Dexamethasone inhibits the stimulation of muscle protein synthesis and PHAS-I and p70 S6-kinase phosphorylation

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Dexamethasone inhibits the stimulation of muscle protein synthesis and PHAS-I and p70 S6-kinase phosphorylation. Am J Physiol Endocrinol Metab 280: E570–E575, 2001.—Glucocorticoids inhibit protein synthesis in muscle. In contrast, insulin and amino acids exert anabolic actions that arise in part from their ability to phosphorylate ribosomal p70 S6-kinase (p70S6k) and eukaryotic initiation factor (eIF)4E binding protein (BP)1 (PHAS-I), proteins that regulate translation initiation. Whether glucocorticoids interfere with this action was examined by giving rats either dexamethasone (DEX, 300 μg·kg−1·day−1, n = 10) or saline (n = 10) for 5 days. We then measured the phosphorylation of PHAS-I and p70S6k in rectus muscle biopsies taken after an 180-min infusion of either insulin (10 mU·min−1·kg−1 euglycemic insulin clamp, n = 5 for both DEX- and saline-treated groups) or a balanced amino acid mixture (n = 5 for each group also). Protein synthesis was also measured during the infusion period. The results were that DEX-treated rats had higher fasting insulin, slower glucose disposal, less lean body mass, and decreased protein synthetic rates during insulin or amino acid infusion (P < 0.05 each). DEX did not affect basal PHAS-I or p70S6k phosphorylation but blocked insulin-stimulated phosphorylation of PHAS-I and amino acid-stimulated phosphorylation of both PHAS-I and p70S6k (P < 0.01, for each). DEX also increased muscle PHAS-I concentration. These effects can, in part, explain glucocorticoid-induced muscle wasting.

glucocorticoid; translation initiation; eukaryotic initiation factor 4E

GLUCOCORTICOIDS EXERT a well recognized protein catabolic action on skeletal muscle. Both protein synthesis and degradation can be affected. In the rat, muscle protein synthesis is inhibited as early as 4 h after glucocorticoid administration (23, 27). Synthesis reverses to normal by 24 h, at least with short-acting glucocorticoids (27). Inhibition of mRNA translation initiation appears to be a major site for glucocorticoid inhibition of protein synthesis (22). This decline in protein synthesis is paralleled by increases in plasma insulin and glucose, findings consistent with a general increase in insulin resistance. In a recent study, Shah et al. (25) observed that, in ad libitum-fed rats, dexamethasone (DEX) acutely (within 4 h) inhibited protein synthesis, decreased the phosphorylation of both PHAS-I eukaryotic initiation factor (eIF)4E binding protein (BP)1 (PHAS-I) and ribosomal p70 S6-kinase (p70S6k), and lowered the amounts of 4E bound to eIF4G. These observations provide one potential mechanism for the inhibitory effect of glucocorticoids on protein synthesis.

Interestingly, in humans, the dominant short-term effect of glucocorticoid infusion is an acceleration of whole body (1, 5, 11, 26) and skeletal muscle (17) protein degradation. Protein synthesis, measured either as whole body nonoxidative leucine disposal or as phenylalanine disappearance into muscle, is typically not affected. The majority, though not all (10), of these clinical studies were performed in postabsorptive subjects. By contrast, the recent studies by Shah et al. (25) and earlier studies by Rannels and Jefferson (23) were performed in ad libitum-fed rats.

Recent data suggest that the stimulatory effect of insulin (14, 15, 21) and amino acids (9, 28) on protein synthesis results, at least in part, from the phosphorylation of two key proteins involved in the regulation of translation initiation, i.e., PHAS-I and p70S6k. Phosphorylation of PHAS-I frees eIF4E, which is then available to associate with eIF4G to form the 40S preinitiation complex and stimulate protein synthesis by accelerating translation initiation. Phosphorylation of p70S6k can increase the phosphorylation of ribosomal protein S6. This facilitates the synthesis of some ribosomal proteins and other selected proteins with oligopyrimidine clusters near the transcription start site. In previous studies, we observed that glucocorticoids increase postabsorptive protein degradation by skeletal muscle but also inhibit the antiproteolytic action of insulin. Whether glucocorticoids affect the basal phosphorylation state of these regulatory proteins or diminish their activation has not been defined.

