IGF-I/IGFBP-3 ameliorates alterations in protein synthesis, eIF4E availability, and myostatin in alcohol-fed rats

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Am J Physiol Endocrinol Metab 286: E916–E926, 2004. First published January 28, 2004; 10.1152/ajpendo.00554.2003.—Chronic alcohol consumption decreases the concentration of the anabolic hormone IGF-I, and this change is associated with impaired muscle protein synthesis. The present study evaluated the ability of IGF-I complexed with IGF-binding protein (IGFBP)-3 to modulate the alcohol-induced inhibition of muscle protein synthesis in gastrocnemius. After 16 wk on an alcohol-containing diet, either the IGF-I/IGFBP-3 binary complex (BC) or saline was injected twice daily for three consecutive days. After the final injection of BC (3 h), plasma IGF-I concentrations were elevated in alcohol-fed rats to values not different from those of similarly treated control animals. Alcohol feeding decreased the basal rate of muscle protein synthesis by limiting translational efficiency. BC treatment of alcohol-fed rats increased protein synthesis back to basal control values, but the rate remained lower than that of BC-injected control rats. The BC partially reversed the alcohol-induced decrease in the binding of eukaryotic initiation factor (eIF)-4E with eIF4G. This change was associated with reversal of the alcohol-induced dephosphorylation of eIF4G but was independent of changes in the phosphorylation of either 4E-BP1 or eIF4E. However, BC reversed the alcohol-induced increase in IGF-I and muscle myostatin, known negative regulators of IGF-I action and muscle mass. Hence, exogenous IGF-I, administered as part of a BC to increase its circulating half-life, can in part reverse the decreased protein synthesis observed in muscle from chronic alcohol-fed rats by stimulating selected components of translation initiation. The data support the role of IGF-I as a mediator of chronic alcohol myopathy in rats.

EXCESSIVE ALCOHOL CONSUMPTION produces numerous biochemical, morphological, and functional changes in skeletal muscle (24, 31). Progressive proximal weakness and muscle atrophy are well-recognized characteristics of prolonged alcohol abuse. Histochemical and biochemical analyses reveal a preferential atrophy of muscle composed predominantly of fast-twitch type II fibers (e.g., gastrocnemius) with a relative sparing of slow-twitch type I fibers (e.g., soleus; see Refs. 28, 34, and 35). The prevalence of chronic alcoholic myopathy has been reported to range between 45 and 70% in alcoholics (38). The etiology of the myopathy is unclear but is undoubtedly multifactorial, with disturbances in electrolytes, peripheral neuropathy, oxidative damage, and inactivity being implicated (24, 38). Malnutrition per se is unlikely to be the sole cause of the myopathy, but nutritional deficiencies undoubtedly exacerbate the disease (37). A sustained erosion of lean body mass (LBM) increases morbidity and mortality in other catabolic conditions (6, 18) and remains a serious concern in those individuals that chronically abuse alcohol.

Tissue protein content is normally maintained by the dynamic balance between the opposing processes of protein synthesis and degradation. The ability of alcohol to modulate muscle proteolysis appears variable depending upon the study (24). In contrast, results from our laboratory (28, 51) and others (34, 36) consistently demonstrate that chronic alcohol consumption decreases muscle protein synthesis. Furthermore, this alcohol-induced decrease appears primarily related to an impairment in translational efficiency of mRNA (28).

Translation of mRNA on the ribosome consists of the following three phases: 1) initiation, whereby the initiator methionyl-tRNA (met-tRNAi) associates with mRNA bound to the 40S ribosomal subunit and the subsequent binding of the 40S ribosome to the 60S subunit, to form a complex capable of translation; 2) elongation, during which tRNA-bound amino acids are incorporated into growing polypeptide chains; and 3) termination, where the completed protein is released from the ribosome (42). The reduced translational efficiency observed after chronic alcohol feeding results primarily from an inhibition of peptide-chain initiation (28). In turn, the process of translation initiation involves two major regulatory steps. The first step controls binding of met-tRNA, to the 40S ribosomal subunit to form the 43S preinitiation complex. This reaction is mediated by eukaryotic initiation factor (eIF)-2 and is regulated by the activity of another initiation factor, eIF2B (39). The second tightly regulated step in translation initiation involves the binding of mRNA to the 43S preinitiation complex. This step requires the participation of the multifimeric eIF4F complex that is composed of eIF4A, eIF4E, and eIF4G (41). Of these proteins, eIF4E appears limiting in skeletal muscle; hence, the availability of eIF4E is important in stimulating the formation of the active eIF4F complex, mRNA translation, and, ultimately, protein synthesis. Under basal conditions, chronic alcohol consumption impacts selected elements of both regulatory steps in skeletal muscle, thereby limiting mRNA translation initiation (28).

Insulin-like growth factor (IGF)-I is an anabolic hormone that plays an essential role in the accretion of LBM (5). Chronic alcohol feeding in rats reduces the concentration of eukaryotic initiation factor-4E; eukaryotic initiation factor-4G; protein synthesis; myostatin; insulin-like growth factor I; insulin-like growth factor-binding protein
IGF-I in blood and muscle (21, 45). Furthermore, the decrease in IGF-I is proportional to the reduction in muscle protein synthesis and the formation of the functional eIF4F complex (24). Acute alcohol intoxication also induces an IGF-I resistance in skeletal muscle (19). Hence, alcohol feeding may impair muscle protein synthesis not only by reducing the circulating and local concentrations of IGF-I but also by diminishing the bioactivity of IGF-I.

