TNF-binding protein ameliorates inhibition of skeletal muscle protein synthesis during sepsis

ROBERT COONEY, SCOT R. KIMBALL, REBECCA ECKMAN, GEORGE MAISH III, MARGARET SHUMATE, AND THOMAS C. VARY

Department of Cellular and Molecular Physiology and Department of Surgery, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Sepsis induces profound alterations in whole body protein metabolism. Marked weight loss and accelerated nitrogen excretion characterize the host's response to infection. Nitrogen losses equivalent to 5–17% of body protein are commonly observed in septic patients despite adequate nutritional support. Much of this nitrogen loss occurs secondary to the net catabolism of skeletal muscle protein. Muscle protein wasting in sepsis results from both a global decrease in the rate of protein synthesis and an increase in protein degradation (for review see Refs. 6, 30). Inflammatory cytokines may regulate the changes in protein metabolism observed during sepsis. Tumor necrosis factor (TNF) has been hypothesized to be an important mediator of the septic response. One strategy to delineate the role of TNF in modulating protein metabolism is to administer recombinant TNF to healthy animals with the aim of reproducing an inhibition of muscle protein synthesis similar to that observed in septic states. Administration of TNF in vivo increases nitrogen efflux from skeletal muscle (17) and loss of body protein (13, 17). Furthermore, a tumor-secreting TNF is associated with severe muscle atrophy in mice (32). Although it provides evidence for involvement of TNF in muscle wasting, this approach has yielded conflicting results regarding the effects of TNF on protein synthesis in muscle. Incubation of rat muscle in vitro with buffer containing TNF does not cause an inhibition of muscle protein synthesis (12, 25). In contrast, other studies provide evidence for an inhibition of skeletal muscle protein synthesis after infusion of TNF in vivo (1, 3, 12, 20, 30) or exposure of human myoblasts in culture to TNF (14). However, these studies do not specifically address the role of TNF in the catabolism of muscle protein after a septic insult, nor do they address potential mechanisms to account for the loss of muscle protein.

An alternative approach to elucidating the role of TNF in regulating skeletal muscle protein synthesis is to modify the release and/or biological activity of TNF during the septic insult. Phosphodiesterase inhibitors, such as pentoxifylline or amrinone, inhibit the secretion of TNF from activated macrophages (8, 15). These compounds decrease the serum TNF concentration of endotoxin-treated mice, rats, and humans and improve survival after a lethal dose of endotoxin (15). Pentoxifylline prevents the fall in protein synthesis in gastrocnemius of rats injected with live Escherichia coli (1). Likewise, daily injections of amrinone for 5 days completely prevent the loss of muscle mass and diminished rates of protein synthesis during chronic abdominal sepsis (19). Although the beneficial effects of pentoxifylline or amrinone on protein metabolism are ascribed to its inhibition of TNF production by macrophages (1, 19), both these compounds were developed to treat cardiac failure or peripheral vascular disease. Therefore, the possibility exists that the effects of these compounds on protein metabolism are mediated by increasing cAMP concentrations in nonmuscle cells, with subsequent release or inhibition of other compounds that potentially modulate protein metabolism.

The biological activity of TNF is modulated in vivo by the proteolytic shedding of the extracellular domain of the p55 and p75 TNF receptors. An increase in soluble TNF receptors in the bloodstream neutralizes circulat-
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No control animals died. The mortality rate in the Septic group (26%) was not significantly different from the Septic+TNFBP group (33%). All of the sepsis-related deaths occurred within 48 h of implantation of the infected fecal-agar pellet. Septic animals examined 5 days after the introduction of the fecal-agar pellet show an intra-abdominal abscess, with accumulation of bacteria and polymorphonuclear leukocytes in the abscess itself. Bacteremia is observed in 75% of the septic animals (41). Septic animals show an increased cardiac output and decreased total peripheral resistance indicative of a hyperdynamic cardiovascular state (26). Skeletal muscle from septic rats exhibits muscle wasting (38), increased glucose utilization, increased lactate production (41), but no change in the tissue high-energy phosphate content (39). All of these are characteristics of a stable hypermetabolic, hyperdynamic septic condition without evidence of hypoperfusion. The Institutional Animal Care Committee at the Pennsylvania State University, College of Medicine approved the experimental protocol.

