

Glucocorticoids and TNF α Interact Cooperatively to Mediate Sepsis-Induced Leucine Resistance in Skeletal Muscle

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Sepsis blunts the ability of nutrient signaling by leucine to stimulate skeletal muscle protein synthesis by impairing translation initiation. The present study tested the hypothesis that overproduction of either tumor necrosis factor (TNF)- α or glucocorticoids mediate the sepsis-induced leucine resistance. Prior to producing peritonitis, rats received either vehicle, TNF binding protein (TNF_{BP}) to inhibit endogenous TNF α action, and/or the glucocorticoid receptor antagonist RU486. Leucine was orally administered to all rats 24 h thereafter and the gastrocnemius removed 20 min later to assess protein synthesis and signaling components important in controlling peptide-chain initiation. Muscle protein synthesis was 65% lower in septic rats administered leucine than in leucine-treated control animals. This reduction was not prevented by either TNF_{BP} or RU486 alone, but was completely reversed by the combination. This sepsis-induced leucine resistance was associated with an 80% reduction in the amount of active eIF4E-eIF4G complex, a 5-fold increase in the formation of the inactive eIF4E-4E-BP1 complex as well as markedly reduced (at least 70%) phosphorylation of 4E-BP1, eIF4G, S6K1, S6, and mTOR. Pretreatment of septic rats with either TNF_{BP} or RU486 individually only nominally improved the leucine action as assessed by the above-mentioned endpoints. In contrast, when TNF_{BP} and RU486 were co-administered, the ability of sepsis to impair the leucine-stimulated phosphorylation of 4E-BP1, eIF4G, S6K1, and S6 as well as the redistribution of eIF4E was essentially prevented. No differences in the total amount or phosphorylation of eIF2 α and eIF2B ϵ were detected between the different groups, and changes could not be attributed to differences in the prevailing plasma concentration of insulin or leucine. Our data demonstrate the sepsis-induced leucine resistance in skeletal muscle results from the cooperative interaction of both TNF α and glucocorticoids.

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INTRODUCTION

Leucine availability represents an important nutritional signal responsible for postprandial stimulation of muscle protein synthesis (1). Amino acid refeeding after short-term starvation increases muscle protein synthesis, and physiologically-relevant concentrations of amino acids enhance synthesis in the perfused hindlimb, in incubated muscle, and cultured myocytes (2–8). The branched-chain amino acid leucine accounts for all or most of the ability of a mixture of amino acids to stimulate protein synthesis. Moreover, the anabolic

effects of leucine are mediated by cell signaling pathways which stimulate translation initiation via activation of mammalian target of rapamycin (mTOR) (2–10). This protein kinase represents a point of signal amplification because stimulation of mTOR phosphorylates multiple substrates which enhance translation, including eukaryotic initiation factor 4E-binding protein (4E-BP1) and the ribosomal protein (rp) S6 kinase-1(S6K1) (11).

The leucine-induced phosphorylation of 4E-BP1 positively modulates the formation of the functional eukaryotic initi-

ation factor (eIF)-4F complex (1,2,9). This complex is necessary for cap-dependent translation and is composed of 3 subunits (i.e., eIF-4E, -4G and -4A). In muscle eIF4E is the least abundant of these subunits and considered rate-limiting in the binding of mRNA to ribosomes (12). During initiation the eIF4E-mRNA complex binds to eIF4G and eIF4A forming the active eIF4F complex and thereby enhances translation. The assembly of the functional eIF4F complex is controlled in part by 4E-BP1 functioning as a cap-dependent translational repressor. The non-phosphorylated form of this binding protein obstructs the interaction of eIF4G with eIF4E limiting assembly of the active eIF4F complex (12,13). Increased phosphorylation of 4E-BP1 by leucine releases eIF4E permitting its binding to eIF4G and stimulating mRNA translation

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(1). Additionally, leucine stimulates phosphorylation and activation of S6K1 (1,2,9,10). This leucine-induced hyperphosphorylation of S6K1 is largely inhibited by rapamycin implicating mTOR in the activation of this downstream kinase (2,14). Leucine-induced activation of S6K1 leads to phosphorylation of the physiological substrate rpS6 and is tightly correlated with a proportional increase in in vivo protein synthesis (5,15)

A defining characteristic of sepsis is the negative nitrogen balance which leads to the erosion of lean body mass (16). This sepsis-induced muscle catabolism results from an increase in protein degradation and a decrease in protein synthesis (15,17,18). The decreased muscle protein synthesis produced by acute peritonitis results from a defect in translation initiation characterized by the reduced constitutive phosphorylation of 4E-BP1 and S6K1/rpS6 (15). A reduction in peptide-chain initiation and protein synthesis is also observed in response to elevated tumor necrosis factor (TNF)- α and glucocorticoids, both of which are increased by sepsis (19–22). Furthermore, sepsis and endotoxin impair the normal protein synthetic response to leucine (15,23). Therefore, because sepsis increases secretion of both TNF α and glucocorticoids and because these immunomodulators impair protein synthesis, the purpose of the present study was to determine their potential role in mediating the sepsis-induced leucine resistance. Hence, septic rats were pretreated with TNF binding protein (TNF_{BP}) and/or the glucocorticoid receptor antagonist RU486 to block the endogenous actions of these catabolic stimuli and determine whether they prevented the sepsis-induced decrement in leucine action via alterations in cell signaling pathways known to regulate translation initiation.

