Acute attenuation of translation initiation and protein synthesis by glucocorticoids in skeletal muscle

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Shah, O. Jameel, Scot R. Kimball, and Leonard S. Jefferson. Acute attenuation of translation initiation and protein synthesis by glucocorticoids in skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 278: E76–E82, 2000.—Glucocorticoids are diabetogenic factors that not only antagonize the action of insulin in target tissues but also render these tissues catabolic. Therefore, in rats, we endeavored to characterize the effects in skeletal muscle of glucocorticoids on translation initiation, a regulated process that, in part, governs overall protein synthesis through the modulated activities of eukaryotic initiation factors (eIFs). Four hours after intraperitoneal administration of dexamethasone (100 µg/100 g body wt), protein synthesis in skeletal muscle was reduced to 59% of the value recorded in untreated control animals. Furthermore, translation initiation factor eIF4E preferentially associates with its endogenous inhibitor 4E-BP1 rather than eIF4G. Dexamethasone treatment resulted in phosphorylation of both 4E-BP1 and the 40S ribosomal protein S6 kinase concomitant with enhanced phosphorylation of eIF4E. Moreover, the guanine nucleotide exchange activity of eIF2B was unaffected as was phosphorylation of the α-subunit of eIF2. Hence glucocorticoids negatively modulate the activation of a subset of the protein synthetic machinery, thereby contributing to the catabolic properties of this class of hormones in vivo.
dexamethasone; eukaryotic initiation factor; eIF4E; eIF2B; 4E-BP1; p70S6k

Translation initiation, the first and rate-determining event governning protein synthesis, is mediated by the eukaryotic initiation factors (eIFs), a class of proteins whose function is to load initiator methionyl-tRNA (Met-tRNAi) onto the translational start site of appropriate transcripts and thereby conduct the synthesis of cognate peptides. It is well established that two operant biochemical mechanisms underlie control of translation initiation (reviewed in Refs. 8 and 23). First, the eIF4F complex, composed of the scaffolding protein eIF4G, the mRNA cap-recognition protein eIF4E, and the ATP-dependent RNA helicase eIF4A, recruits the 43S preinitiation complex (eIF2-GTP-Met-tRNAi, 40S ribosomal subunit, eIF1A, and eIF3) to the 5′ end of eukaryotic mRNAs by virtue of an eIF3-eIF4G interprotein bridge. The assembly of eIF4F is, however, prevented by relatively dephosphorylated species of eIF4E-binding proteins (4E-BPs), which exhibit homology with regions of eIF4G responsible for eIF4E recognition and consequently compete with eIF4G for association with eIF4E. However, the 4E-BPs are keenly susceptible to phosphorylation, which serves to minimize their affinity for eIF4E. Thus regulation of the phosphorylation state of 4E-BPs confers modulated expression of capped mRNA. A second event influencing flux through the initiation cycle involves the guanine nucleotide exchange activity of eIF2B. Before assembly of the 80S initiation complex and in a process requiring eIF5, eIF2-bound GTP is hydrolyzed, and several eIFs are released from the now active elongation complex. The resultant eIF2-GDP is a substrate for eIF2B-mediated nucleotide exchange allowing regeneration of eIF2-GTP and subsequent initiation events. However, phosphorylation of the α-subunit of eIF2 augments the affinity of eIF2 for eIF2B ~100-fold but prevents the nucleotide exchange essential for initiation. Moreover, eIF2B is thought to be regulated directly by phosphorylation and perhaps by allosteric mechanisms.

The 70-kDa 40S ribosomal protein S6 kinase (p70S6k) is a unique serine/threonine kinase that is crucial for cell cycle progression through G1 (2) and has been implicated in translation control. However, until recently, its physiological role in these processes has remained speculative. It has now been shown that phosphorylation of its substrate, ribosomal protein S6, correlates with the preferential translation of a novel class of transcripts containing a terminal oligopyrimidine (TOP) tract adjacent to the 5′ terminus. This rapidly growing transcript family includes those mRNAs.

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encoding factors generally involved in the translational apparatus such as poly(A)-binding protein (9), elongation factors, and ribosomal proteins (11). The pathway proximal to phosphorylation of p70S6K is shared by that of 4E-BP1 and accounts for the rapamycin-sensitive phosphorylation of both factors. The mammalian target of rapamycin (mTOR) is the putative upstream kinase of 4E-BP1 and accounts for the rapamycin-sensitive activation of translation initiation and global protein synthesis in psoas muscle. In an effort to address this issue, an investigation of the role of the eIF4F system, eIF2B, and p70S6K in the regulation of translation initiation and global protein synthesis was undertaken.