In this study, we examined whether DEX inhibits protein synthesis by blocking the signaling pathway that regulates synthesis via phosphorylation of PHAS-I and p70S6k. We reasoned that, if this were due to a proximal action of DEX on the insulin-signaling pathway...
pathway [e.g., the insulin receptor or insulin receptor substrate (IRS) proteins] (8, 24), it should not affect the amino acid-induced activation of protein synthesis. This activation arises from actions beyond protein kinase B (or Akt), perhaps at the mammalian target of rapamycin kinase (2, 9). We observed that protein synthesis rates were diminished during either insulin or amino acid infusion in DEX-treated animals. DEX did not affect basal postabsorptive phosphorylation of PHAS-I or p70S6k but blocked the effects of insulin on PHAS-I and of amino acids on both PHAS-I and p70S6k phosphorylation.

MATERIALS AND METHODS

Animal preparation. Twenty male Sprague-Dawley rats, weighing 225–250 g, were maintained on a 12:12-h light-dark cycle with food and water provided ad libitum. One group of animals (n = 10) received DEX 300 μg·kg⁻¹·day⁻¹ subcutaneously for 5 days, while a control group (n = 10) was given an equivalent volume of saline. Rat body weight was measured daily, and on the 5th day, rats were fasted overnight. The next morning, rats were anesthetized using pentobarbital sodium (50 mg/kg ip; Abbott Laboratories, North Chicago, IL), and a midline neck incision was made and the external jugular vein, internal carotid artery, and trachea were exposed and cannulated. The arterial catheter was connected through a three-way stopcock to a pressure probe, and heart rate and mean arterial pressure were monitored throughout the study (Transonic Systems, Ithaca, NY). Pentobarbital sodium was infused at a variable rate to maintain a steady level of anesthesia throughout the study. From the time the animal was anesthetized until the completion of the study, 5 h were required. Four infusion studies were performed each week (2 DEX treated and 2 control rats).

Experimental protocols. After a 45-min baseline period (used to assure hemodynamic stability and stable level of anesthesia), the 10 DEX- and 10 saline-treated rats were given either insulin (10 mU·kg⁻¹·min⁻¹ euglycemic clamp, n = 5) or an amino acid infusion (10% Travalosol 10 μl·kg⁻¹·min⁻¹, n = 5) for 3 h, respectively. Along with the insulin or amino acids, [³H]phenylalanine was infused continuously at 2 μCi/min for 3 h. Whole blood glucose was monitored every 10 min (YSI model 27 glucose analyzer, glucose oxidase method) throughout the insulin infusion, and 30% dextrose was infused at a variable rate to maintain blood glucose within 10% of basal (6). In rats not receiving insulin, an equivalent volume of saline was given. Immediately before and at the end of the infusion period (3 h), rectus muscle was biopsied, freeze-clamped in liquid nitrogen, and then stored at −70°C. Extensor digitorum longus (EDL) muscle was also obtained at the end. Plasma insulin was measured by immunoassay in plasma obtained at the beginning and at the end of the infusion period (3 h), respectively. A homogenate (50 mg protein) was diluted with an equal volume of SDS sample buffer and run on an 8% SDS-PAGE. For p70S6k, the protein content of the homogenate was measured, and one aliquot of the muscle homogenate (50 μg protein) was diluted with an equal volume of SDS sample buffer and run on an 8% SDS-PAGE. For PHAS-I, another aliquot was heated to 100°C for 5 min and centrifuged at 2,000 rpm for 2 min, the protein content of the supernatant was determined, and an aliquot containing 60 μg of protein was diluted with an equal volume of SDS sample buffer and electrophoresed on a 15% polyacrylamide gel. Proteins on both gels were electrophoretically transferred to nitrocellulose membranes. After being blocked with 5% low-fat milk in Tris-buffered saline plus Tween 20, membranes were incubated with rabbit anti-rat PHAS-I (kindly provided by Dr. J. Lawrence, University of Virginia) or rabbit anti-p70S6k (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. This was followed by a donkey anti-rabbit IgG coupled to horseshadish peroxidase, and the blot was developed using an enhanced chemiluminescence Western blotting kit (Amersham Life Sciences, Piscataway, NJ). Autoradiographic film was scanned densitometrically (Molecular Dynamics, Piscataway, NJ) and quantitated using Imagequant 3.3. The fraction of protein migrating more rapidly (PHAS-I) or more slowly (p70S6k) was quantified as a fraction of the total protein (see RESULTS). In preliminary studies, we had examined the time course over which insulin affected the phosphorylation of PHAS-I and p70S6k as visualized by altered electrophoretic migration on Western blots. An effect was seen within 30 min that increased further by 1 h and then persisted for ≥3 h (data not shown). To quantify the extent of phosphorylation in PHAS-I, we measured the ratio of the intensity of the most rapidly migrating species α (which represents the eIF4E-associated form of PHAS-I) to that of the total intensity (α + β + γ). For p70S6k, we determined the ratio of the more slowly migrating forms (β + γ) to the total (α + β + γ). In preliminary experiments, we observed good reproducibility of the fractional phosphorylation ratios for PHAS-I and p70S6k after loading gels with different amounts of protein (Fig. 1). This suggests that the ratios can be used for quantitative comparisons between samples. Statistical analysis (SigmaStat 3.0) was based on

