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TNFα Mediates Sepsis-Induced Impairment of Basal and Leucine-Stimulated Signaling via S6K1 and eIF4E in Cardiac Muscle

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Abstract Decreased translation initiation adversely impacts protein synthesis and contributes to the myocardial dysfunction produced by sepsis. Therefore, the purpose of the present study was to identify sepsis-induced changes in signal transduction pathways known to regulate translation initiation in cardiac muscle and to determine whether the stimulatory effects of leucine can reverse the observed defects. To address this aim, sepsis was produced by cecal ligation and puncture (CLP) in anesthetized rats and the animals studied in the fasted condition 24 h later. Separate groups of septic and time-matched control rats also received an oral gavage of leucine. To identify potential mechanisms responsible for regulating cap-dependent mRNA translation in cardiac muscle, several eukaryotic initiation factors (eIFs) were examined. Under basal conditions, hearts from septic rats demonstrated a redistribution of the rate-limiting factor eIF4E due to increased binding of the translational repressor 4E-BP1 with eIF4E. However, this change was independent of an alteration in the phosphorylation state of 4E-BP1. The phosphorylation of mTOR, S6K1, the ribosomal protein (rp) S6, and eIF4G was not altered in hearts from septic rats under basal conditions. In control rats, leucine failed to alter eIF4E distribution but increased the phosphorylation of S6K1 and S6. In contrast, in hearts from septic rats leucine acutely reversed the alterations in eIF4E distribution. However, the ability of leucine to increase S6K1 and rpS6 phosphorylation in septic hearts was blunted. Sepsis increased the content of tumor necrosis factor (TNF)-α in heart and pre-treatment of rats with a TNF antagonist prevented the above-mentioned sepsis-induced changes. These data indicate that oral administration of leucine acutely reverses sepsis-induced alterations eIF4E distribution observed under basal conditions but the anabolic actions of this amino acid on S6K1 and rpS6 phosphorylation remain blunted, providing evidence for a leucine resistance. Finally, TNFα, either directly or indirectly, appears to mediate the sepsis-induced defects in myocardial translation initiation. J. Cell. Biochem. 94: 419–431, 2005.

Key words: sepsis; heart; eIF4E; eIF4G; S6K1; S6

One hallmark of gram-negative bacterial infection is the accompanying cardiovascular complications, which include an early and profound defect in intrinsic myocardial performance [McDonough et al., 1985; Kumar et al., 2000]. The sepsis-induced myocardial dysfunction adversely affects morbidity and mortality in this patient population. Based on the available evidence, the etiology for the cardiac dysfunction appears to be multifactorial. For example, data from several studies have implicated increased circulating or tissue levels of tumor necrosis factor (TNF)-α and other cytokines, calcium dyshomeostasis, and altered adrenergic signaling, as central in the pathogenesis regarding this defect [Finkel et al., 1992; Omachi et al., 2002; Wu et al., 2003]. Despite the uncertainty of the underlying mechanism, the sepsis-induced decrease in cardiac contractility is associated with alterations in myocyte ultrastructure [Solomon et al., 1994] and a reduction in cardiac protein synthesis [Lang et al., 2000]. These results suggest that derangements in signal transduction pathways, important in the control of protein

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synthesis are ultimately responsible for at least part of the observed cardiomyopathy.

Cardiac protein synthesis is under tight regulatory control and can be influenced by altering either the number of ribosomes or the efficiency of translation [Russo and Morgan, 1989]. Data from previous studies indicate that the total ribosome number in heart was unchanged after sepsis [Lang et al., 2000], thereby indicating a decreased translational efficiency. The translation of mRNA into protein can be broadly subdivided into three steps: initiation, elongation, and termination. The decreased protein synthesis observed in skeletal muscle in response to various catabolic conditions, is strongly associated with defects in mRNA translation initiation [Cooney et al., 1997; Lang et al., 2000, 2002; Shah et al., 2000]. Currently it is unknown whether the sepsis-induced decrease in protein synthesis is mediated via the same pathways in both skeletal muscle and heart.

Translation initiation is facilitated and regulated by a number of soluble proteins referred to as eukaryotic initiation factors (eIFs). The stable ternary complex referred to as eIF4F, plays a pivotal role in the control of peptide-chain initiation by regulating the recruitment of the 43S pre-initiation complex to the mRNA [Pain, 1986]. The eIF4F complex is heterotrimeric (e.g., eIF-4E, -4G, and -4A) with each subunit having a discrete function. Of these subunits, eIF4E appears least abundant and is considered to be rate-limiting in the binding of mRNA to ribosomes [Duncan and Hershey, 1987]. eIF4E has high affinity for the m^7GTP cap structure present at the 5′-end of all nuclear transcribed mRNAs and is necessary for the formation of the eIF4E · mRNA complex. During translation initiation the eIF4E · mRNA complex interacts with eIF4G and eIF4A to form the active eIF4F holoenzyme, thereby allowing cap-dependent translation to proceed. In muscle, the interaction between eIF4E and the scaffold protein eIF4G is controlled in part by eIF-4E binding protein (BP)-1 that functions as a cap-dependent translational repressor [Karim et al., 2001]. This BP obstructs the interaction of eIF4G with eIF4E and thereby limits the assembly of the active eIF4F complex. The increased phosphorylation of 4E-BP1 releases it from eIF4E and consequently facilitates the binding of eIF4E with eIF4G. It is not known whether sepsis alters 4E-BP1 phosphorylation or impairs eIF4F complex formation in cardiac muscle.

Regulation of mRNA translation is also exerted by activation of the serine (Ser)/threonine (Thr) protein kinase S6K1 (aka p70 S6 kinase) [Avruch et al., 2001]. Multisite interdependent phosphorylation of S6K1 leads to full and complete activation of the kinase. This activation appears to play a role in the translational regulation of the mRNA family that encodes proteins containing a terminal oligopyrimidine tract downstream of their transcription initiation site [Dufner and Thomas, 1999]. Currently, there is little information pertaining to the effect of sepsis on the basal phosphorylation of either S6K1 or its physiological downstream substrate, ribosomal protein (rp) S6, in cardiac muscle.