**MATERIALS AND METHODS**

Materials. Mouse monoclonal anti-eIF4E antibody was raised against recombinant human eIF4E by the method described earlier (15). Rabbit polyclonal anti-eIF4G and rabbit monoclonal anti-4E-BP1 antibodies were raised against the corresponding human peptides that were expressed in and purified from SF9 insect cells using the baculovirus expression system as detailed elsewhere (3, 15). Rabbit polyclonal anti-p70S6K antibody was purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-eIF2α and rabbit polyclonal anti-phospho-Ser51-eIF2α antibodies were generous gifts from Dr. Edgar C. Henshaw and Dr. Gary S. Krause (Wayne State University School of Medicine), respectively. Polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad. Enhanced chemiluminescence (ECL) detection reagents and horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit immunoglobulins were purchased from Amersham Life Sciences.

Animals. Male Sprague-Dawley rats weighing 200–300 g were maintained on a 12:12-h light-dark cycle and were allowed free access to food (Harlan-Teklad Rodent Chow, Madison, WI) and water. Dexamethasone sodium phosphate (American Reagent Laboratories, Shirley, NY) was administered intraperitoneally in doses of 100 µg/100 g body wt. All controls received equivalent volumes of 0.15 M NaCl (vehicle).

Administration of metabolic tracer. Essentially, 100 µCi/ml of L-[2,3,4,5,6-3H]phenylalanine were injected via the tail vein at a concentration of 150 mM diluted in saline. Doses of 1 ml/300 g body wt were administered. Psoas muscle was excised 10 min after administration of the radiolabel and was immediately frozen in liquid nitrogen. The muscle was then stored at -80°C for subsequent analysis. The time elapsed from injection of the phenylalanine tracer until freezing of the tissue was recorded as the true infusion time.

Serum analyses. Serum insulin was measured using an RIA kit for rat insulin (Linco Research, St. Charles, MO). Phenylalanine specific radioactivity was determined as described elsewhere (16). Determination of rates of protein synthesis. Fractional rates of protein synthesis in psoas muscle were estimated based upon the rate of incorporation of radiolabel into protein. The specific radioactivity of serum phenylalanine was used as an estimate of the precursor pool (16).

Quantitation of eIF4E, 4E-BP1-eIF4E, and eIF4G-eIF4E complexes. Quantitation of the respective factors and complexes was carried out exactly as outlined previously (37). eIF4E, 4E-BP1-eIF4E, and eIF4G-eIF4E complexes were immunoprecipitated from 10,000-g supernatants of whole psoas muscle homogenate using a mouse monoclonal anti-eIF4E antibody. The immune complexes were isolated by incubation with goat anti-mouse BioMag IgG beads (PerSep Diagnostics). In preparation for incubation with antigen-antibody complexes, the beads were washed in 1% nonfat, dry milk in 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% β-mercaptoethanol, 0.5% Triton X-100, 50 mM NaF, 50 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM sodium vanadate (buffer A). With the use of a magnetic stand, the beads were captured and washed twice with buffer A and one time with buffer A containing 500 mM NaCl instead of 150 mM. After incubation of the immune conjugates with magnetic beads, the resultant complexes were eluted in SDS sample buffer and then boiled for 5 min. The beads were pelleted by centrifugation, and the supernatants were collected and subjected to SDS-PAGE. After proteins were electrophoretically separated, they were transferred to PVDF membranes. The membranes were then incubated with a mouse monoclonal anti-eIF4E antibody, a rabbit polyclonal anti-4E-BP1 antibody, or a rabbit polyclonal anti-eIF4G antibody overnight at 4°C. The blots were then developed using an ECL Western blotting kit.

Quantitation of phosphorylated and unphosphorylated eIF4E in skeletal muscle homogenates. Quantitation of the percentage of eIF4E in the phosphorylated and unphosphorylated forms was performed using slab gel isoelectric focusing with subsequent protein immunoblot analysis exactly as previously described (37).

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 (37). Briefly, the phosphorylated and unphosphorylated forms of 4E-BP1 were collected by immunoprecipitation of 4E-BP1 from 10,000-g supernatants of whole muscle homogenate using a mouse monoclonal anti-4E-BP1 antibody. The immunoprecipitate was collected, and proteins were separated by SDS-PAGE and then immunoblotted with a rabbit polyclonal anti-4E-BP1 antibody. Because the rate of migration of 4E-BP1 inversely correlates with its degree of phosphorylation, three species referred to as α, β, and γ readily resolve with SDS-PAGE; the most highly phosphorylated form is denoted γ, whereas the least phosphorylated form is denoted α.

