Insulin stimulates muscle protein synthesis in neonates during endotoxemia despite repression of translation initiation

Renan A. Orellana,1 Scot R. Kimball,2 Agus Suryawan,1 Jeffery Escobar,1
Hanh V. Nguyen,1 Leonard S. Jefferson,2 and Teresa A. Davis1

1United States Department of Agriculture/Agricultural Research Service Children’s Nutrition Research Center and Critical Care Section, Department of Pediatrics, Baylor College of Medicine, Houston, Texas; and 2Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania

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Orellana RA, Kimball SR, Suryawan A, Escobar J, Nguyen HV, Jefferson LS, Davis TA. Insulin stimulates muscle protein synthesis in neonates during endotoxemia despite repression of translation initiation. Am J Physiol Endocrinol Metab 292: E629–E636, 2007. First published October 17, 2006; doi:10.1152/ajpendo.00214.2006.—Skeletal muscle protein synthesis is reduced in neonatal pigs in response to endotoxemia. To examine the role of insulin in this response, neonatal pigs were infused with endotoxin (LPS, 0 and 10 μg·kg−1·h−1), whereas glucose and amino acids were maintained at fasting levels and insulin was clamped at fasting or fed (2 or 10 μU/ml) levels. Fractional rates of protein synthesis and translational control mechanisms were examined in longissimus dorsi muscle and liver. In the presence of fasting insulin, LPS reduced muscle protein synthesis (−29%), and increasing insulin to fed levels accelerated muscle protein synthesis in both groups (controls, +44%; LPS, +64%). LPS, but not insulin, increased liver protein synthesis by +28%. In muscle of fasting neonatal pigs, LPS reduced 4E-BP1 phosphorylation and eIF4E to eIF4F binding. In muscle of controls, but not LPS pigs, raising insulin to fed levels increased 4E-BP1 and S6K1 phosphorylation and eIF4E to eIF4G binding. In muscle and liver, neither LPS nor insulin altered eIF2B activity, eIF2 phosphorylation decreased in response to insulin in both LPS and control animals. The results suggest that, in endotoxemic neonatal animals, the response of protein synthesis to insulin is maintained despite suppression of mTOR-dependent translation initiation and eIF4E availability for eIF4F assembly. Maintenance of an anabolic response to the feeding-induced rise in insulin likely exerts a protective effect for the neonate to the catabolic processes induced by sepsis.

eukaryotic elongation factor 2; sepsis; mammalian target of rapamycin; eukaryotic initiation factors 4G and 4E

SEPSIS, A SYSTEMIC INFLAMMATORY RESPONSE to infection, is known to promote insulin resistance for protein metabolism and elicit a profound and immediate reduction in muscle protein synthesis in adult mammals (13, 44). In adult rats, the prominent reduction in muscle protein synthesis by endotoxin (LPS) is associated with a marked depression in the translation initiation process, an effect that can be reproduced in diverse animal models of sepsis (20, 22, 45). LPS is known to consistently reproduce some of the immune and metabolic effects seen during sepsis. LPS administration has been used as a surrogate to elicit a systemic inflammatory response in animals that triggers the release of inflammatory mediators (32, 46). Some mediators of the systemic inflammatory response, such as TNF-α, have been implicated in the depression of translation signaling (21, 41).

Our studies using the healthy neonatal pig as an animal model (9, 47) have demonstrated that neonates maintain elevated rates of protein synthesis in skeletal muscle as a consequence of a high sensitivity and responsiveness to stimulation of protein synthesis by insulin and amino acids. The higher sensitivity of muscle protein synthesis to insulin arises as a result of acceleration in the rate of translation of mRNA through activation of eukaryotic translation initiation factors (16, 19). An early event in the translation initiation pathway is the association of a ternary complex consisting of eukaryotic initiation factor eIF2, the initiator methionyl-tRNA (met-tRNA), and a molecule of GTP to the 40S ribosomal subunit to form the 43S preinitiation complex. This step is modulated by the guanine nucleotide exchange factor eIF2B. The 43S preinitiation complex facilitates translation through identification of the AUG start codon and, in some cases, can trigger internal initiation by binding directly to an internal ribosomal entry site (IRES) (6). A second step regulating peptide-chain initiation is the binding of mRNA to the 43S preinitiation complex, and this process is mediated by the assembly of the eIF4F complex of proteins. Insulin and amino acids stimulate translation initiation by induction of eIF4E-binding protein-1 (eIF4E-BP1) phosphorylation and 70-kDa ribosomal protein S6 kinase (S6K1) phosphorylation through activation of a signaling pathway involving the mammalian target of rapamycin (mTOR), a signaling protein downstream of protein kinase B (PKB) in the insulin signal transduction pathway. 4E-BP1 is a repressor protein that competes with eIF4G for binding to eIF4E. The availability of eIF4E is dependent upon decreased affinity of eIF4E for 4E-BP1 when 4E-BP1 is phosphorylated. Phosphorylation and availability of eIF4E increase the association of eIF4E with eIF4G and eIF4A, and the formation of the eIF4F complex, which mediates the binding of mRNA to the 43S ribosomal complex (12).