Amino acids function as nutrient signals and influence many cellular processes including translation initiation and protein synthesis [Jefferson and Kimball, 2001; Lynch, 2001]. The overwhelming majority of these studies have been performed in either skeletal muscle or liver, and there is a paucity of data pertaining to cardiac tissue. Early studies demonstrated that leucine was unique among the naturally occurring amino acids in being able to stimulate myocardial protein synthesis and initiation, at least when studies were performed under in vitro conditions [Morgan et al., 1971a,b; Chua et al., 1979]. The anabolic effects of amino acids clearly activate cell signaling pathways that enhance translational efficiency predominantly via stimulating the rate of translation initiation, although the mechanism by which the cell senses a change in amino acid availability remains poorly defined [Li and Jefferson, 1978; Anthony et al., 1999; Jefferson and Kimball, 2001; Lynch, 2001; Xu et al., 2001].

Therefore, the purpose of the present study was three-fold: (a) determine defects in the signal transduction pathways that are consistent with the previously observed reduction in cardiac protein synthesis, (b) determine whether leucine retains its anabolic actions during sepsis and is able to stimulate the phosphorylation of S6K1 and/or 4E-BP1 in cardiac muscle, and (c) determine whether the overproduction of one of the inflammatory cytokines, TNF-α, is an important endogenous regulator of the sepsis-induced changes in signal transduction.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 200–225 g were purchased from Charles River Breeding Laboratories (Cambridge, MA). Rats were acclimated for 1 week in a light controlled room (12 h light:12 h dark cycle) under constant temperature. Water and standard rat chow were provided ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine and adhered to the National Institutes of Health (NIH) guidelines for the use of experimental animals.

Experimental Protocols

Rats were anesthetized with pentobarbital sodium (50–60 mg/kg), a midline laparotomy was performed, and sepsis was produced by cecal ligation and puncture (CLP). Briefly, the cecum was ligated at its base and punctured twice using a 20-G needle. The cecum was then returned to the peritoneal cavity and the muscle and skin layers were individually closed. Rats were resuscitated with 10 ml of 0.9% sterile saline administered subcutaneously. Time-matched non-septic control animals were subjected to a midline laparotomy with intestinal manipulation and were then resuscitated with the same volume of saline. During the operation, rats were placed on a warming pad to maintain body temperature. After surgery, food was withheld but the animals were permitted free access to water for the reminder of the study. Hence, any observed changes between septic and non-septic rats cannot be attributed to differences in food intake or nutritional status.

Approximately 24 h after induction of sepsis, animals in both the septic and non-septic (e.g., control) groups were administered either saline (0.155 mol/L) or 1.35 g/kg body weight (BW) leucine (prepared as 54.0 g/L of L-amino acid in distilled water) by oral gavage. This dose of leucine was selected because it is equivalent to that consumed in a 24-h period when rats of this age and strain are provided free access to food [Anthony et al., 1999]. Saline was administered to time-matched control rats, because previous studies indicated that isonitrogenous administration of other non-branched chain amino acids failed to stimulate either protein synthesis or peptide-chain initiation [Anthony et al., 1999].

A separate study was performed to determine the potential role of endogenously produced TNF-α as a mediator of the sepsis-induced changes in signal transduction. Approximately 4 h prior to CLP or sham surgery, rats were injected subcutaneously with TNF-binding protein (TNF_{BP}; 1 mg/kg, 1 ml/rat; Amgen, Inc., Boulder, CO), which antagonizes the actions of TNF. TNF_{BP} is a dimeric, polyethylene glycol-linked form of the human p55 soluble TNF receptor [Espat et al., 1995]. The timing and dose of the synthetic TNF receptor are based on previous work demonstrating its ability to prevent the sepsis-induced loss of skeletal muscle protein mass [Cooney et al., 1999].

Analysis of 4E-BP1 · eIF4E and eIF4G · eIF4E Complexes

The association of eIF4E with either 4E-BP1 or eIF4G was determined, as previously described [Lang et al., 2000, 2002]. Briefly, fresh cardiac tissue was homogenized and eIF4E as well as 4E-BP1 · eIF4E and eIF4G · 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 with BioMag goat anti-mouse IgG beads (Perseptive Biosystems, Framingham, MA). The beads were collected by centrifugation and the supernatants were subjected to electrophoresis either on a 7.5% polyacrylamide gel for analysis of eIF4G, or on a 15% gel to quantify 4E-BP1 and eIF4E. Proteins were then electrophoretically transferred to nitrocellulose. The membranes were incubated with a mouse anti-human eIF4E antibody, a rabbit anti-rat 4E-BP1 antibody, or a rabbit anti-eIF4G antibody (Bethyl Laboratories, Montgomery, TX). The blots were developed using ECL. The blots were exposed to X-ray film in a cassette equipped with a DuPont Lightning Plus intensifying screen. After development, the film was scanned (Microtek ScanMaker IV) and analyzed using National Institutes of Health Image 1.6 software.

Phosphorylation State of 4E-BP1, eIF4G, S6K1, S6, and mTOR

The phosphorylated forms of 4E-BP1 were measured following immunoprecipitation of 4E-BP1 from tissue homogenates, and the various phosphorylated forms of 4E-BP1 were separated by SDS–PAGE and analyzed by protein immunoblotting as described above.

Other Western blots were performed using either primary antibodies to total S6K1 (no. 230, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-specific S6K1 (Thr421/Ser424 or Thr389; Cell Signaling Technology, Beverly, MA), total 4E-BP1 (Bethyl Laboratories, Montgomery, TX), phospho-specific 4E-BP1 (Thr37; Cell Signaling), total and phosphorylated (Ser 2448) mTOR (Bethyl Laboratories), total and phosphorylated (Ser 1108) eIF4G (Cell Signaling), or total and phosphorylated-S6 (Ser235/Ser236; Cell Signaling) primary antibodies. Autoradiographs were scanned and quantified as described above.

Isolation of mRNA, Nuclease Protection Assay, and Cytokine Determination

Total RNA was extracted from heart in a mixture of phenol and guanidine thiocyanate (TRI Reagent, Molecular Research Center, Cincinnati, OH) using the manufacturer's protocol. RNA was separated from protein and DNA by the addition of bromocholoropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in formamide, RNA samples were quantified by spectrophotometry. Ten micrograms of RNA were used for each assay, except where noted. Riboprobes were synthesized from a multi-probe rat template set (rCK-1; Pharmingen, San Diego, CA) using an in vitro transcription kit (Pharmingen). The labeled riboprobe was hybridized with RNA overnight using a ribonuclease protection assay (RPA) and the manufacturer's protocol (Pharmingen). Protected RNAs were separated using a 5% acrylamide gel (19:1 acrylamide/bisacrylamide). Gels were transferred to blotting paper and dried under vacuum on a gel dryer. Dried gels were exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) and the resulting data were quantified using ImageQuant software and normalized to the rat rp L32 mRNA signal in each lane, as previously described [Lang et al., 2003b].

