Phosphorylation of eukaryotic initiation factor eIF2Bε in skeletal muscle during sepsis

THOMAS C. VARY, GINA DEITER, AND SCOT R. KIMBALL
Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Vary, Thomas C., Gina Deiter, and Scot R. Kimball. Phosphorylation of eukaryotic initiation factor eIF2Bε in skeletal muscle during sepsis. Am J Physiol Endocrinol Metab 283: E1032–E1039, 2002; 10.1152/ajpendo.00171.2002.—We reported that the inhibition of protein synthesis in skeletal muscle during sepsis correlated with reduced eukaryotic initiation factor eIF2B activity. The present studies define changes in eIF2Bε phosphorylation in gastrocnemius of septic animals. eIF2B kinase activity was significantly elevated 175% by sepsis compared with sterile inflammation, whereas eIF2B phosphatase activity was unaffected. Phosphorylation of eIF2Bε-Ser535 was significantly augmented over 2-fold and 2.5-fold after 3 and 5 days and returned to control values after 10 days of sepsis. Phosphorylation of glycogen synthase kinase-3 (GSK-3), a potential upstream kinase responsible for the elevated phosphorylation of eIF2Bε, was significantly reduced over 36 and 41% after 3 and 5 days and returned to control values after 10 days of sepsis. The phosphorylation of PKB, a kinase thought to directly phosphorylate and inactivate GSK-3, was significantly reduced ~50% on day 3, but not on days 5 or 10, postinfection compared with controls. Treatment of septicecum rats with TNF-binding protein prevented the sepsis-induced changes in eIF2Bε and GSK-3 phosphorylation, implicating TNF in mediating the effects of sepsis. Thus increased phosphorylation of eIF2Bε via activation of GSK-3 is an important mechanism to account for the inhibition of skeletal muscle protein synthesis during sepsis. Furthermore, the study presents the first demonstration of changes in eIF2Bε phosphorylation in vivo.

glycogen synthase kinase-3; protein kinase B; tumor necrosis factor-binding protein; gastrocnemius; psoas; infection; eukaryotic initiation factor 2B phosphatase; eIF2Bε kinase

SEPSIS INDUCES PROFOUND ALTERATIONS in whole body protein metabolism. Marked weight loss and accelerated nitrogen excretion characterize the host’s response to severe systemic bacterial infection. Nitrogen losses equivalent to 5–17% of total body protein may be observed in septic patients despite aggressive nutritional support. Much of the whole body negative nitrogen balance occurs secondary to a net catabolism of skeletal muscle proteins. Muscle protein wasting in sepsis results from both a prolonged decrease in protein synthesis and an increase in protein degradation (for review see Refs. 2, 31, 33, 41). In contrast, the magnitude and duration of muscle wasting are usually short-lived in trauma or sterile inflammation, with the restoration of lean body mass and skeletal protein metabolism occurring within days of the insult (2, 3, 31, 40, 41, 47, 51).

Regulation of protein synthesis occurs predominantly through changes in the abundance of ribosomes, translational efficiency, and/or concentration of translatable mRNA. The sepsis-induced inhibition of protein synthesis in skeletal muscle results from a defect in translational efficiency (3, 14, 44, 47) rather than changes in total mRNA (24, 48) or the number of ribosomes (3, 47). Our previous studies have shown that sepsis diminished the translational efficiency secondary to a defect in translation initiation rather than reductions in elongation (47).

Two steps (the binding of met-tRNAMet to the 40S ribosomal subunit to form the 43S preinitiation complex and the binding of mRNA to the 43S preinitiation complex) in the translation initiation pathway are important regulatory loci in the overall control of protein synthesis during sepsis. We have previously reported that the formation of the 43S preinitiation complex is markedly reduced in skeletal muscle during sepsis (45). The step involving the binding of met-tRNAMet to the 40S ribosomal subunit forming the 43S preinitiation complex is mediated by eukaryotic initiation factor-2 (eIF2) and is regulated by the activity of another initiation factor, eIF2B. Furthermore, we have shown that decreased formation of the 43S preinitiation complex is associated with a diminished eIF2B guanine nucleotide exchange activity (12, 44, 45). Importantly, eIF2B activity is reduced only in muscle types exhibiting decreased rates of protein synthesis during sepsis, i.e., muscles composed of mixed fast-twitch fibers (44, 47), suggesting that reductions in eIF2B activity may cause the defect in translation initiation.