In mature animals, acute inflammation elicits a profound reduction in muscle protein synthesis by simultaneously suppressing translation initiation signaling downstream of mTOR and eIF2B signaling pathways (22, 44), although particular emphasis has been given to the formation of the active eIF4E·eIF4G complex as a crucial regulatory step affected by inflammation (22, 41, 45). In neonatal animals, acute endotoxin infusion induces only a moderate decrease in muscle protein

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METHODS

Augmenting the translation initiation process. To address this protein synthesis in neonates during early endotoxemia by translation is largely maintained during acute endotoxin infusion that the response of muscle protein synthesis to insulin stimulation. However, in neonatal animals, we found that the response of muscle protein synthesis to insulin stimulation is largely maintained during acute endotoxin infusion (31). Therefore, we hypothesized that insulin stimulates muscle protein synthesis in neonates during early endotoxemia by augmenting the translation initiation process. To address this hypothesis, we infused neonatal pigs with Escherichia coli (E. coli) endotoxin LPS, whereas insulin was maintained at fasting (2 μU/mL) and fed (10 μU/mL) plasma concentrations and plasma amino acids and blood glucose levels were maintained in the fasting range. To evaluate the effect of insulin on translation initiation signaling, we measured eIF4G to form the active mRNA-binding complex in muscle (31). The RNA-to-protein ratio (mg RNA/g protein) was used as an estimate of the protein synthetic capacity, i.e., ribosome number. Fractional rates of protein synthesis were determined as previously described (31). The RNA-to-protein ratio (mg RNA/g protein) was used as an estimate of the protein synthetic capacity of ribosomes. Protein synthetic efficiency was estimated as the total protein synthesized per total RNA (g protein·day⁻¹·g RNA⁻¹).

Plasma hormones and substrate assays. Whole blood glucose concentrations were determined by a glucose oxidase reaction (YSI 2300 STAT Plus; Yellow Springs Instruments, Yellow Springs, OH). Plasma concentrations of total BCAA were measured by analysis of leucine, isoleucine, and valine deamination by leucine dehydrogenase with stoichiometric reduction of NAD measured by spectrophotometry. Nitrogen, and stored at 70°C until protein immunoblot analysis. mTOR SIGNALING IN ENDOTOXEMIC NEONATAL PIGS

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Animals. Two crossbred (Landrace × Yorkshire × Hampshire × Duroc) pregnant sows (Agriculture Headquarters, Texas Department of Criminal Justice, Huntsville, TX) were housed in lactation crates, provided with water ad libitum for 1 to 2 wk before farrowing. After farrowing, piglets remained with the sow. Three days prior to the infusion, piglets were anesthetized and underwent sterile catheter insertion into a jugular vein and a carotid artery. Piglets were then returned to the sow and allowed to suckle freely until they were studied. The previously described protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals. Experimental design. Thirty piglets (5–6 days of age, 1.9 ± 0.31 kg body wt) were assigned randomly to control (n = 15) and LPS (n = 15) treatment groups. After an overnight fast, the animals were placed in a sling restraint system. Pancreatic glucose-amino acid clamps were then performed in each animal after average basal concentrations of whole blood glucose and plasma branched-chain amino acids (BCAA) were obtained, following techniques previously described (31). The clamp was initiated with a primed (20 μg/kg, continuous (100 μg·kg⁻¹·h⁻¹) somatostatin (Bachem, Torrance, CA) infusion to block endogenous insulin secretion (−60 t). A physiological replacement glucagon infusion (150 ng·kg⁻¹·h⁻¹; Eli Lilly, Indianapolis, IN) was provided 10 min after the initiation of somatostatin and was continued to the end of the clamp period. Simultaneously with the glucagon infusion, insulin was infused at 175 μU·kg⁻0.66·min⁻¹ to achieve plasma insulin concentrations of 2–5 μU/mL to simulate a fasting insulinenic state. Glucose and amino acids were clamped to the individual basal fasting levels achieved during the first hour (−60 to 0 t) and maintained during the 8-h infusion by monitoring the blood glucose and serum BCAA at 5–to-10-min intervals and adjusting the infusion rates of dextrose and a balanced amino acid mixture to maintain plasma BCAA and blood glucose within 10% of the desired level. One hour after the initiation of the somatostatin infusion, the LPS group received a continuous infusion (10 μg·kg⁻¹·h⁻¹) of E. coli endotoxin (lyophilized E. coli Serotype 0111-B4; Sigma Chemical, St. Louis, MO) that was continued for 8 h, whereas the control group received an equal volume of sterile normal saline solution.

