Amino acids regulate skeletal muscle PHAS-I and p70 S6-kinase phosphorylation independently of insulin

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Long, W., L. Saffer, L. Wei, and E. J. Barrett. Amino acids regulate skeletal muscle PHAS-I and p70S6k phosphorylation independently of insulin. Am J Physiol Endocrinol Metab 279: E301–E306, 2000.—Refeeding reverses the muscle protein loss seen with fasting. The physiological regulators and cellular control sites responsible for this reversal are incompletely defined. Phosphorylation of phosphorylated heat-acid stabled protein (PHAS-I) frees eukaryotic initiation factor 4E (eIF4E) and stimulates protein synthesis by accelerating translation initiation. Phosphorylation of p70 S6-kinase (p70S6k) is thought to be involved in the regulation of the synthesis of some ribosomal proteins and other selected proteins with polypyrimidine clusters near the transcription start site. We examined whether phosphorylation of PHAS-I and p70S6k was increased by feeding and determined the separate effects of insulin and amino acids on PHAS-I and p70S6k phosphorylation in rat skeletal muscle in vivo. Muscle was obtained from rats fed ad libitum or fasted overnight (n = 5 each). Other fasted rats were infused with insulin (3 μU·min⁻¹·kg⁻¹, euglycemic clamp), amino acids, or the two combined. Gastrocnemius was freeze-clamped, and PHAS-I and p70S6k phosphorylation was measured by quantifying the several phosphorylated forms of these proteins seen on Western blots. We observed that feeding increased phosphorylation of both PHAS-I and p70S6k (P < 0.05). Infusion of amino acids alone reproduced the effect of feeding. Physiological hyperinsulinemia increased p70S6k (P < 0.05) but not PHAS-I phosphorylation (P = 0.98). Addition of insulin to amino acid infusion was no more effective than amino acids alone in promoting PHAS-I and p70S6k phosphorylation. We conclude that amino acid infusion alone enhances the activation of the protein synthetic pathways in vivo in rat skeletal muscle. This effect is not dependent on increases in plasma insulin and simulates the activation of protein synthesis that accompanies normal feeding.

messenger ribonucleic acid translation initiation; protein synthesis; insulin clamp; mammalian target of rapamycin

BRIEF STARVATION DECREASES the rate of protein synthesis in skeletal muscle, and this is rapidly reversed on refeeding (11). The discrete contributions by nutrients themselves and by the hormonal responses that accompany feeding to the stimulation of protein synthesis with refeeding are only beginning to be unraveled. For example, recent in vitro experiments have shown that amino acids, beyond furnishing substrate for protein synthesis, perform a signaling role to enhance protein synthesis by phosphorylating a heat-acid stabilized protein (PHAS-I) or eukaryotic initiation factor 4E-binding protein 1 (eIF4E-BP1) and p70 S6-kinase (p70S6k), two key regulatory proteins involved in initiation of mRNA translation. These actions, at least in the in vitro systems studied, appear independent of insulin availability (14, 26, 36).

In recent in vivo studies, refeeding mice starved for brief or more prolonged times increased p70S6k and PHAS-I phosphorylation (32). These changes are also seen in a murine model of type 1 diabetes, suggesting that a rise in plasma insulin may not be required (32). Interestingly, refeeding with a protein-deficient diet does not increase PHAS-I phosphorylation or protein synthesis (37). In aggregate, these findings suggest an effect of amino acids, independent of insulin, to stimulate muscle protein synthesis in vivo.

The signaling pathway by which insulin activates protein synthesis in a variety of cell systems has been clarified in the past decade. After insulin receptor autophosphorylation and tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, the phosphatidylinositol (PI) 3-kinase pathway is activated, and protein kinase B (Akt), mammalian target of rapamycin (mTOR), eIF4E, PHAS-I, and p70S6k (among other proteins) are each phosphorylated (20, 21). Recent studies in Chinese hamster ovary-insulin receptor (CHO-IR) cells showed that amino acid withdrawal does not significantly alter insulin stimulation of receptor or IRS protein tyrosine phosphorylation, PI 3-kinase activity, c-Akt/protein kinase B activity, or mitogen-activated protein kinase activity, but that it results in rapid dephosphorylation of p70S6k and PHAS-I (2). This suggests a link between amino acid availability and phosphorylation of the more distal signaling events involved in the regulation of protein synthesis at the translational level. These findings imply that amino acids can regulate the activity of the p70S6k and PHAS-I through an insulin-independent mechanism in vitro and in vivo.