On the basis of in vitro experiments, eIF2B activity is regulated by both direct and indirect mechanisms involving allosteric binding, competitive inhibitory processes, and/or phosphorylation (11, 22). However,
not all of these mechanisms appear important for controlling eIF2B activity in skeletal muscle during sepsis. The best-characterized regulatory mechanism involves the phosphorylation of eIF2 on its α-subunit [eIF2α (p)] (23, 30). Phosphorylation of eIF2α converts the protein from a substrate into an inhibitor of eIF2B (7, 23, 30). The resulting eIF2α-GDP⋅eIF2B complex is highly stable and inactive (7, 23, 30). Thus phosphorylation of eIF2α results in a reduction in the amount of eIF2B available for GTP exchange. Under a variety of conditions in nonmuscle cells, the proportion of eIF2α in the phosphorylated form inversely correlates with rates of protein synthesis (16, 23, 30, 44). We could not detect a change in the extent of eIF2α (p) in skeletal muscle during chronic sepsis (44). eIF2B is also subject to allosteric regulation by a variety of effectors (6, 16, 26). Inactivation of eIF2B by NAD⁺ and NADPH is reversed by addition of equimolar amounts of NADH or NADP⁺ (6, 16). However, we have shown that the NADPH-to-NADP⁺ concentration ratio is not significantly altered in gastrocnemius of septic rats (6, 16, 45). Thus diminished eIF2B activity does not appear to be regulated by changes in the redox state during sepsis. These findings suggest that mechanisms other than the phosphorylation state of eIF2α or cytosolic redox state regulate eIF2B activity in skeletal muscle during sepsis.

In addition to the above mechanisms for regulating eIF2B activity, the catalytic e-subunit of eIF2B (eIF2Bε) is a substrate for several protein serine/threonine kinases, which either enhance or inhibit its guanine nucleotide exchange activity after phosphorylation (7, 26, 32, 33, 56, 57). For example, phosphorylation of eIF2Bε by casein kinase (CK)-1 or CK-2 stimulates the activity of the protein in vitro (7, 32, 33), although one group has been unable to reproduce the stimulatory effect of CK-1 (27). In contrast, phosphorylation of eIF2Bε by glycogen synthase kinase (GSK)-3 in cells in culture results in inactivation of the guanine nucleotide exchange activity (12, 54–57). There is no information available concerning the potential role of altered phosphorylation of eIF2Bε in regulating eIF2B activity in any tissue in vivo.

Here, we define the temporal changes in extent of phosphorylation in eIF2Bε in gastrocnemius of septic animals and determine whether anti-cytokine therapy can prevent the sepsis-induced alterations observed. In addition, we examine the role of GSK-3 as a potential upstream kinase responsible for the sepsis-induced alterations in eIF2Bε phosphorylation. The findings suggest that phosphorylation of eIF2Bε may be an important regulator of eIF2B activity, limiting translation initiation in skeletal muscle during sepsis.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats weighing 150–225 g were maintained on a 12:12-h light-dark cycle and fed ad libitum. Chronic abdominal sepsis was created by implantation of a fecal-agar pellet (1.5 ml) inoculated with 10⁴ colony-forming units (CFU) of Escherichia coli and 10⁴ CFU of Bacteroides fragilis into the peritoneal cavity as previously described (3, 4, 13–15, 44, 45, 47, 50–53). The animals develop an abdominal abscess resulting in a hyperdynamic, hypermetabolic septic condition. Control animals underwent the intra-abdominal implantation of a sterilized fecal-agar pellet to which sterile saline had been added to allow for the generation of a sterile abscess (14, 46, 47, 50). Both groups of animals consumed the same amount of rat chow over the course of the experiment (49).

