IGF-I/IGFBP-3 binary complex modulates sepsis-induced inhibition of protein synthesis in skeletal muscle

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Svanberg, Elisabeth, Robert A. Frost, Charles H. Lang, Jorgen Isgaard, Leonard S. Jefferson, Scot R. Kimball, and Thomas C. Vary. IGF-I/IGFBP-3 binary complex modulates sepsis-induced inhibition of protein synthesis in skeletal muscle. Am J Physiol Endocrinol Metab 279: E1145–E1158, 2000.—The present study evaluated the ability of insulin-like growth factor I (IGF-I) complexed with IGF binding protein-3 (IGFBP-3) to modulate the sepsis-induced inhibition of protein synthesis in gastrocnemius. Beginning 16 h after the induction of sepsis, either the binary complex or saline was injected twice daily via a tail vein, with measurements made 3 and 5 days later. By day 3, sepsis had reduced plasma IGF-I concentrations ~50% in saline-treated rats. Administration of the binary complex provided exogenous IGF-I to compensate for the sepsis-induced diminished plasma IGF-I. Sepsis decreased rates of protein synthesis in gastrocnemius relative to controls by limiting translational efficiency. Treatment of septic rats with the binary complex for 5 days attenuated the sepsis-induced inhibition of protein synthesis and restored translational efficiency to control values. Assessment of potential mechanisms regulating translational efficiency showed that neither the sepsis-induced change in gastrocnemius content of eukaryotic initiation factor 2B (eIF2B), the amount of eIF4E associated with 4E binding protein-1 (4E-BP1), nor the phosphorylation state of 4E-BP1 or eIF4E were altered by the binary complex. Overall, the results are consistent with the hypothesis that decreases in plasma IGF-I are partially responsible for enhanced muscle catabolism during sepsis.

Gastrocnemius; eukaryotic initiation factors; translation initiation

Sepsis causes profound alterations in protein metabolism in many tissues. The earliest recognizable alteration in protein metabolism is an excessive urea nitrogen excretion, indicative of a loss of body protein and a negative nitrogen balance. The major source of the excreted nitrogen is derived from the net catabolism of proteins in skeletal muscle. The catabolism of protein in skeletal muscle during sepsis results from an increased rate of protein degradation and a decreased rate of protein synthesis (for review, see Refs. 23, 60). When prolonged, the erosion of lean body mass has potential deleterious effects in septic patients, including sustained muscle fatique, weakness, and poor wound healing.

Several treatment modalities have been advanced to prevent and/or modulate the sepsis-induced alterations in protein metabolism (64), including 1) provision of sufficient nutrients, 2) modulation of the cytokine/inflammatory response, or 3) administration of anabolic hormones. Provision of exogenous nutritional support is not sufficient to completely arrest muscle wasting in septic patients (5). In contrast, modulating the cytokine response (7, 8, 30) prevents sepsis-induced inhibition of protein synthesis in muscle. In these studies, anticytokine therapies were provided either prophylactically or shortly after the induction of the septic focus, a situation which is controllable in the laboratory but is of limited benefit in the clinical setting.

Information regarding the role of anabolic hormones in augmenting protein synthesis in muscle from septic patients or animals is limited. The major anabolic hormones modulating protein synthesis in skeletal muscle include insulin, growth hormone (GH), and insulin-like growth factor-I (IGF-I). The effects of insulin on protein synthesis in skeletal muscle have been well documented (35). However, skeletal muscle is resistant to the anabolic actions of insulin on protein synthesis during chronic intra-abdominal sepsis (29) or after infusion of tumor necrosis factor (19), indicative of an insulin resistance regarding protein synthesis.

Like insulin, the effects of other anabolic hormones on protein metabolism are well documented. GH promotes nitrogen retention and improves nitrogen balance in a variety of conditions, including burn and surgical postoperative patients (22, 36, 64). The anabolic actions of GH on protein metabolism are mediated primarily through an increase in protein synthesis. However, GH has been shown to have reduced effectiveness in retarding protein catabolism in septic
patients, potentially limiting the usefulness of this hormone during sepsis (9, 49). Moreover, administration of GH is associated with an increased morbidity and mortality in critically ill patients (54).

The protein anabolic actions of GH are mediated indirectly by the hepatic synthesis and release of another hormone, IGF-I (44). Systemically administered IGF-I results in weight gain in normal rats, reduces weight loss during starvation or diabetes (47), and attenuates protein loss during cachetic states (55, 56). Chronic IGF-I administration improves nitrogen balance in rats injected with endotoxin (10).

Part of the anabolic action of IGF-I may be mediated through an increase in protein synthesis in muscle (3, 27, 51). In humans, intravenous infusion of IGF-I directly increases protein synthesis in skeletal muscle, provided adequate substrate supply is also present (17, 50). Recombinant human IGF-I (rhIGF-I) stimulates protein synthesis in freshly isolated myocytes from adult rats or in hearts perfused in vitro (18). In myotubes or myoblasts from the L8 or L6 cell lines in culture, IGF-I is a more potent stimulator of protein synthesis than insulin (2, 21). Likewise, IGF-I stimulates protein synthesis in vitro in either incubated muscles (51, 61) or perfused hindlimb (31).

We have previously demonstrated that the ability of IGF-I to stimulate skeletal muscle protein synthesis in vitro was unimpaired during the hypermetabolic phase of sepsis (31). Likewise, we have shown that IGF-I stimulates protein synthesis in incubated epichondral muscle during either the anorexic or the hypermetabolic phase of the host’s response to injection of live bacteria in vitro (61). IGF-I also stimulates protein synthesis in incubated extensor digitorum longus muscle from rats with severe acute peritonitis in vitro (26). However, there are no reports concerning the ability of IGF-I to stimulate protein synthesis in muscles of septic patients or animals in vivo.

Single intravenous injections of this growth factor may have limited therapeutic potential for several reasons. First, exogenously administered IGF-I has a short half-life in vivo (37), making a sustained elevation of IGF-I difficult. Second, IGF-I injections are associated with hypoglycemia, necessitating administration of exogenous glucose (6). Third, an intraperitoneal bolus injection of IGF-I, which transiently elevates plasma IGF-I concentrations, does not cause a clear stimulation of protein synthesis in overnight-starved mice (53). Finally, single injections of IGF-I lose their efficacy over time (41).

The vast majority of IGF-I is bound to one of several binding proteins (IGFBPs) in vivo, the most abundant of which is IGFBP-3. Administration of IGF-I prebound to nonglycosylated IGFBP-3 is being studied as an alternative to administration of free IGF-I. Binding of IGF-I to IGFBP-3 and the acid-labile subunit (ALS) forms a ternary complex that maintains circulating levels of IGF-I by slowing its rate of clearance (37, 40). Furthermore, the IGF-I/IGFBP-3 complex is less likely to cause hypoglycemia compared with IGF-I (1, 24). The purpose of the present investigation was to establish whether a binary complex, consisting of equal molar amounts of IGF-I and IGFBP-3, could modulate the sepsis-induced inhibition in skeletal muscle protein synthesis in vivo during the anorexic (day 3 postinfection) and hypermetabolic (day 5 postinfection) phases of septic response. Furthermore, we wished to determine whether injection of the IGF-I/IGFBP-3 complex could modulate the inhibition of translation efficiency responsible for diminished rates of protein synthesis during sepsis (8, 31, 58, 60). The results provide evidence that in vivo administration of the binary complex stimulates protein synthesis in skeletal muscle during sepsis.

