

Elevated plasma free fatty acids decrease basal protein synthesis, but not the anabolic effect of leucine, in skeletal muscle

Charles H. Lang

Departments of Cellular and Molecular Physiology, and Surgery,
Pennsylvania State University College of Medicine, Hershey, Pennsylvania

Submitted 7 February 2006; accepted in final form 3 May 2006

Lang, Charles H. Elevated plasma free fatty acids decrease basal protein synthesis, but not the anabolic effect of leucine, in skeletal muscle. *Am J Physiol Endocrinol Metab* 291: E666–E674, 2006. First published May 9, 2006; doi:10.1152/ajpendo.00065.2006.—Elevations in free fatty acids (FFAs) impair glucose uptake in skeletal muscle. However, there is no information pertaining to the effect of elevated circulating lipids on either basal protein synthesis or the anabolic effects of leucine and insulin-like growth factor I (IGF-I). In chronically catheterized conscious rats, the short-term elevation of plasma FFAs by the 5-h infusion of heparin plus Intralipid decreased muscle protein synthesis by ~25% under basal conditions. Lipid infusion was associated with a redistribution of eukaryotic initiation factor (eIF)4E from the active eIF4E·eIF4G complex to the inactive eIF4E·4E-BP1 complex. This shift was associated with a decreased phosphorylation of eIF4G but not 4E-BP1. Lipid infusion did not significantly alter either the total amount or phosphorylation state of mTOR, TSC2, S6K1, or the ribosomal protein S6 under basal conditions. In control rats, oral leucine increased muscle protein synthesis. This anabolic response was not impaired by lipid infusion, and no defects in signal transduction pathways regulating translation initiation were detected. In separate rats that received a bolus injection of IGF-I, lipid infusion attenuated the normal redistribution of eIF4E from the active to inactive complex and largely prevented the increased phosphorylation of 4E-BP1, eIF4G, S6K1, and S6. This IGF-I resistance was associated with enhanced Ser³⁰⁷ phosphorylation of insulin receptor substrate-1 (IRS-1). These data indicate that the short-term elevation of plasma FFAs impairs basal protein synthesis in muscle by altering eIF4E availability, and this defect may be related to impaired phosphorylation of eIF4G, not 4E-BP1. Moreover, hyperlipidemia impairs IGF-I action but does not produce leucine resistance in skeletal muscle.

leucine; insulin-like growth factor I; eukaryotic initiation factor 4E-binding protein-1; eukaryotic initiation factor 4G; mammalian target of rapamycin; tuberous sclerosis complex 2

IN CONTRAST TO THE EXTENSIVE LITERATURE on the relationship between circulating free fatty acids (FFAs) and glucose metabolism, there is a paucity of information pertaining to the impact of FFAs on protein metabolism. Early studies where plasma FFAs were acutely increased by infusion of Intralipid plus heparin (9) reported a generalized hypoaminoacidemia. This response demonstrates a mismatch between the rates of appearance and disappearance of amino acids and suggests that elevated plasma FFAs increase protein synthesis and/or decrease proteolysis. This protein-sparing effect of lipid was confirmed in isotope dilution studies where radiolabeled leucine was used, which concluded that increased FFA availability was inversely related to the rate of whole body prote-

olysis (e.g., decreased rate of leucine appearance) but did not change protein synthesis (e.g., rate of nonoxidative disposal of leucine) (45). However, neither of the above-mentioned studies examined the effect of FFAs on protein metabolism in skeletal muscle per se. In this regard, a more recent study (6) demonstrated in vivo inhibition of fatty acid oxidation decreased protein synthesis in heart but did not significantly alter the protein synthetic rate in skeletal muscle. Hence, whether elevated plasma FFAs alter protein synthesis in skeletal muscle under basal conditions has still not been directly addressed.

Elevated plasma FFAs also produce insulin resistance in skeletal muscle, which is characterized by an impaired insulin-stimulated glucose uptake and glycogen synthesis (3, 33, 35, 49). The mechanism for this blunted insulin response on carbohydrate metabolism is partly due to accumulation of intramyocellular fat, which impairs insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and IRS-1-associated phosphatidylinositol (PI) 3-kinase activity (4, 21, 49). However, whether elevated FFAs also blunt the ability of growth factors to stimulate protein synthetic pathways has not been directly investigated.

Amino acid refeeding after brief starvation increases the synthesis of both myofibrillar and sarcoplasmic proteins in skeletal muscle (42, 46). In addition to serving as a substrate for protein synthesis, amino acids stimulate cell signaling pathways important in the regulation of translational efficiency (20, 25, 28). Under in vivo conditions and in the isolated perfused muscle preparation, amino acids enhance protein synthesis by increasing the rate of translation initiation (27, 30). Furthermore, the presence of the branched-chain amino acid leucine is largely responsible for a full complement of amino acids to increase the protein synthetic rate (5, 27, 46). However, there is no information on whether elevated lipids impair signal transduction in response to nutrient (e.g., amino acid) stimulation in skeletal muscle.

Regulatory control of translation initiation involves recruitment of the 43S preinitiation complex to the mRNA and is mediated by the eukaryotic initiation factor (eIF)4F complex (32). This complex is heterotrimeric, being composed of eIF4E, -4G, and -4A. During initiation, the eIF4E·mRNA complex binds to the scaffold protein eIF4G and the RNA helicase eIF4A to form the active eIF4F complex, thereby allowing translation to proceed. The binding of eIF4E to eIF4G is controlled in part by the cap-dependent translational repressor molecule eIF4E-binding protein (4E-BP)1. This binding

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Address for reprint requests and other correspondence: C. H. Lang, Dept. Cell. Molec. Physiology, H166, Penn State College of Medicine, 500 Univ. Drive, Hershey, PA (e-mail: clang@psu.edu).

protein acts as a molecular mimic of eIF4G and obstructs the interaction of eIF4G with eIF4E, limiting assembly of the active eIF4F complex (14). Hyperphosphorylation of 4E-BP1 liberates it from eIF4E and thereby facilitates binding of eIF4E with eIF4G. Elevating amino acids in general, leucine in particular, or IGF-I modulates eIF4F complex assembly by regulating the phosphorylation status of 4E-BP1 in cultured myocytes and in skeletal muscle in vivo (20, 22, 23, 24, 28). Leucine and IGF-I also phosphorylate and activate the Ser/Thr protein kinase S6K1 (14, 22, 47) and its physiologically relevant substrate, the ribosomal protein (rp)S6 (23, 24). Although the proximal elements of the canonical signaling pathways differ for nutrients and growth factors, these anabolic stimuli do apparently share common distal signaling intermediates (12, 14, 19, 20). Hence, it is unclear whether elevated FFA concentrations will have similar or differential effects on growth factor- and leucine-stimulated protein synthesis.