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The major purpose of the present study was to assess whether phosphorylation of p70S6K and PHAS-I was increased by feeding in normal rats and to determine the separate effects of insulin and amino acids on p70S6K and PHAS-I phosphorylation in vivo. The results show that, in rat skeletal muscle, feeding promotes phosphorylation of both PHAS-I and p70S6K, and that this is mimicked by physiological increments in plasma amino acids. In contrast, physiological increases of insulin alone enhance phosphorylation of p70S6K but not PHAS-I. Furthermore, the combination of insulin and amino acids is no more effective than amino acids alone. These findings suggest that amino acids play a significant signaling role in the increased mRNA translation that accompanies feeding.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 230–300 g were studied after an overnight 14-h fast or were fed ad libitum before study. The study protocol was approved by the University of Virginia Animal Care and Use Committee. PHAS-I antibody was generated in rabbit with recombinant His-tagged rat PHAS-I and was kindly provided by Dr. J. Lawrence, Univ. of Virginia. p70S6K antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-rabbit antibody linked to horseradish peroxidase and enhanced chemiluminescence (ECL) Western blot kits were purchased from Amersham. Insulin and Travesol (a mixed amino acid solution) were obtained from Lilly and Clintec Nutrition (Deerfield, IL), respectively. All other chemicals were from commercially available sources.

Experimental protocol. Five ad libitum-fed rats and 20 overnight-fasted rats were studied. All rats were anesthetized with pentobarbital sodium (50 mg/kg ip). A midline neck incision was made, and the external jugular vein, intercarotid artery, and trachea were exposed and cannulated. The arterial catheter was connected through a three-way stopcock to a pressure transducer, which was in turn connected to a Transcon Systems detector for heart rate and blood pressure monitoring. Pentobarbital was infused intravenously at a variable rate to maintain a steady level of anesthesia. After an ~30-min baseline period to assure hemodynamic stability (mean arterial pressure = 100–120 mmHg) and level of anesthesia, the fed rats received a saline infusion for a 3-h period. The fasted rats were divided into four groups (n = 5 each). Each of the groups then also received separately a 3-h continuous infusion of saline, insulin (3 mU · kg⁻¹ · min⁻¹), 10% Travesol (10 μl/min), or a combination of 10% Travesol (10 μl/min) and insulin (3 mU · kg⁻¹ · min⁻¹). In rats given insulin, 30% dextrose was infused at a variable rate to keep blood glucose within 10% of basal, and the equivalent volume of saline was given to the remaining rats. Blood glucose was measured every 10 min, and heart rate and mean arterial pressure were monitored throughout the study. At the end of the infusion period, gastrocnemius muscles were rapidly excised and freeze-clamped in liquid nitrogen and subsequently stored at −70°C. Plasma insulin was measured by immunosassay in plasma obtained at the beginning and completion of the study.