MATERIALS AND METHODS

Experimental Protocols

Male Sprague-Dawley rats (200–225 g; Charles River Breeding Laboratories,

Cambridge, MA) were acclimated for 1 week in a controlled environment. Water and standard rat chow were provided *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine and adhered to National Institutes of Health guidelines for the use of experimental animals. Sepsis was induced by cecal ligation and puncture (CLP). Rats were anesthetized with pentobarbital (50–60 mg/kg) and a midline laparotomy was performed. The cecum was ligated at its base and punctured twice using a 20G needle. The cecum was returned to the peritoneal cavity, the muscle and skin layers closed, and rats were resuscitated with 10 mL of 0.9% sterile saline administered subcutaneously. The nonseptic control animals received a laparotomy with intestinal manipulation and were resuscitated. After surgery, animals were fasted but permitted free access to water. Hence, any observed changes between septic and nonseptic rats cannot be attributed to differences in nutritional status.

Septic and nonseptic rats were administered 1.35 g/kg BW leucine (54 g/L) by oral gavage 24 h after CLP. This dose of leucine was selected because it is the amount consumed in a 24-h period when rats of this age and strain are provided free access to food (15). This leucine dose maximally stimulates muscle protein synthesis and indices of translation initiation in nonseptic rats (24). Finally, this leucine dose increases the plasma leucine concentration to approximately 1500 $\mu\text{mol/mL}$ (15), which is 2- to 3-fold greater than seen in meal-fed rats (25); this increase was comparable in control and septic rats (15 and current study).

To determine the role of endogenously produced TNF α , septic rats were injected subcutaneously with TNF_{BP} (1 mg/kg, 1 mL/rat; Amgen, Boulder, CO), an antagonist of TNF α action, 4 h prior to CLP. The dose of TNF_{BP} and timing of the initial injection were chosen based on the peak plasma TNF levels (60–80 pg/mL) 4 h after induction of sepsis in this model

(26) and previous pharmacokinetic studies (data not shown) demonstrating the plasma concentrations of TNF_{BP} remain above 500 ng/mL for at least 12 h after subcutaneous injection of 1 mg/kg TNF_{BP}. Therefore, by injecting the TNF_{BP} 4 h prior to induction of sepsis, we were assured of relatively high concentrations of TNF_{BP} throughout the septic insult. A lower dose of TNF_{BP}, albeit in baboons, was sufficient to completely neutralize the massive increase in circulating TNF α induced by *E. coli* bacteremia (27). The dose of TNF_{BP} used in the current study is sufficient to prevent the sepsis-induced redistribution of eIF4E between active and inactive complexes in cardiac muscle under basal conditions (28). Finally, this dose of TNF_{BP} completely inhibits the increase in atrogin-1, believed to be responsible for the increased protein degradation, after an exogenous infusion of TNF α which raises the plasma TNF α concentration to values above that seen in CLP (29). Collectively, these studies support the contention that the dose of TNF_{BP} was not limiting under the present experimental conditions. To determine the role of glucocorticoids in modulating the sepsis-induced change in leucine action septic rats were injected subcutaneously with the type II glucocorticoid receptor antagonist RU486 (20 mg/kg, Mifepristone; Sigma, St. Louis, MO) 30 min prior to CLP. This or lower doses of RU486 prevents the glucocorticoid-induced increase in muscle proteolysis (18) and the endotoxin-induced decrease in plasma IGF-I (30). Finally, additional septic rats were injected with both TNF_{BP} and RU486 (1 mg/kg and 20 mg/kg, respectively) prior to CLP. Nonseptic animals administered TNF_{BP} and RU486 were not included in the presented study because our preliminary data ($n = 5$ per group) indicated there was no statistically significant difference in the rate of muscle protein synthesis between naive control animals (1.21 ± 0.09 nmol Phe/mg protein/hour) and those treated with TNF_{BP} or RU486 (1.15 ± 0.11 and 1.22 ± 0.08 nmol Phe/mg protein/hour, respectively). Furthermore, these drugs did not

alter the phosphorylation of 4E-BP1 and S6 in muscle, compared with values from untreated control rats (data not shown).

Finally, in a 3rd study, rats were injected via a lateral tail vein with L-[2,3,4,5,6-³H]-phenylalanine (Phe; 150 mM, 30 μ Ci/mL; 1 mL/100 g body weight) 10 min after administration of leucine or saline for in vivo determination of muscle protein synthesis (15,19,20). Rats were then anesthetized with pentobarbital and the gastrocnemius was freeze-clamped 10 min after injection of Phe (i.e., 20 min after leucine). Thereafter, blood was collected from the abdominal aorta to measure plasma Phe specific radioactivity. Blood was centrifuged and plasma was collected. In this study and all others, tissue and plasma samples were stored at -70°C until analyzed. Muscle was powdered and a portion used to estimate the rate of incorporation of [³H]Phe into protein exactly as previously described (15,19,20).

