4E availability, we examined the phosphorylation state of 4E-BP1. Mitogen stimulation increases phosphorylation of 4E-BP1 on multiple sites, resulting in the dissociation of 4E-BP1 from eIF4E and thereby allowing binding of eIF4G to free eIF4E (35). The abovementioned alcohol-induced changes in eIF4E distribution were associated with an 80% decrease in the amount of the hyperphosphorylation.
lated γ-isofrom of 4E-BP1 in muscle from rats not receiving alcohol (Fig. 4A). There are at least six candidate serine and threonine residues in 4E-BP1 that are sites for phosphorylation (12). However, use of one-dimensional SDS-PAGE does not permit identification of site-specific phosphorylation of 4E-BP1, because the phosphorylation of some residues fails to produce visible shifts in electrophoretic mobility. Therefore, we also used phosphospecific antibodies to detect whether alcohol or leucine alters the phosphorylation of specific threonine residues in muscle under in vivo conditions. The data presented in Fig. 4 (B, C, and D, respectively) reveal that the alcohol-induced decrease in 4E-BP1 phosphorylation was attributable to a generalized decrease in the phosphorylation of Thr37, Thr46, and Thr70. Conversely, the γ-phosphorylated form of 4E-BP1 was increased ~70% in the rats administered saline-leucine. This leucine-induced increase was due to a coordinated increase in the phosphorylation of each of the three threonine residues examined (Fig. 4, A-D). In alcohol-treated rats, leucine increased the amount of γ-phosphorylated 4E-BP1, but values returned only to those observed in the saline-saline group (Fig. 4A). Examination of the effect of alcohol on leucine-induced stimulation of 4E-BP1 phosphorylation demonstrated differences depending on the specific threonine residues assessed. That is, in alcohol-treated rats, the ability of leucine to stimulate the phosphorylation of 4E-BP1-Thr37 was unaltered (Fig. 4B), but alcohol resulted in a progressively greater inhibition of phosphorylation of Thr46 and Thr70 in response to leucine (Figs. 4, C and D).

S6K1 and S6 phosphorylation. When S6K1 is subjected to SDS-PAGE, it resolves into multiple bands with different electrophoretic mobilities dependent on the extent of phosphorylation at various serine/threonine sites. In this regard, the most slowly migrating forms represent the heavily phosphorylated, and thus highly active, form of the kinase. There was constitutive S6K1 phosphorylation in muscle from rats in the saline-saline group (Fig. 5A). Acute ethanol alone ap-

![Fig. 3. Effect of acute alcohol (EtOH) intoxication on Leu-stimulated phosphorylation of eIF4G. Top: a representative immunoblot of Ser1108-phosphorylated (p) and total eIF4G. Bar graph, densitometric analysis of eIF4G phosphorylation, where the value from control rats treated with saline (ip) + saline (oral gavage) was set at 1.0 AU. The 4 experimental groups are as in Fig. 1. Values are means ± SE; n = 8–9 per group. Means with different letters are statistically different from each other (P < 0.05).](image3)

![Fig. 4. Effect of acute alcohol (EtOH) intoxication on Leu-stimulated phosphorylation of 4E-BP1. A, top: a representative immunoblot for total 4E-BP1 and positions of the α-, β-, and γ-isofroms. B, C, and D: representative immunoblots of the amount of 4E-BP1 phosphorylation on Thr37, Thr46, and Thr70, respectively. Bar graphs represent densitometric analysis of immunoblots, where the basal value from control rats treated with saline (ip) + saline (oral gavage) was set at 1.0 AU. Note that the bar graph for A quantifies the relative amount 4E-BP1 in the hyperphosphorylated γ-form. The 4 experimental groups are as in Fig. 1. Values are means ± SE; n = 8–9 per group. Means with different letters are statistically different from each other (P < 0.05).](image4)
preciably increased the mobility of the bands, indicating a relative dephosphorylation of S6K1. In the absence of alcohol, oral administration of leucine decreased the mobility of the electrophoretic bands, suggesting an increased phosphorylation of the kinase. In contrast, acute alcohol intoxication largely prevented the nutrient-stimulated phosphorylation of S6K1, as evidenced by the disappearance of slower electrophoretic species (Fig. 5A).

We next examined different phosphorylation sites in S6K1 using phosphospecific antibodies. In muscle from fasted control rats, there was essentially no basal phosphorylation of the Thr$^{389}$ (Fig. 5, B and D) or Thr$^{421}$/Ser$^{424}$ sites (Fig. 5, C and E). There was a marked increase in the phosphorylation of S6K1 at both sites in muscle from rats in the saline-leucine group. In contrast, acute ethanol exposure completely prevented the normal leucine-induced increase in phosphorylation at Thr$^{389}$ and Thr$^{421}$/Ser$^{424}$.