The concentration of TNFα protein in cardiac muscle was assessed as previously described [Lang et al., 2003c]. Briefly, powdered frozen tissue was homogenized in a buffer containing a protease-inhibitor cocktail including 1 mM phenylmethylsulfonylfluoride, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 1 μg/ml leupeptin in phosphate-buffered saline solution, pH 7.2, containing 0.05% sodium azide and 0.5% Triton X-100. Samples were homogenized and subjected to two liquid nitrogen freeze-thaw cycles, sonication, and finally incubated at 4°C for 1 h. The final homogenate was centrifuged at 40,000*g* (Beckman, J2-21) and the supernatant used to determine TNFα using rat-specific enzyme-linked immunoadsorbant assays (Biosource International, Camarillo, CA).

Plasma Determinations

Immediately prior to the excision of the heart, a blood sample was collected from the abdominal aorta into heparinized syringes. Blood was centrifuged and the plasma insulin concentration was measured using a commercial radioimmunoassay (RIA) for rat insulin (Linco Research, St. Charles, MO). The plasma leucine concentration was determined by derivatizing with phenylisothiocyanate, followed by high-performance liquid chromatography analysis [Lang et al., 2002].

Statistics

Data were obtained from three separate experimental series each containing all four experimental groups. For each study rats were randomly assigned to either the experimental or control group. Experimental values are presented as means ± SE. The number of rats in each group is indicated in the figure and table legends. Statistical evaluation of the data was performed using ANOVA followed by the Student–Neuman–Keuls' test to determine treatment effect (Instat, San Diego, CA). Differences between the groups were considered significant at $P < 0.05$.

RESULTS

The mechanistic interactions between sepsis and leucine were investigated under in vivo conditions by analyzing known regulatory steps in the control of translation initiation. To this end, the extent of S6K1 phosphorylation was assessed. When S6K1 is subjected to SDS–PAGE, it resolves into multiple bands with different electrophoretic mobilities dependent on the extent of phosphorylation at various Ser/Thr sites. In this regard, the most slowly migrating forms represent the most heavily phosphorylated and, thus, highly active form of the kinase. There was a low constitutive level S6K1 phosphorylation in cardiac muscle from rats in the non-septic control group (Fig. 1A). Sepsis did not appear to significantly alter the mobility of the bands under basal (e.g., no leucine

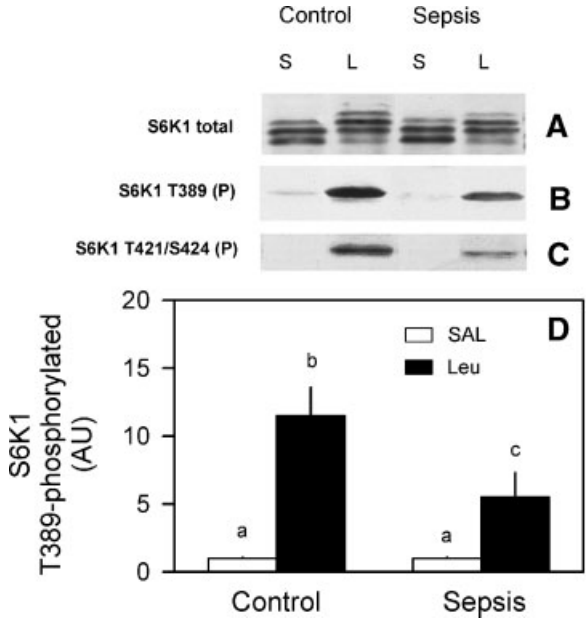

Fig. 1. Sepsis- and leucine-induced changes on the phosphorylation of S6K1 in cardiac muscle. **Panel A**, a representative immunoblot for total S6K1; **panel B**, a representative immunoblot for the phosphorylation (P) of the Thr389 site of S6K1; **panel C**, a representative immunoblot for the phosphorylation of the T421/S424 site of S6K1; and **panel D**, bar graph represents the densitometric analysis of S6K1 phosphorylation of T389 where the value from the control (non-septic) + saline group was set at 1.0 AU (arbitrary units). Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 11–12 per group. Means with different letters are statistically different from each other ($P < 0.05$).

stimulation) conditions. In control animals, oral leucine increased phosphorylation of the kinase as evidenced by the decreased mobility of the electrophoretic bands. A diminution of the leucine-induced S6K1 phosphorylation response was observed in hearts from septic rats administered leucine (Fig. 1A).

S6K1 is activated by undergoing multiple Ser/Thr phosphorylation events. Different phosphorylation sites in S6K1 were examined using phospho-specific antibodies. In heart from saline-treated control rats, there was essentially no basal phosphorylation of either Thr389 or Thr421/Ser424 (Fig. 1B–D). Phosphorylation of S6K1 in heart from control rats administered leucine was markedly increased. Leucine also stimulated S6K1 phosphorylation in hearts from septic rats but the rise was attenuated by approximately 50% (Fig. 1B–D).

The phosphorylation status of the rp S6, a physiologically relevant S6K1 substrate, was also determined using a phosphospecific antibody directed against the first and second phosphorylation sites, Ser236 and Ser235 [Martin-Perez and Thomas, 1983]. Although sepsis tended to decrease the phosphorylation of rpS6 in hearts from septic rats under basal conditions, compared with control values, this difference failed to achieve statistical significance (Fig. 2). However, as seen with S6K1, the ability of leucine to increased rpS6 phosphorylation in hearts from septic rats was markedly blunted. The amount of total rpS6 was not altered by either sepsis or leucine (Fig. 2B).