After 6 h of LPS/saline infusion (6 t), insulin infusion either was increased to 1,000 μU·kg⁻0.66·min⁻¹ (LPS + insulin, n = 8) or remained at replacement levels (LPS, n = 8; control, n = 7). Blood glucose and serum BCAA continued in the targeted fasting range in both groups. All animals were killed 8 h after the LPS infusion began (8 t). I.e., 2 h after increasing the insulin infusion rate in the fed-insulin-level groups (6 t), thereby providing sufficient time to achieve a steady state for the targeted circulating glucose, amino acids, and insulin levels before the animals were subjected to a flooding dose of the tracer.

Measuring protein synthesis in vivo. Tissue protein synthesis was measured in vivo using a modification of the flooding dose technique, as previously described (31). Briefly, 7 h and 30 min after the LPS infusion was initiated, pigs were injected via the jugular vein catheter with 1.5 nmol/kg body wt (1 mCi/kg body wt) of a flooding dose of L-[4-¹³C]phenylalanine (Amersham, Arlington Heights, IL). Pigs were killed 8 h after the LPS infusion began, and longissimus dorsi (LD) muscle and liver tissue samples were rapidly removed. Fractional rates of protein synthesis were determined as previously described (31). The RNA-to-protein ratio (mg RNA/g protein) was used as an estimate of the protein synthetic capacity of ribosomes. Within 60 h of the termination of the injection, the LPS group received a continuous infusion (10 μg·kg⁻¹·h⁻¹) of E. coli endotoxin (lyophilized E. coli Serotype 0111-B4; Sigma Chemical, St. Louis, MO) that was continued for 8 h, whereas the control group received an equal volume of sterile normal saline solution.
Antibody that recognizes phosphorylation at Thr70 (Cell Signaling) was used to resolve into multiple electrophoretic forms by a rabbit polyclonal antibody that recognizes the phosphorylated epitope (18). 4E-BP1 present in tissue extracts was separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were then probed with either anti-4E-BP1 or anti-4E-BP1 antibodies (Bethyl Laboratories, Montgomery, TX) or the mouse monoclonal anti-4E-BP1 antibody. Values obtained using the anti-4E-BP1 antibodies were normalized for the amount of 4E-BP1 present in the sample.

**Determination of PKB phosphorylation.** Muscle and liver homogenates were incubated with an anti-PKB antibody (New England Biolabs, Beverly, MA). The second membrane was incubated with a rabbit polyclonal antibody that recognizes the phosphorylation of PKB on Ser473 (New England Biolabs). Results were normalized for the total amount of PKB on the blot.

**Determination of 4E-BP1 phosphorylation.** Muscle and liver homogenates were subjected to protein immunoblot analysis using an Amersham enhanced chemiluminescence Western blotting kit as previously described (18). 4E-BP1 present in tissue extracts was separated into multiple electrophoretic forms by a rabbit polyclonal antibody that recognizes the phosphorylation of 4E-BP1 on Thr37/46 (Santa Cruz Biotechnology, Beverly, MA) during SDS-PAGE, with the more slowly migrating forms representing more highly phosphorylated 4E-BP1. Phosphorylation of 4E-BP1 was corrected for total 4E-BP1.