Three, five, or ten days after the implantation of the fecal-agar pellet, animals were anesthetized with pentobarbital sodium, and the gastrocnemius and psoas were excised, weighed, and either frozen between clamps precooled to the temperature of liquid nitrogen for immunoblot studies or homogenized for eIF2B kinase assays. The frozen tissue was powdered under liquid nitrogen and stored at −80°C until analyses were performed.

In a second set of experiments, we examined the possible role of cytokines in mediating the sepsis-induced alterations in phosphorylation of eIF2Bε. To accomplish this, saline (1 ml) or tumor necrosis factor (TNF)-binding protein (TNFbp) (1 mg/ml, Amgen, Thousand Oaks, CA) was injected subcutaneously daily beginning 4 h before the implantation of the infected fecal-agar pellet to form a septic abscess (1, 4). TNFbp is a dimeric, polyethylene glycol-linked form of the human p55-soluble TNF receptor (8, 29, 34). The dose and timing of TNFbp injection were based on previously determined plasma TNF levels in the intra-abdominal septic abscess model (14) and pharmacokinetic studies demonstrating inhibitory plasma concentrations of TNFbp (>500 ng/ml) with this dosing regimen (1, 4, 29, 34). Plasma TNFbp concentrations were 500 ng/ml at the early (0–12 h) times after the initial injection of the compound on day 0. On day 5, plasma concentrations of TNFbp in septic rats were −2,500 ng/ml (1). The abscess was allowed to develop for 5 days, and the gastrocnemius was sampled and processed as described above.

eIF2Bε kinase assay. Purified eIF2Bε was used to assay the kinases present in extracts of psoas from sterile inflammatory and septic rats responsible for phosphorylating the protein (12). Fresh psoas was excised, minced with scissors, and homogenized at 4°C in 7 vol buffer A containing (in mM) 50 Tris·HCl (pH 7.4), 150 KCl, 5 magnesium acetate, 6 β-mercaptoethanol, 250 sucrose, and 5 EGTA by use of a Polytron PT10 set to 60% of full power. The homogenate was centrifuged at 10,000 g (4°C) for 10 min, and the supernatant was decanted. The supernatant then was centrifuged at 300,000 g (4°C) for 35 min, and the supernatant was used for eIF2Bε kinase assays. eIF2Bε kinase activity was assayed in buffer containing 20 mM HEPES (pH 7.4), 2.5 mM magnesium acetate supplemented with 10 μl of the 300,000-g supernatant, 5 μg of purified eIF2Bε, and 7.5 μCi [γ-32P]ATP in a total volume of 45 μl at 30°C. At 5, 10, and 15 min, 15 μl were removed from the reaction mixture and placed in a tube containing 15 μl of 2× Laemmli SDS sample buffer warmed to 60°C and heated at 90°C for 2 min. Samples underwent electrophoresis on a 10% polyacrylamide gel at 60 mA. The polyacrylamide gels were dried by use of the Easy Breeze gel dryer (Hoffer Scientific, San Francisco, CA) without heat. The gels were exposed to X-ray film, and the autoradiographs were developed. After development, the film was scanned (Microtek ScanMaker IV) and quantified with the use of NIH Image 1.6 software. eIF2Bε used as substrate was expressed in SF9 insect cells by use of the baculovirus expression system and subsequently purified to >98% homogeneity using immunoaffinity chromatography (9).

Phosphorylation or dephosphorylation of eIF2Bε. A portion of the purified eIF2Bε (15 μg) was dephosphorylated using
λ-phosphatase (3,440 U; New England BioLabs, Boston, MA) by incubation at 37°C for 30 min in the buffer supplied by the manufacturer. After incubation, an equal volume of 2× Laemmli SDS sample buffer was added, and the mixture was heated at 90°C for 5 min. A second portion of the purified protein (15 μg) was phosphorylated using GSK-3 (85 U; New England Biolabs) by incubation at 30°C for 60 min in a solution consisting of (in mM) 35 Tris (pH 7.4), 7 β-mercaptoethanol, 7% glycerol, 0.05 PMSF, 0.7 benzamidine, 12 magnesium chloride, and 0.25 ATP. An equal volume of 2× Laemmli SDS sample buffer was then added, and the mixture was heated at 90°C for 5 min.