The phosphorylation state of rpS6, a physiologically relevant S6K1 substrate, was also determined. rpS6 is a component of the 40S ribosome, and previous studies report that phosphorylation of rpS6 occurs concomitantly with an increase in protein synthesis (19). In the current study, rpS6 exhibited a constitutive level of phosphorylation in muscle from control rats (Fig. 6, top). Acute alcohol intoxication decreased basal S6 phosphorylation by almost 50%. In contrast, S6 phosphorylation was increased 3.9-fold in the saline-leucine group (Fig. 6). Moreover, S6 phosphorylation was only modestly increased in muscle obtained from alcohol-treated rats in response to leucine. Values in this group were increased to those observed in the saline-saline group but remained markedly depressed compared with values from the saline-leucine group.

**mTOR.** mTOR is a proline-directed serine-threonine protein kinase and is believed to be a common intermediate in the phosphorylation of both 4E-BP1 and S6K1 that is produced by growth factors and amino acids (13, 43). Its activity in part is regulated by phosphorylation. As illustrated in Fig. 7, there was a slight constitutive phosphorylation of mTOR (Ser$^{2448}$) in muscle from rats in the saline-saline group. Acute alcohol intoxication decreased the basal content of mTOR phosphorylation by ~50%. Leucine administration increased mTOR phospho-Ser$^{2448}$ immunoreactivity by about threefold, and this stimulation was diminished by ~50% in alcohol-treated rats. Neither alcohol nor leucine altered the content of total mTOR in muscle at the time point examined.

**Plasma determinations.** Table 1 presents data on selected plasma hormone concentrations as well as the plasma concentrations of leucine and alcohol in the
Fig. 7. Effect of acute EtOH intoxication on Leu-stimulated phosphorylation of mammalian target of rapamycin (mTOR). Top: a representative immunoblot of Ser2448-phosphorylated (p) and total mTOR. Bar graph, densitometric analysis of mTOR phosphorylation normalized for total mTOR, where value from control rats treated with saline (p) + saline (oral gavage) was set at 1.0 AU. The 4 experimental groups are as in Fig. 1. Values are means ± SE; n = 8–9 per group. Means with different letters are statistically different from each other (P < 0.05).

four experimental groups. Insulin and IGF-I are anabolic hormones, and changes in their prevailing concentration can affect translation initiation (24). Alcohol had no significant effect on the plasma insulin concentration compared with values from control (saline-saline) rats. The plasma insulin concentration was increased approximately threefold in the saline-leucine group, and a comparable elevation was observed in alcohol-treated rats in response to leucine. There was no significant difference in the plasma IGF-I concentration between groups.

Elevations in glucocorticoids are known to adversely influence translation initiation (44). Acute alcohol intoxication increased the plasma corticosterone concentration compared with that from rats not receiving alcohol; the elevation in corticosterone was similar in both alcohol-treated groups.

There was no significant difference in the plasma leucine concentration between the saline-saline and alcohol-saline groups. Furthermore, although oral leucine administration increased the plasma leucine concentration, this increase was comparable in both groups of animals.

The plasma alcohol concentration at the time animals were killed averaged ~350 mg/dl and did not differ significantly between rats in the alcohol-saline and alcohol-leucine groups (Table 1).

Role of endogenous glucocorticoids. Figure 8 illustrates that the ability of alcohol to prevent or attenuate the leucine-induced increase in the phosphorylation of 4E-BP1, S6K1, and mTOR was not significantly ameliorated by RU-486 pretreatment. Previous studies (21) have also indicated that the glucocorticoid receptor antagonist RU-486 does not prevent the alcohol-induced decrease in 4E-BP1 phosphorylation or the alteration in eIF4E availability; hence, an RU-486 + alcohol group was not included in the current study.

DISCUSSION

Basal alcohol-induced changes. We (23) and others (38) have previously demonstrated that acute alcohol intoxication decreases the rate of muscle protein synthesis and that this defect is believed responsible, at least in part, for the erosion of lean body mass and the proximal myopathy observed in animals and humans in response to chronic alcohol abuse (24). Moreover, the acute effects of alcohol on muscle protein synthesis are due to an impairment in the rate of translational efficiency and not the number of functioning ribosomes (23, 38). We have previously reported that the alcohol-induced decrease in basal (e.g., no exogenous stimulus) translation initiation results from a decrease in the phosphorylation of both 4E-BP1 and S6K1 (21). The reduction in 4E-BP1 phosphorylation is accompanied by the expected redistribution of eIF4E from the active eIF4E·eIF4G complex to the inactive eIF4E·4E-BP1 complex. Furthermore, the ability of alcohol to acutely impair the constitutive phosphorylation of S6K1 was associated with a concomitant reduction in the phosphorylation of rpS6. These changes are consistent with the observed reduction in the synthetic rate of both total and myofibrillar proteins in response to acute alcohol intoxication (38).