**Determination of S6K1 phosphorylation.** Muscle and liver homogenates were subjected to protein immunoblot analysis using an Amersham enhanced chemiluminescence Western blotting kit as previously described (18), using a rabbit polyclonal antibody that recognizes S6K1 when it is phosphorylated at the activating residue Thr421/422 (Santa Cruz Biotechnology, Santa Cruz, CA). Results were normalized for the total amount of PKB on the blot.

**Determination of eukaryotic elongation factor-2, eIF2α, and p42/44 phosphorylation.** Aliquots of muscle and liver homogenates were subjected to protein immunoblot analysis using a rabbit polyclonal antibody that recognizes the site-specific phosphorylation of eukaryotic elongation factor-2 (eEF2) at Thr56 or total eEF2. Phosphorylation of eEF2 was corrected for total eEF2. Rabbit polyclonal antibodies were used for protein immunoblot analysis to determine eEF2α phosphorylation and dual p42/44 phosphorylation. The results were normalized for the total amount of eEF2α and total p42/44 on the blot, respectively.

**Data analysis.** Treatment with insulin was the grouping factor for different parameters. A general linear model of ANOVA was used to determine the effect of LPS, insulin, and their interaction on protein synthesis and translation initiation factor activation using statistical software (Minitab for Windows). T-tests were also performed to examine the specific effects of each treatment group. Results are presented as means ± SE. Probability values of <0.05 were considered statistically significant.

**RESULTS**

**Hormones and substrates during pancreatic-substrate clamps.** C-peptide was not detectable in plasma of either LPS or control animals, indicating endogenous insulin blockade. Baseline fasting insulin levels were obtained for each individual animal (controls, 3 ± 0.6 μU/ml; LPS, 3 ± 0.4 μU/ml; P > 0.05), and those fasting levels were maintained during the period of LPS infusion in both control and experimental animals (P > 0.05, baseline vs. fasting insulin levels). In the insulin-stimulated group, insulin levels similar to those observed in the fed state were obtained by providing exogenous insulin in both control and LPS-infused animals during the last 2 h of LPS infusion (controls, 12 ± 3; LPS, 13 ± 0.8 μU/ml; P > 0.05), and they were different from those in the fasting condition in both control and LPS-infused animals (P < 0.05, baseline vs. fed insulin levels). In controls and LPS-infused animals, whole blood glucose was maintained at fasting levels during the entire experiment (controls, 75 ± 4; LPS, 82 ± 4 mg/dl; P > 0.05), even when exogenous insulin was supplemented (P > 0.05). In fasting LPS-infused animals, plasma BCAA were maintained at baseline levels during the entire experimental period (controls, 515 ± 34; LPS, 509 ± 36 mmol/ml; P > 0.05). When exogenous insulin was supplemented, LPS-infused animals maintained baseline levels of blood glucose and plasma BCAA (P > 0.05).

**Protein synthesis in muscle and liver during endotoxemic pancreatic-substrate clamps.** In the presence of fasting insulin levels and fasting blood glucose and plasma amino acid levels, LPS reduced protein synthesis in the LD (−29%, P < 0.05; Fig. 1A), a muscle composed primarily of fast-twitch muscle fibers. Raising insulin to fed levels, while maintaining fasting blood glucose and plasma amino acid levels, accelerated muscle protein synthesis in both groups (controls, +44%; LPS, +64%; P < 0.05; Fig. 1A). The protein synthetic capacity of the tissue, measured by the RNA-to-protein ratio, was not affected by LPS or insulin in muscle (P > 0.05; Fig. 1B). In
animals maintained at fasting insulin levels, the protein synthetic efficiency, as indicated by the total protein synthesized per total RNA, was significantly decreased by LPS in LD muscle (−28%, P < 0.05; Fig. 1C). Increasing insulin to fed levels enhanced protein synthetic efficiency in muscle (controls by 38%, LPS by 60%, P < 0.05; Fig. 1C). The effect of insulin on protein synthesis in muscle was proportionally similar in LPS-infused and control animals, and no interaction between LPS and insulin was detected. LPS increased protein synthesis in muscle by +28% compared with controls (P < 0.05; Fig. 1D). Raising insulin to fed levels did not alter protein synthesis in either group (P > 0.05; Fig. 1D). LPS increased the protein synthetic efficiency, but not the protein synthetic capacity, of the liver of neonatal animals (P < 0.05; Fig. 1D).