Exogenous administration of free or unbound IGF-I stimulates muscle protein synthesis (13), and this effect is most pronounced in muscle composed predominantly of fast-twitch glycolytic fibers (3). Moreover, the ability of IGF-I to accelerate protein synthesis is mediated via an increased rate of translation initiation (49). However, the bioavailability of exogenously administered IGF-I is limited because of the rapid clearance (half-life <10 min) of this peptide hormone from the circulation (9). Thus achieving a sustained elevation of plasma IGF-I is difficult, and the resulting stimulation of protein synthesis is often relatively transient. The majority of IGF-I is bound to one of several binding proteins (e.g., IGFBP’s) in vivo, the most abundant of which is IGFBP-3 (40). Administration of IGF-I complexed to nonglycosylated IGFBP-3 has been used as an alternative to the injection of free IGF-I (16, 46, 47, 53). Once administered, the IGF-I/IGFBP-3 binds to the acid-labile subunit present in the blood, forming the naturally occurring ternary complex. Because of the size of this complex (e.g., 150 kDa), IGF-I administered in conjunction with IGFBP-3 has a slower rate of disappearance from the circulation, resulting in a more sustained bioactivity than injection of free IGF-I. Injection of this IGF-I/IGFBP-3 binary complex reverses the inhibition of muscle protein synthesis induced by semistarvation, bacterial infection, and thermal injury (16, 46, 47).

The primary purpose of the present investigation was to determine whether a binary complex, consisting of equal molar amounts of IGF-I and IGFBP-3, could modulate the inhibition of muscle protein synthesis produced by chronic alcohol feeding in rats and whether such changes were mediated via concomitant alterations in various initiation factors that regulate peptide-chain initiation. A secondary aim of the current study was to investigate the ability of this binary complex to reverse the alcohol-induced changes in several potential catabolic mediators, such as IGFBP-1, tumor necrosis factor (TNF)-α, and myostatin, which are known to adversely impact muscle protein synthesis. Finally, the net effect of the binary complex on protein balance was assessed by determining the increase in the total protein content of muscle.

METHODS AND MATERIALS

Experimental protocol. Specific pathogen-free, male Sprague-Dawley rats (Charles River Breeding Laboratories, Cambridge, MA) were housed at a constant temperature and exposed to a 12:12-h light-dark cycle. After a 1-wk period of quarantine, animals were maintained on an ethanol-containing agar block diet for 16 wk. Initially all rats were provided agar without alcohol for 2 days. Thereafter, half of the animals were fed agar containing 10% alcohol ad libitum, whereas the remaining rats were provided agar containing an equal caloric amount of dextrin-maltose. The ethanol content of the agar was increased to 20% and then 30% at 1-wk intervals. Rats were maintained on the 30% ethanol-agar block for the duration of the experimental protocol. Animals were also supplemented with ethanol-containing (10%) drinking water. Based on the average weight of the alcohol-containing agar consumed the previous day, control animals were provided approximately the same amount of agar without alcohol, but with the isocaloric substitute of dextrin-maltose. Control rats were also provided with alcohol-free water. The nutrient intake for both groups of animals was supplied by consumption of standard solid rodent chow (no. 8604; Harlan, Madison, MI) because the agar block, while providing a source of water and/or ethanol, has nominal nutritional value. Alcohol-fed rats were provided standard rat chow ad libitum. Control rats were pair-fed to match the average solid food consumption of the alcohol-fed rats. Thus the total caloric intake was not different between the two groups (Table 1). This model of chronic alcohol consumption has been previously reported to decrease circulating and tissue concentrations of the anabolic hormone IGF-I (25) and decrease muscle protein synthesis (28, 50).

Binary complex. The recombinant human binary complex was a kind gift provided by Celtrix Pharmaceuticals (now Insmed, San Jose, CA). Each protein was produced separately in Escherichia coli and then purified before complex formation, as previously described (2). The two proteins were mixed in a 1:1 molar ratio, corresponding to the naturally occurring protein complex, and the complex was further purified by ion exchange chromatography. Purity of the complex was verified by reversed-phase-high-pressure liquid chromatography (HPLC) and SDS-PAGE and was estimated to be >95%. Beginning on the morning of week 17, animals were randomly divided into two groups (i.e., binary complex treated and saline treated). Rats treated with binary complex were injected via a tail vein two times daily (0900 and 1800) at a dose of 5 μg/g body wt of the complex per

| Table 1. Weight gain, EtOH consumption, and plasma concentrations of IGF-I, insulin, glucose, and EtOH in control and EtOH-fed rats treated with the IGF-I/IGFBP-3 binary complex |
|---------------------------------------------------------------|---------------------------------|------------------------------|------------------------------|
| Body wt gain, g                                               | Control + BC                    | EtOH                         | EtOH + BC                     |
| 445±16*                                                       | 451±17*                        | 391±13*                      | 397±19†                      |
| Total energy consumption, kcal·kg⁻¹·day⁻¹                    | 269±21                         | 271±16                       | 275±19                       | 268±15                      |
| EtOH consumption, g·kg⁻¹·day⁻¹                               | 0*                             | 0*                           | 16±2*                        | 17±3*                       |
| EtOH, mM                                                      | ND                             | ND                           | 19.3±2.9                     | 21.9±3.2                    |
| IGF-I, ng/ml                                                  | 812±51*                       | 1.057±119*                   | 554±34†                      | 899±94*                     |
| Insulin, μU/ml                                                | 15±2                           | 17±2                         | 14±2                         | 15±3                        |
| Glucose, mM                                                   | 8.4±0.5                        | 8.1±0.6                      | 8.2±0.4                      | 8.0±0.5                     |

Values are means ± SE; n = 8–9 rats/group. BC, IGF-I/IGFBP-binding protein (IGFBP)-3 binary complex; EtOH, alcohol; ND, not detectable. Rats were provided the EtOH-containing diet ad libitum or pair-fed an isocaloric isoinosotrogenous diet for 16 wk. Thereafter, both groups of animals were subdivided, and half of the rats in each group were treated with IGF-I/IGFBP-3 or saline for the final three consecutive days of the study. Body weight was determined immediately before death, and weight gain was determined as the difference between the starting weight on day 1 and the final body weight. Total energy consumption and EtOH consumption were calculated on basis of intake during the final 3 days of feeding. However, total energy consumption determined at weekly intervals throughout the 16-wk protocol was not different between control and alcohol-fed rats (data not shown). Means with different superscripts for a specific parameter are statistically significantly different from each other (P < 0.05).
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injection. This dose of the binary complex was selected on the basis of previous studies by our laboratories indicating that this agent is capable of reversing the defects in muscle protein synthesis induced by sepsis and food restriction (46, 47). Time-matched alcohol-fed and pair-fed control rats each received an equal volume (1 ml) of saline. Treatment was continued for three consecutive days. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine.

