Acute Oral Leucine Administration Stimulates Protein Synthesis during Chronic Sepsis through Enhanced Association of Eukaryotic Initiation Factor 4G with Eukaryotic Initiation Factor 4E in Rats\textsuperscript{1,2}

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Abstract
Sepsis induces the loss of muscle proteins by impairing skeletal muscle protein synthesis through an inhibition of messenger RNA (mRNA) translation initiation. Amino acids and Leu (Leu) in particular stimulate mRNA translation initiation. The experiments were designed to test the effects of Leu on potential signal transduction pathways that may be important in accelerating mRNA translation initiation in skeletal muscle of rats with chronic (5–6 d) septic intra-abdominal abscess. Gastrocnemius from male Sprague Dawley rats gavaged with Leu or water were sampled 5–6 d following development of an intra-abdominal sterile or septic abscess. Gavage with Leu stimulated protein synthesis and enhanced the assembly of the active eukaryotic initiation factor (eIF)4G-eIF4E complex. Increased assembly of the active eIF4G-eIF4E complex was associated with a robust rise in phosphorylation of eIF4G(Ser\textsuperscript{1108}) and a decreased assembly of inactive eIF4E binding protein-1 (4E-BP1)-eIF4E complex in both sterile inflammatory and septic rats. The reduced assembly of 4E-BP1-eIF4E complex was associated with an increase in phosphorylation of 4E-BP1 in the y-form following Leu gavage. Phosphorylation of 70-kDa ribosomal protein S6 kinase on Thr\textsuperscript{389} was also increased following Leu gavage, as well as the phosphorylation of mammalian target of rapamycin on Ser\textsuperscript{2448} or Ser\textsuperscript{2481}. In contrast, phosphorylation of protein kinase B (PKB) on Thr\textsuperscript{308} or Ser\textsuperscript{473} was not augmented following Leu gavage in septic rats. We conclude that Leu stimulates a PKB-independent signal pathway elevating the eIF4G-eIF4E complex assembly through increased phosphorylation of eIF4G and decreased association of 4E-BP1 with eIF4E in skeletal muscle during sepsis. J. Nutr. 137: 2074–2079, 2007.

Introduction
Sepsis, the systemic response to bacterial infection, remains a serious complication contributing to a high (20–45\%) mortality rate. A major complication contributing to the morbidity and mortality in septic patients is the development of diffuse tissue injury referred to as multiple organ system dysfunction. Multiple organ system dysfunction can lead to the failure of major organ systems of the body to maintain homeostasis and is manifested in skeletal muscle by a loss of skeletal muscle mass producing diminished muscle strength (1–3). Muscle weakness in septic patients contributes to a continued dependence on mechanical respirators, an increased risk of pneumonia, and the complications associated with an inability for ambulation. These clinical complications prolong hospitalization and convalescence, thereby increasing health-care costs (4).

Protein wasting during sepsis results from an imbalance between the rate of protein synthesis and protein degradation (5). The relative contribution of protein synthesis and proteolysis to the overall net catabolic state in muscle varies depending upon the severity of the septic insult. Protein degradation varies and becomes accelerated as the septic episode worsens. In contrast, rates of protein synthesis are reduced to a similar extent regardless of the severity of the septic insult (6,7). Furthermore, the synthesis of both myofibrillar and sarcoplasmic proteins is equally diminished, indicating that sepsis affects the overall process of protein synthesis (8). As the septic process wanes, skeletal muscle protein mass and protein synthesis return toward control values (9). In contrast to sepsis, the decrease in protein synthesis and increase in proteolysis in skeletal muscle is short-lived in nonseptic, traumatic insults and restoration of positive nitrogen balance and lean body mass occurs quickly (9–11). The sepsis-induced inhibition in protein synthesis results from a restraint in the process of initiation of messenger RNA (mRNA)\textsuperscript{3} translation. Translation initiation is a complex process in which initiator transfer RNA, 40S, and 60S ribosomal subunits

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\textsuperscript{3} Abbreviations used: eIF, eukaryotic initiation factor; 4E-BP1, eIF4E binding protein-1; mTOR, mammalian target of rapamycin; PAGE, polyacrylamide gel electrophoresis; PKB, protein kinase B; PKC, protein kinase C; S6K1, 70-kDa ribosomal protein S6 kinase; SP, septic; ST, sterile.
are assembled by eukaryotic initiation factors (eIF) into an 80S ribosome at the initiation codon of mRNA. In eukaryotes, protein synthesis initiates with the binding of the multimeric translation initiation complex eIF4F to the monomethylated cap present on the 5' end of most mRNAs (12).