Another potential site for control of peptide-chain initiation involves the regulation of eIF4E availability [Pain, 1986]. Hence, the ability of sepsis to modify the distribution of eIF4E in heart was subsequently determined. Figure 3, panels A and C, illustrates that the α- and β-isoforms of 4E-BP1 were detected in eIF4E immunoprecipiates of heart and the content of the inactive eIF4E · 4E-BP1 complex was increased approximately 60% in hearts from septic rats under basal conditions. Although oral administration of leucine did not significantly alter the binding of eIF4E to 4E-BP1 in hearts from control rats, it was capable of lowering the amount of the eIF4E · 4E-BP1 complex to

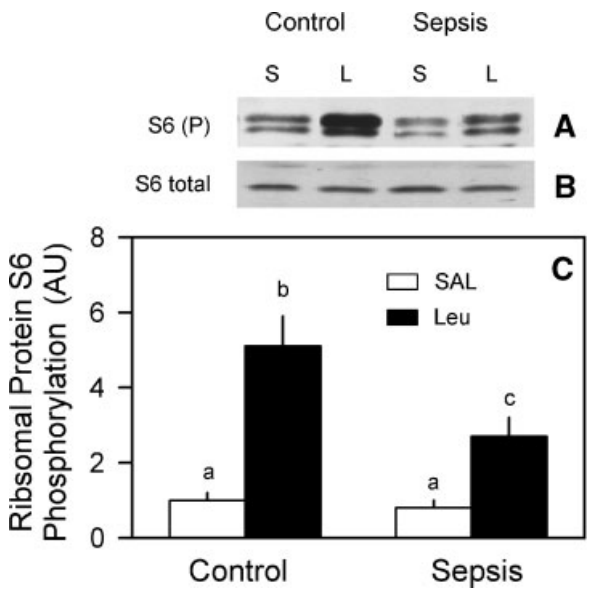

Fig. 2. Sepsis- and leucine-induced changes in the phosphorylation of ribosomal protein (rp) S6. **Panels A and B**: A representative immunoblot of phosphorylated (P) and total rpS6, respectively; and **panel C**, bar graph represents densitometric analysis of rpS6 phosphorylation where the value from the control + saline group was set at 1.0 AU. Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 11–12 per group. Means with different letters are statistically different from each other ($P < 0.05$).

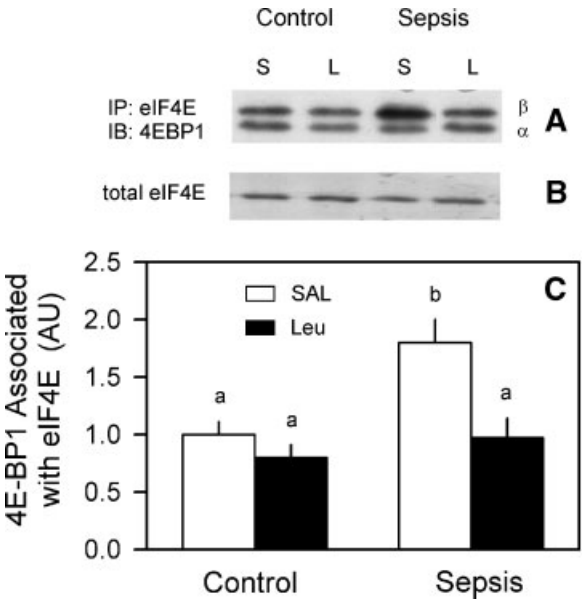

Fig. 3. Sepsis- and leucine-induced changes in the binding of 4E-BP1 to eIF4E in cardiac muscle. **Panel A**, eIF4E was immunoprecipitated (IP) and the amount of 4E-BP1 bound to eIF4E assessed by immunoblotting (IB), and the positions of the α- and β-isoforms are so indicated; **panel B**, representative Western blot of total eIF4E demonstrating no differences among groups; and **panel C**, bar graph represents densitometric analysis of immunoblots of 4E-BP1 associated with eIF4E, where the value from the control + saline group was set at 1.0 AU. Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 11–12 per group. Means with different letters are statistically different from each other ($P < 0.05$).

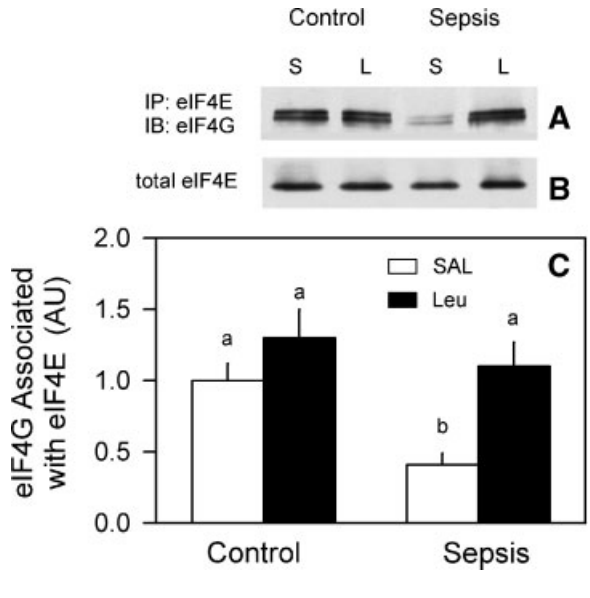

Fig. 4. Sepsis- and leucine-induced changes in the association of eIF4G with eIF4E in cardiac muscle. **Panel A**, eIF4E was immunoprecipitated (IP) and the amount of eIF4G bound to eIF4E assessed by immunoblotting (IB); **panel B**, representative Western blot of total eIF4E; **panel C**, bar graph represents densitometric analysis of immunoblots of eIF4G associated with eIF4E, where the value from control rats treated with saline was set at 1.0 AU. Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 11–12 per group. Means with different letters are statistically different from each other ($P < 0.05$).

basal levels in hearts from septic rats. Neither sepsis nor leucine significantly altered the total amount of eIF4E in heart (Fig. 3B).

When eIF4E is bound to 4E-BP1, it is unable to interact with eIF4G to form the active eIF4E · eIF4G complex. Figure 4 illustrates that under basal conditions the amount of the eIF4E · eIF4G complex was reciprocally reduced by approximately 60% in response to the septic insult, compared with values from time-matched non-septic control animals. Leucine had no detectable effect on eIF4E association with eIF4G in hearts from control rats. However, leucine acutely increased the binding of eIF4E to eIF4G in hearts from septic rats so the resulting value was not different from either of the two control groups.

Many catabolic conditions lead to the redistribution of eIF4E between the active and inactive eIF4F complex via the actions of the translational repressor molecule 4E-BP1 [Lang et al., 2000, 2002, 2003a; Shah et al., 2000]. One-dimensional SDS–PAGE allows the various phosphorylated forms of 4E-BP1 to be resolved into three bands (Fig. 5A). Hyper-phosphorylation of 4E-BP1 decreases the association of the BP with eIF4E and generally increases translation [Karim et al., 2001]. Figure 5, panels A and C, illustrates that the amount of 4E-BP1 in the hyper-phosphorylated γ-form was not statistically altered by either sepsis or leucine. Similar results were obtained when a phospho-specific antibody was used to detect sepsis- and leucine-induced changes in the phosphorylation of Thr37/Thr46 in 4E-BP1 (Fig. 5B).