Protein synthesis. On the morning of the 4th day, rats were injected with 0.5 mg/kg IGF-I/IGFBP-3 complex or saline. After this final treatment (3–4 h), rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Thereafter, the in vivo rate of global protein synthesis was determined in the gastrocnemius muscle using the flooding-dose technique, as originally described by Garlick et al. (14) and slightly modified by our laboratory (46, 49, 50). Catheters were placed in the jugular vein and carotid artery. Rats were injected intravenously with [1-2,3,4,5,6-3H]phenylalanine (150 nM, 30 μCi/ml; 1 ml/100 g body wt). Blood samples, collected from the arterial catheter in heparinized syringes, were obtained at 2, 6, and 10 min thereafter. The whole gastrocnemius was excised and weighed. A portion of the fresh muscle from each animal was taken for measurement of eIF2B activity and analysis of the eIF2B and the eIF4E system, and the remaining tissue was frozen between aluminum plates and stored at −70°C until analyzed.

A portion of the powdered muscle was used to estimate the rate of incorporation of [3H]phenylalanine in protein exactly as described previously (46, 50, 51). The specific radioactivity of the plasma phenylalanine was measured by HPLC using supernatant from TCA extracts of plasma. The specific radioactivity was calculated by dividing the amount of radioactivity in the peak corresponding to phenylalanine by the concentration of the amino acid in the same fraction. The rate of protein synthesis was expressed as nanomoles of phenylalanine incorporated per hour per gram wet weight of tissue. Qualitatively similar trends were observed when data were expressed per milligram protein.

Total RNA and translational efficiency. Total RNA was measured from muscle homogenates as previously described (46, 50, 51). The concentration of RNA in the supernatant was determined by measuring the absorbance at 260 nm and correcting for the absorbance at 232 nm. Values for RNA content were expressed per gram wet weight of muscle. These data were then used to calculate translational efficiency, which equals the rate of protein synthesis divided by the RNA content.

Amount of eIF2 and eIF2B and eIF-2B activity. The total amount and the phosphorylation state of the α-subunit of eIF-2 (eIF-2α) and the ε-subunit of eIF-2B (eIF-2Be) in muscle was estimated by protein immunoblot analysis, as described previously (23, 28, 50). eIF2 and eIF2Be were chosen because a change in the content and/or activity of these initiation factors correlates with alterations in protein synthesis (33). eIF2 consists of three subunits, of which the α-subunit appears important in regulating protein synthesis. Likewise, eIF2B is a multimeric protein consisting of five subunits, with the ε-subunit being the catalytic subunit. Previous studies have established that the expression of the ε-subunit is representative of the other subunits and the eIF2B holoenzyme. Tissues were homogenized and proteins separated by SDS-PAGE. Immunoblotting for eIF2α was performed with a rabbit polyclonal antibody raised against the phosphorylated form of eIF2α (anti-Ser51) or an antibody recognizing both phosphorylated and unphosphorylated eIF2α (Cell Signaling Technologies, Beverly, MA). Similarly, immunoblotting for eIF2Be was determined using either a rabbit polyclonal antiphosphopeptide antibody specific for eIF2Bε phosphorylated on Ser535 or a monoclonal anti-eIF2Be antibody that recognizes both the phosphorylated and unphosphorylated forms of the protein (Biosource International, Camarillo, CA). Anti-bodies were visualized using an enhanced chemiluminescence (ECL) procedure with the secondary antibody linked to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were exposed to X-ray film in a cassette equipped with a DuPont Lightning Plus intensifying screen. After being developed, the film was scanned (Microtek ScanMaker IV) and analyzed using NIH Image 1.6 software.

The eIF2B activity in tissue was measured in postmitochondrial supernatants using a [3H]GDP-GDP exchange assay, as previously described (23, 28).

Analysis of 4E-BP1/eIF4E and eIF4G/eIF4E complexes. Muscle was homogenized, and eIF4E was immunoprecipitated using an anti-eIF4E monoclonal antibody (Drs. Jefferson and Kimball, Pennsylvania State University College of Medicine). The association of eIF4E with either 4E-BP1 or eIF4G was determined using SDS-PAGE, as previously described (23, 28). The association of eIF4E with 4E-BP1 was measured after immunoprecipitation of 4E-BP1. The various phosphorylated forms of 4E-BP1 were separated by SDS-PAGE and analyzed by protein immunoblotting. Other blots were incubated with primary antibodies to phosphorylated (Ser112) and total eIF4G (Bethyl Laboratories, Montgomery, TX). The blots were developed using ECL, and autoradiographs were scanned and analyzed as described above.

Phosphorylation state of 4E-BP1, eIF4E, and eIF4G. The phosphorylated form of eIF4E in tissue extracts was determined by protein immunoblot analysis using a phosophospecific Ser209 antibody. The phosphorylated forms of 4E-BP1 were measured after immunoprecipitation of 4E-BP1. The various phosphorylated forms of 4E-BP1 were separated by SDS-PAGE and analyzed by protein immunoblotting. Other blots were incubated with primary antibodies to phosphorylated (Ser112) and total eIF4G (Bethyl Laboratories). Autoradiographs were scanned and quantified as described above.