Translation initiation appears regulated through the formation of the eIF4G-eIF4F complex. A positive linear relationship exists between rates of protein synthesis and the amount of eIF4G associated with eIF4E in muscle in vivo (13). Consistent with this relationship, assembly of the eIF4G-eIF4E complex is significantly diminished in skeletal muscle from septic rats (13,14). The diminished assembly of eIF4G-eIF4E complex is not the result of a reduced amount of eIF4G in muscles from septic rats (13). However, eIF4G-eIF4E complex assembly appears regulated through the availability of eIF4E for binding to eIF4G in part through the association of eIF4E with a family of translational repressor proteins [eIF4E binding proteins (4E-BP)] (13) and phosphorylation of eIF4G (15) during chronic hypermetabolic, hyperdynamic sepsis. Endotoxin administration (16–18) or infusion of cytokines (15,19,20) cause similar changes. Reduced amounts of eIF4E associated with eIF4G following chronic sepsis would be expected to diminish the association of mRNA with the ribosome and, hence, limit protein synthesis.

Amino acids, Leu in particular, have the ability to regulate protein synthesis (21,22). Emerging evidence indicates that amino acids acting as nutrient signals themselves modulate cellular processes leading to an acceleration of protein synthesis through augmented mRNA translation initiation (12). Formation of the active eIF4E-eIF4G complex is dependent upon amino acids in postprandial state (23). Oral administration of Leu enhances skeletal muscle protein synthesis through changes in the regulation of proposed effectors of mRNA translation initiation, as evidenced by upregulated phosphorylation of the translational repressor, 4E-BP1, the association of eIF4G with eIF4E, and the phosphorylation of the 70-kDa ribosomal protein S6 kinase (S6K1) (24). Likewise, protein synthesis in gastrocnemius is stimulated by short-term (30 min) perfusion with buffer supplemented with amino acids at 10 times greater than the plasma concentration by enhancing peptide-chain initiation (25).

The purpose of this study was to test the hypothesis that Leu stimulates protein synthesis in gastrocnemius of septic rats by increasing the formation of the active eIF4E-eIF4G complex.

Materials and Methods

Animals and experimental design. Adult male Sprague-Dawley rats weighing 150–225 g were maintained on a 12-h-light:12-h-dark cycle and maintained on a standard rodent chow (Teklad Rodent Diet no. 8818) and water ad libitum. Chronic abdominal sepsis was created by implantation of a fecal-agar pellet (1.5 mL) inoculated with 10^4 colony forming units of Escherichia coli and 10^3 colony forming units of Bacteroides fragilis into the peritoneal cavity as previously described (10,13,14,25,26). Replacing the bacterial inoculum with an equal concentration by enhancing peptide-chain initiation (25).

TABLE 1 Effect of oral Leu gavage on plasma Leu concentrations and rates of protein synthesis in chronic (5 d) sterile inflammatory and septic rats

<table>
<thead>
<tr>
<th></th>
<th>ST</th>
<th>ST+Leu</th>
<th>SP</th>
<th>SP+Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu (mmol/L)</td>
<td>153 ± 34</td>
<td>1593 ± 129</td>
<td>165 ± 21</td>
<td>1824 ± 224</td>
</tr>
<tr>
<td>Protein synthesis, %/d</td>
<td>20 ± 2</td>
<td>34 ± 4</td>
<td>11 ± 3</td>
<td>33 ± 3</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 8–12. Gavage with Leu (+Leu) was significant for all variables, P < 0.0001.
2 P < 0.05 vs. ST.
antibodies that recognize either phospho-eIF4G (Ser1108), phospho-mTOR(Ser2448), or phospho-mTOR(Ser2481) (Cell Signaling Technology) (15,43,44). The phosphorylated eIF4G or mTOR signal densities were normalized to the respective total eIF4G or mTOR signal to reflect the relative ratio of phosphorylated eIF4G or mTOR to total eIF4G or mTOR, respectively.