Therefore, on the basis of results from previous studies, we hypothesize that acute increases in the plasma concentration of FFAs will not alter muscle protein synthesis under basal conditions but impair the normal anabolic response produced by leucine and/or IGF-I. Because protein synthesis is primarily regulated at the level of peptide chain initiation, activation (e.g., phosphorylation) of key elements in the signal transduction pathway central to the control of translation initiation was also assessed.

MATERIALS AND METHODS

Animal preparation and experimental protocols. Male Sprague-Dawley rats weighing 200–225 g were purchased from Charles River Breeding Laboratories (Cambridge, MA). Rats were acclimated for 1 wk in a light-controlled room (12:12-h light-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 National Institutes of Health guidelines for the use of experimental animals.

On the day prior to the experiment, rats were anesthetized with intramuscular injections of ketamine and xylazine (90 and 9 mg/kg, respectively) and a jugular vein catheter surgically implanted under sterile conditions. Rats were provided water ad libitum and fasted overnight and were randomized into two groups the following morning. Half of the rats received an intravenous priming dose of heparin (10 U) followed by a constant intravenous infusion of 20% Intralipid plus 40 U heparin/h (1.2 ml/h). This regime increases plasma FFA levels above the physiological range and decreases insulin-mediated glucose uptake by skeletal muscle (33, 49). Time-matched control rats were infused intravenously with the same volume of saline. After 5 h, rats were administered either saline (0.115 mol/l) or 1.35 g/kg body wt leucine by oral gavage. This amount of leucine approximates that consumed in a 24-h period when rats of this age and strain are provided free access to chow (1). Furthermore, this amount of leucine increases muscle protein synthesis by stimulating translation initiation (1, 23, 25).

In a separate group of rats, we also evaluated the ability of elevated FFA levels to alter responses in translation initiation produced by IGF-I. This study used three groups of rats: control animals infused intravenously with saline for 5 h and then injected intravenously with either IGF-I (25 nmol/kg body wt) or an equivalent volume (0.5 ml) of isotonic saline, and rats infused intravenously with heparin plus Intralipid for 5 h followed by acute IGF-I stimulation. This dose of IGF-I transiently elevates the plasma IGF-I concentration to pharmacological levels and produces a robust phosphorylation of the IGF-I receptor with only negligible activation (e.g., phosphorylation) of the

insulin receptor (22). In this second study, rats infused with heparin plus Intralipid and then injected with saline were not included in the protocol because comparable data were obtained from the first experimental series.

In the first study only, rats were injected intravenously with L-[2,3,4,5,6-³H]phenylalanine (Phe; 150 mM, 30 Ci/ml; manufacturer-determined purity 99.8% by HPLC, 1 ml/100 g body wt) 10 min after administration of leucine or saline for the in vivo determination of muscle protein synthesis (23, 24, 26). Rats were then anesthetized with intravenous pentobarbital sodium, and the gastrocnemius muscle was freeze-clamped in vivo 10 min after injection of Phe. Thereafter, an arterial blood sample was collected from the abdominal aorta for measurement of plasma Phe-specific radioactivity and substrate concentrations. Blood was centrifuged, and plasma was collected. All tissue and plasma samples were stored at -70°C until they were analyzed. Muscle was powdered and a portion used to estimate the rate of incorporation of [³H]Phe into protein (23).

Plasma determinations. Blood was collected into heparinized syringes and centrifuged, and the plasma was collected for determination of insulin (Linco Research, St. Charles, MO) or total IGF-I by radioimmunoassay (23, 24, 26). Plasma FFA and triglyceride concentrations were measured colorimetrically (Wako Industrials, Osaka, Japan, and Boehringer Mannheim, Mannheim, Germany). The glucose concentration was determined using an Analox rapid analyzer (Lunenburg, MA). Amino acid concentrations were determined using reverse-phase HPLC after precolumn derivatization of amino acids with phenylisothiocyanate (26).

Immunoprecipitation and Western blotting. The tissue preparation was the same as previously described by our laboratory (22–26). Briefly, a homogenate of fresh muscle was prepared with a 1:5 ratio of ice-cold homogenization buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DTT, 0.1 mM PMSF, 1 mM benzamide, 0.5 mM sodium vanadate), using a Polytron homogenizer, and clarified by centrifugation (10,000 g). The supernatant was aliquoted into microcentrifuge tubes and 2 sample buffer (2 ml of 0.5 M Tris, pH 6.8, 2 ml of glycerol, 2 ml of 10% SDS, 0.2 ml of mercaptoethanol, 0.4 ml of a 4% solution of bromophenol blue, 1.4 ml of water to a final volume of 8 ml) added in a 1:1 ratio. The samples were boiled before being used for Western blot analysis. The samples were subjected to electrophoresis on either a 6% polyacrylamide gel for mammalian target of rapamycin (mTOR), 7.5% gel for S6K1 and tuberous sclerosis complex (TSC)2, a 12% gel for protein kinase B (PKB), and a 15% polyacrylamide gel for phosphorylated rpS6 and 4E-BP1. Proteins were electrophoretically transferred to nitrocellulose membranes. The blots were incubated with either primary antibodies to total and phosphorylated (Thr³⁰⁸ or Ser⁴⁷³) PKB (Cell Signaling Technology, Beverly, MA), total (C-20) and Thr¹⁴⁶²-phosphorylated tuberin (Cell Signaling), total S6K1 (no. 230; Santa Cruz Biotechnology, Santa Cruz, CA), phosphospecific S6K1 (Thr³⁸⁹; Cell Signaling Technology), total 4E-BP1 (Bethyl Laboratories, Montgomery, TX), phosphospecific 4E-BP1 (Thr³⁷/Thr⁴⁶; Cell Signaling), total and phosphorylated (Ser²⁴⁴⁸ and Ser²⁴⁸¹) mTOR (Bethyl Laboratories), total eIF4G (Cell Signaling), and total and phosphorylated S6 (Ser²³⁵/Ser²³⁶; Cell Signaling Technology). The blots were washed with Tris-buffered saline-Tween 20 (TBS-T; 1× TBS, including 0.1% Tween) and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit) at room temperature. The blots were developed with enhanced chemiluminescence Western blotting reagents according to the manufacturer's (Amersham) instructions. 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 (Molecular Dynamics ScanMaker IV) and analyzed using National Institutes of Health Image 1.6 software.