IGF-I and IGFBP-1. The concentration of total IGF-I in plasma was determined using a modified acid-ethanol (0.25 N HCl, 87.5% ethanol) procedure with cryoprecipitation. Samples for total IGF-I were analyzed by RIA as previously described (9, 21, 28).

To quantify the relative amount of IGFBP-1 in plasma, samples were separated on SDS-PAGE under nonreducing conditions (25). The membranes were incubated with antiseraum against rat IGFBP-1 (Upstate Biotechnology). Antigen-antibody complexes were identified with goat anti-rabbit IgG tagged with horseradish peroxidase (Sigma, St. Louis, MO) and with the ECL detection system. Bands were scanned and analyzed using NIH Image 1.6 software.

Northern blotting. Northern blot analysis was performed to quantify the content of IGF-I, IGFBP-1, and myostatin in various tissues. Total RNA was isolated using TRI Reagent TR-118, as outlined by the manufacturer (Molecular Research Center, Cincinnati, OH). Samples of total RNA (20–100 μg) were electrophoresed under denaturing conditions in 1% agarose-6% formaldehyde gels, as described previously (23, 25). A 800-bp probe from rat IGF-I and a 407-bp probe from rat IGF-I and IGFBP-1 were labeled using a Random Primed DNA Labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Oligonucleotides for myostatin 5’-CAGCCCATCTTCCTGTCCTGGAGAAGTACAGC-3’ (26) were radioactively end-labeled using T4 polynucleotide kinase (Promega, Madison, WI). A rat 18S oligonucleotide was used for normalization and was labeled in the same manner. Membranes were exposed to a PhosphorImager screen, and the resultant data were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Relative mRNA abundance was expressed as the ratio between the particular mRNA and 18S mRNA. This ratio was arbitrarily set at 1.0 for tissues from control animals injected with saline.

Other assays. The plasma insulin concentration was determined using a rat-specific RIA (Linco Research, St. Louis, MO). The glucose and alcohol concentrations in plasma were determined using a rapid analyzer (model GL5; Analox Instruments, Lunenburg, MA). The plasma concentration of rat TNF-α was measured using a solid-phase sandwich enzyme linked-immunosorbent assay (BioSource International).
RESULTS

Body weight and alcohol consumption. There was no difference in the starting weight of animals assigned to either the control (100 ± 5 g) or alcohol-fed group (107 ± 6 g). The gain in body weight of the rats consuming alcohol was 12% less than that of pair-fed control animals (Table 1). Injection of IGF-I/IGFBP-3 did not significantly increase the body weight of either control or alcohol-fed rats after 3 days of treatment. Injection of the binary complex in alcohol-fed rats did not alter their alcohol consumption, compared with that consumed by saline-injected animals (Table 1). Likewise, there was no difference in the blood ethanol concentrations at the time of death between the two groups of alcohol-fed rats (Table 1).

Plasma IGF-I, insulin, and glucose concentrations. After 16 wk of alcohol feeding, rats demonstrated a 32% decrease in the plasma concentration of IGF-I (Table 1). Administration of the IGF-I/IGFBP-3 complex in control rats tended to increase (+30%) the circulating concentration of IGF-I, but the change did not reach statistical significance (P = 0.079). Injection of the binary complex in alcohol-fed rats, however, significantly increased the plasma concentration of IGF-I (+62%) so that levels were not different from those in either group of control animals.

Exogenous administration of IGF-I can reduce insulin secretion and induce hypoglycemia, which may indirectly influence protein balance. Therefore, we also measured the plasma concentration of insulin and glucose in rats treated with the binary complex (Table 1). The plasma insulin concentration was not altered in alcohol-fed rats compared with pair-fed control animals. Furthermore, the administration of IGF-I/IGFBP-3 did not alter insulin levels at the time of death in either control or alcohol-fed rats. Similarly, the plasma glucose concentration was not significantly altered by either alcohol feeding or administration of the binary complex (Table 1).

IGF-I mRNA content. Under basal conditions, alcohol-fed rats demonstrated a reduction in the IGF-I mRNA content of both liver (46%) and skeletal muscle (28%), compared with pair-fed control rats (Fig. 1). Administration of the binary complex did not alter the steady-state IGF-I mRNA content of liver or muscle in either control or alcohol-consuming rats.

Muscle protein content and protein synthesis. Initially, the amount of total protein per whole muscle was calculated [e.g., total organ weight × protein content (mg/g wet wt)]. Feeding an alcohol-containing diet for 16 wk decreased the total protein per muscle by 27%, compared with values from pair-fed control rats (Fig. 2). This alcohol-induced decrease was partially reversed by the 3-day treatment with IGF-I/IGFBP-3. Although the total protein per muscle also tended to be increased in control rats, the increase was smaller in magnitude than that observed in alcohol-fed rats and did not achieve statistical significance (P = 0.067).

One possible mechanism for the alcohol- and binary complex-induced changes in muscle protein content is a change in the rate of protein synthesis. In this regard, the in vivo-determined rate of protein synthesis in gastrocnemius was decreased 34% in saline-injected alcohol-fed rats compared with similarly treated control animals (Fig. 2). Administration of the binary complex increased muscle protein synthesis by 35% in control animals and by 49% in alcohol-fed rats. As a result of these changes, the rate of protein synthesis in alcohol-fed rats injected with the binary complex was increased to the same level seen in saline-treated control rats but remained lower than in control rats injected with IGF-I/IGFBP-3.