Immunoprecipitation and Western Blotting

The tissue preparation was exactly as described by our laboratory (15,19,20,23). Briefly, muscle homogenates were prepared in a 1:4 ratio of ice-cold homogenization buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β -glycerophosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, 0.5 mM sodium vanadate) and clarified by centrifugation. The supernatant was aliquoted and mixed with an equivalent volume of sample buffer. The samples were subjected to electrophoresis on either a 7.5% polyacrylamide gel for S6K1, a 15% polyacrylamide gel for phosphorylated S6 and 4E-BP1, and 6% gel for mTOR, and proteins electrophoretically transferred to PVDF. The blots were incubated with either primary antibodies to total S6K1 (no. 230, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-specific S6K1 (Thr421/Ser424 or Thr389; Cell Signaling Technology, Beverly, MA), total 4E-BP1 (Bethyl Laboratories, Montgomery, TX), phospho-specific 4E-BP1

(Thr37; Cell Signaling), total and phosphorylated (Ser 2481) mTOR (Bethyl Laboratories), total and phosphorylated (Ser 1108) eIF4G (Cell Signaling) or phosphorylated-S6 (Ser235/Ser236; Cell Signaling). The total amount and the phosphorylation state of the α -subunit of eIF2 (Ser51 eIF2 α ; Cell Signaling) and the ϵ -subunit of eIF2B (Ser535 eIF2B ϵ ; Biosource International, Camarillo, CA) in muscle were estimated by protein immunoblot analysis, as described previously (19). All blots were washed, incubated with secondary antibody (horseradish peroxidase conjugated goat anti-mouse or goat anti-rabbit), and developed with enhanced chemiluminescence (ECL) Western blotting reagents as per the manufacturer's (Amersham) instructions. The blots were exposed to X-ray film, developed, and finally 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 (15,19, 20,23). Briefly, eIF4E was immunoprecipitated using an anti-eIF4E monoclonal antibody (gift from Drs. Jefferson and Kimball; Hershey, PA) and subjected to polyacrylamide gel electrophoresis. The blots were incubated with a mouse anti-human eIF4E antibody, a rabbit anti-rat 4E-BP1 antibody,

or a goat anti-eIF4G antibody. The phosphorylated forms of 4E-BP1 were measured after immunoprecipitation of 4E-BP1. The blots were developed with ECL and the autoradiographs scanned for analysis as described above.

Plasma Determinations

The plasma insulin concentration was measured using a commercial radioimmunoassay (RIA) for rat insulin (Linco Research, St. Charles, MO). The plasma leucine concentration was determined by derivatizing with phenylisothiocyanate, followed by high-performance liquid chromatography analysis (15).

Statistical Analysis

Experimental data for each condition are summarized as means \pm SE where the number of animals was 7-8 for each treatment group. Statistical evaluation of the data was performed using ANOVA followed by Student-Neuman-Keuls test to determine treatment effect. Differences between the groups were considered significant when $P < 0.05$.

RESULTS

Alteration in Muscle Protein Synthesis

Protein synthesis in the gastrocnemius was 65% lower in septic rats administered leucine, compared with values from the

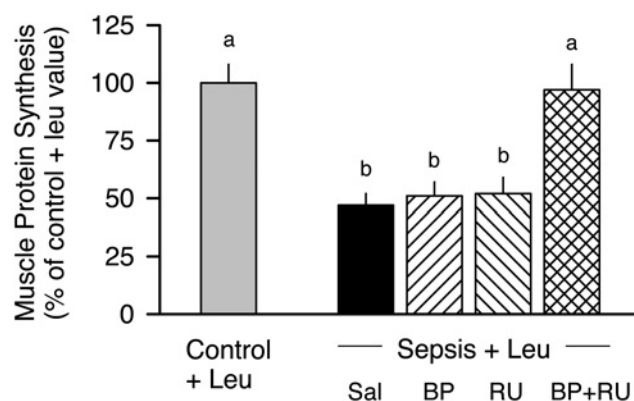


Figure 1. Effect of sepsis on leucine (Leu)-stimulated muscle protein synthesis Sal=saline; BP= TNF_{BP} ; and RU=RU486. Values are means \pm SEM; $n = 6-7$ rats per group. Means with different letters are statistically different from each other ($P < 0.05$).