**Determination of extent of 4E-BP1 phosphorylation.** The various phosphorylated forms of 4E-BP1 (designated α, β, and γ) were separated by SDS-PAGE electrophoresis and quantitated by protein immunoblot analysis as described previously (37,43,44).

**Determination of extent of S6K1 and PKB phosphorylation.** Ly- sates from gastrocnemius muscle were mixed with 2x Laemml-SDS sample buffer and subjected to electrophoresis on 12.5% SDS-PAGE Criterion gels (Biorad) as described previously using phospho-specific antibodies that recognize either phospho-S6K1(Thr389), phospho-PKB (Thr347, 35), or phospho-PKB(Ser473) (Cell Signaling Technology) and quantified as described above for eIF4G phosphorylation (13,39,44). Results are presented as the ratio of the densitometric analysis of blot for phosphorylated form divided by total (phosphorylated + unphosphorylated) on same gel.

**Statistical analysis.** Values shown are means ± SEM. Data were analyzed using a 2-way ANOVA with condition (ST vs. SP) and Leu gavage ST + Leu vs. SP + Leu as factors (Graphpad Prism4 Software). We performed a Sidak post hoc test to determine differences between means for all significant interactions and main effects. For all analyses, differences were considered significant at P < 0.05.

## Results

**Rates of protein synthesis.** Gavage with a solution containing Leu raised the plasma concentration to 10-fold that in rats with either a sterile or septic intra-abdominal abscess (Table 1). The plasma Leu concentration did not differ between ST and SP rats following either saline or Leu gavage. Sepsis depressed rates of protein synthesis by 40% (Table 1), consistent with our previous findings in gastrocnemius (8,10,13,45). Elevating the plasma concentration to 10-fold that in rats with either a sterile or septic intra-abdominal abscess (Table 1). The amount of the inactive eIF4E-4E-BP1 complex in ST+Leu rats 47-fold compared with ST and SP, respectively (Table 2).

**Effect of oral Leu gavage on phosphorylation of eIF4G and association of eIF4E with eIF4G**

<table>
<thead>
<tr>
<th></th>
<th>ST</th>
<th>ST+Leu</th>
<th>SP</th>
<th>SP+Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4G associated with eIF4E</td>
<td>18 ± 7</td>
<td>58 ± 18</td>
<td>2 ± 1</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>eIF4G(Ser1108) phosphorylation + eIF4E</td>
<td>36 ± 8</td>
<td>91 ± 11</td>
<td>41 ± 6</td>
<td>146 ± 19</td>
</tr>
<tr>
<td>eIF4G(Ser1108) phosphorylation + total eIF4E</td>
<td>36 ± 8</td>
<td>91 ± 11</td>
<td>41 ± 6</td>
<td>146 ± 19</td>
</tr>
<tr>
<td>4E-BP1 associated with eIF4E</td>
<td>17 ± 2</td>
<td>8 ± 3</td>
<td>21 ± 3</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>4E-BP1 phosphorylation + total 4E-BP1</td>
<td>5 ± 2</td>
<td>41 ± 6</td>
<td>11 ± 3</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>S6K1(Thr389) phosphorylation + total S6K1</td>
<td>5 ± 1</td>
<td>28 ± 5</td>
<td>6 ± 1</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>mTOR(Ser2448) phosphorylation + total mTOR</td>
<td>11 ± 2</td>
<td>40 ± 4</td>
<td>14 ± 2</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>mTOR(Ser2481) phosphorylation + total mTOR</td>
<td>7 ± 1</td>
<td>12 ± 1</td>
<td>8 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>PKB(Ser473) phosphorylation + total PKB</td>
<td>10 ± 1</td>
<td>15 ± 3</td>
<td>7 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>PKB(Thr347) phosphorylation + total PKB</td>
<td>5 ± 1</td>
<td>8 ± 1</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 7-14. Gavage with Leu (+Leu) was significant for all variables, P < 0.005, with exception of PKB phosphorylation, which did not show a Leu gavage effect.