The 4E-BP1·eIF4E and eIF4G·eIF4E complexes were quantified as described previously (22–26). Briefly, eIF4E was immunoprecipitated from aliquots of supernatants using an anti-eIF4E monoclonal

antibody (Drs. Jefferson and Kimball; Pennsylvania State University College of Medicine, Hershey, PA). Antibody-antigen complexes were collected using magnetic beads and subjected to electrophoresis with the use of a 7.5 or a 15% polyacrylamide gel. Proteins were then electrophoretically transferred to a nitrocellulose membrane. The blots were incubated with a mouse anti-human eIF4E antibody, a rabbit anti-rat 4E-BP1 antibody, or a goat anti-eIF4G antibody. The phosphorylated forms of 4E-BP1 were measured after immunoprecipitation of 4E-BP1 from the tissue homogenates after centrifugation. The various phosphorylated forms of 4E-BP1 were separated by SDS-PAGE and analyzed by protein immunoblotting. The blots were then developed with enhanced chemiluminescence, and the autoradiographs were scanned for analysis as described above.

To measure tyrosine-phosphorylated IRS-1, serine-phosphorylated IRS-1, and total IRS-1, muscle homogenates were immunoprecipitated, and subsequent immunoblotting was performed. Gastrocnemius was homogenized in ice-cold lysis buffer, and the protein concentration was determined. IRS-1 protein was immunoprecipitated using anti-IRS-1 antibody (anti-rat COOH-terminal IRS-1; Upstate Biotechnology, Lake Placid, NY) and protein A-agarose (Upstate Biotechnology). After PAGE, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) and blotted with mouse monoclonal antiphosphotyrosine (PY-20; Transduction Laboratories, Lexington, KY) or IRS-1 serine phosphorylation measured using a site-specific antibody, phospho-Ser³⁰⁷ (49). Blots were stripped and reblotted with anti-IRS-1 antibody to assess total protein.

Statistical analysis. Experimental data for each condition are summarized as means \pm SE, where the number of animals for each treatment group is indicated in the legends to the figures or tables. Unless otherwise indicated, statistical evaluation of the data was performed using ANOVA followed by Student-Neuman-Keuls test to determine treatment effect. Differences between the groups were considered significant when $P < 0.05$.

RESULTS

Plasma substrate and hormone concentrations. The infusion of heparin plus Intralipid produced a pharmacological increase in the circulating concentration of FFAs of approximately sevenfold (Table 1). There was a concomitant elevation in the plasma triglyceride concentration in lipid-infused rats. Although oral leucine tended to decrease the plasma concentration of both FFAs and triglycerides in lipid-infused rats, this reduction did not achieve statistical significance. The plasma insulin concentration was comparable in control and lipid-infused rats under basal conditions. As expected, leucine significantly increased insulin in both groups; however, the magnitude of the elevation tended ($P > 0.05$) to be lower in the lipid-infused rats. Neither lipid infusion or oral leucine altered the plasma glucose concentration at the time point examined.

The plasma leucine concentration did not differ in saline- and lipid-infused rats under basal conditions, and the increase in

leucine was comparable in both groups administered the amino acid (Table 1). The plasma concentrations of other amino acids were also determined in saline- and lipid-infused rats (Table 2). Of the 18 amino acids quantified, only the plasma concentration of alanine was significantly reduced (18%).

Muscle protein synthesis. Under basal conditions (e.g., no leucine stimulation), global muscle protein synthesis was decreased 23% compared with time-matched control rats infused with saline (Fig. 1). In contrast, leucine increased protein synthesis in the gastrocnemius by 41% in saline-infused control rats. There was a comparable leucine-induced increase in protein synthesis in muscle of lipid-infused rats.

Alterations in eIF4E distribution. Protein synthesis is regulated, at least in part, by alterations in the rate of translation initiation (32). To determine whether elevated FFAs were associated with concomitant changes in peptide-chain initiation, relevant signal transduction pathways controlling translation initiation were investigated. In this regard, neither lipid infusion nor leucine significantly altered the total amount of eIF4E in gastrocnemius (Fig. 2C). In contrast to total eIF4E, lipid infusion acutely altered the distribution of eIF4E between the active eIF4E·eIF4G complex and the inactive eIF4E·4EBP1 complex. In control animals, Intralipid decreased the amount of eIF4E bound to eIF4G in muscle (35%; Fig. 2, A and D). Conversely, the lipid infusion reciprocally increased eIF4E bound to the translation repressor molecule 4E-BP1 (Fig. 2, B and E). Because the hyperphosphorylated γ -isoform of 4E-BP1 cannot bind to eIF4E, the eIF4E in the immunoprecipitate contains the two nonphosphorylated α - and β -isoforms of 4E-BP1 that are resolved as a doublet on Western blot analysis. As expected, oral leucine increased the amount of eIF4E·eIF4G and decreased eIF4E·4EBP1 in saline-infused rats. The magnitude of the leucine-induced change was comparable in saline- and lipid-infused rats.

To define the mechanism through which the lipid infusion modulates eIF4E availability, the phosphorylation state of 4E-BP1 was examined. Mitogen activation and amino acids stimulate hierarchical multisite phosphorylation of 4E-BP1, starting with Thr³⁷ and Thr⁴⁶ (10). This phosphorylation is a priming event that ultimately leads to the dissociation of 4E-BP1 from eIF4E, thereby permitting the binding of free eIF4E to eIF4G. Under control conditions, Intralipid did not alter 4E-BP1 phosphorylation (Fig. 3, A and B). Moreover, the incremental increase in 4E-BP1 phosphorylation seen in response to leucine was comparable in muscle from both saline- and lipid-infused rats. There was no lipid- or leucine-induced change in the total amount of 4E-BP1 (data not shown).

Table 1. Plasma concentrations of FFAs, triglycerides, insulin, leucine, and glucose

	Control		Intralipid + Heparin	
	Saline	Leucine	Saline	Leucine
FFA, meq/l	0.49 \pm 0.06 ^a	0.47 \pm 0.05 ^a	3.42 \pm 0.57 ^b	2.89 \pm 0.31 ^b
Triglyceride, mM	0.27 \pm 0.12 ^a	0.25 \pm 0.19 ^a	1.16 \pm 0.21 ^b	0.91 \pm 0.34 ^b
Insulin, pM	61 \pm 8 ^a	125 \pm 17 ^b	65 \pm 8 ^a	109 \pm 12 ^b
Leucine, μ mol/l	146 \pm 21 ^a	1,189 \pm 346 ^b	152 \pm 25 ^a	1,207 \pm 288 ^b
Glucose, mM	6.1 \pm 0.3 ^a	5.8 \pm 0.2 ^a	6.3 \pm 0.4 ^a	6.1 \pm 0.3 ^a

Values are means \pm SE; $n = 9$ –10/group. FFAs, free fatty acids. Means with different letters for specific parameters are statistically different from each other ($P < 0.05$).