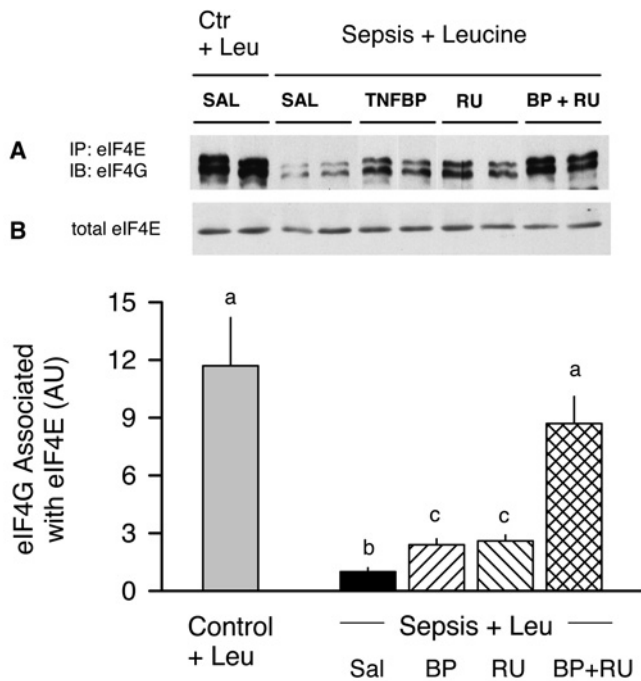


Figure 2. Effect of sepsis on leucine (Leu)-stimulated association of eIF4G with eIF4E in skeletal muscle. *Panel A*: eIF4E was immunoprecipitated (IP) and the amount of eIF4G bound to eIF4E assessed by immunoblotting (IB); *Panel B*: representative Western blot of total eIF4E; *bar graph*: densitometric analysis of immunoblots of eIF4G associated with eIF4E, where the value from septic rats treated with Leu and without additional drug administration (Sal) was set at 1.0 AU (arbitrary units). Values are means \pm SEM; $n = 7-8$ per group. Means with different letters are statistically different from each other ($P < 0.05$).

control + leucine group (Figure 1). This lower synthetic rate was not prevented by either TNF_{BP} or RU486 alone, but was completely reversed by the combination.

Alterations in eIF4E Distribution

The cellular mechanism by which sepsis induces muscle leucine resistance was investigated by analyzing known regulatory steps in translational control. The ability of leucine to increase the amount of the active eIF4E-eIF4G complex in muscle was reduced by 90% in septic rats, compared with values from leucine-treated control rats (Figure 2). Pretreatment of septic rats with either TNF_{BP} or RU486 alone slightly, albeit significantly, increased the action of leucine on this association. However, the amount of the

eIF4E-eIF4G complex was still reduced more than 80%, compared with control rats administered leucine. In contrast, the ability of leucine to increase eIF4G binding with eIF4E was not different in septic rats treated with both TNF_{BP} + RU486, compared with values in the control + leucine group. None of the changes in eIF4E-eIF4G complex formation could be ascribed to sepsis-, drug-, or nutrient-induced changes in the total amount of eIF4E in muscle (Figure 2B).

Conversely, the amount of the inactive eIF4E-4E-BP1 complex was 5-fold greater in septic rats administered leucine, compared with values from control rats given leucine (Figure 3). Because the hyperphosphorylated γ -isoform of 4E-BP1 cannot bind to eIF4E, the eIF4E immuno-

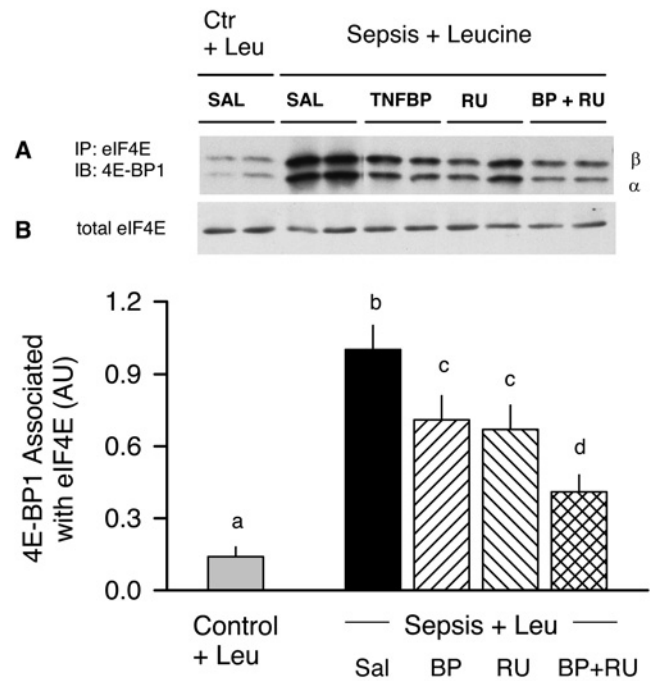


Figure 3. Effect of sepsis on leucine (Leu)-induced changes in the binding of 4E-BP1 to eIF4E in skeletal muscle. *Panel A*: eIF4E was immunoprecipitated (IP) and the amount of 4E-BP1 bound to eIF4E assessed by immunoblotting (IB); *Panel B*: representative Western blot of total eIF4E; and *bar graph*: densitometric analysis of immunoblots of 4E-BP1 associated with eIF4E, where the value from septic rats treated with Leu and without additional drug administration (Sal) was set at 1.0 AU. Values are means \pm SEM; $n = 7-8$ per group. Means with different letters are statistically different from each other ($P < 0.05$).

precipitate contains the 2 nonphosphorylated α - and β - isoforms of 4E-BP1 which resolve as a doublet on Western blot analysis (Figure 3A). Pretreatment of septic rats with either TNF_{BP} or RU486 individually reduced the amount of the inactive complex by 30%, but the amount remained elevated compared with control + leucine group. A further reduction in eIF4E-4E-BP1 was seen in septic rats co-administered both TNF_{BP} and RU486; however, the amount of inactive complex was still greater than values in leucine-gavaged control animals.