The phosphorylation of eIF4G has been associated with an increased interaction between eIF4E and eIF4G reflecting increased eIF4F complex formation [Morley et al., 1997]. In control rats there was constitutive phosphorylation of eIF4G in cardiac muscle and the extent of this phosphorylation event was not altered by sepsis under basal conditions (Fig. 6). Leucine administration increased eIF4G phosphorylation in hearts from both control and septic rats, but the increment was approximately 50% of that detected in the control group. Somewhat unexpectedly, sepsis almost doubled

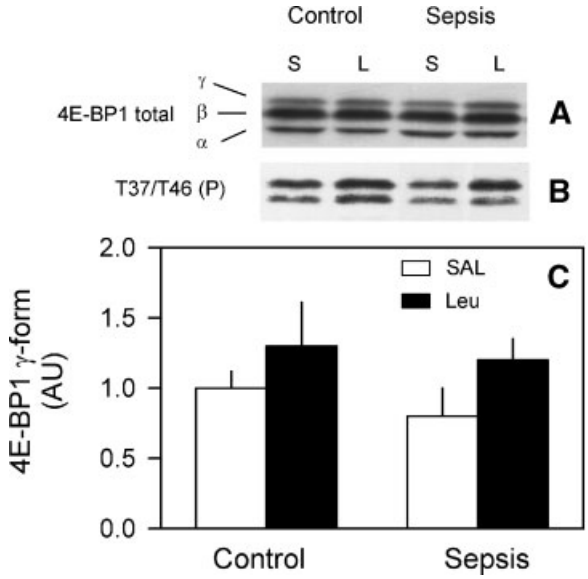

Fig. 5. Sepsis- and leucine-induced changes in the phosphorylation of 4E-BP1 in cardiac muscle. **Panel A**, inset is a representative immunoblot for total 4E-BP1 with the positions of the α-, β-, and γ-isoforms indicated; **panel B**, representative immunoblot of phosphorylated (P) 4E-BP1 using a phospho-specific antibody to T37/T46; **panel C**, bar graph represents densitometric analysis of the hyperphosphorylated γ-isoform where the value from control + saline group was set at 1.0 AU. Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 11–12 per group. Means with different letters are statistically different from each other ($P < 0.05$).

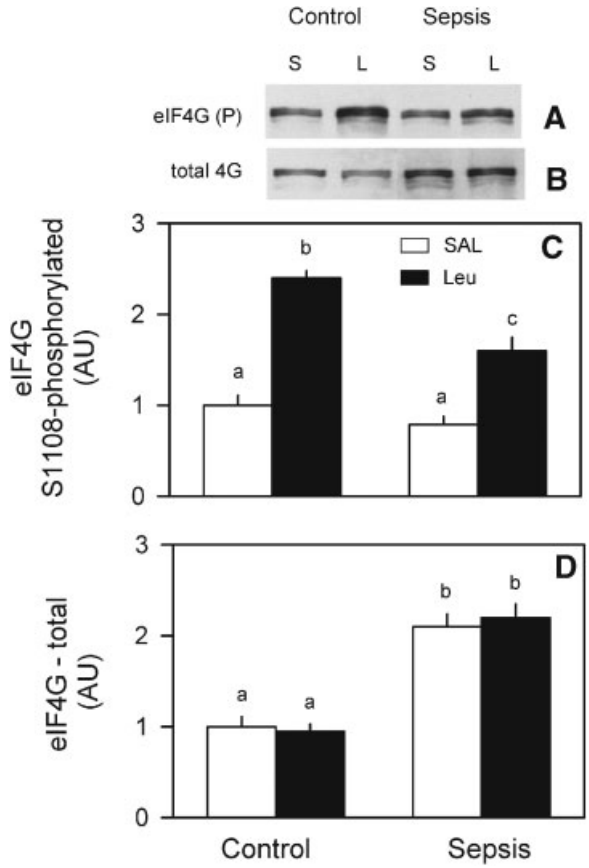

Fig. 6. Sepsis- and leucine-induced changes on the amount of phosphorylated and total eIF4G in cardiac muscle. Autoradiographs are representative immunoblots of phosphorylated (P) eIF4G using an antibody specific for residue Ser1108 (**panel A**) and total (**panel B**) eIF4G. Bar graphs (**panels C and D**) are densitometric analysis of all of the autoradiographs for phosphorylated and total eIF4G, respectively, where the value from the control + saline group was set at 1.0 AU. Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 11–12 per group. Means with different letters are statistically different from each other ($P < 0.05$).

the total amount of eIF4G in cardiac muscle (Fig. 6B,D).

The proline-directed Ser/Thr protein kinase referred to as mammalian target of rapamycin (mTOR) is believed to be a common upstream mediator responsible at least in part for the phosphorylation of two translational components, 4E-BP1 and S6K1 [Gingras et al., 2001]. Although sepsis did not consistently alter the cellular content of either total or phosphorylated mTOR protein (Fig. 7), leucine modestly but consistently increased mTOR S2448-phosphorylation by 50%–60% in hearts from both control and septic rats.

Under basal conditions, there was no difference in the plasma concentration of insulin between control and septic rats (Table I). Although leucine administration increased the plasma insulin concentration, the degree of hyperinsulinemia was comparable in both groups. Sepsis alone did not alter the plasma leucine concentration. Oral leucine increased the plasma leucine concentration to a similar magnitude in both control and septic rats.

Inflammatory cytokines impair protein synthesis and translation initiation in striated muscle [Frost et al., 1997; Lang et al., 2002]. Therefore, the steady-state mRNA content of various cytokines was determined in hearts from control and septic rats. Figure 8A illustrates that 24 h after induction of sepsis, the myocardial mRNA content of TNF-α, interleukin (IL)-6, and IL-1β was consistently increased. Subsequently, it was determined that septic rats also had an elevated concentration of TNFα protein in heart (Fig. 8B) but not plasma (data not shown) 24 h after induction of sepsis. Because of the acute nature of the anabolic stimulus, cytokines were not assessed in tissues from leucine-treated rats.