The results of the present study not only confirm the abovementioned findings but also provide novel insights into the possible mechanism for these alterations. First, acute alcohol intoxication decreased mTOR phosphorylation, and this reduction was independent of a change in the total mTOR content in muscle. However, the role phosphorylation plays in

Table 1. Effect of acute alcohol intoxication on plasma concentrations of hormones, leucine, and alcohol

<table>
<thead>
<tr>
<th></th>
<th>Saline-Saline</th>
<th>Saline + Leu</th>
<th>EtOH-Saline</th>
<th>EtOH + Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, ng/ml</td>
<td>0.23 ± 0.02a</td>
<td>0.91 ± 0.14b</td>
<td>0.32 ± 0.06a</td>
<td>0.87 ± 0.12b</td>
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<tr>
<td>IGF-1, ng/ml</td>
<td>816 ± 25</td>
<td>841 ± 32</td>
<td>719 ± 43</td>
<td>825 ± 33</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>128 ± 10a</td>
<td>137 ± 15a</td>
<td>445 ± 78b</td>
<td>496 ± 52b</td>
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<tr>
<td>Leucine, μmol/l</td>
<td>169 ± 12a</td>
<td>1,782 ± 204b</td>
<td>175 ± 13a</td>
<td>1,844 ± 186b</td>
</tr>
<tr>
<td>Ethanol, mg/dl</td>
<td>ND</td>
<td>ND</td>
<td>387 ± 41</td>
<td>362 ± 44</td>
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Values are means ± SE; n = 8–9/group. EtOH, alcohol; ND, not detectable. Means with different letters for a specific parameter are statistically significantly different from each other (P < 0.05).

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controlling mTOR activity is unclear (13). Because mTOR is believed to represent a point of divergence for the regulation of both 4E-BP1 and S6K1 phosphorylation, these data may suggest that the kinase activity of mTOR has directly or indirectly been suppressed by alcohol. However, previous in vivo studies (21) have failed to demonstrate an alcohol-induced decrease in protein synthesis or in the phosphorylation of S6K1 (Thr389), and mTOR (Ser2448), where the value from control rats treated with saline (ip) + saline (oral gavage) was set at 1.0 AU. Values are means ± SE; n = 8–9 per group. Means with different letters are statistically different from each other (P < 0.05).

Alcohol-induced inhibition of translational effects of leucine. In the present study, orally administered leucine increased protein synthesis in gastrocnemius ~25%. A comparable stimulation of protein synthesis has been previously reported for leucine in fasted rats (2, 31). In contrast, leucine does not appear to increase muscle protein synthesis in fed rats (44). The present study did not assess whether the leucine-induced changes in eIF4E distribution were a direct effect of leucine or occurred secondary to the concomitant increase in circulating insulin. Numerous studies indicate that the physiological increase in plasma insulin is not sufficient to stimulate muscle protein synthesis in fasted adult rats (10, 37). Furthermore, when fasted rats are administered an oral carbohydrate load, which increases the plasma insulin concentration to that seen in leucine-treated rats, muscle protein synthesis was not increased (3, 31). However, prevention of the leucine-induced hyperinsulinemia by the infusion of somatostatin was efficacious in preventing the normal increase in muscle protein synthesis (2). Therefore, these data and others suggest that the transient increase in insulin may have a permissive effect on enhancing leucine-induced stimulation of protein synthesis.

Alcohol partially prevented the leucine-induced redistribution of eIF4E in skeletal muscle and blunted the increase in the hyperphosphorylated γ-isoform of 4E-BP1. As mentioned above, 4E-BP1 is phosphorylated in a hierarchical manner, progressing from threonine residues 37 to 46 to 70 (12). Acute alcohol intoxication appears to have a progressive inhibitory effect on 4E-BP1 phosphorylation, as evidenced by the relatively normal phosphorylation of Thr37, modest impairment of Thr46 phosphorylation, and the marked suppression of Thr70 phosphorylation. The progressive
impairment in 4E-BP1 phosphorylation may suggest that alcohol alters the kinase or phosphatase activity specifically related to the phosphorylation or dephosphorylation of Thr70. The ability of alcohol to abrogate the stimulating effect of leucine on mTOR phosphorylation was similarly blunted, further suggesting a role for mTOR in the phosphorylation of 4E-BP1. Regardless of the exact mechanism, in alcohol-treated rats, leucine was able only to increase 4E-BP1 phosphorylation and shift eIF4E distribution back to values observed in muscle from rats in the saline-saline control group. These changes are generally consistent with the ability of alcohol to blunt the leucine-induced increase in muscle protein synthesis. Similarly, alcohol has also recently been reported to blunt the stimulation of muscle protein synthesis in fasted rats refed enterally, but not intravenously, with all amino acids, glucose, and lipids (45). The anabolic hormone IGF-I also increases 4E-BP1 phosphorylation and the amount of eIF4E bound to eIF4G (21). However, in contrast to the above-mentioned impairment in leucine stimulation, alcohol did not appear to markedly affect 4E-BP1 phosphorylation or the availability of eIF4E (21). The exact cellular mechanism whereby alcohol adversely influences leucine signaling, but not IGF-I signaling, is unknown.

