Glucocorticoids oppose translational control by leucine in skeletal muscle

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Shah, O. Jameel, Joshua C. Anthony, Scot R. Kimball, and Leonard S. Jefferson. Glucocorticoids oppose translational control by leucine in skeletal muscle. Am J Physiol Endocrinol Metab 279: E1185–E1190, 2000.—Glucocorticoids comprise an important class of hormonal mediators of fuel and protein homeostasis in normal and pathological scenarios. In skeletal muscle, exposure to glucocorticoids is characterized by a reduction in protein synthetic rate coincident with hampered translation initiation. However, it is unclear whether this involves attenuation of anabolic stimuli or is simply due to inhibition of the basally activated translational apparatus. Therefore, this inquiry was designed to determine whether leucine, administered orally, could rescue the translational inhibition induced by glucocorticoids. Dexamethasone, injected intraperitoneally, acutely diminished protein synthetic rates to 80% of control values in skeletal muscle from rat hindlimb. The eukaryotic initiation factor (eIF)4 regulatory element was simultaneously and negatively impacted via sequestration of eIF4E by the hypophosphorylated form of the translational suppressor, eIF4E binding protein 1 (4E-BP1). The 70-kDa ribosomal protein S6 kinase (S6K1) was also dephosphorylated, notably at T389, in response to glucocorticoids. Leucine, administered orally, effectively restored each aforementioned translational parameter to control levels. Inasmuch as leucine’s potency in modulation of the translational machinery, and indeed of protein turnover in general, is widely appreciated, this amino acid may prove useful in normalizing the impairment of mRNA translation associated with various muscle-wasting pathologies, such as glucocorticoid excess.


The eIF4 family of eukaryotic initiation factors is recognized as a critical regulatory element governing the initiation of mRNA translation (reviewed in Ref. 16). The speed at which ribosomes engage mRNA for subsequent peptide synthesis is governed by the efficiency of the translation initiation cycle, which, under some circumstances, is determined by the availability of the mRNA cap recognition factor, eIF4E. A family of translational repressors, the 4E-BPs, are phosphoproteins that, when hypophosphorylated, competitively exclude eIF4E from the remainder of the translational apparatus. However, the inhibition of eIF4E imposed by the 4E-BPs is relinquished on 4E-BP phosphorylation, which liberates eIF4E and facilitates assembly of the heterotrimmeric (eIF4E, eIF4G, and eIF4A) initiation complex known as eIF4F. Glucocorticoids markedly suppress protein synthetic rate in skeletal muscle concomitant with dephosphorylation of 4E-BP1, which is the predominant 4E-BP isoform in this tissue, as well as disassembly of eIF4F (24). S6K1 is similarly impacted, in that exposure to glucocorticoids induces a robust dephosphorylation and, thereby, inactivation of the kinase (18). S6K1 phosphorylates ribosomal proteins such as S6 (13) and S17 (19); importantly, S6K1-mediated phosphorylation of S6 at five carboxy-terminal serine residues may serve to select those mRNAs encoding elements of the translational machinery and thereby influence the overall protein synthetic capacity of the cell (11, 12).
Amino acid sufficiency is demonstrably requisite for efficient propagation of modulatory signals to the translational apparatus. Although various physiological roles have been ascribed to individual amino acids, the branched-chain group, and particularly leucine, has been implicated in the regulation of protein metabolism (2, 17, 29). Indeed, leucine, through as yet undiscovered mechanisms, activates an intracellular signal transduction pathway that requires the protein kinase activity associated with, or intrinsic to, the mammalian target of rapamycin (mTOR) (9). mTOR is a component of an ancient nutrient-sensing pathway that, in mammalian cells, regulates activation of S6K1 and eIF4E availability [the latter through phosphorylation of 4E-BPs (reviewed in Refs. 3 and 28)]. Hence, the mTOR signaling module has emerged as a central element in the regulation of translational homeostasis (Fig. 1).

Leucine, administered as an oral bolus, completely reverses the reduction in protein synthetic rate induced by fasting, an effect that appears to be mediated by eIF4F and not eIF2B (1). Along similar lines, glucocorticoids negatively affect protein synthetic rates in skeletal muscle primarily through inhibition of eIF4F and independently of eIF2B (24). Thus potential counterregulatory control of translation initiation by leucine and glucocorticoids is predicted to occur largely via their respective influences on the assembly of eIF4F. Therefore, we sought to determine whether the adverse effects of glucocorticoids on the eIF4 system could be corrected by oral leucine supplementation and, moreover, whether a parallel restoration of protein synthesis was attainable.