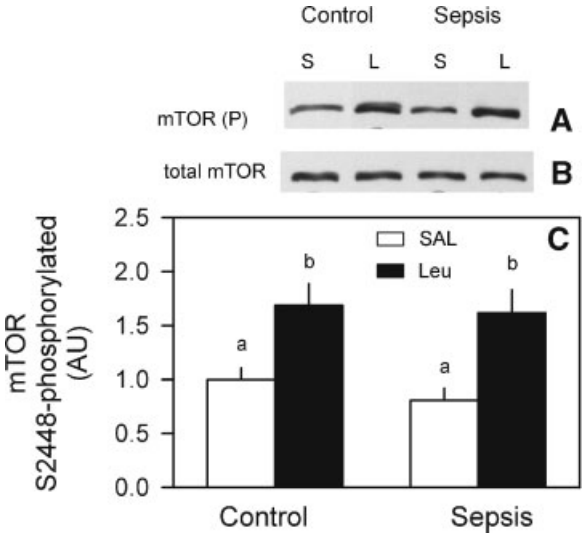

Fig. 7. Sepsis- and leucine-induced changes of mTOR (mammalian target of rapamycin) in cardiac muscle. **Panels A and B**, a representative immunoblot of Ser2448-phosphorylated (P) and total mTOR, respectively; **panel C**, a bar graph represents densitometric analysis of mTOR phosphorylation where the value from the control + saline group was set at 1.0 AU. Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 11–12 per group. Means with different letters are statistically different from each other ($P < 0.05$).

A final study was performed to elucidate the role of the endogenous over expression of TNFα as a potential early-phase mediator for the sepsis-induced changes in the previously described signal transduction pathways. In this study, septic rats were pretreated with TNF_{BP} prior to induction of peritonitis, and their response compared to either untreated septic rats or control animals administered TNF_{BP}. Preliminary studies indicated that there was no difference in any of the measured parameters in hearts from control rats either in the presence or absence of TNF_{BP} and, therefore, the later group was not included in the current investigation. As illustrated in Figure 9, TNF_{BP} prevented the sepsis-induced decrease in S6K1 and rpS6 phosphorylation in rats administered leucine. Hence, values in the TNF_{BP} + sepsis+ leucine group were not different from those in the TNF_{BP} + control + leucine group. In addition, neutralization of TNFα also prevented the sepsis-induced increase in eIF4E · 4E-BP1 and decrease in eIF4E · eIF4G under basal conditions (Fig. 10).

DISCUSSION

The results of the present study indicate that the previously reported sepsis-induced decrease in cardiac protein synthesis [Lang et al., 2000] was not associated with alterations in the phosphorylation and activation of S6K1 under basal conditions. In contrast, sepsis did alter the availability of eIF4E by increasing the amount of this initiation factor in the inactive eIF4E · 4E-BP1 complex and decreasing the amount in the active eIF4E · eIF4G complex. A similar response has been reported in skeletal muscle from septic rats [Lang and Frost, 2004a] as well as in striated muscle from endotoxin- and TNF-treated rats, all of which demonstrate a decreased rate of protein synthesis [Lang et al., 2000, 2002]. However, in contrast to these previous results, the redistribution of eIF4E in heart was not associated with a concomitant reduction in the phosphorylation of the translational repressor molecule 4E-BP1. Moreover, the sepsis-induced decrease in eIF4E · eIF4G occurred despite an increase in the total amount of eIF4G. Such an increase in total eIF4G has not been previously reported and the mechanism and significance of this change are not known. These data appear to be in contrast to those in which increased eIF4G is associated with a loss of growth control [Brass et al., 1997].

A mixture of amino acids or leucine alone is sufficient to increase protein synthesis in striated muscle [Li and Jefferson, 1978; Jurasinski et al., 1995; Anthony et al., 1999; Lang et al.,

TABLE I. Effect of Sepsis on Plasma Concentrations of Insulin and Leucine

	Control + Sal	Control + Leu	Sepsis + Sal	Sepsis + Leu
Insulin, pmol/L	102 ± 18^a	331 ± 45^b	119 ± 18^a	347 ± 36^b
Leucine, μmol/L	164 ± 21^a	$1{,}555 \pm 244^b$	186 ± 33^a	$1{,}687 \pm 205^b$

Values are means ± SEM; n = 11–12 rats per group. Leu, leucine; Sal, saline. Means with different letters for a specific parameter are statistically different from each other ($P < 0.05$). Rats were subjected to cecal ligation puncture to induce sepsis and studied approximately 24 h thereafter; non-septic control rats were time-matched and pair-fed. Arterial blood was collected 20 min after oral administration of either leucine or an equal volume of saline. Means with different letters are statistically different from each other ($P < 0.05$).

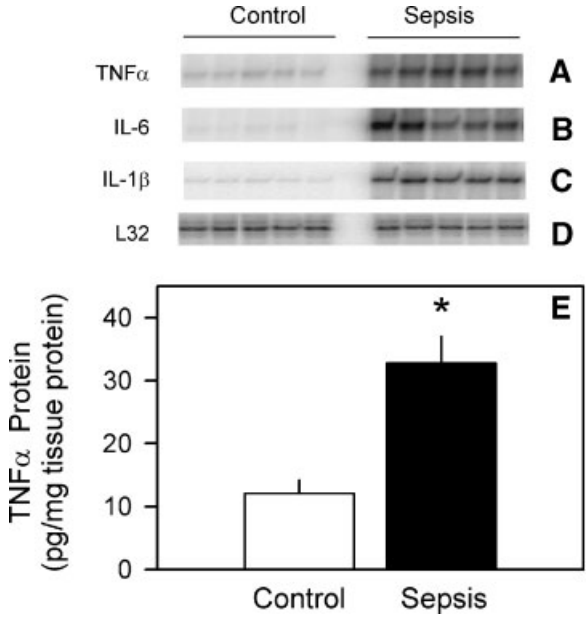

Fig. 8. Cardiac mRNA and protein content for TNFα and other inflammatory cytokines. **Panels A**, **B**, **C**, **and D**, are representative ribonuclease protection assays for TNFα, IL-6 and IL-1β, and L32, respectively. The blank area dividing the five control and five septic samples is an empty lane. **Panel E**, is a bar graph for the TNFα protein content in hearts from non-septic control and septic rats. Hearts excised 24 h after induction of sepsis by CLP. Values are means ± SEM; n = 5 per group. Means with different letters are statistically different from each other ($P < 0.05$).