**MATERIALS AND METHODS**

Experimental animals. Male Sprague-Dawley rats weighing 175-200 g were anesthetized with a combination of ketamine (110 mg/kg body wt) and acepromazine (1 mg/kg body wt). Sepsis was induced by implanting a fecal-agar pellet inoculated with $10^7$ colony-forming units (CFU) of Escherichia coli and $10^8$ CFU of Bacteroides fragilis into the abdominal cavity (29-31, 57-59). After recovery from surgery, all animals were allowed free access to rat chow (Harlan Teklad) and water. Beginning the next morning after induction of sepsis, animals were randomly divided into two groups, binary complex-treated and untreated. Binary complex-treated rats were injected via a tail vein twice daily with 5 µg/g body wt of the binary complex. Untreated septic rats received an equal volume of saline. On day 3 or day 5 after induction of sepsis, binary complex-treated and untreated septic rats were anesthetized, and rates of protein synthesis were measured in vivo. In the first 72 h, animals were recovering from surgery and the implantation of fecal agar pellet. During this period, food consumption was reduced relative to ad libitum-fed controls (30). This stage is referred to as the anorexic phase of the septic response. By day 5 postinfection, septic abscess animals consumed the same amount of food as they did before the surgery but showed muscle loss and other metabolic derangements (8, 30, 57-59).

This period corresponds with the hypermetabolic phase of sepsis. Food consumption in binary complex-treated and untreated septic rats was the same over the course of the experiment. Control animals underwent the implantation of a sterile agar pellet (30, 58, 59) and were pair-fed with respect to the amount of food consumed by the septic rats. The experiments described herein were performed in adherence with the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the approval of the Penn State University College of Medicine Animal Care and Use Committee.

**Binary complex.** The recombinant human (rh) binary complex was provided by Celtrix Pharmaceuticals, Inc, (Santa Clara, CA) in a 1:1 molar ratio of rhIGF-I to rhIGFBP-3 corresponding to the naturally occurring protein complex. The recombinant proteins were purified as previously described in detail (1). The two proteins were mixed together in a 1:1 molar ratio, with the complex being further purified by ion exchange chromatography. Purity of the complex was verified by reversed-phase HPLC and SDS-PAGE and was estimated to be >95%. Bioactivity was monitored using a cell culture assay (proliferation of MG63 cells). Injections were prepared from aliquoted frozen concentrates and performed twice daily at 0900 and 1800.

**Measurement of protein synthesis in vivo.** Rates of protein synthesis in vivo were estimated 3 or 5 days after the im-
plantation of the fecal agar pellet after a bolus infusion of \( \text{L-[3H]} \)phenylalanine (150 mM, 30 ng of the fecal agar pellet after a bolus infusion of \( \text{L-[3H]} \)phenylalanine (150 mM, 30 mg of body wt) described by Garlick et al. (20) and used previously in our laboratory (7, 8, 30, 57, 58). Ten minutes after injection of the radioisotope, a second blood sample (3 ml) was withdrawn for measurement of phenylalanine concentrations and specific radioactivity. Soleus, gastrocnemius, liver, and kidney were excised, weighed, and frozen to the temperature of liquid nitrogen or homogenized immediately for assay of eukaryotic initiation factors (eIFs). The frozen muscle samples were subsequently powdered under liquid nitrogen.

A portion of the frozen powdered tissue (0.5 g) was homogenized in ice-cold 3.6% (wt/vol) perchloric acid (PCA) to estimate the rate of incorporation of radioactive phenylalanine into protein, as described previously (7, 8, 29–31, 57, 58). A portion of this sample was assayed for protein concentration by the Biuret method, using crystalline bovine serum albumin as a standard. Another portion of the sample was used for the measurement of radioactivity in protein by liquid scintillation spectrometry using the proper corrections for quenching (dpm).

The specific radioactivity of phenylalanine was measured in deproteinized plasma samples by HPLC analysis according to the method of Drnević and Vary (11). The specific radioactivity of the phenylalanine was then calculated by dividing the disintegrations per minute of the phenylalanine peak by the concentration of phenylalanine in the sample. Rates of protein synthesis were calculated as described earlier with the use of the specific radioactivity of the plasma phenylalanine as the precursor pool (7, 8, 29–31, 57, 58). The assumption in using this technique to estimate the rate of protein synthesis in vivo is that the intracellular phenylalanine concentration is elevated to high concentrations, thereby limiting any dilution effect of nonradioactive phenylalanine derived from proteolysis. The plasma phenylalanine concentration was increased from \( \sim 70 \) nmol/ml to 1,344 ± 76 nmol/ml at the time of tissue sampling (average of all conditions). At perfuse concentrations >800 nmol/ml, the specific radioactivity of tRNA-bound phenylalanine is the same as that of the extracellular and intracellular pools of free phenylalanine (4, 28). Therefore, the specific radioactivity of plasma phenylalanine provides an accurate estimate of the specific radioactivity of phenylalanine bound to the tRNA.

**Determination of total RNA.** Total RNA was measured from homogenates of muscle samples. Briefly, 0.3 g of frozen powdered tissue was homogenized in 5 volumes of ice-cold 10% TCA. The homogenate was centrifuged at 10,000 \( \times \)g for 11 min at 4°C. The supernatant was discarded, and the remaining pellet was mixed in 2.5 ml of 6% (wt/vol) PCA. The sample was centrifuged at 10,000 \( \times \)g for 6 min at 4°C, the supernatant was discarded, and the procedure was repeated. Then, 1.5 ml of 0.3 N KOH was added to the pellet, and the samples were placed in a 50°C water bath for 1 h. Samples were then mixed with 5 ml of 4 N PCA and centrifuged at 10,000 \( \times \)g for 11 min. The concentration of RNA in the supernatant was determined as previously described (7, 8, 57, 58).

**Determination of plasma IGF-I concentrations.** Plasma samples were acid–ethanol extracted and then subjected to an additional cryoprotection step to remove IGFBPs; this procedure quantitatively removes IGFBP5 from serum (15). IGF-I was assayed by RIA as described previously (14). Human and rat IGF-I in plasma were determined with either human- or rat-specific RIA (Diagnostic Systems Laboratories, Webster, TX).

**Measurement of plasma IGFBP-3 by ligand and Western blot techniques.** Relative amounts of the various IGFBPs in plasma were determined by ligand blot analysis as previously described. Plasma samples were electrophoresed on 10% denaturing polyacrylamide gels, as described previously (15). Proteins were transferred electrophoretically to nitrocellulose membranes with a semidy blotter (Bio-Rad Laboratories, Melville, NY). The membranes were blocked with 5% nonfat dry milk for 1.5 h and subsequently incubated with antibodies against human IGFBP-3 (Upstate Biotechnology, Lake Placid, NY), ALS (Diagnostic Systems Laboratories, Webster, TX), or [\(^{125}\)I]IGF-I (Amersham, Arlington Heights, IL). After several washes, the membranes were then incubated with anti-rabbit immunoglobulin conjugated to horseradish peroxidase. The blots were then developed by use of an ECL Western blotting kit as per the manufacturer’s (Amersham) instructions. Proteins were visualized by exposing X-ray film to the blots.

**Separation of ternary complexes by Sephadex chromatography of plasma.** To establish that injection of the binary complex formed a ternary complex in vivo, plasma samples (100 \( \mu l \)) were mixed with a tracer amount of \([^{125}\text{I}]\)IGF-I and fractionated on a Sephadex G100 column equilibrated with 0.1 M Tris-buffered saline. The radioactivity in each fraction was determined with a gamma counter, and an aliquot of each fraction was subjected to Western blot analysis of IGFBP-3 and ALS after electrophoresis with a SDS-PAGE gel. The radioactivity of the fractions was measured, and selected fractions were electrophoresed on a 12% polyacrylamide gel for Western blot analysis of human IGFBP-3, as described above.