Effects of insulin on PKB signaling and biomarkers of mRNA translation in muscle and liver during endotoxemic pancreatic-substrate clamps. In skeletal muscle, insulin, but not LPS, increased the phosphorylation of PKB (P < 0.05; Fig. 2A). In liver, neither LPS nor insulin altered PKB phosphorylation (P > 0.05; Fig. 2B). In muscle of control animals, insulin increased 4E-BP1 and S6K1 phosphorylation, decreased the association of the inactive eIF4E-4E-BP1 complex, and increased the abundance of the active eIF4E-eIF4G complex (P < 0.05; Figs. 2B and 3A) but did not alter S6K1 phosphorylation or the abundance of the inactive eIF4E-4E-BP1 complex (P > 0.05; Figs. 2C and 3B). Insulin failed to increase 4E-BP1 or S6K1 phosphorylation in muscle of LPS-infused animals (P > 0.05; Figs. 2B and 3B). In addition, insulin failed to decrease the abundance of the inactive eIF4E-4E-BP1 complex or to increase the abundance of the active eIF4E-eIF4G complex in LPS-infused animals (P > 0.05; Figs. 2C and 3A). In liver, LPS, but not insulin, increased 4E-BP1 and S6K1 phosphorylation and decreased the formation of the inactive eIF4E-4E-BP1 complex (P < 0.05; Figs. 2, D and F, and 3E).

In muscle of controls, insulin decreased eIF2α and eEF2 phosphorylation (P < 0.05; Figs. 3C and 4A) and tended to increase the relative p42/p44 phosphorylation (P > 0.05; Fig. 4C). eIF2α and eEF2 phosphorylation did not change in response to LPS infusion (P > 0.05; Figs. 3C and 4A), and p42/p44 phosphorylation in muscle of fasting animals increased in response to LPS (P < 0.05; Fig. 4C). Raising insulin to fed levels in LPS-infused animals did not affect eIF2α phosphorylation (P > 0.05; Fig. 4A), did not decrease p42/p44 phosphorylation (P > 0.05; Fig. 4C), and decreased eEF2 phosphorylation in muscle (P < 0.05; Fig. 3C). In liver, LPS, but not insulin, decreased eIF2α phosphorylation (P < 0.05; Fig. 4D), decreased eEF2 phosphorylation (P < 0.05; Fig. 3F), and increased p42/p44 phosphorylation (P < 0.05; Fig. 4F).

![Fig. 2. Effects of insulin on PKB (A and D) and eukaryotic initiation factor (eIF) binding protein-1 (eIF4E-4E-BP1) phosphorylation (B and E) and eIF4E-4E-BP1 association (C and F) in longissimus dorsi muscle and liver of LPS-infused neonatal pigs. The phosphorylated forms were normalized to the total content. Values are means ± SEM; n = 7–8/group. a,b,c,dValues with different superscripts differ significantly (P < 0.05).](http://ajpendo.physiology.org/)

![Fig. 3. Effects of insulin on the abundance of the active eIF4E-eIF4G complex (A and D) and phosphorylation of S6K1 (B and E) and eukaryotic elongation factor-2 (eEF2; C and F) in longissimus dorsi muscle and liver of LPS-infused neonatal pigs. The phosphorylated forms were normalized for the total content. Values are means ± SEM; n = 7–8/group. a,b,c,dValues with different superscripts differ significantly (P < 0.05).](http://ajpendo.physiology.org/)
eIF2B activity was not affected by insulin or LPS infusion in muscle or liver (P > 0.05; Fig. 4, B and E).

**DISCUSSION**

Recently, the beneficial role of insulin therapy in minimizing the effects of critical illness on muscle wasting has been reported (40). It has also been recognized (1, 35) that insulin resistance plays a major role in the metabolic dysregulation during sepsis. However, the mechanisms that regulate the beneficial effects of insulin in the critically ill are unknown (7), and few reports address the effect of insulin on muscle metabolism during sepsis (13, 22, 42). LPS infusion elicits a consistent response that imitates some of the clinical signs and cytokine production seen in sepsis, and this response has been validated and reliably reproduced in adult and young swine (32, 46). We (31) have demonstrated that the muscle of neonatal pigs retains the ability to respond to the stimulatory action of insulin by increasing protein synthesis after LPS administration. Because the regulation of muscle protein synthesis in the neonate is mediated by key translation initiation factors (16), we wished to define the mechanisms that regulate the effect of insulin on translation initiation during neonatal endotoxemia. As a comparison, we examined the activation of the translation initiation process in the liver of LPS-infused neonatal pigs, since we (29, 31) have demonstrated previously that liver protein synthesis is stimulated by endotoxin but not by insulin infusion.