MATERIALS AND METHODS

Materials. Mouse monoclonal anti-eIF4E antibody was raised against recombinant human eIF4E by the method described earlier (14). Rabbit polyclonal anti-eIF4G antibody was raised against recombinant human eIF4G by the method described earlier (14). Rabbit polyclonal anti-S6K1, anti-phospho-T389-S6K1, and anti-4E-BP1 antibodies were purchased from Santa Cruz Biotechnology. Polyvinylidene difluoride membranes were purchased from Bio-Rad. Enhanced chemiluminescence detection reagents and horseradish peroxide-conjugated sheep anti-mouse and donkey anti-rabbit Igs were purchased from Amersham Life Sciences.

Animals. The animal facilities and protocol were reviewed and approved by the Animal Care and Use Committee of The Pennsylvania State University College of Medicine. Male Sprague-Dawley rats weighing 200–300 g were maintained on a 12:12 h light-dark cycle and allowed free access to food (Harlan-Teklad Rodent Chow, Madison, WI) and water.

Rats were given dexamethasone sodium phosphate (American Reagent Laboratories, Shirley, NY; 100 μg/100 g body wt ip) or an equal volume of vehicle (0.15 M NaCl). Three hours after drug administration, one-half of the control and one-half of the dexamethasone-treated group received an intragastric bolus of 2.5 ml/100 g body wt L-leucine (54.0 g/l) in distilled water.

Administration of metabolic tracer. A metabolic tracer consisting of a flooding dose (1 ml/100 g body wt) of L-[2,3,4,5,6-3H]phenylalanine (150 mM containing 100 μCi/ml) was injected via the tail vein 50 min after leucine administration for the measurement of synthesis of total mixed proteins in skeletal muscle, as described previously (6). Exactly 1 h after leucine administration, animals were killed by decapitation. Trunk blood was collected and centrifuged at 1,800 g for 10 min to obtain serum. The right gastrocnemius and plantaris muscles were excised as a unit 10 min after administration of the radiolabeled amino acid into protein. The contralateral muscles were similarly excised, weighed, and homogenized in seven volumes of buffer A (20 mM HEPES, pH 7.4, 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 50 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM sodium vanadate). The homogenate was immediately centrifuged at 10,000 g for 10 min at 4°C. The supernatant was reserved for Western analysis as described below. All serum and tissue samples were stored at −80°C.

Determination of rates of protein synthesis. Fractional rates of protein synthesis were estimated on the basis of the incorporation of [3H]phenylalanine into muscle proteins, with the specific radioactivity of serum phenylalanine as representative of the precursor pool (15). The elapsed time from injection of the metabolic tracer until freezing of muscle in liquid nitrogen was recorded as the actual time for incorporation of the radiolabeled amino acid into protein.

Quantitation of eIF4E, 4E-BP1-eIF4E, and eIF4G-eIF4E complexes. Quantitation of the respective factors and complexes was carried out exactly as outlined previously (31).
Quantitation of phosphorylated and unphosphorylated 4E-BP1 in skeletal muscle homogenates. Quantitation of the phosphorylation state of 4E-BP1 was carried out exactly as described elsewhere (31). The data were expressed as the percentage of total 4E-BP1 in the \( g \)-form.

Quantitation of phosphorylation state of S6K1. Whole muscle homogenates were centrifuged at 10,000 \( g \) for 10 min at 4°C. The supernatants were collected and subjected to SDS-PAGE and Western blotting essentially as outlined above using a rabbit polyclonal anti-S6K1 antibody. In a manner analogous to 4E-BP1, S6K1 resolves into multiple electrophoretic forms after SDS-PAGE, wherein increasing phosphorylation retards mobility (8). Therefore, more slowly migrating species generally reflect a greater degree of phosphorylation and thus activation. The data were expressed as the percentage of the total S6K1 pool in the hyperphosphorylated, i.e., non-\( a \), forms. Therefore, the data are representative of S6K1 phosphorylation.

Statistical analysis. All data were analyzed using one-way ANOVA and Tukey's posttest comparisons. Statistical significance was defined as \( P < 0.05 \).