Protein synthesis. The rate of protein synthesis in vivo was measured by the incorporation of radioactive phenylalanine by use of a modification of the flooding-dose technique as described previously (19, 22, 36–38, 42). Five days after the induction of sepsis, animals were fasted for 12 h. Saline (1.0 ml) or TNFBP (1 mg/kg; 1.0 ml; Amgen, Boulder, CO) was injected subcutaneously daily starting 4 h before septic surgery. Human recombinant TNFBP demonstrates cross-species biological activity in several experimental models, including endotoxemia in mice (16) and rats (9), E. coli bacteremia in baboons (9), and intra-abdominal sepsis in the rat (5). The dose and timing of TNFBP injection were based on previously determined plasma TNF levels in the intra-abdominal septic abscess model (19) and pharmacokinetic studies demonstrating inhibitory plasma concentrations of TNFBP (>500 ng/ml) with this dosing regimen (5, 9, 29, 31). The peak TNF-α concentration (0.071 ng/ml) occurs within 4 h after implantation of the fecal-agar pellet in this model (19). Thereafter, plasma TNF-α fell progressively from the peak concentration but remained elevated for up to 8 h in the septic rats. Within 24 h after the induction of sepsis, the plasma TNF-α concentration fell to below the level of detection (<0.015 ng/ml) (19). Plasma TNFBP concentrations were 500 ng/ml at the early (0–12 h) times after the initial injection of the compound on day 0. On day 5, plasma concentrations were ~2,500 ng/ml (5).

Animals were anesthetized by injection of ketamine (80 mg/kg) and acepromazine (1 mg/kg). Chronic abdominal sepsis was created in both Septic and Septic+TNFBP groups by implantation of a fecal-agar pellet (1.5 ml) inoculated with 10⁶ colony forming units (CFU) of E. coli and 10⁵ CFU Bacteroides fragilis in the peritoneal cavity, as previously described (19, 36–43). The animals develop an abdominal abscess resulting in a hyperdynamic (26), hypermetabolic septic condition (6, 19, 36–43). Laparotomy was not performed in control rats because laparotomy itself does not modulate gastrocnemius protein content or synthesis after 48 h (27) or 5 days (19, 38). Furthermore, the muscle protein content, muscle weight, and the rates of protein synthesis are not altered 5 days after the formation of a sterile nonseptic abscess compared with the same parameters in nonoperated control rats (19, 38). After recovery from surgery, animals were allowed rat chow and water ad libitum. Previous studies from our laboratory have established that TNFBP does not influence food consumption in septic rats (5).

Materials and Methods

Animals. Four groups of male Sprague-Dawley rats (200–300 g; Charles River Breeding Laboratories, Wilmington, MA) were studied: Control, Control+TNFBP, Septic, and Septic+TNFBP. Saline (1.0 ml) or TNFBP (1 mg/kg; 1.0 ml; Amgen, Boulder, CO) was injected subcutaneously daily starting 4 h before septic surgery. Human recombinant TNFBP demonstrates cross-species biological activity in several experimental models, including endotoxemia in mice (16) and rats (9), E. coli bacteremia in baboons (9), and intra-abdominal sepsis in the rat (5). The dose and timing of TNFBP injection were based on previously determined plasma TNF levels in the intra-abdominal septic abscess model (19) and pharmacokinetic studies demonstrating inhibitory plasma concentrations of TNFBP (>500 ng/ml) with this dosing regimen (5, 9, 29, 31). The peak TNF-α concentration (0.071 ng/ml) occurs within 4 h after implantation of the fecal-agar pellet in this model (19). Thereafter, plasma TNF-α fell progressively from the peak concentration but remained elevated for up to 8 h in the septic rats. Within 24 h after the induction of sepsis, the plasma TNF-α concentration fell to below the level of detection (<0.015 ng/ml) (19). Plasma TNFBP concentrations were 500 ng/ml at the early (0–12 h) times after the initial injection of the compound on day 0. On day 5, plasma concentrations were ~2,500 ng/ml (5).