Table 2. FFA-induced changes in plasma amino acid concentrations

Amino Acid	Control	Intralipid + Heparin
Alanine	334±23	272±21*
Glutamine	889±66	854±54
Glutamate	112±13	106±12
Valine	161±10	153±12
Isoleucine	94±8	91±11
Taurine	68±10	62±10
Tryptophan	9±2	10±3
Arginine	118±12	119±20
Aspartate	27±4	20±2
Asparagine	52±6	57±3
Threonine	172±14	168±20
Serine	123±17	127±11
Proline	124±19	143±20
Glycine	228±18	199±16
Ornithine	51±11	62±6
Methionine	44±5	47±5
Lysine	544±31	516±35
Histidine	87±11	79±5

Values are means ± SE in $\mu\text{mol/l}$; $n = 9/\text{group}$. Values for phenylalanine and tyrosine are not reported because they were artificially elevated by the bolus injection of the flooding dose of phenylalanine. Leucine values are provided in Table 1. Except for leucine, which appears in Table 1, amino acid concentrations were not determined on samples from the 2 leucine-treated groups because the short duration from the time of leucine administration to sampling (e.g., 20 min) was not expected to induce detectable changes in the plasma amino acid concentrations. * $P < 0.05$ vs. control value determined by 2-tailed Student's t -test.

The interaction between eIF4E and eIF4G may also be regulated, in part, by the phosphorylation of eIF4G, which is enhanced by mitogen stimulation and inhibited by the mTOR inhibitor rapamycin (37). The combination of heparin plus Intralipid decreased the constitutive content of Ser¹¹⁰⁸-phosphorylated eIF4G by 45% (Fig. 3, C and E). In contrast, leucine increased eIF4G phosphorylation threefold in both saline- and lipid-infused rats. Neither the lipid- or leucine-induced changes

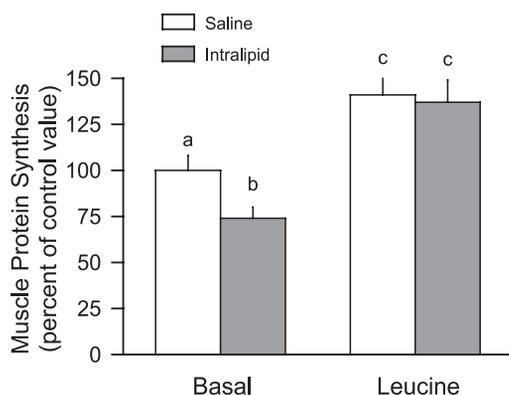


Fig. 1. Effect of short-term elevation of plasma free fatty acids (FFAs) on basal and leucine-stimulated protein synthesis in skeletal muscle. On the morning of the experiment, overnight-fasted rats were infused iv with heparin + Intralipid for 5 h prior to the oral administration of leucine. Protein synthetic rate was measured, 20 min after leucine administration, with incorporation of [³H]phenylalanine into proteins of gastrocnemius. Figs. 1–5 include data from 4 experimental groups of rats: iv saline infused + oral saline, iv saline infused + oral leucine, iv Intralipid infused + oral saline, and iv Intralipid infused + oral leucine. Values are means ± SE for 8–9 rats/group. Means with different letters are statistically different from each other ($P < 0.05$).

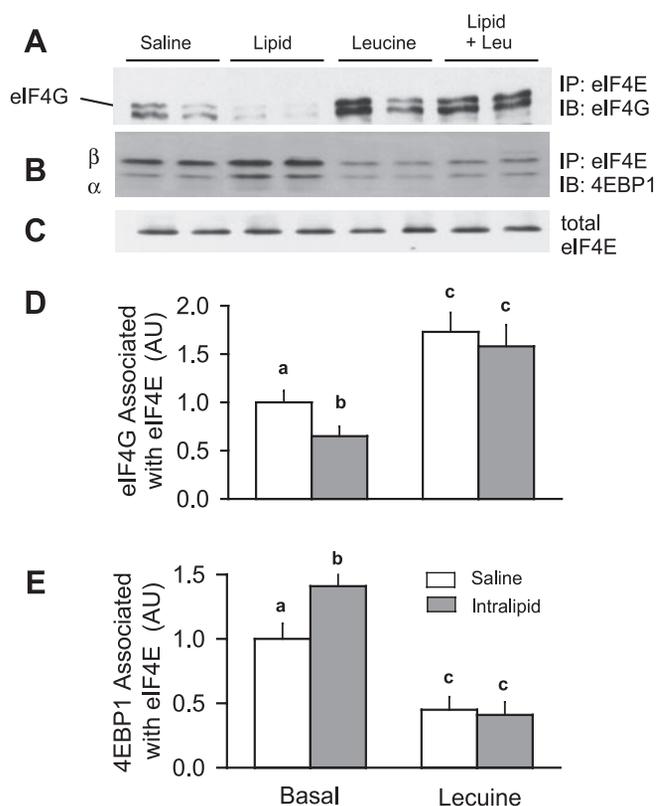


Fig. 2. Effect of short-term elevation of plasma FFAs on basal and leucine-induced changes in the association of eukaryotic initiation factor (eIF)4E with eIF4G and 4E-binding protein 1 (4E-BP1) in skeletal muscle. A: eIF4E was immunoprecipitated (IP), and the amount bound to eIF4G was assessed by immunoblotting (IB). B: representative immunoblot of 4E-BP1 bound to eIF4E in the immunoprecipitate. C: representative Western blot of total eIF4E. D: densitometric analysis of immunoblots of eIF4G associated with eIF4E, as described in MATERIALS AND METHODS. E: densitometric analysis of immunoblots of 4E-BP1 associated with eIF4E. For both bar graphs, the value from rats treated with iv saline + oral saline group was set at 1.0 arbitrary units (AU). Values are means ± SE; $n = 8-9/\text{group}$. Means with different letters are statistically different from each other ($P < 0.05$).

in eIF4G phosphorylation were attributable to a change in total eIF4G content (Fig. 3D).

S6K1, S6, and mTOR phosphorylation. S6K1 is a Thr/Ser kinase that under some, but not all, conditions is associated with increased translation of mRNAs containing a terminal oligopyrimidine (TOP) tract downstream of their transcription initiation site (8). Analysis of the multisite phosphorylation of S6K1 has previously indicated that its full activation is dependent upon phosphorylation of Thr³⁸⁹ (47). In muscle from saline-treated control rats, there was essentially no constitutive phosphorylation of Thr³⁸⁹ (Fig. 4A). However, there was a marked increase in S6K1 phosphorylation in muscle from control rats administered leucine. The magnitude of the leucine-induced increase in Thr³⁸⁹-phosphorylated S6K1 was not different between saline- and lipid-infused rats (Fig. 4A). Comparable changes were also detected in the phosphorylation of S6K1 at residues Thr⁴²¹ and Ser⁴²⁴, which are sites in the autoinhibitory domain of the enzyme (data not shown).