**Effects of endotoxin on protein synthesis in muscle and liver in neonatal pigs.** Sepsis, a systemic inflammatory response syndrome, profoundly decreases protein synthesis in skeletal muscle (22, 44) and represses the insulin-induced stimulation of protein synthesis in mature animals (13, 42). By contrast, we (20, 30) have demonstrated that LPS infusion elicits only a modest reduction in muscle protein synthesis in neonatal animals in the presence of insulin and amino acid stimulation, in part because insulin-stimulated muscle protein synthesis is maintained in neonates faced with acute inflammation generated by LPS administration (31). Similar to adult rats, we found that the decrease in muscle protein synthesis is accompanied by a decrease in translational efficiency rather than changes in the protein synthetic capacity, i.e., ribosome number (45). In the current study, raising insulin to fed levels increased protein synthesis rates as a result of augmenting the protein synthetic efficiency in muscle, similar to our previous report (32). This suggests that insulin stimulates translational efficiency in the muscle of growing organisms even when faced with systemic inflammation, enabling protein deposition in muscle.

In the healthy neonatal pig, insulin does not stimulate hepatic protein synthesis (29), and the production of proteins by the liver (i.e., retinol-binding protein and albumin) responds to amino acid rather than hormonal stimulation (29). In our study, liver protein synthesis in neonatal pigs was enhanced by LPS, but not by insulin, likely because LPS stimulates the hepatic synthesis of acute phase proteins during sepsis (11). Although we did not identify the proteins that were synthesized by the liver in our model, our data suggest that the stimulation of liver protein synthesis during endotoxin infusion is dependent on the inflammatory response triggered by LPS, and this response is not influenced by insulin levels (31, 44). We conclude that maintaining either fasting or fed levels of insulin will not alter the LPS-induced increase in liver protein synthesis.

**Effects of endotoxin on translation initiation during fasting.** Previously, we (32) have shown that muscle protein synthesis decreases only modestly (−11%) in response to LPS infusion if insulin, glucose, and amino acids are maintained at levels similar to those seen in the fed state, and this is associated with substantial reductions in the phosphorylation of 4E-BP1 and S6K1 and a decrease in the binding of eIF4E to eIF4G (20, 32). The fasting animals in the current study had a more profound reduction in protein synthesis (i.e., −29%) than those in our previous study in which insulin, glucose, and amino acid levels of LPS-infused animals were maintained in the fed range. The decrease in muscle protein synthesis with LPS in our fasting neonatal pigs was associated with depression in 4E-BP1 phosphorylation and decreased abundance of the active eIF4E-eIF4G complex. However, we did not find an effect of LPS on other events that also alter the mRNA binding step in translation initiation, such as S6K1 phosphorylation and the assembly of the inactive eIF4E-4E-BP1 complex. We suspect that the LPS-induced decrease in muscle protein synthesis in our study is enhanced by the restriction in substrate and hormonal stimulation, i.e., insulin, glucose, and amino acids, that occurs during fasting. However, the decrease in the abundance of phosphorylated initiation factors in response to fasting may mask a further LPS-induced reduction in translation initiation. Although insulin stimulates translation factor signaling activation, the response of 4E-BP1 phosphorylation and eIF4G-eIF4E formation to insulin stimulation is also dimin-

![Fig. 4. Effects of insulin on eIF2α phosphorylation (A and D), eIF2B activity (B and E), and p42/44 phosphorylation (C and F) in longissimus dorsi muscle and liver of LPS-infused neonatal pigs. The phosphorylated forms were normalized for the total content. Values are means ± SE; n = 7–8/group. a,b,c,dValues with different superscripts differ significantly (P < 0.05).](http://ajpendo.physiology.org/content/292/2/632/F4.large.jpg)
ished in ovine fetuses during maternal fasting (36). Similar studies in neonatal pigs (28) have shown that insulin does not affect 4E-BP1 and S6K1 phosphorylation or eIF4E-4E-BP1 and eIF4G-eIF4E content when plasma insulin was increased from 0 to 2 μU/ml. Furthermore, most studies in adult septic animals have allowed the subjects to feed ad libitum, but they become anorectic, and it is possible that the lack of substrate availability contributes to enhanced protein catabolism (4) and a pronounced decrease in muscle protein synthesis.