Immunoblotting of p70S6K. 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-p70S6K antibody. In a manner analogous to 4E-BP1, p70S6K resolves into multiple electrophoretic forms after SDS-PAGE wherein increasing phosphorylation retards mobility (34). Thus more slowly migrating species generally reflect a greater degree of phosphorylation and thereby activation.

Measurement of eIF2β activity. eIF2β activity in skeletal muscle homogenates was assayed precisely as described elsewhere (13). After excision of the psoas, tissue was rinsed...
in ice-cold saline, weighed, and homogenized in a Polytron in four volumes of buffer consisting of 20 mM triethanolamine, pH 7.0, 2 mM magnesium acetate, 150 mM potassium chloride, 0.5 mM diethiothreitol, 0.1 mM EDTA, 250 mM sucrose, 5 mM ETTA, and 50 mM β-glycerophosphate. Homogenates were then centrifuged for 10 min at 12,000 g at 4°C. Supernatants were then assayed for guanine nucleotide exchange activity. Essentially, 35 µl of a preformed binary complex, assembled by incubation of purified eIF2 with 1.3 µM [3H]GDP (10.7 Ci/mmol), was combined with a mixture consisting of 35 µl of muscle homogenate, 87.5 µl of water, and 140 µl of 50 mM MOPS, pH 7.4, with 209 µM GDP, 2 mM magnesium acetate, 100 mM potassium chloride, 1 mM diethiothreitol, and 200 µg/ml BSA (buffer B). The reaction was initiated by a combination of these reactants and by transfer to a 30°C water bath. At five time points (0, 2, 4, 6, and 8 min), a 75-µl aliquot was removed and placed in a tube containing 2.5 ml of ice-cold wash buffer (buffer B devoid of BSA). The contents were mixed and immediately filtered through a nitrocellulose filter disk. The guanine nucleotide exchange activity was measured as a decrease in eIF2·[3H]GDP complex bound to the filters.

Quantitation of phosphorylated eIF2α. The 10,000-g supernatants of whole tissue homogenate were subjected to SDS-PAGE and Western blotting as described above. The total amount of eIF2α was determined by incubation of membranes with a mouse anti-eIF2α antibody. The phosphorylated form of the protein was detected using a phosphopeptide antibody raised against the sequence surrounding Ser51, the physiological eIF2α phosphorylation site. Values of eIF2α phosphorylation were then expressed as the ratio of the phosphorylated form of the protein to the total amount of eIF2α present.

RESULTS

Acute (4-h) administration of dexamethasone had previously been shown to cause an impairment in translation initiation as evidenced by an accumulation of free 40S and 60S ribosomal subunits; similar results had been obtained with chronic (5-day) drug treatment (27, 28). Moreover, this putative diminution in the rate of translation initiation had been shown to be independent of eIF2-like activity, that is, ternary complex formation. Therefore, in the present study, the influence of dexamethasone treatment on the rate of total protein synthesis in vivo was evaluated. Animals receiving injections of dexamethasone exhibited a protein synthetic rate equivalent to 59% of that recorded in untreated controls (Fig. 1A). Although insulin is known to promote aggregation of ribosomal subunits into translationally active polysomes and thereby diminish the number of free subunits (10), the influence of insulin here is excludable since plasma insulin concentrations were invariant between drug-treated and control animals (data not shown).

In an effort to characterize the underlying biochemical changes contributing to the dexamethasone-induced impairment in translation initiation, the protein-protein interactions governing eIF4F assembly were evaluated. Four hours of treatment with dexamethasone induced dephosphorylation of 4E-BP1 (Fig. 1B) concomitant with stabilization of the cap-dependent inhibitory complex eIF4E·4E-BP1 (Fig. 1C). Not surprisingly, the association of the scaffolding protein eIF4G with eIF4E in drug-treated animals was appre-
ciably less than that of the corresponding controls (Fig. 1D). The observed changes in 4E-BP1 and eIF4G association with eIF4E were not due to an altered expression of eIF4E, since protein levels were similar in control and dexamethasone-treated animals as determined by Western blotting (Fig. 1, C and D).