Phosphorylation of 4E-BP1 and eIF4G

To define the mechanism through which sepsis modulates eIF4E availability we examined the phosphorylation

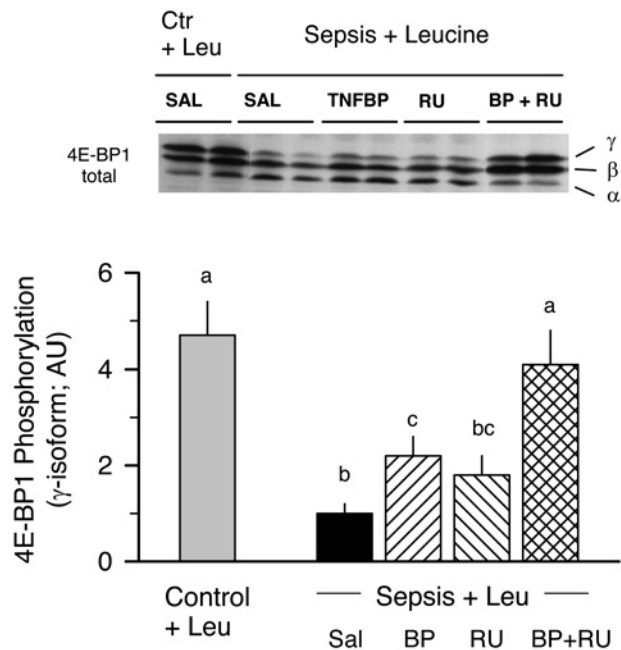


Figure 4. Effect of sepsis on leucine (Leu)-stimulated phosphorylation of 4E-BP1. *Top panel:* is a representative immunoblot for total 4E-BP1 and the positions of the α -, β -, and γ -isoforms are indicated; *bar graph:* represents densitometric analysis of the hyperphosphorylated γ -isoform of 4E-BP1. The value from septic rats treated with Leu and without additional drug administration (Sal) was set at 1.0 AU. Values are means \pm SEM; n = 7-8 per group. Means with different letters are statistically different from each other ($P < 0.05$).

state of 4E-BP1. The above mentioned changes in eIF4E distribution in the sepsis + leucine group were associated with an 80% decrease in the hyperphosphorylated γ -isoform of 4E-BP1, compared with values from the control + leucine group (Figure 4). In septic rats, a modest leucine-induced increase in 4E-BP1 phosphorylation was seen in rats treated with either TNF_{BP} or RU486 alone. However, when both agents were administered together the responsiveness of septic rats to leucine was the same as control animals. Phosphorylation of 4E-BP1 is ordered and hierarchical, with Thr37 and Thr46 being the initial residues phosphorylated (31). The sepsis and/or leucine-induced changes in Thr37-phosphorylation of 4E-BP1 were comparable to those described for the γ -isoform of 4E-BP1 (data not shown).

The interaction between eIF4E and eIF4G can also be regulated in part by the phosphorylation of eIF4G which is enhanced by mitogen and serum stimulation and conversely inhibited by rapamycin (32). The phosphorylation of eIF4G in response to leucine was 80% less in muscle from septic rats than in control animals given leucine (Figure 5). Individually TNF_{BP} or RU486 moderately ameliorated the sepsis-induced inhibition of leucine-stimulated eIF4G phosphorylation. However, muscle from septic rats treated with TNF_{BP} + RU486 in combination exhibited the same extent of eIF4G phosphorylation as control animals given leucine. Changes in eIF4G phosphorylation were not due to a leucine- or sepsis-induced alteration in the content of total eIF4G in muscle (Figure 5B).

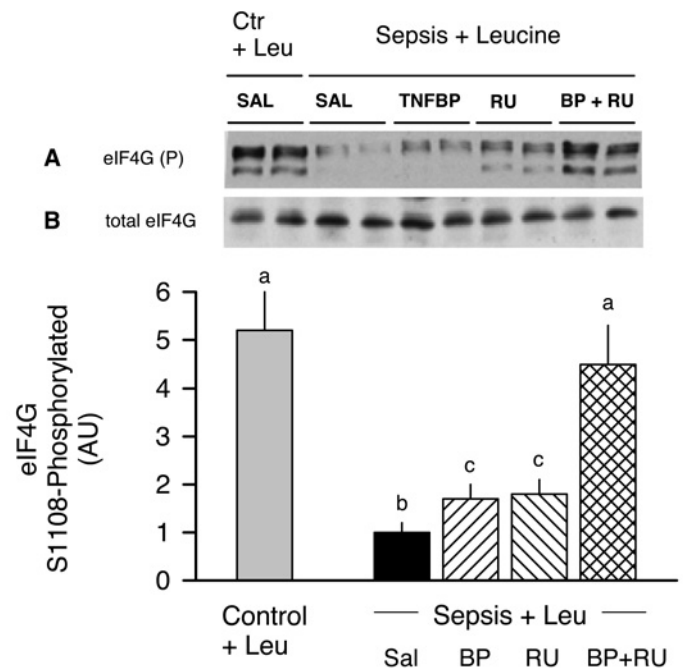


Figure 5. Effect of sepsis on leucine (Leu)-stimulated phosphorylation of eIF4G. *Panels A and B:* a representative immunoblot of Ser1108-phosphorylated (P) and total eIF4G; *bar graph:* densitometric analysis of eIF4G phosphorylation where the value from septic rats treated with Leu and without additional drug administration (Sal) was set at 1.0 AU. Values are means \pm SEM; n = 7-8 per group. Means with different letters are statistically different from each other ($P < 0.05$).