Determination of the phosphorylation state of eIF2Be, GSK-3, and PKB. eIF2Be, GSK-3, and PKB in extracts of gastrocnemius were resolved by electrophoresis, and the phosphorylated and unphosphorylated forms of each enzyme were quantified by Western blot analysis. An aliquot (0.2 g) of the powdered tissue was weighed and homogenized in 7 vol buffer A (20 mM HEPEs (pH 7.4), 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM DTT, 50 mM NaN3, 50 mM β-glycerophosphate, 0.1 mM PMSF, 1 mM benzamidine, 0.5 mM sodium vanadate, and 1 μM microcystin LR) by use of a Polytron homogenizer. The homogenate was centrifuged at 10,000 g for 10 min at 4°C, and the pellet was discarded. Aliquots of the supernatant were mixed with equal volumes of 2× Laemmli SDS sample buffer (60°C), boiled for 3 min, and centrifuged. The samples were subjected to SDS polyacrylamide slab gel electrophoresis followed by transfer of proteins to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Bio-Rad Laboratories, Hercules, CA), as described previously (17, 18). After transfer of the proteins to PVDF membranes, phosphorylation of eIF2Be, GSK-3, or PKB was analyzed by sequential immunoblotting, first with phosphospecific antibodies that specifically recognized the phosphorylated forms of eIF2Be (Biosource International, Camarillo, CA), GSK-3 (Upstate Biotechnology, Lake Placid, NY), or PKB (Cell Signaling Technologies, Vancouver, Canada). After development of the blot, the membranes were treated with a solution containing 62.5 mM Tris-HCl (pH 6.7), 100 mM β-mercaptoethanol, and 2% (wt/vol) SDS to remove antibodies as per the manufacturer’s instructions. Reexposure of the membrane to secondary antibody revealed that this procedure removed the primary anti-phospho-antibodies used. The membranes were then blocked with nonfat dry milk and incubated with an antibody that recognizes eIF2Be (19), GSK-3 (Biosource International), or PKB (Cell Signaling Technologies) independently of the phosphorylation states (total eIF2Be, GSK-3, or PKB). All blots were developed using enhanced chemiluminescence (ECL; Amer sham Pharmacia Biotech, Piscataway, NJ) and then exposed to film. After development, the film was scanned (Microtek ScanMaker IV) and quantified using NIH Image 1.6 software. The phosphorylated eIF2Be, GSK-3, or PKB signal densities were normalized to the respective total eIF2Be, GSK-3, or PKB signal to reflect the relative ratio of phosphorylated eIF2Be, GSK-3, or PKB to total eIF2Be, GSK-3, or PKB, respectively.

eIF2Be phosphatase assay. Gastrocnemius was homogenized in 7 vol of buffer B containing (in mM) 20 HEPEs, pH 7.4, 100 KCl, 0.2 EDTA, 2 EGTA, 1 DTT, 0.1 PMSF, and 1 benzamidine, using a Polytron homogenizer, and centrifuged at 1,000 g at 4°C for 3 min. An aliquot (25 μl) of the supernatant was diluted with 25 μl of homogenization buffer and 60 ng of the phosphorylated 32P-labeled eIF2Be protein at 30°C. At 0, 2, 3, and 5 min, 10 μl of the reaction mixture were added to 10 μl of 2× Laemmli sample buffer warmed to 60°C and boiled for 3 min. The sample was resolved by electrophoresis on a 12.5% polyacrylamide gel. The gel was dried and exposed to X-ray film in a cassette equipped with Du Pont Lightning Plus intensifying screens. The autoradiographs were scanned and analyzed. Phosphatase activity was calculated as the loss of radioactivity over time. Rat eIF2Be was expressed in and purified from SF9 cells as described previously (12). A portion of the purified protein (10 μg) was phosphorylated in vitro by incubation of eIF2Be with purified GSK-3 and [γ-32P]ATP. The phosphorylation reaction was terminated by addition of AMP-PNP to a final concentration of 1 mM.