Because the cap-binding factor eIF4E has been demonstrated to undergo hyperphosphorylation in response to cellular stresses such as exposure to lipopolysaccharide (7), tumor necrosis factor (19), arsenite, anisomycin, and interleukin-1β (32), the effect of dexamethasone on the phosphorylation state of eIF4E was examined. Interestingly, the glucocorticoid analog augmented the percentage of phosphorylated eIF4E from 65 to 80% (Fig. 2A). Although this change was only slight, it was strikingly consistent. In contrast, the phosphorylation state of p70S6k was adversely affected by dexamethasone administration such that the predominant species exhibited only basal activation (Fig. 2B).

Because the enzymatic activity of eIF2B often correlates with, or is rate limiting for, the overall rate of protein synthesis, an investigation of its intrinsic guanine nucleotide exchange activity was undertaken. As depicted in Fig. 3A, the rate of exchange of guanine nucleotides was undiminished by treatment with dexamethasone. Moreover, the phosphorylation state of the α-subunit of eIF2, a potential inhibitor of eIF2B, was not changed with drug administration (Fig. 3B).

DISCUSSION

For over 20 years, the diabetogenic and catabolic properties of glucocorticoids contributing to physiological muscle atrophy have been studied and described. However, the complexity inherent in glucocorticoid
action has hindered elucidation and characterization of the molecular mechanisms that mediate many cellular responses to these hormones. Consequently, few efforts have been undertaken to attempt to address the intracellular events that precipitate muscle wasting. In both acutely (4 h) and chronically treated (5 days) rats, Rannels et al. (27, 28) have demonstrated that, in psoas and gastrocnemius, glucocorticoids induce the disaggregation of actively engaged polysomes thereby depressing protein synthesis through a defect in translation initiation. Interestingly, however, whereas the depression in protein synthetic rate observed in chronically treated animals is partly attributable to diminished total RNA content and attenuated eIF2-like activity, acute administration of dexamethasone evokes a different response (one that is independent of changes in RNA content and ternary complex formation). In the present work, assembly of the cap-recognition holocomplex, eIF4F, is hindered within 4 h, concomitant with a reduction in protein synthesis to 59% of the control value. Meanwhile, the rate of eIF2B-catalyzed guanine nucleotide exchange, a rate determinant of ternary complex formation, is unaffected by acute glucocorticoid administration. Taken together, these data suggest that eIF4F assembly and/or associated circumstances are rate controlling for overall protein synthesis in glucocorticoid-treated psoas muscle.

Recent advances in our understanding of signal transduction pathways influencing the translational apparatus have offered a glimpse not only of the capacity for complex integration of modulatory signals but also of the orchestration of factors that coordinately manifest the protein synthetic response. The eIF4 family of translation initiation factors is integrally a part of this process and, as such, has been extensively characterized. Although much is known of the hormonal and nutritional regulation of the eIF4 system, this is the first inquiry addressing the regulatory properties of glucocorticoids. The initiation complex eIF4F, which specifically recognizes and associates with capped mRNA, plays a critical function in recruitment of the ribosome to those mRNAs selected for translation (reviewed in Refs. 17 and 30). However, the role of eIF4F assembly in the regulation of global protein synthesis remains controversial; contribution of eIF4F to this process appears to be influenced tissue specifically and in a stimulus-dependent manner. With regard to protein synthesis in skeletal muscle, although traditionally promotion of the eIF4F complex has been shown to correlate with increases in protein synthesis (6, 12, 14, 31, 37, 38), such findings are not unequivocal. In fact, in L6 myoblasts, insulin corrects the defect in eIF4F assembly induced by deprivation of leucine without restoration of global protein synthesis (16). Therefore, under those circumstances, eIF4F is not the rate-limiting determinant of overall protein synthesis, but, rather, some other factor, presumably eIF2B, is governing this process. Furthermore, dissociation of eIF4F assembly and protein synthesis is observed under some conditions in other tissues. For example, an amino acid imbalance in the liver created by elevating the circulating concentration of leucine potently augments assembly of eIF4F yet reduces overall protein synthesis concomitant with reduced activity of eIF2B (Shah, unpublished observation).

Although changes in the rate of protein synthesis are often paralleled by changes in the catalytic rate of eIF2B, there is precedent for an eIF2B-independent mechanism of translational regulation. Refeeding 18-h-fasted rats elicits an anabolic response in gastrocnemius characterized by a 30% increase in protein synthetic rate concomitant with eIF4F complex promotion (38). These phenomena occur without a detectable change in eIF2B activity (37). Moreover, 48-h starvation is without effect on the activity of eIF2B although protein synthesis diminishes (1). Thus, although protein synthesis is regulated by eIF2B under a variety of circumstances (8), particular nutritional states and glucocorticoid treatment share a mode of regulation mediated by eIF4F and independent of eIF2B.