RESULTS

Glucocorticoids and the branched-chain amino acid leucine, despite their opposing influences on the protein synthetic apparatus, are similar with regard to selection of regulatory targets, namely, eIF4F. In vivo, neither acute glucocorticoid exposure (24) nor leucine administration (1) affects other known mechanisms involved in regulation of translation initiation such as the phosphorylation state of the \( \alpha \)-subunit of eIF2 or the catalytic activity of eIF2B. Four hours subsequent to intraperitoneal injection of dexamethasone, protein synthetic rates were reduced to 80% of that recorded in control, saline-injected rats (Table 1); this effect was

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein Synthetic Rate, nmol Phe-mg protein(^{-1})-h(^{-1} )</th>
<th>Serum Insulin, ng/ml</th>
<th>Serum Glucose, mM</th>
<th>Serum Leucine, ( \mu )M</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.31 ± 0.05</td>
<td>1.93 ± 0.15</td>
<td>12.83 ± 0.39†‡</td>
<td>159.6 ± 6.92*</td>
</tr>
<tr>
<td>Dex</td>
<td>1.05 ± 0.03</td>
<td>1.55 ± 0.22</td>
<td>14.25 ± 0.48*</td>
<td>264.0 ± 28.38*</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.47 ± 0.21</td>
<td>2.57 ± 0.32</td>
<td>11.10 ± 0.33†</td>
<td>915.1 ± 101.0†</td>
</tr>
<tr>
<td>Dex + Leucine</td>
<td>1.33 ± 0.08</td>
<td>2.69 ± 0.55</td>
<td>12.23 ± 0.37†</td>
<td>1089.4 ± 103.4†</td>
</tr>
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Values are means ± SE of 6 animals/group for protein synthetic rate, serum insulin, and serum leucine and 3 animals/group for serum glucose. Dex, dexamethasone. Means not sharing the same superscript (*, †) display significant difference using one-way ANOVA and Tukey's posttest comparisons.

Fig. 2. Effects of dexamethasone and leucine on phosphorylation status of 4E-BP1. Rats were administered saline, dexamethasone, and/or leucine and then killed. Supernatants (10,000 \( g \)) were subjected to SDS-PAGE and immunoblotting with anti-4E-BP1 antibody. A: representative immunoblot of 10,000-\( g \) supernatants using anti-4E-BP1 antibody. B: each resultant electrophoretic species was densitometrically quantitated; data are expressed as the ratio of 4E-BP1-\( \gamma \) to total 4E-BP1 in arbitrary units. Values are means ± SE; \( n = 6 \) for each condition.

Fig. 3. Effects of dexamethasone and leucine on stability of the eIF4E-4E-BP1 complex. Rats were administered saline, dexamethasone, and/or leucine and then killed. eIF4E was immunoprecipitated from muscle-derived 10,000-\( g \) supernatants using anti-eIF4E antibody. A: immune complex components were separated by SDS-PAGE and subsequently immunoblotted with anti-eIF4E and anti-4E-BP1 antibodies; representative immunoblots are shown. B: respective signals were quantitated and expressed in arbitrary units as the 4E-BP1-to-eIF4E ratio in eIF4E immunoprecipitates. Values are means ± SE; \( n = 6 \) for each condition. Means not sharing the same superscript (a and b) display significant difference using one-way ANOVA and Tukey's posttest comparisons.
entirely reversed after administration of leucine. As reported previously (25), serum insulin concentrations were not significantly altered by any treatment, although administration of leucine slightly reduced circulating concentrations of glucose in dexamethasone-treated animals (Table 1).

To address the mechanistic nature of opposing translational control by dexamethasone and leucine, known regulatory processes involved in mRNA translation were examined. eIF4E recognizes the 5'-methylguanosine cap at the 5' terminus of most eukaryotic transcripts and thereby targets these mRNAs to the ribosome. However, the facility of eIF4E in this process is modulated by phosphorylation of 4E-BPs, which, when hypophosphorylated, sequester eIF4E from the translational machinery, whereas the hyperphosphorylated 4E-BPs lack significant affinity for eIF4E. Whereas dexamethasone diminished the proportion of 4E-BP1 in the highly phosphorylated γ-form, i.e., the disinhibited form, to ~60% of control values, leucine corrected 4E-BP1 dephosphorylation by glucocorticoids, as evidenced by restoration of the percentage of 4E-BP1-γ to near that of the control animal (Fig. 2).

Both 4E-BPs and eIF4G display homology within an eIF4E recognition sequence that renders the association of these factors with eIF4E mutually exclusive. Immunoprecipitation of eIF4E reveals that, although glucocorticoids increase by 50% its association with eIF4G, from muscle-derived 10,000-g supernatants using anti-eIF4E antibody. A: immune complex components were separated by SDS-PAGE and subsequently immunoblotted with anti-eIF4E and anti-eIF4G antibodies; representative immunoblots are shown. B: respective signals were quantitated and expressed in arbitrary units as the eIF4G-to-eIF4E ratio in eIF4E immunoprecipitates. Values are means ± SE; n = 6 for each condition.