Statistical analysis. Values shown are means ± SE. Statistical evaluation of the data was performed using Student’s t-test. Differences among the means were considered significant when P < 0.05.

RESULTS

Effect of sepsis on eIF2Be kinase activity. We have previously established that eIF2B activity is reduced in gastrocnemius and psoas of septic animals (44), but the mechanism responsible for the inhibition remains unknown. One possible mechanism to account for the decreased eIF2B activity would be an increased phosphorylation of eIF2B. Initially, it was important to establish whether or not sepsis increased eIF2Be kinase activity in extracts of psoas. Typical autoradiographs of the eIF2Be kinase assay from psoas of sterile inflammatory and septic rats on day 5 postsurgery are shown in Fig. 1A. A significantly (P < 0.05) greater eIF2Be kinase activity was detected in extracts of psoas obtained from septic rats [226 ± 21 arbitrary units (AU)/min, n = 5] compared with rats with a sterile abscess (130 ± 24 AU/min, n = 6).

![Fig. 1. Phosphorylation of eukaryotic initiation factor eIF2Be. A: eIF2Be kinase activity in psoas from sterile inflammatory and septic rats. Purified eIF2Be was used to assay the kinases present in extracts of psoas from sterile inflammatory and septic rats responsible for phosphorylating the protein, as described in MATERIALS AND METHODS. At 5, 10, and 15 min, 15 μl were removed from the reaction mixture and resolved by electrophoresis. The gels were exposed to X-ray film, and the autoradiographs were developed. After development, the film was scanned (Microtek ScanMaker IV) and quantified using NIH Image 1.6 software. B: rat eIF2Be was dephosphorylated using λ-phosphatase (lane 1), incubated in the absence of added phosphatase or kinase (lane 2), or phosphorylated with the use of glycogen synthase kinase (GSK)-3 (lane 3), as described in MATERIALS AND METHODS. The mixtures (containing 140 ng of eIF2Be) were then analyzed by Western blot analysis, using an affinity-purified anti-phosphopeptide antibody specific for eIF2Be phosphorylated (P) on Ser535 (top) or a monoclonal anti-eIF2Be antibody (bottom).](http://ajpendo.physiology.org/10.1152/ajpendo.00925.2001)
Effect of sepsis on eIF2B phosphorylation. An increase in eIF2B kinase activity would be expected to alter the steady-state phosphorylation state of eIF2B. Therefore, we sought to evaluate the effect of sepsis on the phosphorylation of eIF2B by immunoblot techniques using an antibody raised to a peptide corresponding to the phosphoserine amino acid residue (Ser^535) of eIF2B phosphorylated by GSK-3 (54). This site was chosen because Jefferson et al. (12) have provided evidence that GSK-3 is the predominant kinase present in muscles composed of fast-twitch fibers (gastrocnemius, psoas) that phosphorylates eIF2B (12). GSK-3 phosphorylates eIF2B at a single conserved serine residue (Ser^535 in the rat enzyme) and leads to an inhibition in guanine nucleotide exchange activity (56). The antibody specifically recognized the GSK-3-dependent phosphorylated form of eIF2B [phospho(P)-eIF2B; Fig. 1B]. In these studies, exogenous GSK-3 was used to phosphorylate purified eIF2B in vitro. Therefore, the anti-phospho-eIF2B antibody was used to examine the extent of phosphorylation of eIF2B in skeletal muscle during sepsis. The extent of phosphorylation of eIF2B was increased over 2-fold \((P < 0.01)\) and 2.5-fold \((P < 0.001)\) in gastrocnemius after 3 and 5 days of septic abscess formation, respectively, compared with sterile inflammatory animals (Fig. 2). By 10 days of sepsis, the extent of phosphorylation of eIF2B returned to control values.