Besides indirect, biochemical regulation through interactions with eIF4G and 4E-BPs, the activity of eIF4E is modulated in mammalian cells by phosphorylation. The affinity of eIF4E for the m7GTP cap structure is enhanced three- to fourfold by phosphorylation of Ser209, the physiological phosphorylation site (20). Although, traditionally, phosphorylation of eIF4E has been associated with growth-stimulatory conditions and thus those favoring protein synthesis (reviewed in Ref. 17), there is a growing body of evidence in support of the contrary. In fact, several “stress” conditions such as exposure to lipopolysaccharide (7) or anisomycin (32) augment phosphorylation of eIF4E. In accord with these findings, glucocorticoid treatment increases the percentage of cellular eIF4E in the phosphorylated form. It has been proposed that Mnk1, a kinase constitutively associated with eIF 4G, may represent a physiological eIF4E kinase (24, 32). This serine/threonine kinase is activated by extracellular signal-regulated kinase (Erk) 1, Erk2, and p38 subunit mitogen-activated protein kinase (p38MAPK) both in vitro and in vivo (5, 33) and phosphorylates Ser209 in vitro (33). However, stimuli such as hyperosmolarity and oxidative stress, which activate p38MAPK and Mnk1, are reported to reduce eIF4E phosphorylation (32). The same inquiry reveals that 4E-BP1-associated eIF4E is a poor substrate for Mnk1 in vitro, suggesting that coupling of 4E-BP1 to eIF4E somehow deters eIF4E phosphorylation. Because glucocorticoid treatment enhances the association of 4E-BP1 and eIF4E, yet elevates the percentage of eIF4E in the phosphorylated form, a mechanism whereby the 4E-BP1-eIF4E complex hinders phosphorylation of eIF4E seems unlikely here. Although 4E-BP1, via direct interaction, prevents eIF4E phosphorylation both by Mnk1 (32) and protein kinase C (35), phosphorylated eIF4E can associate with 4E-BP1 (4). Therefore, although eIF4E is more highly bound to 4E-BPs in glucocorticoid-treated animals, a circumstance could theoretically exist in which the presence of eIF4E in solution is subtle yet sufficient to allow it intermittent access to eIF4G and/or activated Mnk1.
Not unexpectedly, the glucocorticoid-induced signals that inhibit the eIF4F system also reduce phosphorylation of p70S6K. It is currently believed that this protein kinases lies distal to mTOR and, in a signal transduction pathway, parallel to 4E-BP1. Hence, those stimuli that affect the activity of mTOR, under most circumstances, mutually influence the phosphorylation of 4E-BP1 and p70S6K. Mitogen stimulation is known to evoke multistep phosphorylation of p70S6K and involves several distinct kinases. The multiple phosphorylated forms of the kinase resolve on SDS-polyacrylamide gels with the rate of migration retarded by increasing phosphorylation. It is generally accepted that, of the four or five resolvable bands, the most slowly migrating form represents the activated form of the protein (34). In the current study, glucocorticoid treatment promoted dephosphorylation of p70S6K below that observed in controls. Presumably, glucocorticoids activate some p70S6K phosphatase or deactivate a relevant kinase, and it is this effect that accounts for the observed dephosphorylation. Whether the primary contribution is due to a phosphatase, kinase, or both is currently unknown. However, in the CTLL-20 T cell line, incubation with calyculin A, a PP1 and PP2A inhibitor, does not correct the dexamethasone-induced inhibition of p70S6K activation by interleukin-2 (21). Moreover, the calcineurin inhibitor FK506 is similarly ineffective. However, the kinases and/or phosphatases that contribute to attenuation of interleukin-2-stimulated p70S6K activation may indeed be distinct from those that reduce basal activation. Presently, the precise physiological function of the observed reduction in basal activation of p70S6K is unknown. However, it likely reflects up- or downregulation of those phosphatases and/or kinases, respectively, which regulate the initial series of activating phosphorylation events.

This work represents the first effort undertaken to address the biochemical changes contributing to glucocorticoid-induced attenuation of translation initiation in skeletal muscle. Dexamethasone impairs protein synthesis, in part, by downregulating both eIF4F and p70S6K. Moreover, glucocorticoids promote phosphorylation of eIF4E, refuting the notion that eIF4E phosphorylation is reserved exclusively for those circumstances that favor protein synthesis. Finally, the protein synthetic depression observed in dexamethasone-treated animals is independent of eIF2B activity, suggesting that regulation of protein synthesis does not always directly reflect a change in the activity of this exchange factor.

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