Alterations in the number of ribosomes or in the efficiency of mRNA translation may influence tissue protein synthesis. To determine which mechanism was responsible for the above-mentioned changes in protein synthesis, the RNA content and translational efficiency were determined. In muscle, ~85% of the RNA is ribosomal RNA; therefore, changes in total RNA content primarily reflect changes in the number of ribosomes. The RNA content in gastrocnemius was not altered by either
alcohol consumption or IGF-I/IGFBP-3 (Fig. 2). These data suggest that an alteration in the relative abundance of ribosomes was not responsible for the observed changes in protein synthesis.

The efficiency of translation, calculated by dividing the protein synthetic rate by the total RNA content, provides an index of how rapidly the existing ribosomes are synthesizing protein (24). Figure 2 shows that alcohol feeding decreased translational efficiency in muscle and that the binary complex increased efficiency in both control and alcohol-fed rats.

Alterations in the amount of eIF2 and eIF2B, and in eIF2B activity. A possible mechanism for the changes in translational efficiency in muscle in response to either alcohol or IGF-I/IGFBP-3 is an alteration in the amount, availability, or activity of specific eIF proteins involved in the individual steps of the protein synthetic process (33). The first step in translation initiation is the formation of a ternary complex consisting of eIF4F complex and the inactive eIF4E. Met-tRNAi, 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. However, neither alcohol feeding nor administration of the binary complex altered the total amount of eIF4E in muscle (Fig. 3A). Our laboratory and others have demonstrated that altering the distribution of eIF4E between the active eIF4E and eIF4F complex and the inactive eIF4E-E-BP1 complex modulates rates of protein synthesis (23, 28, 30, 44, 50). In the present study, the amount of eIF4E bound to eIF4F was decreased in muscle from saline-treated alcohol-fed rats compared with muscle from similarly treated control rats.

### Table 2. Effect of EtOH and the IGF-I/IGFBP-3 BC on eIF2 and eIF2B in muscle

<table>
<thead>
<tr>
<th>Protein Synthetic Rate (mmol Phe incorporated into protein)</th>
<th>Translational Efficiency (mg protein/mg RNA)</th>
<th>RNA Content (mg RNA/g)</th>
<th>Total Protein per Muscle (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Alcohol</td>
<td>Control</td>
<td>Alcohol</td>
</tr>
<tr>
<td>eIF2α, AU</td>
<td></td>
<td>447 ± 38</td>
<td>429 ± 28</td>
</tr>
<tr>
<td>eIF2α (P), AU</td>
<td></td>
<td>206 ± 29</td>
<td>212 ± 34</td>
</tr>
<tr>
<td>eIF2B, AU</td>
<td></td>
<td>555 ± 71</td>
<td>497 ± 52</td>
</tr>
<tr>
<td>eIF2B (P), AU</td>
<td></td>
<td>1,147 ± 101</td>
<td>1,053 ± 146</td>
</tr>
<tr>
<td>eIF2B activity, pmol GDP exchanged/min</td>
<td></td>
<td>0.114 ± 0.009*</td>
<td>0.122 ± 0.012*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–9 rats/group. For all measurements of total or phosphorylated eukaryotic initiation factor (eIF2α or eIF2B), units are arbitrary densitometric volume units (AU) determined by Western blot analysis. P, phosphorylated. Means with different superscripts for a specific parameter are statistically significantly different from each other (P < 0.05).

Despite the lack of change in either the amount or phosphorylation state of eIF2B and eIF2α, alcohol-fed rats demonstrated a 32% decrease in eIF2B activity (Table 2). However, treatment with the binary complex was not able to reverse the alcohol-induced decrease in eIF2B activity observed in gastrocnemius. Similarly, injection of IGF-I/IGFBP-3 did not significantly alter eIF2B activity in muscle from control rats.

**Regulation of eIF4F complex.** A second critical locus of translational regulation involves the binding of the 5′-end of cellular mRNA to the 43S preinitiation complex, a reaction mediated by the cap-binding protein complex eIF4F (41). This complex is heterotrimeric, being composed of eIF4E, eIF4G, and eIF4A, 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. However, neither alcohol feeding nor administration of the binary complex altered the total amount of eIF4E in muscle (Fig. 3A). Our laboratory and others have demonstrated that altering the distribution of eIF4E between the active eIF4E-eIF4F complex and the inactive eIF4E-E-BP1 complex modulates rates of protein synthesis (23, 28, 30, 44, 50). In the present study, the amount of eIF4E bound to eIF4F was decreased in muscle from saline-treated alcohol-fed rats compared with muscle from similarly treated control rats.

The ability of eIF-2 to form a ternary complex can also be modified by another eukaryotic initiation factor, eIF2B (39). eIF2 is bound to GDP as an inactive complex when it is released from the ribosome, and this GDP must be exchanged for GTP before a new round of initiation may proceed. eIF2B catalyzes this guanine nucleotide exchange and is required to regenerate the active eIF2-GTP complex. In the present study, there was no detectable change in the relative amount of total and phosphorylated eIF2B resulting from alcohol feeding or IGF-I/IGFBP-3 (Table 2). Hence, it is unlikely that changes in the amount or phosphorylation of eIF2B are responsible for alterations in protein synthesis after administration of IGF-I/IGFBP-3.