**Quantification of IGF-I, IGF-I receptor, and GH receptor mRNA.** Anti-sense IGF-I [\(^{35}\text{S}\)]UTP-labeled RNA was synthesized from an EcoR I linearized pSP64 plasmid carrying a 153-bp mouse genomic subclone corresponding to exon 3 by analogy to the human IGF-I gene and thus recognizing all reported variants of IGF-I mRNAs (14). The probe gives a 147-bp protected band in RNAse protection assays (46). Anti-sense IGF-I receptor (IGF-IR) [\(^{35}\text{S}\)]UTP-labeled RNA was synthesized from an EcoR I linearized pSP64 plasmid carrying a 265-bp BamH I fragment of the rat IGF-IR cDNA (63). The pGEM 3 plasmid contains a 265-bp BamH I fragment of the rat IGF-I receptor cDNA that encodes the putative signal peptide and the first 53 amino acids of the \( \alpha \)-subunit (63). Anti-sense GH receptor (GH-R) [\(^{35}\text{S}\)]UTP-labeled RNA was synthesized from an EcoR I linearized pT7T3 18U plasmid pSP64 plasmid carrying a 560-bp BamH I fragment of the rat GH-R cDNA (43). The GH-R cDNA fragment corresponds to a part of the extracellular domain of the GH-R.

Concentrations of mRNA for IGF-I, IGF-IR, and GH-R were determined in muscle and liver extracts from all groups of rats by solution hybridization. Total nucleic acids for the solution hybridization assays were prepared by homogenization of frozen tissues in buffer containing 20 mM Tris·HCl (pH 7.5), 1% (wt/vol) SDS, and 4 mM EDTA using a Polytron homogenizer. The homogenized muscle tissue was digested by overnight proteinase-K treatment, and total nucleic acids were prepared by subsequent phenol-chloroform extraction according to Durnam and Palmiter (13). The concentration and purity of the extracted RNA was measured spectrophotometrically by absorbance at 260 nm and by measuring the 260:280 nm ratio, respectively. The ratio of 260:280 nm was >2 in all samples analyzed. In addition, an aliquot of the extracted RNA was electrophoresed on an agarose gel before hybridization to ensure that the RNA was intact and had not undergone extensive degradation. The RNAse protection solution hybridization assay was carried out according to the protocol described by Matthews et al. (44). Protected hybrids were precipitated with TCA, collected on glass-fiber filters, and counted in a scintillation counter. The signal was com-
pared with a standard curve obtained by hybridization to known amounts of sense IGF-I mRNA, IGF-1R mRNA, or GH-R mRNA. The probe for GH-R mRNA also contained sequences complementary to the GHBP mRNA. Thus the probe allows for the detection of both GH-R and GHBP mRNA. The results were correlated to the DNA content as measured according to the method of Labarca and Paigen (38).

Quantification of 4E-BP1-eIF4E complex. The association of eIF4E with 4E-BP1 was determined as previously described in our laboratory (34, 52, 65). Briefly, the gastrocnemius was rapidly removed, immediately weighed, and homogenized in 7 volumes of buffer A ([20 mM HEPES, pH 7.4, 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 50 mM β-glycerolphosphate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 0.5 mM sodium vanadate, and 1 μM microcystin LR]) using a Polytron homogenizer. The homogenate was centrifuged at 10,000 g for 10 min at 4°C, and the pellet was discarded. eIF4E and 4E-BP1-eIF4E complexes were immunoprecipitated from aliquots of 10,000-g supernatants using an anti-eIF4E monoclonal antibody. The antibody-antigen complex was collected by incubation for 1 h with goat anti-mouse Biomag IgG beads (PerSeptive Diagnostics, Cambridge, MA). Before use, the beads were washed in 1% nonfat dry milk in buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% β-mercaptoethanol, 0.5% Triton X-100, 50 mM NaF, 50 mM β-glycerolphosphate, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM sodium vanadate). The beads were captured by means of a magnetic stand and were washed twice with buffer B and once with buffer B containing 500 rather than 150 mM NaCl. Resuspension in SDS-sample buffer and boiling for 5 min eluted the protein bound to the beads. The beads were collected by centrifugation, and the supernatants were subjected to electrophoresis on a 15% polyacrylamide gel for quantitation of 4E-BP1 and eIF4E. Proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane as previously described (34, 52, 65). The membranes were incubated with a mouse anti-human eIF4E antibody or a rabbit anti-rat 4E-BP1 antibody for 1 h at room temperature. The blots were then developed with an ECL Western blotting kit. Films were scanned by a Microtek ScanMaker III scanner equipped with a transparent media adaptor connected to a Macintosh computer. Images were obtained with the ScanWizard Plugin (Microtek) for Adobe Photoshop and were quantitated by means of NIH Image 1.60 software.

Determination of phosphorylation state of eIF4E. Phosphorylated and unphosphorylated forms of eIF4E in extracts of gastrocnemius were separated by isoelectric focusing on a slab gel and were quantitated by protein immunoblot analysis, as previously described (34, 52, 65).

Determination of phosphorylation state of 4E-BP1. The various phosphorylated forms of 4E-BP1 were measured after immunoprecipitation of 4E-BP1 from muscle homogenates after centrifugation at 10,000 g (34, 52). 4E-BP1 was immunoprecipitated from the supernatants as described in the previous section for immunoprecipitation of eIF4E. The immunoprecipitates were solubilized with SDS sample buffer. The various phosphorylated forms of 4E-BP1 were separated by electrophoresis and quantitated by protein immunoblot analysis as described previously (32–34, 65).

Biochemical analyses of blood. Blood samples were collected into heparinized syringes and centrifuged, and plasma was separated from cells and frozen until analyses. Insulin was measured with a commercially available RIA kit (Diagnostic Products, Los Angeles, CA). Plasma amino acids were analyzed by HPLC chromatography (Waters Associates Liquid Chromatographic System) with a precolumn derivatization method (25). Plasma glucose concentrations were determined with a model GL5 glucose analyzer (Analox Instruments, Lunenburg, MA).

Statistical analysis. Experimental data for each condition are summarized as means ± SE. Statistical evaluation of the data was performed using ANOVA to test for overall differences among groups, followed by the Sidak test for multiple comparisons to determine significance between means only when ANOVA indicated a significant difference among the group means. Differences among means were considered significant when P < 0.05. The number of animals in each group is indicated in the table and figure legends.

RESULTS

In the present study, we examined the responses to treatment with the binary complex during the anorexic (day 3 postinfection) and hypermetabolic (day 5 postinfection) phases of sepsis. There were no significant differences in the mortality rates between the treated and untreated septic groups (data not shown). Changes in body weights in the different groups are shown in Fig. 1. Pair-fed control animals showed a loss of body weight during the first 48 h, when food restriction was greatest. After that time, control rats gained weight. Both binary complex-treated and untreated septic rats began to show weight gain by day 4 postinfection. Beginning on day 3 after induction of sepsis, the weight of the animals treated with the binary complex tended to be higher than that of the untreated animals, although the increase did not reach statistical significance.

Plasma IGF-I, insulin, and glucose concentrations. Sepsis caused a diminution in plasma IGF-I concentrations by 58% on day 3 and by 47% on day 5 compared with pair-fed control animals (Fig. 2). Injection of the binary complex into septic rats raised the plasma IGF-I on both days compared with untreated septic rats, such
that values were not significantly different from non-septic control rats. As illustrated in Fig. 2, increases in plasma IGF-I in the binary complex-injected rats was entirely the result of an increase in human IGF-I. The plasma concentration of rat IGF-I in binary complex-treated rats was not significantly different from that observed in untreated septic rats.