S6K1 and S6 Phosphorylation

In muscle from leucine-treated control rats, there was an intensive band indicating Thr389-phosphorylation of S6K1 (Figure 6A). Phosphorylation of this residue is necessary for full and functional activation of S6K1 (33). In contrast, there was essentially no S6K1 phosphorylation in muscle of leucine-treated septic rats. Pretreatment of septic rats with either TNF_{BP} or RU486 alone failed to improve leucine responsiveness. However, the extent of S6K1 Thr389-phosphorylation was the same in septic rats administered the TNF_{BP} + RU486 combination and leucine-treated control animals. Comparable changes in the phosphorylation of S6K1 at residue Thr421/Ser424 in the autoregulatory domain were also observed in response to leucine and/or sepsis (data not shown).

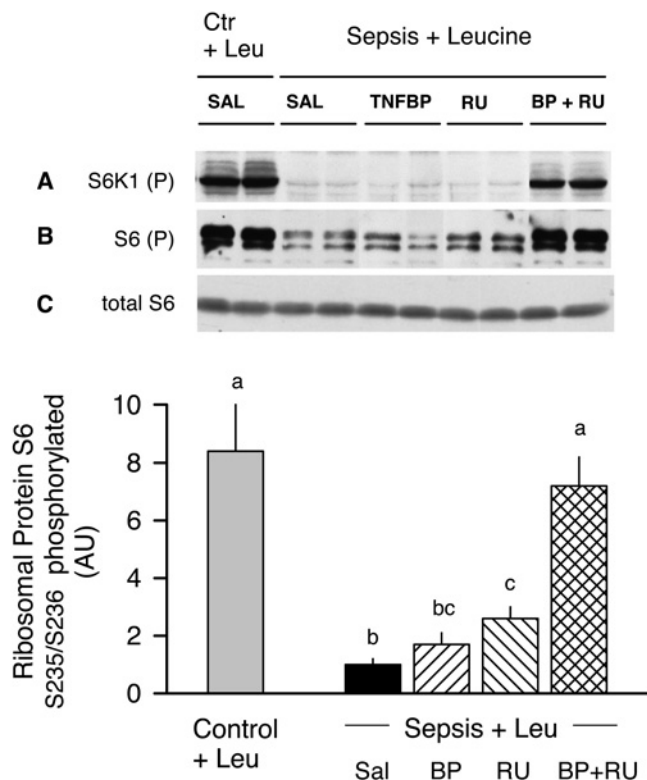


Figure 6. Effect of sepsis on leucine (Leu)-stimulated phosphorylation of S6K1 and ribosomal protein (rp) S6. *Panel A:* a representative immunoblot for the phosphorylation of the Thr389 site of S6K1; *panel B:* a representative immunoblot for the phosphorylation of rpS6 at Ser235/Ser236; *panel C:* representative immunoblot for total rpS6; *bar graph:* the densitometric analysis of rpS6 phosphorylation of Ser235/Ser236 where the value from septic rats treated with Leu and without additional drug administration (Sal) was set at 1.0 AU. Values are means \pm SEM; $n = 7-8$ per group. Means with different letters are statistically different from each other ($P < 0.05$).

The S6 protein is a component of the 40S ribosome and a downstream substrate for S6K1. The leucine-stimulated phosphorylation of rpS6 was 90% lower in septic rats, compared with muscle from control rats provided leucine (Figure 6B and bar graph). Individually TNF_{BP} or RU486 modestly improved leucine-stimulated rpS6 phosphorylation in septic rats. However, together these agents completely prevented the sepsis-induced suppression of rpS6 phosphorylation. Again, these changes were independent of a change in the total amount of rpS6 protein (Figure 6C).

mTOR phosphorylation

When leucine was orally administered to septic rats the extent of mTOR Ser2481-phosphorylation was reduced 70%, compared with the response seen in control rats given leucine (Figure 7). This leucine resistance at the level of mTOR was not ameliorated by either TNF_{BP} or RU486 alone. However, the magnitude of mTOR phosphorylation in muscle from septic rats given both agents in combination was not different from that seen in the control + leucine group. All sepsis-induced changes were independent of a change in total mTOR protein (Figure 7B).

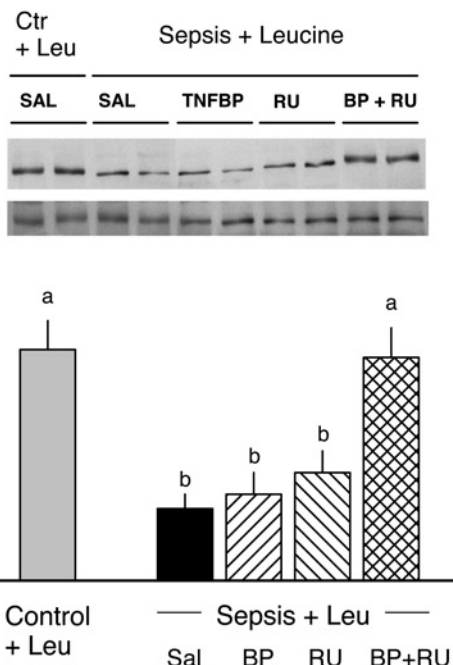


Figure 7. Effect of sepsis on leucine (Leu)-stimulated phosphorylation of mTOR (mammalian target of rapamycin). *Top and middle panels:* a representative immunoblot of Ser2481-phosphorylated (p) and total mTOR, respectively; *bar graph:* densitometric analysis of mTOR phosphorylation where the value from septic rats treated with Leu and without additional drug administration (Sal) was set at 1.0 AU. Values are means \pm SEM; $n = 7-8$ per group. Means with different letters are statistically different from each other ($P < 0.05$).