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HEPES (pH 7.4), 2 EGTA, 50 NaF, 100 KCl, 0.2 EDTA, 1 dithiothreitol (DTT), 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride (PMSF), 1 benzamidine, 0.5 NaVO₄, and 1 microcinin LR] with a Polytron PT10. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was aliquoted and quickly frozen to the temperature of liquid nitrogen. Frozen aliquots were thawed, mixed with 2× Laemml SDS sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Proteins were then electrothermally transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P) and blocked with Tris-buffered saline containing 5% (wt/vol) nonfat powdered dry milk. Membranes were incubated with a polyclonal antibody that recognizes p70S6 kinase (Santa Cruz Biotechnology, Santa Cruz, CA). They were then washed extensively and incubated with a goat anti-rabbit antibody conjugated with horseradish peroxidase. The blots were developed using chemiluminescence detection according to Amersham’s instructions. After development, the radiographic films were subjected to densiometric scanning.

Measurements of eIF2 and eIF2B subunits. The relative content of the α-subunit of eIF2 (eIF2α) and the ε-subunit of eIF2B (eIF2Be) in muscle was determined by protein immunoblot analysis (21, 22, 42, 43). Muscle samples were homogenized in 7 volumes of buffer [in mM: 20 Tris·HCl (pH 7.5), 250 sucrose, 500 KCl, 10 EDTA, 10 magnesium acetate, 1 DTT, 40 NaF, 50 β-glycerophosphate, and 0.5 PMSF]. The samples (150 µl) were mixed with 150 µl of 2× Laemml SDS buffer (60°C), boiled for 3 min, and centrifuged. Equal amounts of protein (160 µg) from skeletal muscle homogenates were electrophoresed in a 10% polyacrylamide gel. After electrophoresis, the gels were transferred electrophoretically onto PVDF membranes (Immobilon P). After blocking with nonfat dry milk, the membranes were incubated with monoclonal antibodies specific for eIF2α or eIF2Be as described previously (21, 22, 42, 43). Antibodies were visualized using an enhanced chemiluminescence procedure as described for the p70S6 kinase.

Statistical methods. Data are expressed as means ± SE for animals in each group. The statistical evaluation of the data was performed using analysis of variance (ANOVA) to test for overall differences among groups. When the ANOVA indicated a significant difference, individual means were compared using the Student-Newman-Keuls test. Differences among means were considered significant at P < 0.05.

RESULTS

Protein metabolism. We examined the effect of sepsis and TNFBP on muscle weight (g), protein content (mg/g wt), and total protein per muscle (mg protein/muscle) of gastrocnemius in the different experimental groups (Table 1). Treatment of control animals with TNFBP did not significantly alter any of these parameters. Sepsis significantly decreased both gastrocnemius weight and total protein relative to control animals (P < 0.001). Septic animals treated with TNFBP demonstrate a significant increase in muscle weight and protein content/muscle when compared with saline-treated septic animals (P < 0.05). Although the administration of TNFBP ameliorated the effects of sepsis on protein content/muscle, the muscle weight in the Septic+TNFBP animals remained diminished relative to the Control group.

Protein synthesis. The effects of sepsis and TNFBP administration on the rate of protein synthesis in gastrocnemius are shown in Fig. 1. Administration of TNFBP to control animals did not alter the rate of protein synthesis in gastrocnemius. Sepsis caused a 39% decrease in the rate of protein synthesis in gastrocnemius compared with control animals. The reduction in gastrocnemius protein synthesis was significantly