Effects of insulin on biomarkers of mRNA translation in skeletal muscle of LPS-infused pigs. Although both glucose and amino acids are required for insulin to stimulate the activation of translation initiation factor signaling in vitro, insulin exerts an independent and profound stimulatory effect on muscle protein synthesis and translation initiation signaling in neonatal animals (8, 16) when levels are above the fasting range. The effects of sepsis on the insulin signaling cascade that leads to translation initiation occur downstream of mTOR in mature animals (20–22, 30, 44, 45). However, we have demonstrated, using rapamycin as an inhibitor of mTOR, that both mTOR-dependent and mTOR-independent pathways are involved in the regulation of protein synthesis in muscle of neonatal animals (19). In the current study, in contrast to reports in adult rats (2, 13), raising insulin to fed levels in control neonatal pigs was associated with an increase in fractional protein synthesis rates in muscle, although glucose and amino acids were maintained in the fasting range, and this response was coupled with increased phosphorylation of PKB, 4E-BP1, and S6K1, reduced binding of eIF4E to 4E-BP1, and increased assembly of the active eIF4E-eIF4G complex. However, in LPS-infused animals, the response to insulin was abrogated.

The eIF4F complex of proteins and S6K1 activation has been considered an important regulator of muscle protein synthesis in adult animals (24, 45). Upon activation of apoptotic pathways, eIF4E and eIF4G undergo cleavage and degradation (5), and these events may play a role in the development of restrictions in translation signaling and its lack of response to insulin (22, 42). Although S6K1-induced phosphorylation of ribosomal protein S6 has been reported to be impaired in rats subjected to experimental intrabdominal sepsis (42), it has been suggested that S6K1 may not be a key regulator for the translation of most mRNAs but regulates the translation of terminal oligopyrimidine mRNAs that encode proteins that are essential for cell growth (18). In our study, LPS restrained the expected increase in eIF4E binding to eIF4G and S6K1 phosphorylation despite an increase in protein synthesis rates in skeletal muscle in response to insulin administration. Inconsistent effects of insulin on signal transduction pathways have been reported (15), but the effect of acute inflammation in different animal models of experimental sepsis suggests a depression in eIF4E to eIF4G binding and S6K1 phosphorylation in muscle (45). In our study in LPS-infused neonatal animals, the stimulation of muscle protein synthesis by insulin occurred independent of change in eIF4E to eIF4G binding and S6K1 phosphorylation, whereas studies in mature animals (13, 42, 44) report a restriction in protein synthesis in response to insulin stimulation. Since the stimulation of muscle protein synthesis by insulin during LPS infusion in neonatal animals cannot be explained by changes in the translation initiation events that lead to protein synthesis, we conclude that the response of muscle protein synthesis to insulin during neonatal endotoxemia may be regulated by a process other than translation initiation.

In our study, eIF2B, a translation initiation factor that does not depend on mTOR activation, was not affected by either LPS or insulin infusion. Previously, we (20) have shown that the lack of effect of LPS on eIF2B activity may account for the near maintenance of global rates of protein synthesis in LPS-infused neonatal pigs with fed insulin and amino acid levels. It has been suggested that eIF2 complex of proteins influence global rates of protein synthesis (20), and the phosphorylation of eIF2α coincides with the inhibition of translation followed by activation of apoptotic pathways (5). In adult rat models, eIF2B activity in skeletal muscle is reduced following infusion of TNF-α, experimentally-induced sepsis with LPS, and peritonial infection (21, 23, 43), and thus eIF2B appears to have a key role in regulating global rates of protein synthesis during inflammation (45). In normal-fasted neonates, eIF2B activity in muscle is not affected by insulin, in contrast to the insulin-stimulated increase in 4E-BP1 phosphorylation, eIF4E-eIF4G content, and S6K1 phosphorylation in these animals (28). In the current study, we found that modulation of eIF2B activity is not involved in the regulation of muscle protein synthesis by insulin during acute endotoxemia in neonates.