Injection of IGF-I can be associated with diminished insulin concentrations and hypoglycemia. Therefore, we measured plasma insulin and glucose in IGF-I/IGFBP-3-treated septic rats. As we have reported previously, sepsis did not alter the plasma insulin concentration compared with controls on either day 3 (9 ± 1 vs. 7 ± 1 μU/ml, respectively) or day 5 (16 ± 1 vs. 18 ± 1 μU/ml, respectively). Plasma insulin concentrations were not significantly altered by injection of the binary complex compared with untreated septic rats on either day 3 (7 ± 2 vs. 9 ± 1 μU/ml, respectively) or day 5 (17 ± 1 vs. 16 ± 1 μU/ml, respectively) postinfection. There were no significant differences in the plasma concentrations of glucose in response to either sepsis and/or injection of the binary complex compared with controls on either day 3 [control (C) = 7.4 ± 0.6, septic (S) = 6.7 ± 0.3, septic + binary complex = 6.4 ± 0.4 mM] or day 5 postinfection [C = 7.8 ± 0.3, S = 7.4 ± 0.3, septic + binary complex = 7.7 ± 0.6 mM]. Thus injection of IGF-I/IGFBP-3 binary complex was not associated with hypoglycemia in septic rats despite an elevation of plasma IGF-I concentrations.

IGFBP-3 complex. It was also important to determine whether the IGFBP-3 was retained in plasma from septic animals after injection of the binary com-
plex. Analysis of the ligand blot (Fig. 3 top) shows the appearance of a 29-kDa band only in the plasma from animals treated with the binary complex. Western blot analysis indicated that this 29-kDa protein was rhIGFBP-3 (Fig. 3 bottom).

The half-life of IGF-I and IGFBP-3 in the circulation is dramatically increased by the presence of another protein, termed ALS. Loss of ALS is also associated with abnormally low IGF-I and IGFBP-3 plasma levels in some pathological states (16, 39). Therefore, we determined whether injection of the binary complex formed a ternary complex in vivo after injection into septic rats. To accomplish this objective, plasma from septic rats was fractionated by chromatographic separation on a Sephadex G100 column in the presence of tracer amounts of [125I]IGF-I. Fractions corresponding to the molecular weight of the binary and ternary complex were electrophoresed using SDS-PAGE and probed for IGFBPs by ligand blotting or ALS and rhIGFBP-3 by Western blotting to determine whether the binary complex formed a ternary complex in vivo. Endogenous rat IGFBP-3 with a molecular mass of 45–50 kDa was found in 150-kDa fraction of plasma (data not shown). More importantly, rhIGFBP-3 and endogenous ALS co-eluted in a ternary complex fraction (Fig. 4, top). rhIGFBP-3 was not detected in the molecular mass range of either free binding protein or binary complexes. [125I]IGF-I co-eluted with rhIGFBP-3 and ALS in the ternary complex (Fig. 4B) but also with small molecular mass binding proteins and as free IGF-I. When the binary complex was fractionated either alone or in the presence of [125I]IGF-I, it eluted in fractions 20–24 (Fig. 4 bottom).

**Fig. 2.** Effect of BC on the plasma concentration of rat and human IGF-I in septic rats. There were 3 groups of rats at day 3 and day 5 postinfection: sham-operated and pair-fed (Control), septic treated with saline (Saline), or septic treated with BC (S + BC). Values are means ± SE; n = 6–7 rats/group. *P < 0.05 septic vs. control, #P < 0.05 saline vs. S + BC, †P < 0.05 rat IGF-I on day 3 vs. day 5.

**Fig. 3.** Effect of BC on plasma IGFBP-3. Top: representative Western ligand blot of rat plasma from animals 3 and 5 days after induction of sepsis. Infected rats were treated with either saline (−) or the IGFBP-3 BC (+). The 3 major glycosylated variants of rat IGFBP-3 and exogenous recombinant human BP-3 (rhBP-3) in the plasma are noted. Molecular mass (M) standards on the left are in kDa. Bottom: plasma from 4 rats injected with saline (saline) or BC were separated by SDS-PAGE and probed for rhBP-3. rhBP-3 was detected only in animals receiving the BC.
Hepatic and muscle IGF-I, IGF-IR, and GH-R mRNA. The hepatic and gastrocnemius IGF-I mRNA content in control, untreated septic, and binary complex-treated septic rats is shown in Table 1. In association with the fall in plasma IGF-I concentrations on day 3 postinfection, hepatic and muscle IGF-I mRNA content fell 34% and 23%, respectively, compared with control. Treatment of septic rats with the binary complex did not affect the hepatic IGF-I mRNA content but did restore the muscle IGF-I mRNA to control values. The expression of hepatic IGF-I mRNA in control rats was augmented on day 5 compared with day 3 in control rats, presumably because food intake was increased. Likewise, the hepatic IGF-I mRNA content was increased on day 5 compared with day 3 in untreated septic rats or in septic rats treated with the binary complex. However, the hepatic IGF-I mRNA contents remained lower in both treated and untreated septic rats compared with controls on day 5. Unlike liver, the muscle IGF-I mRNA content returned to control values by day 5.

The hepatic IGF-IR mRNA content was not altered during sepsis (Table 1). Treatment with the binary complex did not alter IGF-IR mRNA content on day 3 or day 5 postinfection compared with untreated septic rats (Table 2). In muscle, the IGF-IR mRNA was elevated by sepsis at either time point examined. Injection of the binary complex had differential effects on the expression of the hormone’s receptor mRNA. On day 3, the binary complex attenuated the rise in IGF-IR mRNA in untreated septic rats. On day 5, the binary complex was without effect on IGF-IR mRNA in muscle.

GH receptor mRNA content in muscle and liver were reduced in septic rats (days 3 and 5 postinfection) compared with controls (Table 1). Injection of the binary complex did not alter GH receptor mRNA, except in liver on day 5 compared with values obtained in treated septic rats on day 3.

Organ weights and protein content. The organ weights and protein content in muscle and visceral tissues in the presence and absence of the binary complex on day 3 after induction of sepsis are shown in Table 2. Weights of gastrocnemius (−46%), soleus (−38%), and kidney (−13%) were all significantly re-

Table 1. Effect of the binary complex on expression of IGF-I, IGF-I Receptor, and growth hormone (GH) receptor mRNA in liver and muscle during sepsis

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Septic</th>
<th>Septic + IGBP3</th>
<th>Control</th>
<th>Septic</th>
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<tr>
<td><strong>IGF-I mRNA, pg/μg DNA</strong></td>
<td></td>
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<tr>
<td>Liver</td>
<td>7.59 ± 0.35</td>
<td>4.99 ± 0.53a</td>
<td>4.09 ± 0.25a</td>
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<td>5.35 ± 0.39*</td>
<td>5.82 ± 0.52§</td>
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<td>Gastrocnemius</td>
<td>0.86 ± 0.04</td>
<td>0.66 ± 0.03a§</td>
<td>0.97 ± 0.13</td>
<td>0.99 ± 0.08</td>
<td>1.05 ± 0.08†</td>
<td>0.83 ± 0.11</td>
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<td><strong>IGF-I Receptor mRNA, pg/μg DNA</strong></td>
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<tr>
<td>Liver</td>
<td>0.016 ± 0.002</td>
<td>0.016 ± 0.001</td>
<td>0.012 ± 0.002</td>
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<td>Gastrocnemius</td>
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<td><strong>GH Receptor mRNA, pg/μg DNA</strong></td>
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<td>0.58 ± 0.03</td>
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</tbody>
</table>

Values are means ± SE; n = 6–10 animals/group. IGF-I, insulin-like growth factor I; BP3, IGF binding protein-3; GH, growth hormone. *P < 0.05 vs. control on same day; †P < 0.05 vs. 3-day control; ‡P < 0.05 vs. 3-day septic; §P < 0.05 vs. 3-day septic + IGBP3 [IGF-I mRNA Liver ANOVA, F = 20.18, P < 0.001; IGF-I mRNA Muscle ANOVA, F = 5.7, P < 0.005; IGF-I Receptor mRNA Liver ANOVA, NS; IGF-I Receptor mRNA Muscle ANOVA, F = 5.48, P < 0.001; GH Receptor mRNA Liver ANOVA, F = 16.08, P < 0.001; GH Receptor mRNA Muscle ANOVA, F = 17.64, P < 0.001].
duced by sepsis. Treating rats with the binary complex significantly increased the weights of the gastrocnemius and soleus but had no effect on kidney. The magnitude of the increase in weight was similar in both gastrocnemius (19%) and soleus (22%) after treatment with the binary complex. Despite the increase in muscle mass, weights of gastrocnemius and soleus remained significantly lower than those of controls.