4E-BP1 and S6K1 are commonly inhibited by rapamycin and are coordinately regulated under many circumstances. Because S6K1 affects the translation of mRNAs encoding components of the protein synthetic apparatus, such as ribosomal proteins and elongation factors, the phosphorylation state of S6K1 under these conditions was evaluated. In dexamethasone-treated animals, total S6K1 exhibited a bias toward faster mobility as visualized by SDS-PAGE and immunoblotting (Fig. 5A). Since the rate of migration of the kinase is inversely proportional to its degree of phosphorylation (8), these results suggest that glucocorticoids induce dephosphorylation of the enzyme. Indeed, the proportion of S6K1 existing in hyperphosphorylated species (relative to S6K1-α) diminished appreciably in response to glucocorticoids (Fig. 5), whereas leucine exerted an opposing effect. Moreover, the glucocorticoid-induced disappearance of slower electrophoretic species was associated with dephosphorylation of S6K1 at T389 (Fig. 5A), a site at which phosphorylation...
heralds full enzyme activation (3, 30). Administration of leucine after dexamethasone completely reversed the glucocorticoid-induced dephosphorylation of the kinase and returned phospho-T389 immunoreactivity to control levels (Fig. 5).

DISCUSSION

Although the drastic adverse effects of glucocorticoids on protein metabolism, particularly in skeletal muscle, have long been recognized, relatively little information is available regarding the intracellular events involved in this process. The action of glucocorticoids, and indeed many other steroid hormones, is mediated primarily via specific, ligand-activated intracellular receptors. The recognition of glucocorticoid by its receptor (GR) induces a conformational change in the receptor that exposes a nuclear localization signal; the GR is thereby targeted to the nuclear compartment. Once nuclear, the GR functions as a transcriptional activator or repressor, modulating the expression of responsive genes.

Glucocorticoids appear to influence S6K1 and 4E-BP1 function via transcriptional means, i.e., via activation or repression of target genes, since a temporal lag of 2–3 h is required for these effects to evolve (18, 26). These translational pathways appear to be inhibited downstream of phosphatidylinositol 3-kinase, since the activity of this lipid kinase is not reduced in cells treated with dexamethasone (18). Furthermore, activation of protein kinase B (PKB/Akt) as evidenced by phosphorylation of two critical sites, T308 and S473, is not hindered by glucocorticoids (25). In light of recent evidence that PKB/Akt is an authentic upstream effector of 4E-BP1 (7), but not S6K1 (4), these data imply that glucocorticoids act either downstream of or parallel to PKB/Akt in the downregulation of eIF4F and S6K1.

Several scenarios could be envisioned to account for the ability of glucocorticoids to inhibit leucine-induced activation of eIF4F and S6K1. It is likely that glucocorticoids are exerting this regulation by modulating the expression of one or more genes encoding some type of negative regulator(s). Induction of protein phosphatases that directly dephosphorylate 4E-BP1 and/or S6K1 or dephosphorylate other upstream effectors, leading to inactivation of those effectors, could account for the dephosphorylation of 4E-BP1 and S6K1. Conversely, repression of a critical upstream regulator, such as an activating kinase, could also explain this effect. Alternatively, alterations in some protein-protein interaction, which impedes activation of eIF4F and S6K1, or omission of essential upstream effectors (4E-BP1 and S6K1 themselves for that matter) from productive signaling networks by subcellular relocalization could hinder the activities of these translational effectors. The obvious complexity in such translational regulation will undoubtedly prove challenging to resolve.

For all biochemical parameters examined, leucine reversed the effects of glucocorticoids on S6K1 and eIF4F and, in doing so, restored protein synthetic rates to control values. The glucocorticoid-mediated impairment of the translational apparatus is associated with insufficient eIF4F assembly and inactivation of S6K1, despite unperturbed catalytic activity of eIF2B (24). It therefore appears that eIF4F function represents a critical target for translational regulation by glucocorticoids. By comparison, eIF4F assembly in skeletal muscle is attenuated with fasting, again concomitant with undiminished eIF2B activity, suggesting that the eIF4 system is rate controlling under those conditions as well. Interestingly, the fasting-induced reduction in protein synthetic rate and associated diminution of eIF4F function are readily corrected after a single bolus ingestion of leucine (1). The results presented here demonstrate that eIF4F and S6K1 are translational elements subject to counterregulation by glucocorticoids and leucine. Moreover, these data imply that a primary defect in the function of eIF4F, such as that engendered by glucocorticoid exposure, may be correctable by administration of leucine.

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