2 P < 0.05 for effect of SP vs. ST.
S6K1 induced by Leu (12). Its activity is regulated in part by phosphorylation. Oral Leu administration increased the extent of mTOR phosphorylated on Ser\textsuperscript{2448} or Ser\textsuperscript{2481} (from ST rats (Table 2). Likewise, gavaging SP rats with a solution containing Leu increased the extent of mTOR phosphorylated on Ser\textsuperscript{2448} or Ser\textsuperscript{2481} (Table 2). None of the treatments altered the total amount of mTOR in skeletal muscle (data not shown).

**Phosphorylation of PKB.** PKB, also known as AKT, is a Ser/Thr kinase hypothesized to enhance mTOR activity. PKB itself is activated by reversible phosphorylation. Consequently, we also examined the response of PKB phosphorylation to Leu gavage following sterile inflammation or sepsis. The level of PKB phosphorylation on Thr\textsuperscript{308} was not significantly affected by sepsis and did not increase with a gavage containing Leu (Table 2). Sepsis decreased (~34%) the phosphorylation of PKB on Ser\textsuperscript{473} and the extent of phosphorylation did not recover to values observed in sterile inflammatory rats when gavaged with a solution containing Leu (Table 2).

**Discussion**

The results of this study indicate that raising the plasma Leu concentration 10 times fasting Leu concentrations following gavage with a solution containing Leu enhanced factors associated with accelerating translational control in gastrocnemius of rats possessing a chronic, intra-abdominal septic abscess. Control of mRNA translation initiation posits assembly of active eIF4G-eIF4E complex as a central regulator of protein synthesis. Indeed, assembly of active eIF4G-eIF4E complex positively correlates with rates of protein synthesis in skeletal muscle (13). In this study, raising the plasma Leu enhanced the assembly of an active eIF4G-eIF4E complex and stimulated protein synthesis during sepsis.

Assembly of an active eIF4G-eIF4E complex depends upon both the availability of eIF4E and on phosphorylation of eIF4G. Phosphorylation of eIF4G on residues in the C-terminal region of the eIF4G protein including Ser\textsuperscript{1108} fully activates eIF4G (48). Increased phosphorylation of eIF4G on Ser\textsuperscript{1108} enhances formation of active eIF4G-eIF4E complex in cells in culture (48,49), leading to an increased rate of protein synthesis. Likewise, elevated phosphorylation of eIF4G correlates with mRNA translation in skeletal muscle during perfusion of hind limb with buffer containing Leu (50) or following oral administration of a single bolus of Leu (24). In this study, Leu gavage increased the extent of eIF4G phosphorylation in septic rats. Enhancing the availability of eIF4E to bind with eIF4G represents another mechanism for increasing assembly of eIF4G-eIF4E complex. eIF4E availability is controlled through interactions with 4E-BP1 (46,51,52) that compete with eIF4G for a common binding site on eIF4E (53,54). Hence, reduced binding of eIF4G with 4E-BP1 would be expected to enhance availability for eIF4E to associate with eIF4G. Indeed, gavage with a solution containing Leu markedly reduced the binding of the eIF4E to 4E-BP1 in rats with either a sterile or septic abscess. Taken together, the results are consistent with a combined role of eIF4G phosphorylation and increased availability of eIF4E as a means to increase the assembly of active eIF4G-eIF4E complex following Leu gavage.

Amino acids, and Leu in particular, consistently activate the S6K1 and the translation repressor 4E-BP1 through enhanced phosphorylation both in vitro (55,56) and in vivo in control rats (23,35,38,39,57,58). Phosphorylating S6K1 and 4E-BP1 are associated with an acceleration of mRNA translation initiation, leading to a stimulation of protein synthesis. Enhanced phosphorylation of 4E-BP1 lowers the interactions between eIF4E and 4E-BP1, allowing increased availability of eIF4E. On the other hand, phosphorylation of S6K1 promotes translation of mRNA containing 5'-terminal oligopyrimidine tract sequences. In both sterile inflammatory and septic rats, Leu was able to cause an increase in phosphorylation of S6K1 and 4E-BP1. The S6K1 and 4E-BP1 are phosphorylated by a common upstream kinase, mTOR, suggesting a role for mTOR in mediating in part the effects of Leu to phosphorylate these 2 proteins (37,59). Indeed, in this investigation, Leu increased the phosphorylation of mTOR in gastrocnemius of septic rats, consistent with Leu's role in stimulating mTOR.