Western blotting. Pieces (~20 mg) of frozen gastrocnemius muscle were weighed and powdered in liquid nitrogen, then mixed with ice-cold 25 mM Tris · HCl buffer (26 mM KF and 5 mM EDTA, pH 7.5), and disrupted by sonication with a microtip probe, 0.5 s on/0.5 s off for 45 s total, at a 3.0 power setting on the Fisher XL2020 sonicator. For p70S6K, one aliquot of the muscle homogenate (~60 μg protein) was diluted with an equal volume of SDS sample buffer and run on an 8% SDS-PAGE. For PHAS-I, another aliquot was heated to 100°C for 5 min and centrifuged at 2,000 rpm × 2 min, and an aliquot of supernatant (~60 mg protein) was diluted with an equal volume of SDS sample buffer and electrophoresed on a 15% polyacrylamide gel. Proteins on both gels were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). After blocking with 5% low-fat milk in Tris-buffered saline-Tween-20, membranes were incubated with rabbit anti-rat PHAS-I or rabbit anti-p70S6K for 1 h at room temperature. This was followed by incubation with a donkey anti-rabbit IgG coupled to horseradish peroxidase, and the blot was developed using an ECL Western blotting kit. Autoradiographic film was scanned densitometrically (Molecular Dynamics) and quantitated using ImageQuant software version 3.3. PHAS-I, a 12-kDa protein, migrates in this system anomalously at ~20 kDa. One to three bands are seen on Western blotting that correspond to the unphosphorylated and several more slowly migrating phosphorylated forms of the protein. Likewise, p70S6K in extracts from unstimulated muscle migrates predominantly as a single band, with several faint bands at a higher molecular weight. However, with further phosphorylation, electrophoretic mobility is retarded, and the intensity of these more slowly migrating forms increases. For PHAS-I we quantified the ratio of the most rapidly migrating (nonphosphorylated) band (a) to the total immunoreactive material. The α-band was selected because it is this form of the protein that binds to eIF4E and prevents the association of 4E with the initiation complex. Conversely, for p70S6K we quantified the ratio of the more heavily phosphorylated (more slowly migrating) forms to the total immune reactivity, because it is the phosphorylated forms that possess kinase activity. In preliminary experiments, we examined the effect of varying the amount of protein loaded on the SDS-PAGE on the measured ratios of the several phosphorylated forms of PHAS-I and p70S6K in Western blots. Over the concentration range of protein used in the current study, there was no significant effect. Statistical analysis (Sigmastat 3.0) was based on one-way ANOVA with post hoc testing, as indicated in RESULTS.

RESULTS

Body weight, blood glucose, blood pressure, plasma insulin characteristics of animals. Table 1 shows the animal body weights, the blood glucose concentrations, the mean arterial pressures, and the glucose infusion rates in the five groups. There was no difference between groups in any of these variables except for the amount of glucose infused. The steady-state (120- to 180-min) glucose infusion rate required to maintain euglycemia was significantly higher in the rats given insulin and insulin plus amino acids than in animals given amino acids or saline infusion. Although the glucose infusion rate in the rats given insulin infusion alone seemed higher than in the rats given combination infusion, this was not statistically significant. Figure 1 illustrates the plasma insulin concentration at baseline and at the end of the 3-h infusion period for each of the five study groups. Basal insulin concentrations were higher in the fed group than in each of the fasted groups. At 3 h, the insulin concentrations in the
animals receiving insulin or insulin with amino acids, and in the fasted animals, were significantly higher than those in the fasted saline-infused animals. Plasma insulin in the amino acid-infused animals was not different from that in the saline-infused animals.

**PHAS-I and p70\(^{S6k}\) phosphorylation.** In these studies we elected to use electrophoretic behavior on SDS-PAGE and Western blotting as indexes of the biological effect of PHAS-I and p70\(^{S6k}\). Inasmuch as this method allows the simultaneous quantitation of multiple forms of both proteins, as well as internal normalization for both the recovery of target proteins from tissue and for loading of gels, it is well suited to the quantitative approach required in these studies. Available data support an inverse correlation between electrophoretic mobility and kinase activity for p70\(^{S6k}\) (29) and a direct correlation with PHAS-I bound to eIF4E (7, 27). Because the change in apparent molecular weight induced by phosphorylation of either protein exceeds that expected from simple stoichiometry of the added phosphate groups, factors other than changes in mass appear to be involved in the altered electrophoretic mobility.

Figure 2 illustrates typical patterns of PHAS-I observed on Western blots of gastrocnemius muscle at 3 h of infusion in each of the five study groups. The blots appear as three distinct bands marked α, β, and γ. To quantify the extent of phosphorylation of PHAS-I, we measured the ratio of the intensity of the most rapidly migrating species (α), which is the form associated with eIF4E, to the total intensity (α+β+γ). The lesser the amount of the α form, the greater is the availability of free eIF4E. In gastrocnemius muscle, feeding and amino acid infusion had an obvious effect to decrease the ratio of α/α+β+γ by decreasing the amount of rapidly migrating species (α) and increasing the density of the more slowly migrating forms (γ) of PHAS-I (P < 0.05 for each, ANOVA), whereas a physiological-dose insulin infusion (3 mU ⋅ kg\(^{-1}\) ⋅ min\(^{-1}\)) did not affect the phosphorylation state of PHAS-I when compared with fasted saline-infused rats (P = 0.98). In the combined amino acid and insulin animals, insulin did not enhance the amino acid effect on phosphorylation on PHAS-I when compared with the amino acid alone infusion group.