The phosphorylation state of the rpS6, a physiologically relevant S6K1 substrate, was also determined. The S6 protein is multiply phosphorylated on five Ser residues in an ordered manner (29). Using a phosphospecific antibody directed

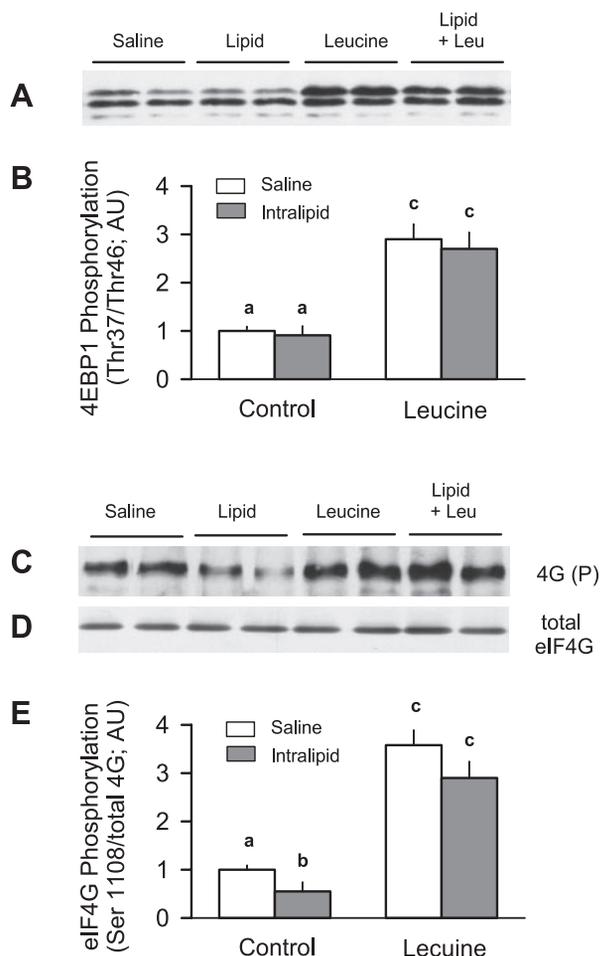


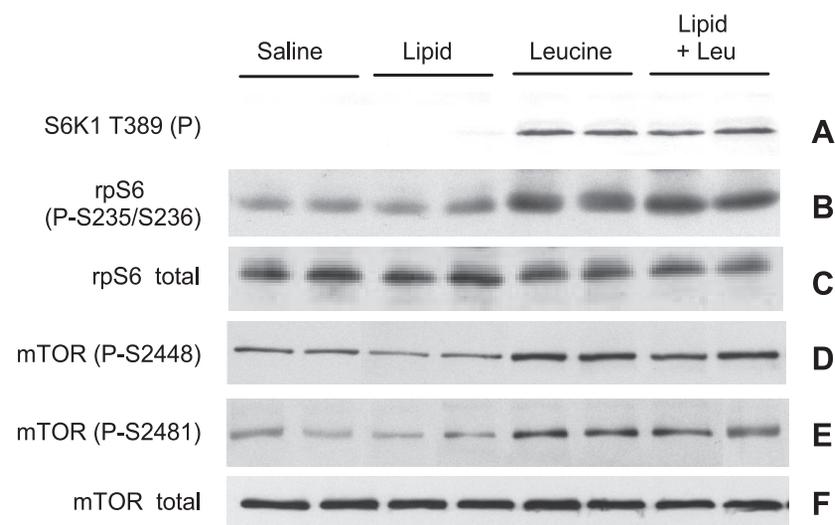
Fig. 3. Effect of short-term elevation of plasma FFAs on basal and leucine-induced changes in the phosphorylation state of 4E-BP1 and eIF4G in skeletal muscle. *A*: representative immunoblot of Thr³⁷/Thr⁴⁶-phosphorylated 4E-BP1. *B*: densitometric analysis of immunoblots of phosphorylated 4E-BP1. *C*: representative immunoblot of Ser¹¹⁰⁸-phosphorylated (P) and total eIF4G. *D*: representative immunoblot of total eIF4G. *E*: densitometric analysis of immunoblots of phosphorylated eIF4G normalized for the total amount of eIF4G. For both bar graphs, the value from rats treated with iv saline + oral saline group was set at 1.0 AU. Values are means \pm SE; $n = 8$ –9/group. Means with different letters are statistically different from each other ($P < 0.05$).

Fig. 4. Effect of short-term elevation of plasma FFAs on basal and leucine-induced changes in the phosphorylation of S6K1, ribosomal protein (rp)S6, and mTOR in skeletal muscle. *A*, *B*, and *C*: representative immunoblots for the effect of FFAs and leucine on phosphorylation (P) of the Thr³⁸⁹ site of S6K1, Ser²³⁵/Ser²³⁶ of rpS6, and total S6 protein, respectively. *D*, *E*, and *F*: representative immunoblots of Ser²⁴⁴⁸-phosphorylated mTOR, Ser²⁴⁸¹-phosphorylated mTOR, and total mTOR, respectively. Blots are representative of $n = 8$ –9 rats/group. For phosphorylated S6K1, rpS6, and mTOR there was no statistical difference ($P > 0.05$) between saline- and lipid-infused values under basal conditions. Additionally, whereas leucine significantly ($P < 0.05$) increased the phosphorylation of S6K1, rpS6, and mTOR, the increment was the same in saline- and lipid-infused rats (data not shown).

against the first and second phosphorylation sites, Ser²³⁶ and Ser²³⁵, a constitutive level of phosphorylation in muscle from control rats was detected (Fig. 4*B*). Short-term infusion of Intralipid did not alter the basal level of rpS6 phosphorylation. In response to leucine, rpS6 phosphorylation significantly increased three- to fourfold in gastrocnemius from both saline- and lipid-infused rats (Fig. 4*B*) and was independent of a change in total S6 protein (Fig. 4*C*).

The proline-directed Ser/Thr protein kinase, referred to as mTOR, regulates the phosphorylation of both 4E-BP1 and S6K1 by integrating signals from nutrients (e.g., amino acids and energy) and growth factors (11). Phosphorylation of mTOR on residues Ser²⁴⁴⁸ and Ser²⁴⁸¹ has been used as an index of mTOR activation. There was constitutive phosphorylation of mTOR (both Ser²⁴⁴⁸ and Ser²⁴⁸¹) in muscle from rats in the saline-saline group (Fig. 4, *D* and *E*, respectively). Lipid infusion tended to decrease mTOR phosphorylation, but because of the variability of the response, statistical significance was not achieved. Leucine administration approximately doubled the extent of mTOR phosphorylation in muscle from control rats. A comparable increase in phosphorylated mTOR was noted in gastrocnemius from Intralipid-treated rats in response to leucine. Neither Intralipid or leucine altered the total amount of total mTOR protein in muscle (Fig. 4*F*).