eIF2α phosphorylation, an event that can mediate IRES-dependent translation, has been reported to regulate in vitro global rates of protein synthesis and offers an appealing link between the regulation of translation initiation, control of gene expression, and apoptosis (34, 38). Phosphorylation of the α-subunit of eIF2 has been reported to occur during oxidative stress and apoptosis (5, 39). Enhanced IRES-dependent translation is believed to function as a protective homeostatic mechanism through increased synthesis of several proteins critically involved in apoptosis, cell cycle, development, amino acid availability, and endoplasmic reticulum stress, and IRESs are found preferentially in the mRNAs of genes involved in the control of cellular proliferation, survival, and death (27). In the current study, insulin stimulated muscle protein synthesis, concomitant with a decrease in eIF2α phosphorylation in control animals. However, LPS blunted an insulin-induced reduction in eIF2α phosphorylation despite an increase in protein synthesis in response to insulin stimulation. In response to cellular stress, hepatic GADD34 (growth arrest and DNA damage-inducible protein 34) promotes dephosphorylation of eIF2α, suggesting a protective role for the recovery of protein synthesis after stress (33). Since eIF2B exhibits little or no binding to the α-subunit of eIF2 regardless of whether or not the α-subunit of eIF2 has been phosphorylated, and in rat muscle only about 2–4% of eIF2α is basally phosphorylated (14), we suggest that a decrease in eIF2α phosphorylation would leave little, if any, effect on eIF2B activity (17).

The translation of mRNA to protein occurs in three distinct phases: initiation, elongation, and termination (26). All three of these phases can undergo regulation, but initiation and elongation seem to be the most tightly controlled (37). The elongation process occurs after translation initiation and requires that eEF2 couples to GTP hydrolysis (26), and thus the phosphorylation of eEF2 is associated with decreased elongation. In our study, insulin, in the presence of fasting levels of amino acids and glucose, reduced the phosphorylation of eEF2. This effect of insulin on eEF2 phosphorylation was present in both
control and LPS animals. This suggests that the response of muscle protein synthesis to insulin during endotoxemia may be mediated by the elongation step in peptide formation.

In vitro, TNF-α has been reported to stimulate P42/44 phosphorylation, an event associated with transcriptional alterations (10, 41), and activation of P42/44 MAPK has been associated with cell growth arrest (25). LPS has been shown to elicit a predictable TNF-α response in swine, even in a neonatal model (32). In the adult rat liver, Maira et al. (25) reported an increase in p42/44 activation following cecal ligation and puncture, and insulin pretreatment appeared to amplify this response. It has been postulated (41) that events that mediate transcription, such as p42/44 activation, are related to suppression of translation initiation signaling by decreasing availability of eIF4E for eIF4F complex assembly. In the present study, we found that LPS increased P42/44 phosphorylation in muscle during fasting, and this response was reduced by insulin, suggesting that insulin may modify protein metabolism in muscle during endotoxemia by affecting some transcriptional processes that in turn may regulate protein synthesis and translational events.

Effects of insulin on biomarkers of mRNA translation in liver of LPS-infused pigs. The results of the current study demonstrated that liver protein synthesis was stimulated by endotoxin but not by insulin infusion. The LPS-induced increase in liver protein synthesis was associated with an increase in S6K1 phosphorylation and reductions in the abundance of the inactive eIF4E-4E-BP1 complex, phosphorylation of eIF2α, and activation of p42/44. These events suggest a differential regulation of protein synthesis by insulin during acute inflammation in different organs and that the effects of LPS in the liver occur independent of insulin administration.

PERSPECTIVES

The results of the current study suggest that insulin stimulates muscle protein synthesis in LPS-infused neonates despite a suppression of the insulin-stimulated mTOR-dependent translation initiation signaling. In our study, insulin appears to modulate protein synthesis in muscle of neonates during endotoxemia by altering the elongation process. It is possible that age and the duration and intensity of the septic model may result in different alterations in the translational signaling pathway and other processes that regulate cell growth, such as transcription and gene expression. Despite the profound changes in cellular signaling in muscle, neonatal animals appear to maintain an anabolic response to insulin during LPS infusion, which may exert a protective effect for the neonate to the catabolic processes induced by sepsis. Understanding of the interface between insulin and the molecular mechanisms that govern amino acid metabolism during acute inflammation will facilitate the design of appropriate nutritional and hormonal therapies for use during neonatal sepsis.

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REFERENCES