Figure 3 illustrates typical patterns of p70\(^{S6k}\) observed on Western blots of gastrocnemius muscle at 3 h of infusion in each of the five study groups. The blots appear as two or three distinct bands. The top one or two bands represent the more active forms of p70\(^{S6k}\). To quantify the extent of phosphorylation of p70\(^{S6k}\), we measured the ratio of the intensity of the slowly migrating species (top one or two bands) to the total intensity. In gastrocnemius muscle feeding, amino acid

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**Table 1. Body wts, blood glucose concentrations, blood pressures, and glucose infusion rates of animal groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Wt, g</th>
<th>Blood Glucose, mg/dl</th>
<th>Mean Arterial Pressure, mmHg</th>
<th>Glucose Infusion Rate, mg ⋅ kg(^{-1}) ⋅ min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed saline</td>
<td>258 ± 24</td>
<td>63 ± 8</td>
<td>115 ± 13</td>
<td>0</td>
</tr>
<tr>
<td>Fasted saline</td>
<td>268 ± 23</td>
<td>57 ± 4</td>
<td>117 ± 18</td>
<td>0</td>
</tr>
<tr>
<td>Insulin</td>
<td>251 ± 18</td>
<td>52 ± 9</td>
<td>116 ± 11</td>
<td>9.3 ± 3.7*</td>
</tr>
<tr>
<td>Amino acids</td>
<td>251 ± 30</td>
<td>65 ± 9</td>
<td>112 ± 7</td>
<td>0</td>
</tr>
<tr>
<td>Insulin + amino acids</td>
<td>269 ± 18</td>
<td>63 ± 4</td>
<td>119 ± 7</td>
<td>5.9 ± 3.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 5 animals in each group. See full description of treatment groups in **Experimental protocol**. *P < 0.01 vs. fasted saline rats.
infusion and physiological-dose insulin infusion had an obvious effect to increase the ratio of $\beta + \gamma/\alpha + \beta + \gamma$ of p70$^S6K$ ($P < 0.05$ for each, ANOVA) when compared with fasted saline-infused rats. In the amino acid + insulin combination-infused animals, insulin and amino acid did not further enhance the effect on phosphorylation of p70$^S6K$ when compared with the amino acid alone or insulin alone infusion groups.

**DISCUSSION**

The current results suggest that, in the rat as in the mouse (32), feeding promotes the phosphorylations of both PHAS-I and p70$^S6K$. In overnight-fasted rats, infusion of insulin, at a rate that increments plasma insulin to levels that match those of fed animals while maintaining euglycemia, reproduced the phosphorylation of p70$^S6K$ seen with feeding. However, this insulin infusion did not mimic the effect of feeding on the phosphorylation of PHAS-I. It is of considerable interest that amino acid infusion alone enhanced the phosphorylation of both p70$^S6K$ and PHAS-I and appeared to fully mimic the pattern of phosphorylation of both proteins seen in muscle from the ad libitum-fed rats. This occurred despite the absence of a significant increment in plasma insulin. Indeed, plasma insulin concentrations in the amino acid-infused rats were no greater than those in the fasted animals. We cannot eliminate the possibility that small or transient increments in plasma insulin occurred during the amino acid infusion and were not captured by our blood sampling regimen, and these might account for the phosphorylation of p70$^S6K$. However, these would not explain the phosphorylation of PHAS-I seen in these animals. Moreover, it appears clear that greater increments in plasma insulin, as seen in the insulin + amino acid-infused animals, had no further effect on the phosphorylation state of p70$^S6K$ than that seen with amino acid infusion alone. We emphasize that the insulin concentrations in all of these animals are in the low physiological range. We cannot exclude, on the basis of these studies, an effect of much greater concentrations of insulin on the degree of phosphorylation of PHAS-I. Indeed, in vivo, large doses of insulin (5 U given to <300-g rats) increase phosphorylation of PHAS-I and promote its dissociation from eIF4E (18). Thus the dose-response relationship between insulin and PHAS-I phosphorylation in vivo remains to be defined. Suffice it to say that the low insulin concentrations used here differentially stimulate the phosphorylation of p70$^S6K$.