Intralipid-induced changes in IGF-I signal transduction. We (23, 24) previously demonstrated that other conditions can differentially effect the ability of nutrients (e.g., leucine) and growth factors (e.g., IGF-I or insulin) to regulate signaling pathways important for translation initiation. Hence, in the second study, the ability of elevated plasma FFA concentrations to suppress IGF-I-stimulated translation initiation in skeletal muscle was assessed. As illustrated in Fig. 5, in control rats IGF-I acutely increased the amount of the active eIF4E·eIF4G complex and decreased the inactive eIF4E·4E-BP1 complex. These changes were associated with an increased phosphorylation of both 4E-BP1 and eIF4G. Additionally, IGF-I also increased the phosphorylation of S6K1 and rpS6. All of these responses are consistent with previous observations in muscle (22–24). However, in lipid-infused rats, the ability of IGF-I to alter eIF4E distribution between the active and inactive complex was attenuated, as was the increased phosphorylation of



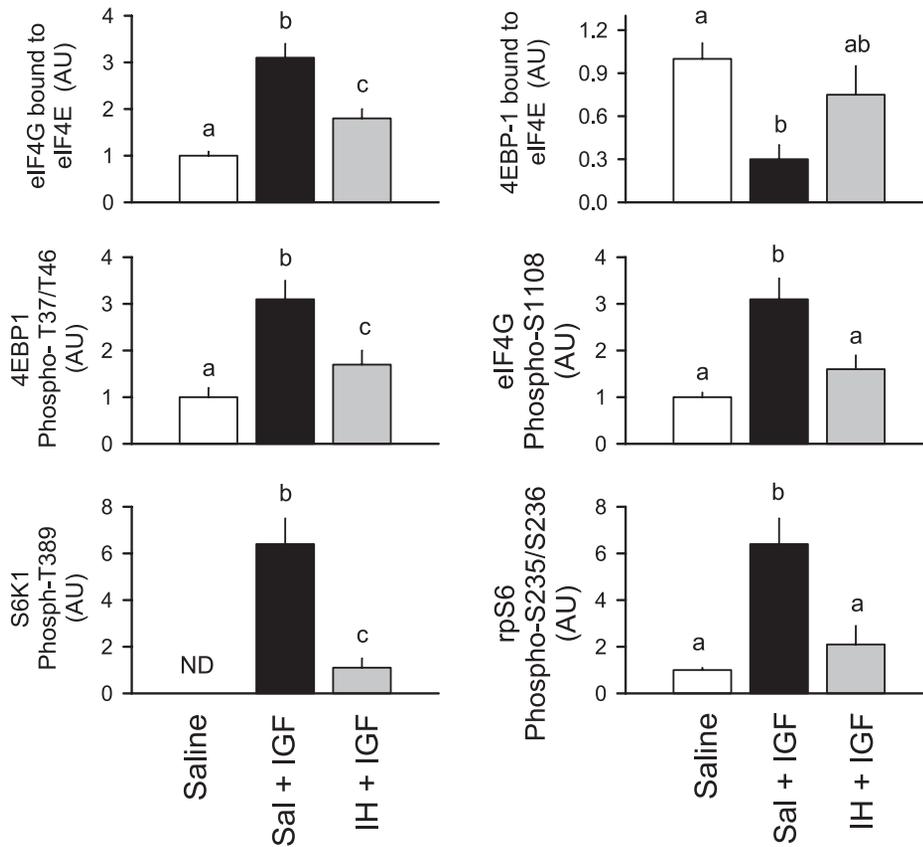


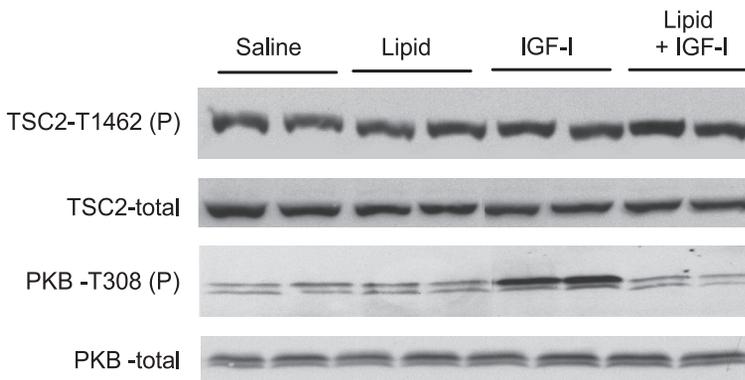
Fig. 5. Short-term elevation of plasma FFAs blunts changes in signal transduction pathways for protein synthesis produced by insulin-like growth factor I (IGF-I). The 3 experimental groups used in this separate study include animals infused iv with saline for 5 h and then administered either a bolus iv injection of IGF-I or saline (Sal), and rats infused with Intralipid + heparin (IH) and then challenged with IGF-I. Gastrocnemius was excised 20 min after IGF-I administration. Bar graphs quantify densitometric analysis of distribution of eIF4E between eIF4G and 4E-BP1 as well as the phosphorylation states of 4EBP1, eIF4G, S6K1, and rpS6. Value from control rats treated with saline + saline was set at 1.0 AU. Values are means \pm SE; $n = 7-8$ rats/group. Means with different letters are statistically different from each other ($P < 0.05$). ND, not detectable.

4E-BP1. In addition, lipid infusion essentially completely prevented the IGF-I-induced phosphorylation of eIF4G, S6K1, rpS6, and mTOR (data not shown). These differences in IGF-I action between saline- and lipid-infused rats were not due to differences in the prevailing concentration of total IGF-I (saline + IGF-I = 824 ± 55 ng/ml vs. Intralipid + IGF-I = 847 ± 61 ng/ml; $P =$ not significant).

One potential mechanism by which mTOR might mediate the phosphorylation of S6K1 and 4E-BP1 in response to nutrients and growth factors is by altering the activity of the TSC, consisting of the TSC1 (hamartin) and TSC2 (tuberin) proteins (38). The TSC complex functions as a suppressor of mTOR activity. Data presented in Fig. 6, A and B, indicate that neither the lipid infusion or IGF-I consistently altered either the total amount or the extent of Thr¹⁴⁶²-phosphorylation of TSC2 in skeletal muscle at the time point assessed. Because only a single time point was assessed for

TSC2 phosphorylation, we cannot exclude the possibility that a transient response was missed.