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Gastrocnemius Weight, g</th>
<th>Protein Content, mg/g</th>
<th>Total Protein/Muscle, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.79 ± 0.2</td>
<td>143 ± 6</td>
<td>540 ± 34</td>
</tr>
<tr>
<td>Control + TNFBP</td>
<td>3.87 ± 0.3</td>
<td>146 ± 8</td>
<td>576 ± 58</td>
</tr>
<tr>
<td>Septic</td>
<td>2.38 ± 0.1*†</td>
<td>136 ± 5</td>
<td>325 ± 16*†</td>
</tr>
<tr>
<td>Septic + TNFBP</td>
<td>3.00 ± 0.1†</td>
<td>142 ± 6</td>
<td>441 ± 19</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6–11 animals/group. TNFBP, tumor necrosis factor-binding protein. Gastrocnemius weight represents the combined weight of individual muscles taken from each leg. Protein/Muscle was calculated by multiplying mg protein/g times muscle weight. *P < 0.05 vs. Septic + TNFBP; †P < 0.01 vs. Control and Control + TNFBP. [Weight by ANOVA F = 16.22, P < 0.001; Protein Content by ANOVA F = 0.39, P = 0.75, not significant (NS); Total Protein/Muscle by ANOVA F = 8.9, P < 0.001.]
ameliorated by treatment of septic animals with TNFBP. The rate of gastrocnemius protein synthesis was not significantly different in Control, Control + TNFBP, and Septic+TNFBP groups.

Changes in the number of ribosomes or in the efficiency of mRNA translation may cause the reduction in gastrocnemius protein synthesis. To determine which mechanism was responsible for the alterations in muscle protein synthesis, the RNA content and translational efficiency were determined. Because ~80% of the RNA in muscle is ribosomal RNA, changes in total RNA content presumably reflect changes in the number of ribosomes. The RNA content of gastrocnemius from control rats was 7.02 ± 0.06 µg RNA/mg protein. There were no significant differences in RNA content in any of the conditions examined (data not shown). Therefore, alterations in the relative abundance of ribosomes were not responsible for changes in gastrocnemius protein synthesis in either untreated or TNFBP-treated septic rats.

The efficiency of translation, calculated by dividing the protein synthesis rates by the total RNA content, provides an index of how rapidly the existing ribosomes synthesize protein. In muscles from untreated septic animals, the translational efficiency was reduced by ~45% compared with control animals (Fig. 2). Treatment of septic rats with TNFBP restored the translational efficiency in gastrocnemius to values observed in controls (Fig. 2). Treatment of control rats with TNFBP did not alter the translational efficiency compared with untreated control rats. Thus daily injections of TNFBP for 5 days prevented the sepsis-induced inhibition of translational efficiency in gastrocnemius.

Effect of sepsis and TNFBP on p70S6 kinase phosphorylation.

We examined the extent of phosphorylation of the p70S6 kinase to determine whether this was an important regulatory mechanism in gastrocnemius during sepsis. Activation of p70S6 kinase has been implicated in regulating translation by its ability to phosphorylate ribosomal S6 protein. Phosphorylation of ribosomal protein S6 correlates with selective upregulation of protein synthesis after stimulation of cells by mitogens (for review see Refs. 10, 24). The linkage of p70S6 kinase activation to protein synthesis would be interesting in defining potential downstream regulators of the septic response in skeletal muscle. p70S6 Kinase is activated by multisite phosphorylation that results in phosphorylated forms exhibiting retarded electrophoretic mobility when subjected to SDS-PAGE (4, 7, 10, 24). We used this property (assessed by immunoblotting techniques) as an indicator of the effect of sepsis on the activation of the kinase (Fig. 3). Immunoblotting of gastrocnemius homogenates with antibodies raised against p70S6 kinase revealed a band at ~70 kDa and three other bands with retarded electrophoretic mobilities. Sepsis did not appreciably reduce the prominence of the electrophoretically retarded bands. Thus the phosphorylation state of p70S6 kinase was not altered in skeletal muscle during sepsis. Furthermore, treatment of either controls or septic rats with TNFBP did not influence the phosphorylation of p70S6 kinase in gastrocnemius.

Western blot analysis of eIF2α and eIF2Bε.