Lastly, an upstream activator of both mTOR and S6K1 phosphorylation appears to be PKB (12). PKB is activated by phosphorylation of 2 critical Ser/Thr residues (i.e. Thr\textsuperscript{308} and Ser\textsuperscript{473}). Gavage with a solution containing Leu did not significantly increase PKB phosphorylation in sterile inflammatory rats. During sepsis, PKB(Ser\textsuperscript{473}) phosphorylation was reduced 30%, a defect in cell signaling that was not corrected by gavage with a solution containing Leu. Studies by Orellana et al. (18) did not observe a significant reduction in phosphorylation of PKB(Ser\textsuperscript{473}) during a 20-h endotoxin infusion in 5-6-d-old piglets provided amino acids and dextrose for 2 h prior to sampling of the longissimus dorsi. The differences between that study and our set of investigations may relate to the relative lack of insulin resistance in muscle of neonatal pigs (18) compared with adult rats (13). Similarly, Lang (17) observed a defect in the ability of Leu to enhance phosphorylation of PKB in adult rats. Therefore, increased phosphorylation of mTOR, S6K1, 4E-BP1, and eIF4G induced by Leu administration during sepsis occurred without evidence of activation of the PKB pathway. These observations are consistent with Leu acting downstream of PKB.

The effects of Leu on translational control reported in this study are in contrast to previous reports in the literature showing a blunted response to oral Leu gavage in fasted rats subjected to perfusion of the intestinal tract (cecal ligation and puncture) for 24 h in cardiac (20) or skeletal muscle (60). Likewise, Lang et al. (17) showed that LPS administration partially or completely abrogated the Leu-induced phosphorylation of 4E-BP1, S6K1, ribosomal S6, and mTOR and changes in eIF4E distribution after 4 h. There are several potential reasons for these discrepancies. First, our model shows a defined bacteremia (27) contrasting with the endotoxin model (17) where a sustained (chronic) bacteremia cannot be induced. Models of sepsis induced by endotoxin administration, while allowing for quantifiable administration, represent an acute insult initiating a depression of cardiac index, mean arterial pressure, and total leukocyte count (61), which contrasts with the human septic condition characterized by an increased cardiac output (30). Second, models involving perforation of the intestinal tract (cecal ligation and puncture) suffer in that the bacterial insult is (chronic) bacteremia cannot be induced. Models of sepsis induced by endotoxin administration, while allowing for quantifiable administration, represent an acute insult initiating a depression of cardiac index, mean arterial pressure, and total leukocyte count (61), which contrasts with the human septic condition characterized by an increased cardiac output (30). The variability of the response is shown by the studies of Hasselgren et al. (62–64), who reported that sepsis had either no effect or resulted in decreased rate of protein synthesis in muscle, and our studies, showing the metabolic response appears dependent upon the invading organism (27). The cecal ligation and puncture model reflects an acute peritonitis rather than a chronic septic process. In this regard, animals subjected to cecal ligation and puncture are anorexic, become dehydrated, and expire within 72 h. In contrast, all rats implanted with an infected fecal agar pellet that survive the abscess formation stage (48 h) live. Hence, both the
LPS injection model and cecal ligation and puncture model produce preterminal septic shock rather than a hemodynamically stable chronic sepsis.

In summary, oral administration of a solution composed of Leu raised plasma Leu concentrations ~10 times more than plasma concentrations. Oral administration of Leu to septic rats stimulated phosphorylation of both 4E-BP1 and eIF4G, maximizing the assembly of active eIF4G-eIF4E complex. The increased formation of active eIF4G-eIF4E complex and activation of S6K1 pathway was associated with accelerated rates of protein synthesis to values observed in rats with a sterile, nonseptic abscess. Thus, an enteral Leu treatment modality apparently overcomes the inhibition of protein synthetic process through acutely augmenting eIF4G-eIF4E during chronic sepsis.

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Literature Cited