PKB is believed to lie upstream of mTOR and to be activated by phosphorylation of two critical Ser/Thr residues (e.g., Thr³⁰⁸ and Ser⁴⁷³). Furthermore, PKB also appears to be the kinase responsible for TSC2 phosphorylation (15). Activation of PKB was determined by immunoblotting, with phospho-specific antibodies recognizing the T-loop phosphoinositide-dependent kinase 1 phosphorylation site (Thr³⁰⁸) as well as the hydrophobic motif (Ser⁴⁷³). Figure 6, C and D, illustrates the total amount of PKB, and the extent of Thr³⁰⁸ phosphorylation in muscle was not altered by lipid infusion alone. In contrast, the ability of IGF-I to stimulate Thr³⁰⁸ phosphorylation of PKB was impaired in lipid-infused rats. Although phosphorylation at Thr³⁰⁸ partially activates PKB, full activation of PKB requires phosphorylation on a second site (Ser⁴⁷³



A
B
C
D

Fig. 6. Effect of short-term elevation of plasma FFAs on the phosphorylation of PKB and tuberous sclerosis complex 2 (TSC2) in skeletal muscle under basal conditions and in response to IGF-I. A and B: representative immunoblot of Thr¹⁴⁶²-P and total TSC2, respectively. C and D: representative immunoblot of Thr³⁰⁸-phosphorylated and total PKB, respectively. Blots are representative of $n = 7$ /group. There was no statistically significant change in either total or phosphorylated TSC2 or PKB in skeletal muscle in response to lipid infusion. However, IGF-I increased PKB phosphorylation in control rats at the same time point (control + saline = 1.0 ± 0.14 AU vs. control + IGF-I = 2.80 ± 0.34 AU, $P < 0.05$), and this increase was markedly blunted in lipid-infused rats receiving IGF-I (1.3 ± 0.33 AU; $P < 0.05$ vs. control + IGF-I).

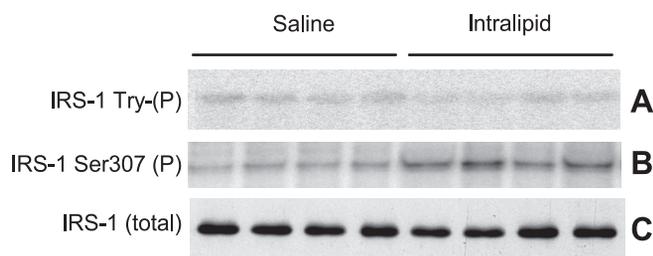


Fig. 7. Effect of elevated plasma FFAs on the tyrosine and serine phosphorylation of insulin receptor substrate-1 (IRS-1) in skeletal muscle. Muscle homogenates were IP, and IRS-1 was immunoblotted for either tyrosine phosphorylation (A), Ser³⁰⁷ phosphorylation (B), or total protein (C). Samples are from 4 control rats and 4 rats infused for 5 h with heparin + Intralipid. Data are representative of $n = 7-8$ /group. Intralipid significantly ($P < 0.05$) increased IRS-1 Ser³⁰⁷ phosphorylation (2.11 ± 0.31 AU) compared with control values (1.00 ± 0.09 AU).

in PKB α /Akt1) located in the regulatory tail of the protein. Elevation of plasma FFAs also coordinately downregulated Ser⁴⁷³-phosphorylated PKB in response to IGF-I (data not shown).

Finally, to address the mechanism by which the lipid infusion impaired IGF-I action on protein-synthetic signaling pathways, we assessed the extent of tyrosine and serine phosphorylation of IRS-1. Figure 7 illustrates that there was a low basal expression of tyrosine-phosphorylated IRS-1 in saline-infused control rats. Because of the low constitutive expression of tyrosine-phosphorylated IRS-1 in muscle under postabsorptive conditions, it was not possible to detect any consistent change produced by Intralipid. In contrast, Ser³⁰⁷ phosphorylation of IRS-1 was increased approximately twofold in muscle from lipid-infused rats compared with time-matched control animals infused with saline.

DISCUSSION

The present data demonstrate that elevating the plasma FFA concentration above the physiological range decreases muscle protein synthesis in the basal postabsorptive state. This result was largely unexpected because previous work by others (45) failed to detect a change in the rate of whole body nonoxidative leucine disappearance in response to elevated FFAs. Moreover, no significant change in skeletal muscle protein synthesis was detected in studies where FFA oxidation was reduced (6) or in the isolated hindlimb perfused with buffer containing palmitate, instead of glucose, as the sole substrate (16, 36). The lipid-induced decrease in basal muscle protein synthesis does not appear to be caused by a reduction in amino acid availability because the plasma concentrations of all amino acids, with the exception of alanine, were well maintained. These data differ from those reported previously by Ferrannini et al. (9), where a generalized hypoaminoacidemia was observed in response to lipid infusion. There are several important differences between this published report and the present data: 1) species differences (humans vs rats), 2) different duration of lipid infusion (7 vs. 5 h), and 3) the presence of mild hyperinsulinemia in humans in response to the lipid infusion and the lack of change in plasma insulin in rats. Regardless of the reason for the difference, the ability of elevated FFAs to decrease basal muscle protein synthesis in rats appears independently of a significant change in either amino acid availability or the prevailing insulin concentration.

Although protein synthesis is a multistep process, it is most often regulated at the level of translation initiation. Hence, our study aimed to elucidate the mechanism for the lipid-induced decrease in protein synthesis by examining known regulatory elements of this pathway. During mRNA translation, the eIF4E·mRNA complex binds to eIF4G and eIF4A to form the active eIF4F complex (32). One mechanism for modulating the formation of the eIF4F complex is by altering the distribution of eIF4E between inactive and active protein complexes. Our data clearly demonstrate the FFA-induced decrease in protein synthesis is associated with altered distribution of eIF4E characterized by a decreased binding of eIF4E to eIF4G (e.g., less active complex) and increased binding to 4E-BP1 (e.g., more inactive complex). Such a redistribution of eIF4E has also been reported in catabolic conditions manifesting a decrease in protein synthesis (20, 23, 24, 25, 40).

The assembly of the functional eIF4F complex is controlled in part by 4E-BP1, which functions as a cap-dependent translational repressor (18). Changes in the phosphorylation of 4E-BP1 are often directly related to changes in protein synthesis and translation initiation. For example, nutrients and mitogens increase 4E-BP1 phosphorylation, whereas catabolic stimuli, such as glucocorticoids and inflammatory cytokines, generally decrease phosphorylation (19, 26, 40). However, unexpectedly, the redistribution of eIF4E and reduced amount of the active eIF4F complex produced by elevated FFAs were independent of a change in 4E-BP1 phosphorylation. Alternatively, the phosphorylation state of eIF4G represents a secondary mechanism possibly regulating the interaction between eIF4E and eIF4G (31). In this regard, treatment with rapamycin, an mTOR inhibitor, decreases eIF4G phosphorylation and translation, whereas nutrient and growth factor stimulation reciprocally increase the phosphorylation of this protein (37). In the present study, the decreased muscle protein synthesis and eIF4F complex formation seen in muscle from lipid-infused rats were associated with a reduction in Ser¹¹⁰⁸-phosphorylated eIF4G. Hence, these data support the concept that a reduction in protein synthesis may occur independently of a change in 4E-BP1 phosphorylation and instead be dependent upon changes in the phosphorylation state of eIF4G.