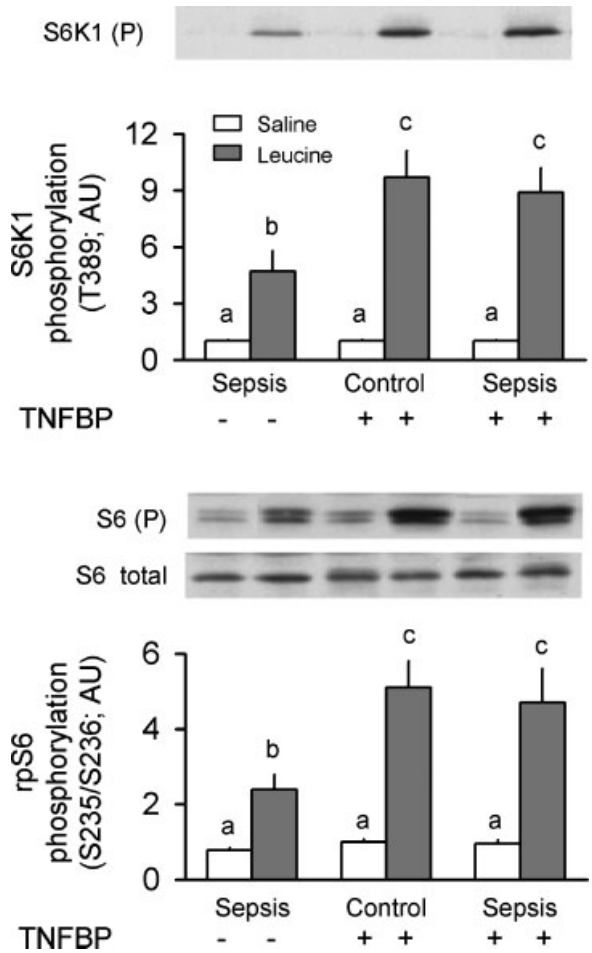

Fig. 9. TNFα neutralization prevents sepsis-induced reduction in S6K1 and rpS6 phosphorylation in response to leucine. **Top panel**, autoradiograph is a representative immunoblot of Thr389-phosphorylated (P) S6K1 with the densitometric analysis of all immunoblots quantified in first bar graph. **Bottom panel**, autoradiograph is a representative immunoblot of Ser235/Ser236-phosphorylated (P) or total rpS6, with the densitometric analysis of all immunoblots for P-rpS6 quantified in the bar graph. There was no difference in the total S6 protein among various groups. The value from the TNFBP + control + saline group was set at 1.0 AU. Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 7–8 per group. Means with different letters are statistically different from each other ($P < 0.05$).

2003a]. Moreover, this anabolic effect appears to be a direct effect of leucine and is not mediated via the secondary rise in insulin [Kimball et al., 1999]. Leucine and other branched chain amino acids stimulate protein synthesis in the isolated working heart and this response is mediated by an increased peptide-chain initiation as evidenced by increased ribosomal aggregation [Morgan et al., 1971a,b; Chua et al., 1979]. The present study extends these original observations by identifying the specific signal transduction pathways important in stimulating translation initiation in hearts under in vivo conditions. Specifically, leucine stimulated the phosphorylation S6K1 and rpS6. The phosphorylation and activation of S6K1 is believed to be an important element of the protein synthetic signaling pathway because it phosphorylates a number of proteins including the small ribosomal subunit protein S6 [Dufner and Thomas, 1999]. The phosphorylation of S6K1 and S6 are tightly associated with accelerated rates of mRNA translation initiation and a stimulation of skeletal muscle protein synthesis under in vivo conditions where other components of the translational apparatus have not been overexpressed or altered as a compensatory response to their deletion [Anthony et al., 1999; Shah et al., 2000; Kumar et al., 2002; Lang et al., 2003a]. The ability of leucine to stimulate S6K1 phosphorylation under in vivo conditions in heart has not been previously reported. However, myocardial S6K1 phosphorylation is enhanced by the growth factors insulin-like growth factor (IGF)-I and insulin [Lang et al., 2003b]. The activation of S6K1 and the phos-

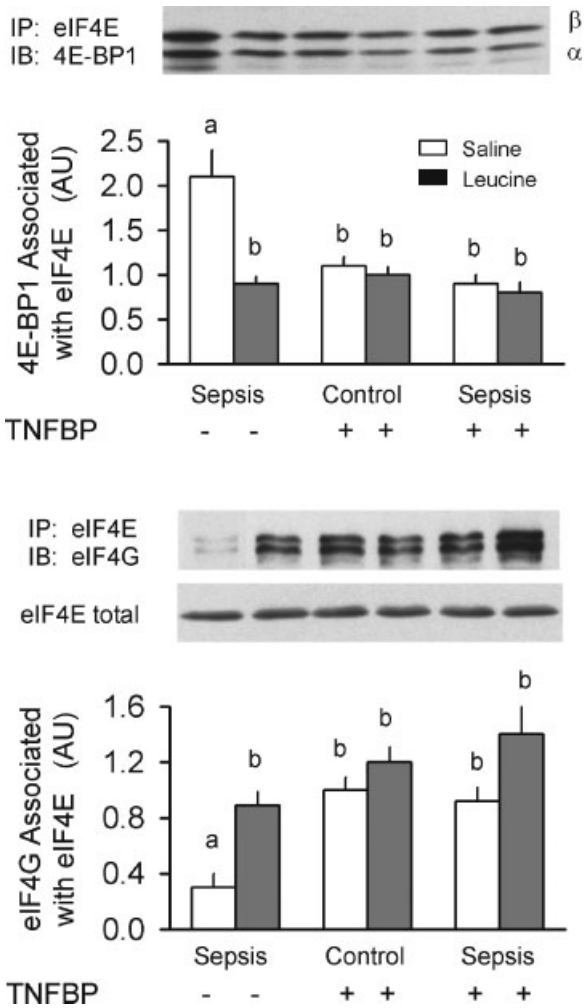

Fig. 10. TNFα neutralization prevents sepsis-induced changes in eIF4E availability under basal conditions in cardiac muscle. **Top panel**, eIF4E was immunoprecipitated (IP) and the amount of 4E-BP1 bound to eIF4E assessed by immunoblotting (IB), where the positions of the α- and β-isoforms are so indicated. The first bar graph represents densitometric analysis of immunoblots of 4E-BP1 associated with eIF4E. **Bottom panel**, eIF4E was IP and the amount of eIF4G bound to eIF4E assessed by IB; a representative Western blot of total eIF4E demonstrating no differences among groups is also included. The second bar graph represents densitometric analysis of immunoblots of eIF4G associated with eIF4E. The value from the TNFBP + control + saline group was set at 1.0 AU. Saline, Sal or S; Leucine, Leu or L. Values are means ± SEM; n = 7–8 per group. Means with different letters are statistically different from each other ($P < 0.05$).

phorylation of rpS6 was originally believed to be the principle mechanism through which the translation of a subset of mRNAs containing 5′-terminal tracts of oligopyrimidines (e.g., 5′-TOP mRNAs) was enhanced [Terada et al., 1994]. However, more recent studies indicate that redundancy may exist between S6K1, S6K2, and a mitogen-activated protein kinase-dependent kinase in the mitogen stimulated phosphorylation of rpS6 at least under conditions in which S6K1 and/or S6K2 have been conditionally deleted [Pende et al., 2004]. Furthermore, there are reports suggesting that rpS6 phosphorylation alone may not be sufficient for translational activation of TOP mRNAs [Stolovich et al., 2002]. Therefore, the physiological significance of these changes under in vivo conditions remains to be determined.