The eIF2α and eIF2B proteins consist of three and five subunits, respectively, each of which is present in equimolar amounts in the holoenzymes (21). Previous results have established that alterations in the expression of
eIF2α and eIF2Bε are representative of changes in expression of the holoenzymes during sepsis (22, 37, 42, 43). In the present study, the relative abundance of eIF2α and eIF2Bε in skeletal muscle was measured by densitometric analysis of immunoblots from the different experimental groups. Equal amounts of protein (160 µg) were electrophoresed on each lane. Therefore, a change in intensity on the immunoblot indicates that the relative amount of the eukaryotic initiation factor is different in one condition compared with another. Representative immunoblots from studies examining the effects of TNFBP on the content of eIF2α and eIF2Bε protein in gastrocnemius are shown in Fig. 4. The densitometric analyses from several immunoblots are summarized in Table 2. Consistent with our previous reports (36, 43), there was no significant decrease in the relative abundance of the eIF2α protein during sepsis compared with control. TNFBP did not affect expression of eIF2α in either control or septic rats.

We previously established that the expression of the ε-subunit of eIF2B is reduced during sepsis (42, 43). Therefore, we investigated whether injection of TNFBP prevented the sepsis-induced reduction in eIF2Bε protein. As described previously, the amount of eIF2Bε (expressed relative to total tissue protein) was reduced by 50% in muscle from septic rats (P < 0.01 vs. Control; Table 2). We hypothesized that the sepsis-induced alterations in expression of eIF2Bε should be prevented by injection of TNFBP. Injection of septic rats with TNFBP limited the sepsis-induced decrease in gastrocnemius eIF2Bε expression and restored eIF2Bε to values that were not significantly different from those of control rats.

Table 2. Effects of chronic abdominal sepsis and TNFBP on relative abundance of eIF2α and eIF2Bε protein in gastrocnemius

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>eIF2α</th>
<th>eIF2Bε</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>23 ± 3</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Control + TNFBP</td>
<td>6</td>
<td>28 ± 4</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>Septic</td>
<td>8</td>
<td>28 ± 2</td>
<td>10 ± 2*</td>
</tr>
<tr>
<td>Septic + TNFBP</td>
<td>7</td>
<td>31 ± 3</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed in arbitrary units/mg protein; n, nos. of animals in each group. Muscles from Control, Control + TNFBP, Sepsis, and Sepsis + TNFBP animals were homogenized. Equal amounts (160 µg) of protein were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies to eukaryotic initiation factors (eIF) eIF2α and eIF2Bε. Immunoblots were visualized with chemiluminescence technique per manufacturer’s instructions. Relative amounts of proteins were obtained from densitometric analysis of immunoblots. eIF2Bε (ANOVA) F = 6.09; P < 0.005, *P < 0.01 vs. Control, †P < 0.05 vs. Septic + TNFBP.

DISCUSSION

We examined the effect of TNFBP, a p55 TNF receptor on skeletal muscle protein synthesis during chronic abdominal sepsis. TNFBP comprises a polyethylene glycol-linked dimer of the recombinant human TNF p55-soluble receptor that exhibits high affinity for TNF. The TNFBP-PEGylated dimer is up to 50-fold more potent than the shed soluble p55 TNF receptor in its ability to block TNF. TNFBP acts as a specific inhibitor of TNF bioactivity by binding TNF and sequestering cytokine in a TNF-TNFBP complex (9, 29, 33). In the present experiments, administration of TNFBP to septic rats significantly ameliorated the sepsis-induced net catabolism of gastrocnemius protein, as evidenced by an increased muscle weight and protein content per gastrocnemius compared with untreated septic rats. The loss of protein during the septic insult results from an increase in protein degradation and a decrease in protein synthesis. Therefore, it seems likely that the protein-sparing effects of TNFBP stem from its ability to limit protein degradation and enhance protein synthesis. Administration of antisera to TNF reduces protein degradation in skeletal muscle during acute peritonitis (44). However, similar measurements on protein synthesis in vivo have not been reported after administration of TNF antisera. In the present set of investigations, sepsis caused a 39% decrease in the rate of protein synthesis in gastrocnemius. Treatment of septic rats with TNFBP prevented the sepsis-induced inhibition of protein synthesis and translational efficiency in gastrocnemius, such that no significant differences were observed between TNFBP-treated septic and control rats. We expressed the rate of protein synthesis as nanomoles of Phe incorporated into mixed muscle proteins per milligram of muscle protein per hour. Quantitatively similar percent decreases in protein synthesis (−39%) were obtained when the results were expressed as nanomoles of Phe incorporated into mixed muscle proteins per gram wet weight per hour (−35%) or nanomoles of Phe incorporated into mixed muscle proteins per milligrams of RNA per hour (−42%) (see explanation that follows). Therefore, part of the
protein-sparing effects of TNFBP resulted from its ability to abrogate the inhibitory effects of sepsis on gastrocnemius protein synthesis. This finding appeared to be independent of the parameters used to calculate the rate of protein synthesis.