The phosphorylation and activation of S6K1 may be an important element of the protein-synthetic signaling pathway because it phosphorylates a number of proteins, including the small ribosomal subunit protein S6 (8). The phosphorylation of S6K1 and rpS6 is tightly associated with accelerated rates of mRNA translation initiation and a stimulation of skeletal muscle protein synthesis under *in vivo* conditions. However, other data indicate that rpS6 is not essential for the translation of 5'-TOP mRNAs under some experimental conditions where components of the translational apparatus have been either overexpressed or deleted (34, 41, 43). In the basal condition, there was no consistent change in the constitutive phosphorylation of rpS6 in muscle from lipid-infused rats. These data are internally consistent with our finding that constitutive phosphorylation of mTOR was also not altered by elevated FFA concentrations. Because mTOR represents the bifurcation point in the distal signaling pathways between the phosphorylation of 4E-BP1 and S6K1, the lipid-induced decrease in muscle protein synthesis appears to be independent of changes in mTOR activity. These data are particularly noteworthy because other conditions that impair muscle protein synthesis

appear to be largely mediated via mTOR-dependent mechanisms (23–25, 40).

It has been previously demonstrated that either a mixture of amino acids (17) or leucine alone (25) is sufficient to increase muscle protein synthesis in fasted naive control rats. This increase results from a stimulation of translation initiation and was associated with an altered availability of eIF4E, as evidenced by the increased amount of this initiation factor bound to eIF4G and the decreased amount of eIF4E bound to the translational repressor molecule 4E-BP1. This redistribution of eIF4E was associated with the hyperphosphorylation of 4E-BP1. In addition, in muscle from control rats, leucine also increased the phosphorylation of eIF4G, mTOR, S6K1, and rpS6. These changes in eIF4F complex assembly are consistent with results from earlier reports (23–25, 40). In contrast to the above-mentioned changes in translation initiation observed in the basal state in response to elevated FFAs, short-term lipid infusion did not significantly alter the anabolic response of muscle to oral leucine. Muscle from lipid-infused rats receiving leucine demonstrated an increase in protein synthesis as well as changes in eIF4E availability and S6K1/S6 phosphorylation that were comparable to the response observed in saline-infused rats. Hence, there is no evidence that elevated plasma lipid levels produce a leucine resistance in skeletal muscle.

It is well accepted that elevated circulating FFAs impair the ability of insulin to stimulate glucose transport and intracellular glucose metabolism in skeletal muscle (3, 33, 35, 39, 49). However, whether elevated FFA concentrations can also impair growth factor-stimulated increases in protein synthesis has not been investigated. The exogenous administration of IGF-I increases muscle protein synthesis (2, 23) because of the enhanced rate of peptide-chain initiation (2, 22–25). The accelerated rate of protein synthesis in response to IGF-I has been attributed to the concomitant increase in the amount of active eIF4E·eIF4G complex and the increased phosphorylation and activation of S6K1 and rpS6. Our data clearly demonstrate that lipid infusion markedly impairs the IGF-I-induced activation of selected end points of cell signaling relevant to the translational control of protein synthesis. In this regard, the short-term elevation of FFAs blunted the increase in eIF4E·eIF4G and decrease in eIF4E·4E-BP1 observed in response to IGF-I. The decreased formation of the active eIF4F complex was associated with a reduced phosphorylation of 4E-BP1, eIF4G, and mTOR. Moreover, elevated FFA concentrations essentially completely prevented the normal increase in both S6K1 and rpS6 phosphorylation. Collectively, these data indicate the presence of severe IGF-I resistance in skeletal muscle of lipid-infused rats, suggesting that short-term elevation of FFAs impairs the ability of IGF-I to stimulate cap-dependent translation as well as possibly translation of those mRNAs containing TOP sequences at their 5'-end.

The TSC1/2 heterodimer appears to function primarily in an mTOR-dependent manner to regulate phosphorylation of S6K1 and 4E-BP1 (38). Alterations in the amount of TSC1/2 appear inversely proportional to the extent of phosphorylation of these downstream signaling intermediates (46). Our data indicate that lipid infusion did not alter the constitutive phosphorylation of TSC1/2, and this finding is consistent with the lack of a detectable change in the phosphorylation states of mTOR, 4E-BP1, and S6K1 under basal conditions. Moreover, although

lipid-infusion impaired the ability of IGF-I to stimulate PKB phosphorylation, we detected no consistent alteration in Thr¹⁴⁶² phosphorylation of TSC2 under these conditions. In contrast, both insulin and IGF-I have been reported to stimulate TSC2 phosphorylation in HeLa cells by a PI 3-kinase-dependent mechanism (7). Hence, we were unable to confirm a similar response in skeletal muscle when IGF-I was administered in vivo.

For end points related to carbohydrate metabolism, lipid infusion produces insulin resistance by decreasing IRS-1 phosphorylation, IRS-1-associated PI 3-kinase activity, and PKC-2 activation (4, 21). Moreover, elevated plasma FFAs have also been reported (49) to increase Ser³⁰⁷ phosphorylation of IRS-1. Numerous lines of evidence support a comparable mechanism mediating the insulin resistance produced by the inflammatory cytokine tumor necrosis factor- α . (48). Our data confirm that the short-term infusion of Intralipid increases Ser-phosphorylated IRS-1 in skeletal muscle.

In conclusion, novel data are provided demonstrating the ability of elevated FFA concentrations to decrease basal muscle protein synthesis. This impairment is independent of a change in the prevailing plasma insulin concentration and appears mediated by defects in the formation of a functional eIF4F complex. Moreover, the redistribution of eIF4E from the active to inactive protein complex appears independent of a change in 4E-BP1 phosphorylation but may be dependent upon a reduced phosphorylation of eIF4G. In addition, elevated FFAs did not appear to alter the phosphorylation of either mTOR or S6K1 under basal conditions. Finally, hyperlipidemia selectively impaired signaling pathways central to the increased translation initiation in response to the anabolic hormone IGF-I but not from the nutrient signal leucine.

ACKNOWLEDGMENTS

We express thanks to Drs. Leonard S. Jefferson and Scot R. Kimball (Pennsylvania State University College of Medicine) for the eIF4E antibody. Additionally, we thank Anne Pruznak and Nobuko Deshpande for expert technical assistance.

GRANTS

This work was supported in part by National Institute of General Medical Sciences Grant GM-38032 and AA-11290.

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