**Fig. 2.** Effect of EtOH feeding and administration of the IGF-I/IGFBP-3 BC on protein synthesis, RNA content, translational efficiency, and protein content in muscle. The experimental protocol is the same as described in Fig. 1. Rates of protein synthesis were determined after iv injection of [3H]phenylalanine (Phe). Values are means ± SE; n = 8–9 rats/group. The rate of protein synthesis was expressed as nmol Phe incorporated into protein. Values with different letters are significantly (P < 0.05) different from each other. Values with the same letter are not significantly different.
IGF-I/IGFBP-3 IMPROVES MUSCLE PROTEIN BALANCE

Potential negative regulators of muscle mass. There are numerous factors that may adversely impact protein balance in skeletal muscle and, therefore, may be modulated by either alcohol or IGF-I/IGFBP-3. Our laboratory has previously demonstrated that, under in vivo and in vitro conditions, elevations in one of the IGF-binding proteins, IGFBP-1, decreases muscle protein synthesis (28, 27). Data in Fig. 6 show that alcohol feeding markedly increases the plasma concentration of IGFBP-1. Moreover, this increase is consistent with the elevation in IGFBP-1 mRNA observed in the liver (e.g., the synthetic site for the majority of blood-borne IGFBP-1) in response to either alcohol consumption or administration of IGF-I/IGFBP-3 (Fig. 5, C, D, and F). Alternatively, the recruitment of the translational machinery to the 5′-end of mRNA can be modulated by changes in eIF4G phosphorylation (32). The extent of eIF4G phosphorylation was decreased by 68% by alcohol feeding. Conversely, the binary complex increased the phosphorylation of eIF4G in muscle from both control and alcohol-fed rats (Fig. 5, A, B, and E). Hence, changes in the phosphorylation state of eIF4G may possibly influence the assembly and activity of the eIF4F complex. As evidence, in Fig. 5B, the changes in the phosphorylation of eIF4G produced in response to alcohol consumption or IGF-I/IGFBP-3 were not the result of alterations in the abundance of total eIF4G.

The binding of eIF4E to eIF4G is controlled in part by a family of cap-dependent translational repressors, and, in skeletal muscle, the most prominent member of the family is 4E-BP1 (41). Hyperphosphorylation of 4E-BP1 liberates it from eIF4E and thereby facilitates binding of eIF4E with eIF4G and the stimulation of protein synthesis. The amount of the hyperphosphorylated γ-isof orm of 4E-BP1 was decreased 46% in muscle from alcohol-fed rats (Fig. 4). However, administration of IGF-I/IGFBP-3 did not alter the phosphorylation of 4E-BP1 in either control or alcohol-consuming rats. Hence, the abovementioned increase in the association of eIF4E with eIF4G in response to the binary complex cannot be attributed to changes in the phosphorylation state of 4E-BP1.

Covalent modifications of eIF4E and eIF4G also have the potential to regulate eIF4F assembly. For example, the phosphorylation of eIF4E at Ser209 increases the binding affinity of eIF4E for the m7GTP cap on mRNA and thereby promotes assembly of a functional eIF4F complex (43). However, we could not detect a consistent change in the phosphorylation state of eIF4E in response to either alcohol consumption or administration of IGF-I/IGFBP-3 (Fig. 3, B and D). Conversely, the incremental increase in the amount of eIF4E-eIF4G in response to the binary complex was similar in control and alcohol-fed rats. However, the amount of eIF4E-eIF4G in muscle of binary complex-treated alcohol-fed rats returned only to the level seen in control rats under basal conditions. Alcohol feeding also increased the binding of eIF4E with the translation repressor molecule 4E-BP1 (Fig. 3, C and E). However, somewhat unexpectedly, there was no change in the amount of eIF4E-4E-BP1 in muscle from either control or alcohol-fed rats in response to IGF-I/IGFBP-3.

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response to alcohol. Administration of the binary complex appeared to decrease the plasma and hepatic IGFBP-1 in control rats, although only the latter change was statistically significant. The binary complex also decreased IGFBP-1 in plasma and liver of alcohol-fed rats, and, as a result, levels did not differ from those detected in saline-treated control rats.

Myostatin is a negative regulator of muscle mass and has been demonstrated to decrease protein synthesis (29). The myostatin mRNA content was elevated more than twofold in muscle obtained from rats consuming alcohol for 16 wk (Fig. 7). In alcohol-fed rats administered binary complex for 3 days, the myostatin mRNA content was reduced to values comparable to those detected in saline-treated control rats.

Finally, elevated levels of the inflammatory cytokine TNF-α can dramatically decrease muscle protein synthesis under both in vitro and in vivo conditions (12, 23), and chronic alcohol feeding in rats has been reported to increase plasma TNF-α concentrations (7). However, the plasma concentration of TNF-α was below the limit of detection (~15 pg/ml) for all four experimental groups, and the TNF-α mRNA content was not elevated in muscle from alcohol-fed rats (data not shown).

Hence, it would appear that neither the systemic nor local overproduction of TNF-α is responsible for the impairment in muscle protein balance observed in alcohol-fed rats.

**DISCUSSION**

IGF-I is a pleiotropic anabolic hormone that is essential for the accretion of LBM in the young and maintenance of muscle mass in adults (5). Reduction in the bioavailability or bioactivity of IGF-I has been postulated to be in part responsible for the muscle wasting that characterizes several catabolic conditions (10), including chronic alcohol consumption (24). Previous work by several laboratories demonstrates a marked reduction in the concentration of IGF-I in the circulation and the muscle of alcohol-fed rats (21, 24, 45). Moreover, the plasma concentration of free or unbound IGF-I, believed to be the bioactive form of the peptide, is also reduced by alcohol feeding (25). Therefore, we hypothesized that restoration of IGF-I would reverse the negative impact of alcohol feeding on muscle protein balance.

To raise the plasma IGF-I concentration, rats were injected with an equal molar complex of IGF-I and IGFBP-3. This binary complex was used because it has several advantages compared with the in vivo administration of unbound IGF-I. First, the binary complex delays the clearance of IGF-I from plasma.
the circulation and thereby prolongs the bioavailability of IGF-I (1). Second, elevations in free IGF-I decrease plasma insulin concentrations (4) and thereby potentially diminish the anabolic actions of IGF-I. Third, injection of free IGF-I may produce hypoglycemia that stimulates the secretion of various counterregulatory hormones, such as glucocorticoids and catecholamines (17). In this regard, administration of the binary complex did not significantly alter either the plasma concentration of insulin or glucose in either control or alcohol-fed rats at the time point examined in the present study. Finally, the injection of the binary complex did not downregulate the synthesis of IGF-I, as evidenced by the comparable expression of IGF-I mRNA in tissues from vehicle- and binary complex-treated rats. Hence, the administration of IGF-I and IGFBP-3 in a preformed complex permits potentiation of the anabolic actions of IGF-I while minimizing those “side-effects” that may exacerbate the catabolic state. These results are consistent with the findings of other studies demonstrating an anabolic effect of the binary complex in sepsis, burn, and semistarvation (16, 46, 47).