eIF2 and eIF2B

The 1st step in translation initiation is the formation of a ternary complex consisting of eIF2, GTP, and met-tRNA_i (as reviewed in 34). The binding of met-tRNA_i to the 40S subunit to form the 43S pre-initiation complex is mediated by eIF2, of which the α -subunit appears important in regulating protein synthesis. However, the content of eIF2 α in gastrocnemius did not differ between leucine-treated control and septic rats (541 ± 46 vs 522 ± 39 AU, respectively; $n = 5-6$ per group, $P = \text{NS}$). eIF2 α also undergoes reversible phosphorylation and the extent of phosphorylation is inversely propor-

tional to changes in protein synthesis (34). However, there was no difference in eIF2 α phosphorylation between control + leucine and septic + leucine groups (224 ± 32 vs 231 ± 27 AU, respectively; $P = \text{NS}$). The ability of eIF2 to form a ternary complex is also modified by eIF2B which catalyzes guanine nucleotide exchange and is required to regenerate the active eIF2-GTP complex. In the present study, there was no significant difference in the relative amount of total (755 ± 62 vs 791 ± 58 AU; $P = \text{NS}$) and phosphorylated (334 ± 21 vs 344 ± 32 AU; $P = \text{NS}$) eIF2B ϵ in muscle from control and septic rats administered leucine. Finally, pretreatment of septic rats with TNF $_{\text{BP}}$ or RU486 either alone or in combination also did not significantly alter the total amount or phosphorylation of eIF2 α or eIF2B ϵ (data not shown). Collectively, these data suggest an alteration in the eIF2/eIF2B system is an unlikely locus for the sepsis-induced leucine resistance.

Plasma Insulin and Leucine

The prevailing plasma concentration of the anabolic hormone insulin affects translation initiation via alterations in eIF4E distribution and the phosphorylation of S6K1 (1,2,5–10). However, the insulin concentration of rats in the control + leucine group (1.01 ± 0.13 ng/mL) was not significantly different from septic rats administered leucine without pretreatment (1.12 ± 0.11 ng/mL) or septic rats pretreated with TNF $_{\text{BP}}$, RU486, or TNF $_{\text{BP}}$ + RU486 (1.24 ± 0.21 , 1.09 ± 0.14 and 1.28 ± 0.16 ng/mL, respectively). Likewise, differences in the rate of leucine absorption or clearance would alter the plasma leucine concentration between groups and might account for differences in the phosphorylation of signaling intermediates. However, again, the leucine concentration was not statistically different between animals in the control + leucine group (1323 ± 319 $\mu\text{mol/L}$) and the septic + leucine group (1524 ± 211 $\mu\text{mol/L}$), or septic rats pretreated with TNF $_{\text{BP}}$, RU486, or TNF $_{\text{BP}}$ + RU486 (1487 ± 231 , 1515 ± 198 , and 1299 ± 240 $\mu\text{mol/L}$, respectively). Hence, the ability of TNF $_{\text{BP}}$ +

RU486 to prevent the sepsis-induced leucine resistance could not be attributed to differences in the prevailing blood concentration of insulin or leucine.

DISCUSSION

The present study assessed the relative importance of two known catabolic mediators, TNF α and glucocorticoids, as physiological regulators of the leucine resistance produced acutely by peritonitis. Our data confirm sepsis produced by CLP decreases the anabolic action of leucine as evidenced by the decreased phosphorylation of 4E-BP1, eIF4G, S6K1, and rpS6 as well as the redistribution of eIF4E from the active eIF4E-eIF4G complex to the inactive eIF4E-4E-BP1 complex. Collectively, these changes are consistent with the diminished ability of leucine to stimulate protein synthesis in muscle of septic rats (current study and 4,15) as well as the diminished anabolic response to nutritional support observed in septic patients (35). This sepsis-induced leucine resistance appears independent of changes in translation initiation mediated by the eIF2/eIF2B system. However, we cannot exclude the potential importance of this system in more chronic (for example, 5 days) septic models where eIF2B ϵ has been shown to be reduced (36).

Among amino acids, leucine occupies a central role in the regulation of postprandial stimulation of muscle protein synthesis (1). While the mechanism by which myocytes “sense” leucine is poorly defined, the nutrient signal clearly stimulates mTOR activity (2,5,6,10,23). Our current data indicate that individually TNF $_{\text{BP}}$ and RU486 statistically improved leucine signaling via both the 4E-BP1 and S6K1 pathways in septic animals, although this was not reflected in a significant increase in the autophosphorylation of mTOR. The reason for this apparent discrepancy is unknown but may indicate mechanisms other than Ser2481-phosphorylation are important in controlling mTOR activity. Regardless, the large majority of the sepsis-induced decrement in leucine respon-

siveness still remained when TNF α or glucocorticoids were individually inhibited. In contrast, when the *in vivo* actions of both TNF α and glucocorticoids were inhibited in tandem, the leucine responsiveness of septic rats was comparable to that seen in nonseptic control animals. Overall, these novel findings suggest TNF α and glucocorticoids interact in a cooperative manner to regulate nutrient signaling in skeletal muscle.