![Fig. 1. Effect of protein loading on the quantitation of the ratio of phosphorylated species of eukaryotic initiation factor (eIF4E) binding protein (BP)1 (PHAS-I) and ribosomal p70 S6-kinase (p70S6k). Various amounts of protein were loaded on gel for PHAS-I (30–80 μg) and p70S6k (40–140 μg) Western blotting. Muscle was obtained from rats that had received a combined insulin and amino acid infusion that results in marked increases in phosphorylation of both PHAS-I and p70S6k. □, Ratio of α/total in PHAS-I; ●, phosphorylated fraction of p70S6k.](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.1999.81.5.571)
one-way ANOVA with post hoc testing, as indicated in RESULTS.

RESULTS

General. Rats treated with DEX lost 3–5 g body wt each day, whereas rats treated with saline gained 3–5 g daily, resulting in a significant weight difference at day 5 ($P < 0.01$; Fig. 2). The blood glucose concentration and the mean arterial pressure were not different between any groups either at baseline or during the insulin or amino acid infusions. The baseline insulin concentrations were higher in the DEX-treated rats (143 ± 19 vs. 70 ± 10 pM, $P < 0.001$), and insulin concentrations were comparable during the insulin clamp (3,000 ± 67 vs. 2,748 ± 708 pM) and during amino acid infusion (219 ± 51 vs. 192 ± 24 pM) in the two groups of rats. During the insulin clamp the steady-state (120–180 min) glucose infusion rate required to maintain euglycemia was significantly ($P < 0.001$) lower in the DEX-treated rats (26.3 ± 1.2 vs. 14.6 ± 0.9 mg·min$^{-1}$·kg$^{-1}$).

Protein synthesis. Figure 3 illustrates the rates of protein synthesis observed in rats infused with insulin or amino acids as well as the effects of DEX on protein synthesis in rats given these infusions. With DEX treatment, the rate of protein synthesis decreased ~25% during either insulin or amino acid infusion compared with those groups without DEX treatment.

Phosphorylation of PHAS-I and p70S6k. In these studies, we elected to use electrophoretic behavior on SDS-PAGE and Western blotting as indexes of the biological effect of insulin and amino acid. Inasmuch as this method allows the simultaneous quantitation of multiple forms of both proteins, as well as internal normalization for both the recovery of target proteins from tissue and loading of gels, it is well suited to the quantitative approach required in these studies. As noted in MATERIALS AND METHODS and seen in Fig. 1, the fraction of PHAS I observed as more rapidly migrating species was invariant over a range of gel-loading conditions. Likewise, differing amounts of protein loaded onto gels used for immunoblots for p70S6k did not affect the ratio observed for the more highly phosphorylated (slowly migrating) to the total p70S6k over the range of gel-loading conditions studied. For both p70S6k and PHAS-I, the available data support a good correlation between activity and electrophoretic mobility (7, 16, 29). Because the total shift in electrophoretic mobility induced by phosphorylation of either protein exceeds that expected from simple stoichiometry of the added phosphate groups, factors other than changes in mass appear to be involved.

Figure 4, top, illustrates the alterations of PHAS-I, and Fig. 4, bottom, illustrates the changes of p70 S6k migration observed on representative Western blots of rectus muscle in one rat from each of the four study groups. For PHAS-I, both insulin and amino acid infusions had an obvious effect of decreasing the amount of rapidly migrating species and increasing the density of the more slowly migrating forms of PHAS-I in the absence of DEX treatment. This is presumed to be
secondary to enhanced phosphorylation of PHAS-I (16). A similar result was seen with p70S6k.