Effect of sepsis on GSK-3 phosphorylation. These studies clearly raised the question as to the possible role for GSK-3 in mediating the sepsis-induced changes in phosphorylation of eIF2B. Studies performed in cells in culture suggest that phosphorylation of eIF2B by GSK-3 may result in a decreased activity of the guanine nucleotide exchange function of the protein (33, 56, 57). GSK-3 activity itself is regulated by reversible phosphorylation, where phosphorylation of GSK-3 results in inactivation of the enzyme. We have used changes in phosphorylation (assessed by immunoblotting techniques) as an indicator of the effect of sepsis on the activation of GSK-3 in gastrocnemius from sterile inflammatory and septic rats (Fig. 3). The extent of phosphorylation of GSK-3 was decreased over 36% \((P < 0.001)\) and 41% \((P < 0.01)\) in gastrocnemius after 3 and 5 days of abscess formation in septic rats, respectively, compared with sterile inflammatory animals (Fig. 3). By 10 days of sepsis, the extent of phosphorylation of GSK-3 was not significantly different between control and septic rats.

Effect of sepsis on PKB phosphorylation. The protein kinase PKB has been shown to phosphorylate GSK-3 in response to insulin in L6 cells in culture (5). PKB itself is activated by phosphorylation. We therefore investigated the phosphorylation state of PKB as a possible upstream mediator of the changes in phosphorylation of GSK-3 (Fig. 4). The phosphorylation state of PKB was significantly reduced \(\approx 50\% \ (P < 0.001)\) on day 3 postinfection compared with sterile inflammatory rats. There were no significant differences between the two groups in the phosphorylation state of PKB on day 5 or day 10 postinfection.

Effect of modulation of cytokine response during sepsis on phosphorylation of eIF2B, GSK-3, and PKB. The mediators responsible for the changes in eIF2B and GSK-3 phosphorylation during sepsis remain unknown. Cytokines, which are polypeptides produced by cells of the immune system, have been implicated as potential mediators of the septic response, because...
they elicit various and often overlapping effects designed to protect the host in response to an inflammatory or bacterial insult. The natural induction of cytokines during inflammation is beneficial, but overproduction, as occurs in sepsis, is detrimental to the host (37). Three proinflammatory cytokines, namely, TNF, interleukin (IL)-1, and IL-6 may play a role in mediating the effects of sepsis on skeletal muscle protein metabolism. One approach to understanding the role for these cytokines in mediating the inhibition in skeletal muscle protein synthesis is to modify their release and/or biological action during a septic insult. In this regard, the biological activity of TNF is modulated in vivo by the proteolytic shedding of the extracellular domain of the p55 and p75 TNF receptors. An increase in soluble TNF receptors in the bloodstream neutralizes circulating TNF, thereby lowering the biologically active concentration of TNF in the plasma (10, 21, 28, 34, 38). TNFbp is a dimeric, polyethylene glycol-linked form of the human p55-soluble TNF receptor (8, 29, 34). This synthetic TNF antagonist, TNFbp, is more potent than the native soluble TNF receptor in its ability to block bioactivity of TNF (8, 29, 34). Blocking the bioavailability of the proinflammatory cytokine TNF by use of a specific TNFbp ameliorates the inhibition of skeletal muscle protein synthesis and translational efficiency during sepsis (4). If phosphorylation of eIF2Be is an important mechanism in controlling mRNA translation, then treatment of septic rats with TNFbp should result in a reduction in the phosphorylation of eIF2Be. Indeed, treating septic rats with TNFbp for 5 days significantly reduced ($P < 0.05$) the phosphorylation of eIF2Be compared with saline-treated septic rats (Fig. 5A). We next wished to determine whether TNFbp modulated GSK-3 phosphorylation in septic rats. Treating septic rats with TNFbp for 5 days significantly increased ($P > 0.05$) the phosphorylation of GSK-3 compared with saline-treated septic rats (Fig. 5B). TNFbp did not have any significant effect on the extent of phosphorylation of PKB in septic rats (Fig. 4C).