The current results emphasize the potentially very important role of direct amino acid signaling on skeletal muscle protein synthesis. These findings extend results of a recent study by Yoshizawa et al. (38), which demonstrated that refueling a protein-rich, but not a protein-free, diet reverses the inhibition of protein synthesis seen with fasting. Those authors further showed that refueling the protein-rich, but not the protein-free, diet was associated with increased phosphorylation of PHAS-I and the predicted increase in the formation of the active eIF4G-eIF4E complex involved in translation initiation, strongly suggesting a role for the protein component of the diet (i.e., amino acids). Together with the current results, these findings appear to complement several recent in vitro studies that demonstrated a direct effect of amino acids to stimulate phosphorylation of both PHAS-I and p70$^S6K$. Thus, with use of either CHO cells (14, 36) or a hepatoma cell line (26), amino acid withdrawal led to the dephosphorylation of both proteins, and, conversely, subsequent replacement of amino acids resulted in significant stimulation in the phosphorylation of both. These findings, however, do not exclude a role for other humoral signals [e.g., insulin-like growth factor I (IGF-I) or growth hormone] in the physiological regulation of message translation.

We (13, 22, 39) and many other laboratories (2, 6, 8, 23–25, 30, 35) have previously demonstrated that physiological hyperinsulinemia does not enhance protein synthesis in adult rats or humans. One contrary result has been reported (3). All of these studies are based on the use of tracer methods and are to an uncertain extent compromised by the inaccessibility to repeated sampling of the aminoacyl-tRNA pool that is used for protein synthesis. However, available data in rats (39) and very recently in humans (1), in which aminocarte-tRNA labeling was measured, confirm that physiological hyperinsulinemia alone suppresses protein synthesis in muscle without increasing protein synthesis. This lack of action of insulin on protein synthesis in vivo appears to be the case even when amino acid concentrations in plasma are maintained at basal (13). In contrast, a number of studies have indicated that increasing the plasma concentration of amino acids can enhance whole body (5, 33) and muscle (4, 10, 28) protein synthesis. However, these studies are not without ambiguity, because an effect of amino acid infusion (and raised plasma amino acid concentration) to alter the relationship between tracer enrichment in plasma...
and in the aminoacyl-tRNA pool generally cannot be excluded.

In light of this, the differential effect of insulin on the phosphorylation of p70S6k but not PHAS-I in the current studies is of particular interest. In a variety of studies, phosphorylation of PHAS-I has consistently been observed to accompany increases in protein synthesis (12, 32, 37, 38). Recent in vitro studies in which p70S6k is deleted by a homologous recombination have emphasized that protein synthesis can still be stimulated by insulin and other growth factors (17). p70S6k appears to play a particular role in promoting the translation of messenger RNA with polypyrimidine sequences near the cap site on messenger RNA. These messages code for specific proteins, and among them are a number of ribosomal proteins, as well as initiation and the elongation factors involved in mRNA translation. It is attractive to consider that insulin, by stimulating p70S6k selectively, allows for priming of the protein synthetic apparatus, but initiation of translation requires the additional phosphorylation of PHAS-I, which may respond more sensitively to nutrient signaling. In this manner, specific nutrient (amino acid) signaling is integrated with the more general feeding signal (insulin) to allow message translation to commence. Against this construct is the observation that high doses of insulin in vivo in rats (19) and humans (15) can stimulate muscle protein synthesis. However, it is difficult to exclude an effect of high-dose insulin acting via the IGF-I receptor. The latter hormone has been demonstrated to increase muscle protein synthesis in both humans (9, 31) and animals (16, 34).

In summary, the current studies provide evidence that, in the rat, feeding regular chow sustains PHAS-I and p70S6k in a highly phosphorylated form, and this is lost during fasting but can be fully restored by amino acid infusion. Physiological increments in plasma amino acids, which may respond more sensitively to nutrient signaling, can stimulate muscle protein synthesis in humans (15) can stimulate muscle protein synthesis. In this manner, specific nutrient (amino acid) signaling is integrated with the more general feeding signal (insulin) to allow message translation to commence. Against this construct is the observation that high doses of insulin in vivo in rats (19) and humans (15) can stimulate muscle protein synthesis. However, it is difficult to exclude an effect of high-dose insulin acting via the IGF-I receptor. The latter hormone has been demonstrated to increase muscle protein synthesis in both humans (9, 31) and animals (16, 34).

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