Our laboratory and others have reported that chronic alcohol feeding decreases the rate of protein synthesis in skeletal muscle composed primarily of fast-twitch fibers (28, 34). In the present study, there was no reduction in the number of ribosomes; hence, the decreased protein synthesis was primarily caused by a reduction in the efficiency of mRNA translation. Furthermore, we have previously reported that this alcohol-induced change is associated with defects in both potentially rate-controlling steps of peptide-chain initiation. That is, alcohol feeding not only reduces eIF2B activity but also shifts the distribution of eIF4E from the active eIF4E-eIF4G complex to the inactive eIF4E-eIF4E-BP1 complex under basal conditions (28). The interaction of 4E-BP1 and eIF4E inhibits cap-dependent translation both in vivo and in vitro (33, 41). The results of the present study (e.g., comparison of vehicle-treated control vs. vehicle-treated alcohol-fed rats) are consistent with these original observations. In addition, we have extended these findings and now report that, although alcohol feeding does not alter the total amount of eIF4G, it dramatically reduces the phosphorylation of this polypeptide in muscle. Although various growth factors increase eIF4G phosphorylation and the rate of translation (32), the exact mechanism by which phosphorylated eIF4G stimulates synthesis remains speculative. However, in this study and others, phosphorylation of eIF4G is associated with increased binding of the protein to eIF4E (22).

The alcohol-induced decrease in basal muscle protein synthesis and initiation was also associated with a decrement in IGF-I in both the blood and skeletal muscle per se. We have previously demonstrated a strong linear correlation between the content of IGF-I peptide or mRNA in muscle and the rate of protein synthesis and the amount of the active eIF4E-eIF4G complex (24, 28).

The results of the present investigation indicate that the inhibition of muscle protein synthesis and translational efficiency induced by chronic alcohol feeding is reversed by the in vivo administration of a binary complex. Furthermore, the restoration of protein synthesis and translational efficiency in alcohol-fed rats was associated with an increased muscle protein content. Although there was a comparable increment in muscle protein synthesis in both control and alcohol-fed rats treated with the binary complex, the synthetic rate of the latter group remained significantly reduced. There are two possible interpretations of these data. First, because plasma IGF-I concentrations in alcohol-fed rats treated with binary complex were not significantly different from those detected in similarly treated control rats, the ability of IGF-I to stimulate protein synthesis in the alcohol-consuming rats appears to be reduced. Such data are consistent with the presence of IGF-I resistance in alcohol-fed rats. Alternatively, these data may suggest that alcohol produces a defect that mediates the reduction in the basal rate of protein synthesis but that the ability of IGF-I to stimulate protein synthesis is relatively unimpaired. In this latter scenario, a portion of the rate of protein synthesis is independent of IGF-I and remains depressed after chronic alcohol feeding.

Synthesis of protein in eukaryotic cells is achieved through a complex series of discrete reactions (33). The initial rate-controlling step in translation initiation involves the actions of eIF2, which mediates the binding of met-tRNA<sub>i</sub> to the 40S ribosomal subunit. This step is regulated by eIF2B functioning as a GDP-exchange factor for eIF2 (39). We have also reported that chronic alcohol consumption decreases the activity of eIF2B in skeletal muscle (28). However, the mechanism for this reduction remains unsettled because there was no concomitant decrease in the amount of eIF2B protein or the extent of eIF2α phosphorylation, the latter of which is known to be a potent inhibitor of eIF2B activity. It is noteworthy that the injection of IGF-I/IGFBP-3 in either control rats or animals consuming alcohol failed to increase eIF2B activity (or the amount and phosphorylation of eIF2α and eIF2βe) at the time point examined. These data indicate that the increased muscle protein synthesis induced by the binary complex was not associated with a detectable change in any of the measured parameters in the eIF2/eIF2B system.
The binding of the 43S preinitiation complex represents a second important regulatory step in translation initiation and is mediated by the eIF4F complex (41). Assembly of this complex appears to be most often regulated by changes in the availability of eIF4E rather than the abundance of the factor per se. To function in cap-dependent translation initiation, eIF4E must form an initiation complex with eIF4G, which acts as a scaffold protein and an RNA helicase (eIF4A). The binding of eIF4E with eIF4G occurs via a site that also binds the translational repressor molecule 4E-BP1. Hence, association of eIF4E with eIF4G occurs via a site that also binds the trans-

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ternally consistent in that phosphorylation of 4E-BP lowers the affinity of 4E-BP for eIF4E. This results in the dissocia-
tion of eIF4E from 4E-BP1 and its reciprocal binding to eIF4G. However, in the current study, the IGF-I/IGFBP-3 complex increased the amount of eIF4E bound to eIF4G in muscle of both control and alcohol-fed rats, independent of a reciprocal change in the amount of the eIF4E-eIF4G complex or an increase in the amount of 4E-BP1 in the hyperphosphorylated γ-isofrom. The inability of the binary complex to stimulate 4E-BP1 phosphorylation has also been reported in rats in response to bacterial infection and severe food restriction (46, 47), despite its stimulation of protein synthesis. The reason that rats administered IGF-I complexed with IGFBP-3 have a different response than animals injected with free IGF-I is not known but may be because of 1) differences in the duration between treatment and tissue sampling in the two studies (i.e., 20 min vs. 3 h), 2) the prolonged elevation in plasma IGF-I induced by the binary complex, or 3) the presence of exoge-
nous IGFBP-3 acting in an IGF-1-independent manner. An increase in the amount of eIF4E bound to eIF4G in the absence of a reciprocal change in the binding of eIF4E to 4E-BP1 has been reported previously (46, 47, 49). This somewhat atypical situation may be explained by a separate intracellular pool of “free” eIF4E and/or a possible reciprocal binding of eIF4E to 4E-BP2 that is also present in muscle. Regardless of the exact mechanism, the increased muscle protein synthesis induced by the binary complex (in both control and alcohol-fed rats) was independent of changes in 4E-BP1 phosphorylation and the amount of the elf4E-eIF4G-eIF4 complex.