In contrast to S6K1/S6, there was no detectable change in 4E-BP1 phosphorylation or the distribution of eIF4E in hearts from control rats in response to leucine. The lack of leucine stimulation on the functional eIF4F complex is in marked contrast to the response observed in skeletal muscle [Anthony et al., 1999; Kimball et al., 1999; Lang et al., 2003a]. Moreover, this lack of response is in contrast to the ability of insulin to stimulate 4E-BP1 phosphorylation in heart under both in vivo and in vitro conditions [Tuxworth et al., 1999; Lang et al., 2003b]. The reason for this tissue-specific response to leucine is currently unknown. The differential response in hearts from control and septic rats mentioned above, however, cannot be attributed to differences in the prevailing plasma concentration of leucine between the two groups. Furthermore, although insulin can increase cardiac protein synthesis and translation initiation [Morgan et al., 1971b; Kimball et al., 1997], the circulating concentrations of this particular anabolic hormone also did not differ between control and septic rats administered leucine.

It is noteworthy that data from the current study clearly indicates that leucine acutely increases the formation of the functional eIF4F complex in hearts from septic rats. This response is evidenced by the ability of leucine to redirect eIF4E from the inactive 4E-BP1 · eIF4E complex into the active eIF4E · eIF4G complex. In this regard, although protein synthesis was not directly determined in the present study, the ability of the eIF4E · eIF4G complex to bind to the mRNA cap has been reported to be a rate-limiting determinant of total protein synthesis in cardiomyocytes [Saghir et al., 2001]. Furthermore, such an anabolic response has previously been observed in skeletal muscle of septic rats [Lang et al., 2000]. However, the current results in heart differ from those in skeletal muscle in that they appear to be independent of a change in the 4E-BP1 phosphorylation.

Leucine also increased the phosphorylation of S6K1 and rpS6 in hearts from septic rats. However, this response was attenuated by approximately 50%, compared with the robust response observed in control hearts. Because the prevailing leucine concentration is maximally stimulating for the parameters assessed (Lang, unpublished data), these data imply the presence of a myocardial leucine resistance. According to the prevailing model of activation for S6K1, the sites in the autoinhibitory domain (Ser411, Ser418, Thr421, and Ser424) are phosphorylated by an upstream kinase [Shah et al., 2000]. These phosphorylation events disrupt the interaction between the carboxy-terminal and amino-terminal domains, thereby permitting S6K1 to unfurl and expose additional sites in the linker and kinase domains. Subsequently, the Thr389 residue in the linker domain is phosphorylated and this step is necessary for the full and functional activation of S6K1 [Weng et al., 1998]. Our results indicate that sepsis impairs the ability of leucine to stimulate phosphorylation sites in both the autoregulatory and linker domain in cardiac muscle. The sepsis-induced impairment in leucine-stimulated S6K1 phosphorylation cannot be attributed to diminished kinase activity of mTOR, at least as indirectly assessed by the phosphorylation state of mTOR.

The ability of leucine to stimulate Ser1108-phosphorylation of eIF4G in cardiac muscle from septic rats is potentially noteworthy. Phosphorylation of Ser residues at this site on the protein is associated with enhanced recruitment of the translational machinery to the 5′-end of mRNA [Morley et al., 1997]. This intermolecular interaction might be expected to enhance rates of cap-dependent translation and may represent a secondary mechanism regulating the interaction of eIF4E with eIF4G. Although eIF4G phosphorylation is thought to be rapamycin sensitive (e.g., mTOR-dependent), the kinase action of mTOR on Ser1108-phosphorylation of eIF4G appears to be indirect [Raught et al., 2000]. In the current study, leucine stimulation of eIF4G and mTOR appear coordinately regulated. It remains possible that the ability of leucine to stimulate eIF4G phosphorylation in hearts from septic rats may be at least in part responsible for the increase in the binding of eIF4E with eIF4G in a 4E-BP1-independent manner.

The above-mentioned impairment in mRNA translation, and presumably decreased rate of protein synthesis, occurs at the same time as a marked dysfunction in mechanical function [McDonough et al., 1985]. The etiology of the contractile defect has been extensively investigated and a large number of putative mediators implicated. Previous studies have shown that elevated concentrations of TNFα decrease cardiac protein synthesis and peptide-chain initiation [Lang et al., 2002], produce defects in mechanical function [Mann, 1996], and portends to the maladaptive effects of this multifunctional cytokine in the heart. Data from the current study indicate that sepsis increases the mRNA content for TNFα and several other inflammatory cytokines. Furthermore, at least for TNFα, it was possible to detect an increase in cytokine protein content in myocardium of septic rats. A variety of inflammatory insults and pathological stresses have also been shown to increase cytokine mRNA or protein content in cardiac or skeletal muscle [Torre-Amione et al., 1996; Kapadia et al., 1997; Frost et al., 2002; Lang et al., 2003b, 2004]. Collectively, these data suggest that over production of TNFα, either directly within heart or as a secreted protein from other tissues, mediates the sepsis-induced impairment in protein synthetic signaling pathways. This suggestion was confirmed by the results from the study in which septic rats were pretreated with the TNF antagonist. In these rats, TNF_{BP} prevented the sepsis-induced redistribution of eIF4E under basal conditions and the diminished phosphorylation response of S6K1 and rpS6 after leucine stimulation. Collectively, these data emphasize the importance of TNFα in the sepsis-induced defects in signal transduction. These conclusions are consistent with the role of endogenous TNFα as a mediator of the cachectic response in skeletal muscle [Cooney et al., 1999].

In summary, the results of the present investigation provide evidence that sepsis inhibits mTOR signaling pathways in cardiac muscle under in vivo conditions, and that this defect is evidenced by a redistribution of eIF4E from the active eIF4E · eIF4G complex to the inactive eIF4E · 4E-BP1 complex in the basal condition that is independent of 4E-BP1 phosphorylation. However, hearts from septic rats also appear to have a diminished responsiveness toward the anabolic actions of leucine as evidenced by the smaller increase in S6K1 and

rpS6 phosphorylation. These sepsis-induced defects appear mediated, either directly or indirectly, by the endogenous over production of TNFα.

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