In marked contrast to the partial efficacy of leucine at restoring eIF4F function noted above, alcohol essentially completely prevented the ability of leucine to stimulate the phosphorylation of eIF4G, as well as S6K1 and its substrate, the 40 S ribosome. According to the prevailing model of activation for S6K1, the sites in the autoinhibitory domain (Ser411, Ser418, Thr421, and Ser424) are phosphorylated by an upstream kinase whose identity has not been resolved (13). These phosphorylation events disrupt the interaction between the COOH-terminal and NH2-terminal domains, thereby permitting S6K1 to unfurl and exposing additional sites in the linker and kinase domains. Subsequently, the Thr428 residue in the linker domain is phosphorylated, and this step has been demonstrated to be necessary for the full and functional activation of S6K1 (49). Our results indicate that leucine coordinately upregulates phosphorylation of both Thr428 and Thr421/Ser424, results similar to those reported previously in muscle of both rats (2, 3) and humans (15). Moreover, alcohol prevented the leucine-induced stimulation of phosphorylation sites in both the autoregulatory and linker domains. Similarly, acute alcohol intoxication has also been previously reported to impair S6K1 phosphorylation by insulin or IGF-I in both skeletal (21) and cardiac (25) muscle, potentially suggesting a common mechanism. Finally, while this manuscript was under review, Sneddon et al. (45) reported that acute alcohol intoxication also prevents stimulation of S6K1 in response to an enterally administered complete diet.

The initiation factor eIF4G, which is a scaffolding protein important in the recruitment of the translational machinery to the 5'-end of mRNA, is also phosphorylated. Although growth factors stimulate the phosphorylation of eIF4G (39) and thereby possibly influence its activity, our data appear to be the first report regarding the ability of leucine to stimulate eIF4G phosphorylation in muscle under in vivo conditions. Despite the dramatic effect of leucine on eIF4G phosphorylation in control rats, alcohol treatment completely prevented the normally occurring increase in gastrocnemius. The complete suppression of the leucine-induced increase in eIF4G and S6K1 phosphorylation suggests that alcohol alters the function of a common intermediate.

Possible hormonal mediators. The alcohol-induced alterations in various components of mRNA translation could not be attributed to differences in the prevailing blood alcohol concentration between rats in the saline-leucine and EtOH-leucine groups. Likewise, the plasma concentration of leucine was comparable in both groups of alcohol-treated rats, suggesting that a difference in amino acid availability was also an unlikely causative factor in the divergent responses. Similarly, although leucine is a known insulin secretagogue (8), the extent of hyperinsulinemia was comparable in alcohol-treated and control rats. In contrast, the plasma corticosterone concentration differed markedly between rats in the saline-leucine and alcohol-leucine groups. This alcohol-induced activation of the hypothalamic-pituitary-adrenal axis has been extensively reported (21). Additionally, the potent synthetic glucocorticoid dexamethasone also prevents amino acid-induced hyperphosphorylation of S6K1 and 4E-BP1, as well as the stimulation of protein synthesis in muscle (44). Therefore, we postulated that the elevation in endogenous corticosterone might be a causative factor in the inability of leucine to stimulate S6K1 and eIF4G phosphorylation after acute alcohol intoxication. However, administration of the glucocorticoid receptor antagonist RU-486 failed to prevent or attenuate the alcohol-induced dephosphorylation of either S6K1 or eIF4G and also did not ameliorate the changes in eIF4E distribution or 4E-BP1 and mTOR phosphorylation. Therefore, whereas a pharmacological excess of glucocorticoids clearly impairs translation initiation and protein synthesis in skeletal muscle, the alcohol-induced defects in leucine signaling to the translational apparatus appear largely independent of elevations in endogenous glucocorticoids.

We thank Dr. Morris Birnbaum (University of Pennsylvania) for the generous gift of the anti-phospho-S6 antibody.

DISCLOSURES

This work was supported in part by National Institutes of Health Grants AA-11290 and DK-15658. V. Kumar was supported by Training Grant GM-08619.

REFERENCES