Our results also indicate the mechanism for the sepsis-induced muscle leucine resistance differs from the mechanism mediating the decrease in basal protein synthesis seen in the post-absorptive state. In previous studies, treatment of septic rats with agents which decrease TNF α synthesis or antagonize its actions prevented the decrease in protein synthesis and translation initiation as well as the accompanying loss of muscle protein (36,37). These data are consistent with the ability of a sustained infusion of recombinant TNF α to decrease basal peptide-chain initiation and protein synthesis in fasted rats (20). In contradistinction, in the current study the inhibition of TNF α bioactivity alone only nominally improved leucine signaling. Likewise, inhibition of endogenous glucocorticoids by RU486 also failed to prevent the sepsis-induced leucine resistance. This finding complements earlier reports indicting the sepsis-induced decrease in *basal* muscle protein synthesis is also glucocorticoid-independent (18,38). These results were unexpected because the synthetic glucocorticoid dexamethasone markedly decreases both basal and leucine-stimulated muscle protein synthesis via defects in translation initiation (21,22). Collectively, these data suggest the alterations in protein synthesis produced by large doses of the synthetic glucocorticoid dexamethasone may not faithfully mimic the protein metabolic effects produced by the increased secretion of endogenous glucocorticoids. One possible explanation for the ineffectiveness of RU486 in the current study is the elevation in endogenous corticosterone naturally suppresses the inflammatory in-

sult produced by sepsis thereby limiting severity (39). Hence, RU486 treatment of septic rats potentially unmasks or accentuates inhibitory effects of other mediators, such as TNF α . In this regard, RU486 increases the early sepsis-induced increase in NF- κ B activation in muscle (40) and increases cytokine production in septic or endotoxemic animals (41,42). Collectively, these findings are consistent with reports that adrenalectomized rats and intact rats treated with RU486 are more susceptible to the lethal effects of endotoxin, sepsis, IL-1, and TNF α (41–43).

Alternatively, the need to antagonize both TNF α and glucocorticoid actions to prevent the sepsis-induced leucine resistance may also suggest endogenous glucocorticoids are necessary for the full inhibitory effect of TNF α on skeletal muscle protein synthesis to be manifested. The evidence supporting such a permissive effect of glucocorticoids on enhancing sensitivity of various metabolic and humoral immune responses has been reviewed (39,44). For example, it is generally accepted that the permissive effects of glucocorticoids are necessary for the optimal glycogenolytic effects of catecholamines and the gluconeogenic effects of glucagon which combine to maintain glucose homeostasis under stress conditions (45). Moreover, glucocorticoids enhance selected components of the innate immune system. In this regard, pretreatment of humans with cortisol prior to endotoxin increased circulating concentrations of TNF α and IL-6 (46). Furthermore, glucocorticoids act synergistically with either cytokines or endotoxin to increase other diverse inflammatory mediators, such toll-like receptor-2 mRNA and protein (47), IL-6 receptor number (48), and under some conditions the acute-phase protein response (49).

At the molecular level the cooperative effect between glucocorticoids and TNF α is poorly characterized but, theoretically, could result from an upregulation of the glucocorticoid receptor number in muscle and/or the concentration

of the free intracellular glucocorticoid to which receptors are exposed. In this regard, glucocorticoid receptor mRNA and protein as well as binding activity are increased in skeletal muscle of septic rats (50). Additionally, inflammation-induced alterations in 11 β -hydroxysteroid dehydrogenase (11 β HSD) enzyme activity can alter the equilibrium between the concentration of the active glucocorticoid (i.e., cortisol in humans, corticosterone in rodents) and their inert 11-keto metabolite (i.e., cortisone and 11-dehydrocorticosterone, respectively). Endotoxin and inflammatory cytokines inhibit 11 β HSD type 2 and reciprocally increase the activity of 11 β HSD type 1 thereby increasing the tissue concentration of the active steroid (51,52). Such an increase in the bioactivity of glucocorticoids within muscle would be expected to impair translational control of protein synthesis. In addition, glucocorticoids also increase the expression of a variety of cytokine receptors, including TNF α and IL-6 (53,54), which would be expected to enhance their bioactivity. At this time, it appears that both glucocorticoids and TNF α impair peptide-chain initiation via similar defects in mTOR-mediated mechanisms. However, we cannot exclude the possibility that these immunomodulators could also activate unique and separate signal transduction pathways.

In conclusion, the ability of sepsis to produce this leucine resistance was not prevented by antagonizing the bioactivity of either TNF α or glucocorticoids individually. However, when septic rats were administered TNF α and RU486 in combination, the ability of leucine to stimulate the phosphorylation of 4E-BP1, S6K1, and rpS6, increase the amount of the functional eIF4F complex, and stimulate protein synthesis in muscle was completely normalized. These data suggest TNF α and glucocorticoids function in a cooperative manner to integrate the physiological whole-body response to sepsis and impair leucine-stimulated mTOR-mediated translation initiation.

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