The rat responds to the implantation of an infected fecal-agar pellet in three phases. The first phase is characterized by recovery from abdominal surgery, initiation of a septic focus, and anorexia. Plasma TNF concentrations peak within 4 h of implantation of the fecal-agar pellet and return to control values within 24 h (19). Approximately 20% of the animals expire at various times within 48 h after the induction of sepsis. After that period, all animals survive and form an abscess (26, 38, 41). Therefore, it becomes problematic as to which septic animals are in a preterminal state, and measurement of rates of protein synthesis during the anorexic period can be extremely variable (for review see Refs. 6, 34, 38). During this phase, anorexia induced by surgery and/or infection contributes to the loss of body weight and muscle protein. We have previously established that reduced food intake during the first 48 h after the induction of sepsis masks any additional inhibitory effect of infection per se on protein synthesis in muscle (19). This conclusion is consistent with the findings of Hoshino et al. (17), who reported that muscle protein wasting after infusion of TNF was a consequence of reduced food intake rather than TNF.

The second phase is characterized by a stable, hypermetabolic septic state in which normal growth or positive nitrogen balance is not achieved despite normal food intake (6, 19, 34, 38, 40). We have previously established that, during this period, the rate of protein synthesis in hindlimb muscles measured in vivo or in vitro is reduced ~40% compared with pair-fed control rats (6, 19, 34, 35, 38, 42). Thus the effects of sepsis on protein synthesis can be distinguished from those of decreased food intake. Moreover, ~5 days are required before significant decreases in eIF2B content can be observed in gastrocnemius of septic rats (42, 43). The third phase is characterized by a waning of the hypermetabolic septic state. During this phase there is a partial recovery from the septic insult, as evidenced by decreased leukocytosis, normalization of carbohydrate and protein metabolism, decreased abscess size, and a growth curve indistinguishable from healthy control animals. In the present study, we investigated whether administration of TNFBP modulates skeletal muscle protein metabolism on day 5 postinfection during the hypermetabolic phase of sepsis. Treatment of septic rats with TNFBP attenuated the loss of muscle protein and inhibition of protein synthesis that developed during the hypermetabolic phase of sepsis.

At least three lines of evidence suggest that it is highly unlikely, although not impossible, that the ability of TNFBP to prevent skeletal muscle wasting during the stable, hypermetabolic phase of sepsis is the result of a nonspecific effect of the compound rather than, or in addition to, its ability to neutralize TNF. First, treatment of control animals with TNFBP did not modify muscle weight, protein content, or protein synthesis. TNFBP does not appear to have a direct or indirect effect to stimulate protein metabolism in skeletal muscle from nonseptic animals. If TNFBP acted in a nonspecific manner, we would have expected a stimulation of protein synthesis in muscles from control rats. Second, although daily injection of the TNFBP does induce antibody production to the compound, measurable antibody titers are not observed until 8 days of treatment (31). The present set of experiments was completed within 5 days of the initial injection of TNFBP. Third, there is no evidence that administration of TNFBP reduces the number of TNF receptors on cells.