**Effect of sepsis on eIF2Be phosphorylation activity.** The increase in the phosphorylation state of eIF2Be during sepsis may also result from a diminished eIF2Be phosphatase activity. Therefore, we measured phosphatase activity toward eIF2Be by use of homogenates from

---

**Fig. 4.** Effect of sepsis on phosphorylation of PKB. To determine the relative phosphorylation state of PKB, equal amounts of protein from homogenates of gastrocnemius obtained 3, 5, and 10 days after induction of sterile or septic abscess were immunoblotted with an anti-PKB antibody specific for the phosphorylated form of PKB. The blots were then stripped of antibody and reprobed with an antibody that recognizes both phosphorylated and unphosphorylated PKB. The data are expressed as the amount of PKB in the phosphorylated form divided by the total of all phosphorylated and nonphosphorylated forms of PKB. Results represent means ± SE for 4–10 muscles in each group. *$P < 0.001$ vs. control.

---

**Fig. 5.** Effect of administration of tumor necrosis factor (TNF)-binding protein (TNFbp) on phosphorylation of eIF2Be, GSK-3, and PKB. The relative phosphorylation states of eIF2Be (A), GSK-3 (B), and PKB (C) in homogenates of gastrocnemius obtained 5 days after induction of a septic abscess from animals administered TNFbp (+TNFbp) or saline (−TNFbp) were determined as described in legends to Figs. 1–3. Results represent means ± SE for 6–8 muscles in each group. A: *$P < 0.05$ vs. −TNFbp. B: *$P < 0.05$ vs. +TNFbp.
gastrocnemius in vitro. No significant differences in eIF2B\textsubscript{e} phosphatase activity were observed between sterile inflammatory and septic rats either on day 3 (sterile 31 ± 11 vs. sepsis 30 ± 14 AU/mg protein) or on day 5 (sterile 33 ± 9 vs. sepsis 32 ± 9 AU/mg protein) postinfection. Because no differences were observed on day 3 and day 5 postinfection, we did not examine eIF2B\textsubscript{e} phosphatase activity on day 10 postinfection, when no differences in eIF2B\textsubscript{e} phosphorylation between sterile abscess and septic abscess rats were observed.

DISCUSSION

In the present study, we have investigated whether sepsis modulates the phosphorylation state of eIF2B\textsubscript{e} in skeletal muscle during any of three different phases of the septic response. The first phase (day 3) is characterized by recovery from abdominal surgery and initiation of a septic focus. During this phase, both sterile inflammation and sepsis are associated with elevations in white cell counts (39). Moreover, the gastrocnemius displays a marked protein wasting induced in part by an inhibition of protein synthesis that occurs in sepsis but not sterile inflammation (36). In the present study, phosphorylation of eIF2B\textsubscript{e} was noticeably enhanced in septic rats during this period. During the second phase (day 5), the weight of the abscess is greater in septic rats compared with sterile inflammatory rats (39). Likewise, septic animals manifest a twofold elevation in white cell counts, a low-grade fever, and a hyperdynamic cardiovascular state, indicating a differential host response to sepsis compared with sterile inflammatory abscess rats (25, 39, 50). During this phase, gastrocnemius from septic rats exhibits a striking muscle wasting characterized by a 50% inhibition in rates of protein synthesis (3, 4, 13–15, 43, 47, 48, 51, 53). As observed on day 3 postinfection, phosphorylation of eIF2B\textsubscript{e} is noticeably enhanced in septic rats compared with sterile inflammatory rats 5 days after the induction of sepsis. The third phase (day 10) is characterized by a waning of the hypermetabolic septic state. During this phase, there is recovery from the septic insult, as evidenced by a 40% drop in the size of the abscess compared with day 5 postsurgery, a 50% fall in the white blood cell count, and a restoration of rates of protein synthesis to values observed in nonoperated animals (14). In the present study, there were no significant differences in phosphorylation of eIF2B\textsubscript{e} between the two groups at this time. Taken together, these observations indicate that alterations in the phosphorylation state of eIF2B\textsubscript{e} appear to correlate with the changes of protein synthesis and translation efficiency after induction and recovery from the septic insult.