The protein content (mg protein/g muscle) was significantly reduced only in gastrocnemius (30%) than in controls. In kidney, sepsis augmented the protein content (+20%). The protein per gram of liver was not significantly affected by treatment with the binary complex compared with untreated septic rats but remained significantly elevated compared with controls. There was no significant difference in the protein per gram for either soleus or kidney among the three different groups.

Sepsis lowered the total protein per muscle in gastrocnemius (−47%; Table 2). Likewise, total protein per kidney was reduced by 12% during sepsis. The total protein per soleus was not significantly affected by sepsis. Treatment of septic rats with the binary complex augmented the total protein per gastrocnemius by 30%; however, the total protein per gastrocnemius remained significantly lower (−30%) than in controls.

Treatment with the binary complex was without effect on the total protein per soleus or total protein per kidney.

The organ weights and protein content in muscle and visceral tissues in the presence and absence of the binary complex on day 5 after induction of sepsis are shown in Table 3. The weight of gastrocnemius was significantly reduced (−43%) by sepsis, whereas the weight of soleus was unaffected. Treating septic rats with the binary complex significantly increased the weight of the gastrocnemius (+11%) but had no significant effect on soleus. Like gastrocnemius, the weight of the kidney was diminished (−17%) by sepsis. The weight of the kidney in septic rats treated with the binary complex was intermediate between those of untreated septic rats and controls.

Sepsis lowered the total protein per muscle in gastrocnemius (−37%), and kidney (−14%; Table 3). The total protein per soleus was not significantly affected by sepsis. Treatment with the binary complex augmented the total protein per gastrocnemius by 20%; however, the total protein per gastrocnemius remained significantly lower (−25%) than controls. In kidney, treatment with the binary complex increased total protein per kidney by 20% to values that were not significantly different than that observed in control animals.

Protein synthesis. To further investigate the factors responsible for the changes in tissue weights and pro-

Table 2. Effect of binary complex on protein metabolism in muscle and visceral organs during sepsis (day 3)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Condition</th>
<th>Organ Weight, g</th>
<th>Protein Content, mg/g</th>
<th>Total Protein/Organ, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>Control</td>
<td>1.49 ± 0.04</td>
<td>167 ± 4</td>
<td>219 ± 14</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td>0.80 ± 0.02‡</td>
<td>145 ± 5*</td>
<td>117 ± 4*</td>
</tr>
<tr>
<td></td>
<td>Septic + IGF/BP3</td>
<td>0.95 ± 0.07$$</td>
<td>161 ± 3</td>
<td>152 ± 6$$</td>
</tr>
<tr>
<td>Soleus</td>
<td>Control</td>
<td>1.065 ± 0.007</td>
<td>160 ± 9</td>
<td>17 ± 1</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td>0.065 ± 0.004*</td>
<td>184 ± 9</td>
<td>12 ± 1</td>
</tr>
<tr>
<td></td>
<td>Septic + IGF/BP3</td>
<td>0.079 ± 0.004$$</td>
<td>163 ± 13</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Liver</td>
<td>Control</td>
<td>nd</td>
<td>157 ± 3</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td>nd</td>
<td>188 ± 11*</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Septic + IGF/BP3</td>
<td>nd</td>
<td>204 ± 17*</td>
<td>nd</td>
</tr>
<tr>
<td>Kidney</td>
<td>Control</td>
<td>0.94 ± 0.02</td>
<td>152 ± 9</td>
<td>143 ± 6</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td>0.82 ± 0.02†</td>
<td>154 ± 4</td>
<td>126 ± 5*</td>
</tr>
<tr>
<td></td>
<td>Septic + IGF/BP3</td>
<td>0.83 ± 0.03*</td>
<td>148 ± 4</td>
<td>122 ± 3*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–13 tissues/group. nd, Not determined. *P < 0.05; †P < 0.005; ‡P < 0.001 vs. control; §P < vs. septic.

Table 3. Effect of binary complex on protein metabolism in muscle and visceral organs during sepsis (day 5)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Condition</th>
<th>Organ Weight, g</th>
<th>Protein Content, mg/g</th>
<th>Total Protein/Organ, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>Control</td>
<td>1.68 ± 0.09</td>
<td>164 ± 3</td>
<td>256 ± 18</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td>0.96 ± 0.05†</td>
<td>172 ± 9</td>
<td>162 ± 11**</td>
</tr>
<tr>
<td></td>
<td>Septic + IGF/BP3</td>
<td>1.07 ± 0.06$$</td>
<td>182 ± 6</td>
<td>193 ± 10$$</td>
</tr>
<tr>
<td>Soleus</td>
<td>Control</td>
<td>0.090 ± 0.006</td>
<td>150 ± 3</td>
<td>14 ± 1</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td>0.081 ± 0.003</td>
<td>191 ± 10</td>
<td>15 ± 1</td>
</tr>
<tr>
<td></td>
<td>Septic + IGF/BP3</td>
<td>0.075 ± 0.004</td>
<td>197 ± 11</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Liver</td>
<td>Control</td>
<td>nd</td>
<td>161 ± 3</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td>nd</td>
<td>185 ± 11</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Septic + IGF/BP3</td>
<td>nd</td>
<td>170 ± 14</td>
<td>nd</td>
</tr>
<tr>
<td>Kidney</td>
<td>Control</td>
<td>1.02 ± 0.03</td>
<td>148 ± 3</td>
<td>150 ± 6</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td>0.85 ± 0.03*</td>
<td>151 ± 2</td>
<td>129 ± 6*</td>
</tr>
<tr>
<td></td>
<td>Septic + IGF/BP3</td>
<td>0.96 ± 0.07</td>
<td>148 ± 7</td>
<td>154 ± 13§</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–9 tissues/group. *P < 0.05; **P < 0.005; †P < 0.001 vs. control; ‡P < 0.05; §P < 0.001 vs. septic; nd, not determined.
tein content, rates of protein synthesis were measured in vivo by the incorporation of radioactive phenylalanine into total mixed proteins. In vivo rates of protein synthesis in gastrocnemius and soleus are shown in Fig. 5. Sepsis caused an inhibition of protein synthesis in gastrocnemius on day 3. Treatment with the binary complex significantly increased protein synthesis by ~50%. Similarly, sepsis inhibited protein synthesis in gastrocnemius on day 5. The reduction in gastrocnemius protein synthesis was significantly ameliorated by treatment of septic animals with binary complex. Unlike day 3, the rate of gastrocnemius protein synthesis was not significantly different in control and septic rats treated with the binary complex on day 5. In contrast to gastrocnemius, sepsis was without significant effect on rates of protein synthesis in soleus. Moreover, there was no significant change in the protein synthesis in soleus in response to the binary complex administration.

The rate of protein synthesis in visceral organs is presented in Fig. 6. Sepsis caused an inhibition of protein synthesis in kidney on both days 3 and 5 postinfection. There were no sepsis-induced changes in hepatic protein synthesis at either time point examined. The rate of protein synthesis in liver or kidney was unaffected at either time point by administration of the binary complex.