To determine the potential mechanisms responsible for the maintenance of protein synthesis in septic rats treated with TNFBP, we examined the total muscle RNA content and translational efficiency. Increasing the number of ribosomes enhances protein synthesis. Because ~80% of the total cellular RNA is ribosomal, alterations in muscle RNA content reflect changes in the relative amount of ribosomes. TNFBP treatment of septic rats did not increase total RNA content in muscle. Therefore, the restoration of protein synthesis in TNFBP-treated septic rats did not result from an increased number of ribosomes. Instead, TNFBP prevented the sepsis-induced inhibition of gastrocnemius protein synthesis by increasing translational efficiency. Translational efficiency reflects how well the existing protein synthesis machinery functions and is related to the activity and/or amount of the components involved in the process of protein synthesis. The effect of TNFBP on protein synthesis and translational efficiency was specific for septic animals because TNFBP was without effect on these parameters in muscle from control rats.

One potential mechanism to account for a defect in translation efficiency during sepsis is through regulation of p70S6 kinase (2, 4, 7, 10, 18, 24, 34). Phosphorylation of ribosomal S6 protein enhances translation of mRNA into protein (for review see Ref. 10). Ribosomal S6 protein is uniquely positioned to regulate translation by its location at the tRNA binding site on the 40S ribosome. Ribosomal S6 protein is phosphorylated by a family of 70-kDa protein kinases termed p70S6 kinase (10). Phosphorylation of ribosomal S6 protein results in a modest increase in the overall rate of protein synthesis and a selective increase in translation of mRNA containing a polypyrimidine tract in the 5′-terminus (18). The p70S6 kinase, in turn, activated by phosphorylation catalyzed by the FKBP-rapamycin-associated protein (FRAP)/mTOR pathway (2). Linkage of the FRAP/mTOR pathway with changes in phosphorylation of p70S6 kinase would define potential downstream regulators of response to sepsis. It is not known whether p70S6 kinase plays a role in the inhibition of muscle protein synthesis during sepsis. We anticipated that the phosphorylation state would be dramatically reduced in muscle from septic animals if phosphorylation of p70S6 kinase were important in regulating protein synthesis during sepsis, because dephosphorylation inactivates p70S6 kinase. However, no apparent differences were observed in the prominence of the phosphory-
lated bands of the p70S6 kinase between muscles from control and septic rats, despite a 39% reduction in rates of protein synthesis. Treatment of septic rats with TNFBP did not increase the phosphorylation state of the p70S6 kinase compared with untreated septic rats despite a stimulation of protein synthesis. Thus phosphorylation of p70S6 kinase did not correlate with alterations in skeletal muscle protein synthesis during sepsis. These results suggest that inactivation of p70S6 kinase by dephosphorylation does not play a role in the sepsis-induced inhibition of skeletal muscle protein synthesis.

In contrast, the formation of a 43S preinitiation complex appears to be an important regulatory mechanism for muscle protein synthesis during sepsis. The ability of skeletal muscle to incorporate methionine into a 43S initiation complex is reduced by 65% during sepsis (37). The reduction in 43S initiation complex formation correlates with the inhibitory effect of sepsis on gastrocnemius protein synthesis. Assembly of the 43S preinitiation complex is dependent on the formation of a ternary complex consisting of eIF2, GTP, and met-tRNA<sub>met</sub> (for review see Refs. 6, 34). The primary function of eIF2 is to bind met-tRNA<sub>met</sub> in a GTP-dependent manner. This is important both for providing methionyl-tRNA in the P site on ribosomes and for identifying the initiating codon. eIF2-mediated binding of met-tRNA<sub>met</sub> to the 40S ribosomal subunit is regulated by several mechanisms. First, the cellular content of eIF2 protein can be altered. A correlation between the cellular content of eIF2 and the rate of protein synthesis is observed in nonmuscle tissues (22, 36). However, neither sepsis nor TNFBP decreased the expression of eIF2<sub>x</sub> in gastrocnemius. This observation is consistent with previous reports concerning the effect of sepsis on the expression of eIF2 in muscle (43). Thus alterations in eIF2 expression are probably not an important regulatory mechanism of muscle protein synthesis during sepsis.