Alternatively, enhanced phosphorylation of elf4G might be responsible for the increased binding of elf4E with elf4G in response to the binary complex. Previous work has demon-

strated that increased Ser1108 phosphorylation of elf4G is also associated with enhanced binding of elf4G with elf4E and increased rates of protein synthesis (32), although the exact mechanism remains to be elucidated. elf4E also undergoes reversible phosphorylation, and such posttranslational modification has been associated with an increased rate of protein synthesis independent of changes in the phosphorylation of 4E-BP1. Phosphorylation of elf4E enhances affinity of the protein for m7GTP cap analogs of mRNA (31), whereas reduced phosphorylation of elf4E correlates with the inhibition of protein synthesis observed in response to heat shock or serum depletion (8). Specifically, phosphorylation of residue Ser209 appears to stabilize the binding of m7GTP cap in a hydrophobic pocket of elf4E and thereby increases its stability (43). However, in the present study, we could not detect a significant change in the extent of Ser209 phosphorylation of elf4E by either alcohol feeding and/or treatment with the binary complex. Thus in vivo modulation of the elf4E phos-

phorylation does not appear to represent an important regula-
tory step for the alcohol-induced decrease in basal protein synthesis or the ability of the IGF-I/IGFBP-3 to stimulate protein synthesis in skeletal muscle of rats. In contrast, changes in the phosphorylation state of elf4G are entirely consistent with the binary complex-induced changes in elf4E-eIF4G binding and protein synthesis.

In the present investigation, we also assessed changes in the plasma concentration or tissue expression of several mediators that may negatively regulate muscle protein synthesis. Hence, alcohol- or IGF-I/IGFBP-3-induced changes in these mediators may also directly or indirectly impact muscle protein balance. Both natural and experimental deletions in the myostatin gene markedly increase skeletal muscle mass (29). An inverse cor-

relation exists between myostatin-immunoreactive protein and LBM in HIV-infected patients with weight loss (15), as well as between muscle myostatin mRNA content and muscle protein mass after thermal injury and glucocorticoid excess (26). The potential importance of myostatin as a negative regulator of muscle mass is emphasized by results from a recent study in which the rate of protein synthesis was decreased in cultured myocytes incubated with human recombinant myostatin (48). At this time, it is not known whether a defect in translation initiation is responsible for the myostatin-induced decrease in protein synthesis. Our data indicate that chronic alcohol feeding for 16 wk increases the myostatin mRNA content in the gastrocnemius. Moreover, 3-day treatment of alcohol-fed rats with IGF-I/IGFBP-3 reversed the increase in muscle myostatin mRNA. Constitutive expression of myostatin mRNA in muscle from control rats was relatively low, and we could not reliably detect by Northern blot analysis any effect of the binary complex in control animals. Although the basal and IGF-I-

induced changes in myostatin mRNA are consistent with the observed changes in muscle protein mass determined in alco-

hol-fed rats, their physiological relevance remains to be as-

essed.

Chronic alcohol consumption also increases the plasma concentration of one of the IGF-binding proteins, IGFBP-1 (20). Elevations in this binding protein have catabolic effects. For example, we have demonstrated that addition of IGFBP-1 to cultured myocytes decreases both basal and IGF-I-stimu-

lated increases in protein synthesis (11). Furthermore, an

increased circulating concentration of IGFBP-1 decreases the plasma concentration of free IGF-I and specifically reduces protein synthesis in fast-twitch skeletal muscle (27). Part of the ability of IGFBP-1 to decrease the synthetic rate in muscle may be related to the concomitant reduction in the activity of the ribosomal protein S6 kinase-1 that controls selected aspects of mRNA translation (27). Administration of the binary complex reversed the alcohol-induced increase in the plasma IGFBP-1 concentration. This response was associated with a comparable reduction in the IGFBP-1 mRNA content of liver, the primary synthetic site of blood-borne IGFBP-1. The binary complex also modestly decreased IGFBP-1 mRNA in liver of control rats, which resulted in a trend toward lower plasma concentra-

tions of IGFBP-1. The mechanism by which IGF-I decreases
the hepatic synthesis and secretion of IGFBP-1 appears indirect because this growth factor has not been shown to affect IGFBP-1 expression in primary rat hepatocytes (52).

In summary, the results of the present study clearly indicate that administration of IGF-I as part of a binary complex with IGFBP-3 is capable of reversing the decrement in muscle protein synthesis in alcohol-fed rats. The increase in protein synthesis resulted from a stimulation of translational efficiency and was associated with an increased amount of the active eIF4E-eIF4G complex and an increased phosphorylation of eIF4G. In contrast, the stimulation of translation by IGF-I/IGFBP-3 appears independent of changes in eIF2/2B, and the phosphorylation of either 4E-BP1 or eIF4E. Additionally, the binary complex also reversed alcohol-induced elevations in myostatin and IGFBP-1, two known mediators that adversely influence muscle protein synthesis. Therefore, this response represents an additional mechanism by which the anabolic actions of IGF-I/IGFBP-3 may be manifested in muscle. Collectively, these data further support the role of IGF-I deficiency as a mediator of the myopathy that develops in response to chronic alcohol consumption.

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