**Plasma amino acids.** The ability of IGF-I to modulate protein synthesis can be dependent on the plasma amino acid concentration. To test the possibility that amino acids limited the ability of IGF-I to stimulate protein synthesis in different tissues, plasma amino acid concentrations were measured by HPLC (Table 4). On day 3 postinfection, total amino acids in plasma were reduced by 25% compared with controls. Plasma concentrations of alanine, arginine, asparagine, glutamine, tryptophan, threonine, taurine, and serine were significantly reduced compared with controls. Treatment of septic rats with the binary complex restored the total plasma amino acid content, primarily by augmenting the plasma concentrations of alanine, glutamine, histidine, and serine compared with untreated septic rats. The concentrations of the other amino acids were not significantly different from untreated septic rats.
responsible for changes in gastrocnemius protein synthesis. The number of ribosomes or changes in the efficiency of mRNA translation during sepsis. Changes in the number of ribosomes was increased on day 3 postinfection. In muscles from untreated septic animals, the translational efficiency was reduced by ~50% compared with control animals. Treatment of septic rats with the binary complex increased the translational efficiency in gastrocnemius during sepsis. In muscles from untreated septic animals, the translational efficiency was increased on day 5 compared with day 3 but remained reduced by ~40% compared with control animals. Treatment of septic rats with the binary complex increased the translational efficiency by 50% in gastrocnemius from septic rats. Thus daily injections of the binary complex for 5 days modulated the translational efficiency in gastrocnemius to levels that were not significantly different from control values.

Effect of binary complex on eIF2B protein content during sepsis. We have previously shown that the relative abundance of the e-subunit of eIF2B (eIF2Be) falls during the first 5 days after the septic insult (8, 62). Therefore, we measured the effect of binary complex administration on the expression of eIF2Be. The amount of eIF2Be on day 3 postinfection tended to be diminished, although this change did not reach statistical significance (Table 5). In contrast to day 3, the amount of eIF2Be on day 5 postinfection was significantly reduced by 37% compared with control values (Table 5). Injection of the binary complex did not alter the amount of eIF2Be at either time point examined.

On day 5 postinfection, there were no significant differences in the total amino acid concentration between the three groups. However, changes in individual amino acids were observed. The concentration of alanine, arginine, asparagine, glutamine, leucine, lysine, serine, threonine, ornithine, and valine were significantly higher in untreated septic rats on day 5 compared with day 3 (Table 4). Furthermore, the plasma concentrations of leucine, ornithine, and lysine were elevated in untreated septic rats compared with controls on day 5, whereas asparagine, glutamate, and tryptophan were lower. Administration of the binary complex did not alter individual plasma amino acid concentrations further, except for lysine, which was elevated, and valine, which was diminished, compared with controls.

Translation efficiency. Because administration of the binary complex improved rates of protein synthesis in gastrocnemius, we investigated several potential mechanisms responsible for the stimulation of protein synthesis during sepsis. Changes in the number of ribosomes or changes in the efficiency of mRNA translation may be responsible for the sepsis-induced reduction in gastrocnemius protein synthesis. The number of ribosomes cannot be measured directly, but it is usually estimated from the RNA content of the tissue, where rRNA constitutes ~85% of the total RNA. Therefore, changes in total RNA content presumably reflect changes in the number of ribosomes. There was no significant difference in RNA content in any of the conditions examined (Fig. 7 top). Therefore, alterations in the relative abundance of ribosomes were not responsible for changes in gastrocnemius protein synthesis in either untreated or binary complex-treated septic rats.

The efficiency of translation, calculated by dividing the protein synthesis rate by the total RNA content, provides an index of how rapidly the existing ribosomes synthesize protein. In gastrocnemius from untreated septic animals, the translational efficiency was reduced by ~50% compared with control values (Fig. 7 bottom). Treatment of septic rats with the binary complex increased the translational efficiency in gastrocnemius during sepsis (Fig. 7 bottom). In muscles from untreated septic animals, the translational efficiency was increased on day 5 compared with day 3 but remained reduced by ~40% compared with control animals (Fig. 7 bottom). Treatment of septic rats with the binary complex increased the translational efficiency by 50% in gastrocnemius from septic rats. Thus daily injections of the binary complex for 5 days modulated the translational efficiency in gastrocnemius to levels that were not significantly different from control values.

### Table 4. Plasma concentrations of amino acids in control and septic rats

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Control</th>
<th>Septic</th>
<th>Septic + IGF/BP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>580 ± 31</td>
<td>352 ± 38*</td>
<td>617 ± 56†</td>
</tr>
<tr>
<td>Arginine</td>
<td>132 ± 8</td>
<td>82 ± 5*</td>
<td>106 ± 10</td>
</tr>
<tr>
<td>Asparagine</td>
<td>61 ± 4</td>
<td>33 ± 3*</td>
<td>41 ± 2*</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>62 ± 3</td>
<td>48 ± 10</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>Glutamine</td>
<td>596 ± 17</td>
<td>506 ± 36*</td>
<td>694 ± 44†</td>
</tr>
<tr>
<td>Glycine</td>
<td>280 ± 9</td>
<td>241 ± 20</td>
<td>315 ± 21</td>
</tr>
<tr>
<td>Histidine</td>
<td>67 ± 2</td>
<td>63 ± 4</td>
<td>78 ± 3†</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>67 ± 2</td>
<td>68 ± 6</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>Leucine</td>
<td>111 ± 10</td>
<td>124 ± 14</td>
<td>133 ± 8</td>
</tr>
<tr>
<td>Lysine</td>
<td>338 ± 17</td>
<td>311 ± 43</td>
<td>384 ± 21</td>
</tr>
<tr>
<td>Methionine</td>
<td>40 ± 2</td>
<td>37 ± 3</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>Ornithine</td>
<td>46 ± 3</td>
<td>50 ± 6</td>
<td>53 ± 8</td>
</tr>
<tr>
<td>Phenylalanine (+)</td>
<td>1,154 ± 47</td>
<td>1,284 ± 148</td>
<td>1,421 ± 152</td>
</tr>
<tr>
<td>Serine</td>
<td>300 ± 11</td>
<td>180 ± 15*</td>
<td>276 ± 28†</td>
</tr>
<tr>
<td>Taurine</td>
<td>118 ± 10</td>
<td>57 ± 11*</td>
<td>53 ± 3*</td>
</tr>
<tr>
<td>Threonine</td>
<td>258 ± 18</td>
<td>104 ± 8*</td>
<td>155 ± 20*</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>54 ± 2</td>
<td>31 ± 3*</td>
<td>31 ± 3*</td>
</tr>
<tr>
<td>Tyrosine (+)</td>
<td>404 ± 31</td>
<td>287 ± 32</td>
<td>331 ± 35</td>
</tr>
<tr>
<td>Valine</td>
<td>151 ± 10</td>
<td>134 ± 10</td>
<td>140 ± 10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total Amino Acids</th>
<th>3,267 ± 79</th>
<th>2,427 ± 144*</th>
<th>3,231 ± 214†</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>Septic</th>
<th>Septic + IGF/BP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>500 ± 30</td>
<td>563 ± 40†</td>
</tr>
<tr>
<td>Arginine</td>
<td>127 ± 11</td>
<td>114 ± 7†</td>
</tr>
<tr>
<td>Asparagine</td>
<td>68 ± 4</td>
<td>52 ± 4†</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>78 ± 5</td>
<td>53 ± 6§</td>
</tr>
<tr>
<td>Glutamine</td>
<td>610 ± 39</td>
<td>620 ± 30†</td>
</tr>
<tr>
<td>Glycine</td>
<td>259 ± 12</td>
<td>277 ± 16</td>
</tr>
<tr>
<td>Histidine</td>
<td>65 ± 2</td>
<td>70 ± 3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>65 ± 2</td>
<td>84 ± 6</td>
</tr>
<tr>
<td>Leucine</td>
<td>115 ± 9</td>
<td>163 ± 10‡</td>
</tr>
<tr>
<td>Lysine</td>
<td>346 ± 44</td>
<td>481 ± 22†</td>
</tr>
<tr>
<td>Methionine</td>
<td>41 ± 3</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Ornithine</td>
<td>50 ± 4</td>
<td>70 ± 3†</td>
</tr>
<tr>
<td>Phenylalanine (+)</td>
<td>919 ± 119</td>
<td>1,270 ± 92</td>
</tr>
<tr>
<td>Serine</td>
<td>232 ± 8†</td>
<td>218 ± 12†</td>
</tr>
<tr>
<td>Taurine</td>
<td>98 ± 13</td>
<td>87 ± 15</td>
</tr>
<tr>
<td>Threonine</td>
<td>255 ± 15</td>
<td>223 ± 19†</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>57 ± 2</td>
<td>38 ± 2†</td>
</tr>
<tr>
<td>Tyrosine (+)</td>
<td>378 ± 40</td>
<td>255 ± 41</td>
</tr>
<tr>
<td>Valine</td>
<td>200 ± 9*</td>
<td>182 ± 11†</td>
</tr>
<tr>
<td>Total Amino Acids</td>
<td>3,233 ± 176</td>
<td>3,332 ± 129†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–10/group. All means are expressed as μM. Amino Acid, the sum of the individual amino acids, with the exception of phenylalanine and tyrosine (+), which are artificially elevated as a result of the injection of the bolus of L-phenylalanine. Statistical significance of each amino acid and sum of amino acids was tested by ANOVA followed by the Sidak test for multiple comparisons.