The second mechanism regulating the activity of eIF2 involves another eukaryotic initiation factor, eIF2B. eIF2B is a guanine nucleotide exchange factor required for exchange of GDP for GTP on eIF2 (for review see Refs. 6, 34). Inhibition of eIF2B activity prevents recycling of guanine nucleotides and results in a decreased amount of eIF2-GTP available to form the ternary complex, thereby inhibiting peptide-chain initiation. The activity of eIF2B is decreased in gastrocnemius during sepsis (36), presumably as the result of a reduced expression of eIF2B (42, 43). Changes in eIF2B expression correlate with the overall rate of protein synthesis in muscle. The abundance of eIF2B<sub>x</sub> was significantly reduced in gastrocnemius from septic rats. The decrease in eIF2B<sub>x</sub> may account, in part, for the reduction in gastrocnemius protein synthesis observed in septic rats. Therefore, one goal of the present set of experiments was to establish the effect of TNFBP on the expression of eIF2B<sub>x</sub>. If reduced eIF2B<sub>x</sub> is important in regulating protein synthesis during sepsis, then treatment with TNFBP should prevent the fall in eIF2B<sub>x</sub> protein. Treatment of septic rats with TNFBP limited the decrease in gastrocnemius eIF2B<sub>x</sub> expression observed in septic rats. TNFBP did not have any effect on eIF2B<sub>x</sub> expression in control rats. The maintenance of eIF2B<sub>x</sub> in septic rats treated with TNFBP was associated with enhanced rates of protein synthesis compared with untreated septic rats. The results of the present set of investigations provide further evidence that a decreased eIF2B<sub>x</sub> content may partially account for the observed inhibition of protein synthesis during sepsis.

TNF-α is known to stimulate the secretion of other cytokines, including interleukin (IL)-1 and IL-6, as well as other inflammatory mediators. Furthermore, TNF-α often acts in synergy with other cytokines. Thus it is not possible to ascribe the metabolic effects of TNFBP on protein metabolism in muscle during hypermetabolic sepsis to a primary effect of TNF-α or a secondary effect mediated through another cytokine or inflammatory mediator whose expression is dependent on TNF. TNFBP lowers plasma IL-1β and IL-6 concentrations after an E. coli or endotoxin challenge (28, 31, 33). Like TNF, both of these cytokines have been implicated as mediators of the metabolic response to sepsis. However, mice genetically deficient in IL-6 exhibit the same degree of weight loss as their wild-type controls in response to acute endotoxin administration (11). Thus a role for IL-6 in mediating muscle protein wasting in sepsis is questionable. In contrast, we have previously shown that inhibition of IL-1 bioactivity with a specific IL-1 receptor antagonist abates the reduction in muscle loss and protein synthesis during sepsis (42). However, transient exposure of human myoblast to TNF-α causes an inhibition in protein synthesis that is maintained for ~48 h after removal of the cytokine (14). Thus TNF can directly affect protein synthesis in muscle cells independent of other cytokines. These observations were interpreted to suggest that a transient increase in plasma TNF-α concentrations may impair protein synthesis long after the cytokine has disappeared from the circulation (14). Thus the results of the present study and others (1, 3, 6, 14, 19, 34) are consistent with the hypothesis that TNF-α plays a role, either directly or indirectly, in mediating the inhibition of muscle protein synthesis during chronic sepsis.

In summary, our studies provide evidence that treatment with TNFBP significantly attenuates the catabolism of muscle protein during the stable, hypermetabolic phase of sepsis. This effect was mediated in part by attenuation of the sepsis-induced inhibition of gastrocnemius protein synthesis. TNFBP maintained the translation efficiency of gastrocnemius during the septic insult by preventing the sepsis-induced decrease in eIF2B<sub>x</sub> expression. Our results are consistent with the hypothesis that TNF-α plays a role in mediating the reduction in muscle protein synthesis during chronic sepsis, possibly by modulating the expression of eIF2B<sub>x</sub>.

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