GSK-3 was originally identified as the enzyme responsible for phosphorylating glycogen synthase, leading to its inactivation and thereby reducing glycogen synthesis. More recently, it has become evident that GSK-3 can also phosphorylate a number of proteins involved in the regulation of the other metabolic processes, including eIF2B\textsubscript{e} (56, 57). GSK-3 itself is regulated by phosphorylation, where phosphorylation of GSK-3 leads to inactivation (for review see Refs. 35 and 58). In the present study, the extent of phosphorylation of GSK-3 was decreased in gastrocnemius after 3 and 5 days of abscess formation in septic rats compared with sterile inflammatory animals but returned to control values by 10 days of sepsis. Thus alterations in phosphorylation of GSK-3 during sepsis were inversely related to those of eIF2B\textsubscript{e}. Hence, sepsis may cause an enhanced phosphorylation of eIF2B\textsubscript{e} with an associated inhibition of eIF2B\textsubscript{e} activity through activation of GSK-3 kinase activity secondary to decreased phosphorylation of GSK-3.

In recent years, PKB has emerged as the most likely candidate to phosphorylate and inactivate GSK-3 (5). PKB, like GSK-3, undergoes reversible phosphorylation, with high levels of PKB activity associated with an increased phosphorylation of GSK-3. In the present studies, we provide evidence of a differential response in the phosphorylation of PKB over the course of the septic episode. Initially, the extent of phosphorylation of PKB is depressed, but beyond day 3 postinfection, the phosphorylation of PKB returns to values observed in sterile inflammatory rats. Hence, reductions in PKB phosphorylation may explain the decrease in GSK-3 phosphorylation on day 3 postinfection. However, PKB phosphorylation is not altered in septic rats on day 5 postinfection, a time when GSK-3 phosphorylation is depressed compared with sterile inflammatory rats.

The studies described herein provide strong evidence supporting the hypothesis that phosphorylation of eIF2B\textsubscript{e} by GSK-3 is intimately involved in the down-regulation of eIF2B\textsubscript{e} activity during initiation and progression of the septic process. Administration of TNFbp reduced phosphorylation of eIF2B\textsubscript{e} in gastrocnemius of septic rats. Thus the reduction in the phosphorylation of eIF2B\textsubscript{e} correlates with our previous observation that attenuating the TNF response to infection prevents the sepsis-induced inhibition in protein synthesis (1, 14, 42). In addition, administration of TNFbp to septic rats increased the phosphorylation state of GSK-3, suggesting that TNF may be modifying the phosphorylation state of GSK-3. Hence, the changes in phosphorylation of both eIF2B\textsubscript{e} and GSK-3 may be mediated by a TNF-dependent event initiated by the septic insult. Activation of GSK-3 during the initiation phase of the septic response correlates with changes in PKB activation by phosphorylation. The finding that the time course of changes in PKB phosphorylation differed from that observed for eIF2B\textsubscript{e} and GSK-3, coupled with the observation that administration of TNFbp reversed the sepsis-induced changes in eIF2B\textsubscript{e} and GSK-3 phosphorylation but not PKB phosphorylation, suggests that PKB may not play an important role in the regulation of GSK during the hypermetabolic phase of sepsis. Further studies will be required to delineate the mechanisms responsible for the regulation of GSK-3 activity during the progression of sepsis.
Although the correlation between extent of phosphorylation of eIF2B and protein synthesis in skeletal muscle of septic rats does not necessarily prove cause and effect, the relationship is consistent with the inhibition of translation initiation observed in skeletal muscle of septic rats and suggests a potential role of eIF2B phosphorylation in the regulation of protein synthesis during sepsis. Although we have used a specific anti-phospho-eIF2B antibody with which we can assess the relative level of phosphorylation of Ser535, this approach does not give absolute levels of phosphorylation of the protein. Despite this limitation, the data suggest that changes in phosphorylation of eIF2B may be an important regulatory mechanism controlling eIF2B activity during sepsis.

Anti-phospho-eIF2B antibody was generously provided by Biosource International. TNFbp was kindly provided by Dr. C. Edwards from Amgen (Boulder, CO).

This work was supported in part by National Institute of General Medical Sciences Grant GM-39277.

REFERENCES