*P < 0.05 vs. 3-day controls; †P < 0.05 vs. 3-day septic controls; ‡P < 0.05 vs. 5-day controls; §P < 0.05 vs. 5-day septic control.
Regulation of eIF4E.

To investigate the effect of sepsis and treatment with binary complex on the association of 4E-BP1 with eIF4E, eIF4E immunoprecipitates were analyzed for 4E-BP1 content (Fig. 8 top). Compared with control animals, sepsis increased the amount of 4E-BP1 associated with eIF4E; 4-fold on day 3 postinfection. Treatment of septic rats with the binary complex had no significant effect on the amount of 4E-BP1 associated with eIF4E. Unlike on day 3, there was no significant difference in the amount of 4E-BP1 associated with eIF4E between control and septic rats on day 5. There was no significant difference in the amount of 4E-BP1 bound to eIF4E between untreated or the binary complex-treated septic rats on day 5; however, the amount of 4E-BP1 bound to eIF4E was significantly reduced by ~50% in either treated or untreated septic rats on day 5 compared with day 3.

The interaction between 4E-BP1 and eIF4E is partially regulated by the extent of 4E-BP1 phosphorylation. Phosphorylation of 4E-BP1 releases eIF4E from the 4E-BP1·eIF4E complex and allows the eIF4E·mRNA complex to bind to eIF4G and then to the 40S ribosome. 4E-BP1 undergoes multiple phosphorylations, which are characterized by reduced mobility after electrophoresis. 4E-BP1 can be resolved into multiple electrophoretic bands termed α, β, and γ, representing differentially phosphorylated forms of the protein (42, 48). The most highly phosphorylated form of the protein, γ, does not bind eIF4E (42, 48). Therefore, we investigated whether a similar mechanism could account for inhibition of protein synthesis in skeletal muscle during sepsis (Fig. 8 middle). On day 3, the amount of 4E-BP1 in the γ-form was diminished from 36% to 5% compared with controls. Treatment of septic rats with the binary complex had no significant effect on the extent of γ-phosphorylation of 4E-BP1 compared with untreated septic rats, and the values remained significantly reduced compared with controls. On day 5, the extent of γ-phosphorylation of 4E-BP1 in gastrocnemius from untreated and binary complex-treated septic rats on was increased 3.5- and 7-fold compared with those determined on day 3 (P < 0.05). Furthermore, treatment of septic rats with the binary complex had no significant effect on the extent of γ-phosphorylation of 4E-BP1 compared with untreated septic rats. Even though the extent of 4E-BP1 phosphorylation increased on day 5, it remained significantly reduced by 50% compared with controls.

To further define potential mechanisms responsible for the sepsis-induced inhibition of protein synthesis, the effect of sepsis and treatment with binary complex on phosphorylation of eIF4E was examined (Fig. 8 bottom). In muscles from control rats, ~80% of the eIF4E was in the phosphorylated form. On day 3 postinfection, sepsis reduced the proportion of eIF4E in the phosphorylated form to 33%. Treatment of septic rats with the binary complex had no significant effect on the extent of phosphorylation of eIF4E compared with untreated septic rats, and the percentage remained significantly reduced compared with controls. On day 5, the extent of phosphorylation of eIF4E was not altered on day 5 compared with day 3 and remained significantly depressed relative to control values.

Table 5. Effect of the binary complex on expression of eIF2Bα in gastrocnemius during sepsis

<table>
<thead>
<tr>
<th></th>
<th>3-Day Postinfection</th>
<th>5-Day Postinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Septic</td>
</tr>
<tr>
<td>eIF2Bα AU/mg protein</td>
<td>170 ± 21</td>
<td>143 ± 16</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–9 animals/group. *P < 0.005 vs. 5-day control (ANOVA F = 5.385, P < 0.001).
DISCUSSION

The present results indicate that the sepsis-induced inhibition in protein synthesis in gastrocnemius can be modulated by the in vivo administration of a binary complex consisting of equimolar concentrations of IGF-I and IGFBP-3. Administration of the binary complex-stimulated protein synthesis in gastrocnemius 3 days after initiation of the septic focus but did not restore rates of protein synthesis to values observed in control animals. By day 5 postinfection, administration of the binary complex raised gastrocnemius protein synthesis to a rate comparable with that of control rats. Injection of the binary complex restored the plasma concentrations of IGF-I in septic rats to control values without alterations in either plasma insulin or glucose concentrations. These findings are consistent with reports in human burn patients, when hypoglycemia was not observed after injection of the binary complex (24), and are in contrast to the hypoglycemia observed after injection of free IGF-I (41).

The augmentation of plasma IGF-I after administration of the binary complex was entirely the result of an increase in human IGF-I. There was no detectable depression of endogenous rat IGF-I by administration of the binary complex. These observations concerning rat plasma IGF-I concentrations imply that injection of the binary complex did not downregulate the ability of the liver to synthesize IGF-I. These data are consistent with the comparable expression of hepatic IGF-I mRNA in binary complex-treated and untreated septic rats. Furthermore, exogenous rhIGFBP-3 binary complex formed a ternary complex with rat ALS in vivo. Sephadex chromatography confirmed that infused rhIGFBP-3, which was subsequently isolated from plasma, co-eluted with ALS and IGF-I in a 150-kDa complex. It is likely that complex formation extends the half-life of injected IGF-I beyond that which is seen for the free peptide. Thus binary complex administration most likely accelerates protein synthesis in gastrocnemius by prolonging the availability of IGF-I, although we cannot exclude the possibility of a ligand-independent IGFBP-3 effect.

The enhanced rates of protein synthesis in vivo after elevation of plasma IGF-I concentrations with the binary complex extends our previous findings, whereby IGF-I stimulates the synthesis of mixed muscle proteins in either perfused hindlimb (31) or incubated muscle preparations (26, 61) from septic animals. In the present study, the restoration of protein synthesis was associated with anabolic effects on gastrocnemius weight and protein content on both day 3 and 5 postinfection. In contrast to muscle, administration of the binary complex did not alter protein synthesis or protein content in liver or kidney. These observations are consistent with reports whereby increased rates of protein synthesis after an acute administration of IGF-I were localized to striated muscle in mice fasted overnight (3). Furthermore, the findings are consistent with improved net protein balance and stimulation of...
muscle protein fractional synthetic rates in severely
burned children treated with the binary complex (24).

In contrast to gastrocnemius, administration of the
binary complex was without effect in soleus. Thus
muscles with predominantly fast-twitch fibers (gas-
 trocnemius) were more sensitive to the administration
of binary complex, whereas the muscles with slow-
twitch fibers (soleus) were relatively unresponsive
during sepsis. This finding is consistent with two obser-
vations. First, the ability of IGF-I to stimulate protein
synthesis was greatest in gastrocnemius and plantaris,
and least in soleus and heart, after infusion of IGF-I in
mice (3). Second, sepsis preferentially inhibits protein
synthesis in muscles composed primarily of fast-twitch
fibers (e.g., gastrocnemius), whereas it is without effect
in muscles composed primarily of slow-twitch fibers
(e.g., soleus) (58). Hence, one might expect a greater
effect of the binary complex in gastrocnemius com-
pared with soleus during sepsis.

Synthesis of protein in eukaryotic cells is achieved
through a complex series of discrete reactions. The
process involves the association of the 40S and 60S
ribosomal subunits, mRNA, initiator methionyl-tRNA
(met-tRNA\textsuperscript{met}), other amino acyl-tRNAs, cofactors (i.e.,
GTP and ATP), and protein factors (collectively known
as eIF, elongation factors, and releasing factors)
through a series of reactions resulting in the transla-
tion of mRNA into proteins. Translation of mRNA on
the ribosome is composed of three phases: 1) initiation,
whereby met-tRNA\textsuperscript{met} associates with mRNA bound to
40S ribosomal subunit and subsequent binding of the
40S ribosomal subunit to the 60S subunit to form
a ribosome complex capable of translation; 2) elongation,
during which tRNA-bound amino acids are incorpo-
rated in growing polypeptide chains according to the
mRNA template; and 3) termination, when the com-
pleted protein is released from the ribosome. Regula-
tion of protein synthesis occurs predominantly through
changes in the abundance of ribosomes, translational
efficiency, and/or translatable mRNA.

To determine the potential mechanisms responsible
for the increased rates of protein synthesis in septic
rats treated with the binary complex, we examined the
total muscle RNA content and translational efficiency.
Because \(\sim 85\%\) of the total cellular RNA is ribosomal,
alterations in the muscle RNA content reflect changes
in the relative amount of ribosomes. Treatment of
septic rats with the binary complex did not increase the
total RNA content in muscle. Therefore, the restoration
of protein synthesis in the binary complex-treated septic
rats did not result from an increased number of ribosomes; instead, the binary complex prevented the
sepsis-induced inhibition of gastrocnemius protein
synthesis by increasing translational efficiency. These
observations are consistent with our previous reports
whereby IGF-I was shown to acutely stimulate trans-
lational efficiency by enhancing translation initiation
in perfused hindlimb of septic rats (31).

The relative abundance of the eIF2B\textsubscript{e} decreases
during the first 5 days after the septic insult (8, 62). We
have previously suggested that this is one mechanism
by which sepsis can reduce protein synthesis in skele-
tal muscle. Injection of the binary complex did not alter
the amount of eIF2B\textsubscript{e} in septic rats at either time point
examined. Thus it is unlikely that the binary complex
enhanced protein synthesis during sepsis by increasing
the muscle content of eIF2B\textsubscript{e}. This finding is in con-
trast to the ability of anticytokine modalities to en-
hance protein synthesis during sepsis by preventing
the fall in skeletal muscle eIF2B\textsubscript{e} content (7, 8, 62).
Thus the binary complex was able to enhance protein
synthesis despite a diminished eIF2B\textsubscript{e} content in skel-
etal muscle. This observation is consistent with our
observations in perfused hindlimb, whereby IGF-I
stimulated protein synthesis in gastrocnemius without
increasing the expression of eIF2B (31). Furthermore,
the data suggest that factors regulating eIF2B expres-
sion during sepsis are independent of IGF-I.

We also investigated the ability of the binary com-
plex to modulate the binding of eIF4E to the transla-
tion repressor protein 4E-BP1, a crucial step control-
ing translation initiation in skeletal muscle. The inhibi-
tion of translation initiation during diabetes and
starvation correlates with an increased amount of
eIF4E found in the inactive 4E-BP1-eIF4E complex in
skeletal muscle (33, 34). Conversely, feeding starved
rats or insulin treatment of diabetic rats causes a
dissociation of the 4E-BP1-eIF4E complex, thereby
promoting translation initiation (33, 65). In the present
set of experiments, sepsis increased the amount of
4E-BP1 bound to eIF4E at both time points, albeit to a
lesser extent on day 5 postinfection. The binary com-
plex did not diminish the abundance of eIF4E associ-
ated with 4E-BP1 in septic rats. Thus we did not
observe a reciprocal relationship between eIF4E found
in the inactive 4E-BP1-eIF4E complex and protein
synthesis in muscle. Hence, protein synthesis appears
to be augmented by the binary complex in septic rats
without reductions in the abundance of eIF4E-4E-BP1
complex.

The interaction between 4E-BP1 and eIF4E is regu-
ulated by the extent of 4E-BP1 phosphorylation. Phos-
phorylation of 4E-BP1 releases eIF4E from the 4E-
BP1-eIF4E complex (48). In the present study, sepsis
caused a marked reduction in the amount of 4E-BP1 in
the \(\gamma\)-phosphorylated form compared with controls.
Furthermore, the amount of 4E-BP1 in the \(\gamma\)-phosphor-
ylated form increased from day 3 to day 5 postinfection,
whereas no differences were observed in controls be-
tween the two days. A reciprocal relationship existed
between the extent of binding of 4E-BP1 to eIF4E and
the amount of 4E-BP1 in the \(\gamma\)-phosphorylated form
during sepsis. Treatment of septic rats with the binary
complex did not significantly modulate the phosphory-
lated status of 4E-BP1. Because 4E-BP1 phosphoryla-
tion was unaltered, it is not surprising that the binary
complex did not affect abundance of eIF4E associated
with 4E-BP1.

Phosphorylation of eIF4E enhances affinity of the
factor for m\textsuperscript{7}GTP cap analogs of mRNA (45). Reduced
phosphorylation of eIF4E correlates with an inhibition
of protein synthesis during heat shock or serum deple-
tion (12). Both phosphorylated and nonphosphorylated forms of eIF4E bind to the mRNA cap structure (45). However, the phosphorylated form possesses a fourfold greater affinity for cap analogs and mRNA than does the unphosphorylated form, providing a potential explanation for the correlation between phosphorylation of eIF4E and rates of protein synthesis (45). Sepsis decreased phosphorylation of eIF4E on both day 3 and day 5. Moreover, we were unable to detect a significant increase in phosphorylation of eIF4E at a time when protein synthesis was stimulated by the binary complex. Thus it is unlikely that the stimulation of protein synthesis in gastrocnemius of septic rats induced by binary complex administration resulted from an enhanced phosphorylation of eIF4E. This observation may not be surprising, because insulin failed to increase the phosphorylation of eIF4E in perfused hind-limb at a time when protein synthesis was enhanced (34).

In summary, these results indicate that injection of the binary complex minimizes the loss of muscle protein during sepsis, in part by promoting protein synthesis. The acceleration of protein synthesis occurred secondary to stimulating translational efficiency, rather than to increasing the number of ribosomes. The increase in muscle protein synthesis induced by administration of the binary complex appears to be independent of changes in eIF2B expression or 4E-BP1 regulation. The importance of these findings lies in the observation that the binary complex has a beneficial effect on protein synthesis, even when administered after initiation of the septic focus.

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