Figure 5 presents the mean values for the fraction of PHAS-I present in the most rapidly migrating species \([\alpha/\alpha + \beta + \gamma]\) that migrated most rapidly is indicated on the y-axis. Samples were obtained from both DEX-treated and control animals before and after 3 h of a euglycemic insulin clamp or balanced amino acid infusion. Baseline values for all groups are shown in the filled bars; those treated with insulin or amino acid infusion are shown in the open bars. #Difference from preinfusion baseline \((P < 0.01); *\) difference between control and respective DEX-treated animals \((P < 0.01); n = 5\) for each group.

Figure 6 presents the mean values for the fraction of \(\alpha\) present as the more slowly migrating species \((\beta + \gamma/\alpha + \beta + \gamma)\) and Fig. 6 presents the mean values for the fraction of \(\beta + \gamma\) in the more slowly migrating species to the total \(\alpha + \beta + \gamma\), because this would index the more highly phosphorylated (active) forms of p70S6k. Both insulin and amino acid infusion decreased the \(\alpha/\alpha + \beta + \gamma\) fraction of PHAS-I \((P < 0.01 vs. baseline)\), which would result in more free eIF4E release, and the latter would increase the protein synthesis rate. Again, both insulin and amino acid infusion increased the fraction of p70S6k in the more highly phosphorylated forms \((P < 0.05 vs. baseline)\). DEX treatment blocked these effects. Interestingly, DEX had no effect on the basal phosphorylation state of either PHAS-I or p70S6k that prevailed before the insulin or amino acid infusion was given. We also observed that treatment of rats with DEX led to a selective increase in the amount of PHAS-I protein. Figure 7 shows the alterations of total amount PHAS-I and p70S6k in insulin- and amino acid-infused groups with or without DEX treatment. DEX treatment increased significantly the total protein of PHAS-I compared with the groups without DEX treatment. As we mentioned previously, the amounts of protein loaded onto the gels were the same. Unlike PHAS-I, p70S6k was not increased. This indicates that there is differential regulation of these proteins by DEX. This may have resulted from DEX either enhancing transcription or retarding degradation of PHAS-I.

**DISCUSSION**

The results reported here provide several interesting insights into the mechanisms responsible for glucocorticoid-induced muscle wasting. First, the data demonstrate that short-term administration of glucocorticoids causes a decline in insulin- or amino acid-stimulated protein synthesis in vivo. Previously, Southorn et al. (27) had shown that DEX could blunt the acute effect of insulin to stimulate muscle protein synthesis in the juvenile rat. Second, the findings suggest that DEX’s action to inhibit protein synthesis may be related to an inhibition of nutrient- or insulin-induced phosphorylation of PHAS-I and p70S6k. In addition, an increase in the total amount of PHAS-I in muscle was observed. This would be expected to further limit the availability of eIF4E required to bind to eIF4G to initiate translation.

When the effects of DEX on the phosphorylation of PHAS-I and p70S6k are considered, it is interesting that there was no apparent effect of DEX on the base-

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**Fig. 5.** Effect of treatment on PHAS-I phosphorylation. The fraction of PHAS-I immunoreactivity \([\alpha/\alpha + \beta + \gamma]\) that migrated most rapidly is indicated on the y-axis. Samples were obtained from both DEX-treated and control animals before and after 3 h of a euglycemic insulin clamp or balanced amino acid infusion. Baseline values for all groups are shown in the filled bars; those treated with insulin or amino acid infusion are shown in the open bars. #Difference from preinfusion baseline \((P < 0.01); *\) difference between control and respective DEX-treated animals \((P < 0.01); n = 5\) for each group.

**Fig. 6.** Effect of treatment on p70S6k phosphorylation. The y-axis shows the fraction of p70S6k that migrated in the two most slowly migrating bands \([\beta + \gamma]/[\alpha + \beta + \gamma]\). Baseline values for all groups are shown in the filled bars; those treated with insulin or amino acids are shown in the open bars. #Difference from preinfusion baseline \((P < 0.01); *\) difference between control and respective DEX-treated animals \((P < 0.01); n = 5\) for each group.

**Fig. 7.** Effect of treatment on total amount immunoreactive protein of PHAS-I and p70S6k; \(n = 5\) for each group. Shown here is the total amount of immunoreactive PHAS-I and p70S6k on Western blots from animals given either saline or DEX for 5 days and then acutely given amino acids or insulin.
line phosphorylation of either PHAS-I or p70S6k seen in the postabsorptive state. Rather, DEX specifically blocked the ability of either insulin or amino acids to phosphorylate these regulatory proteins. In a previous study, Shah et al. (25) reported that DEX given for 4 h blocked the baseline phosphorylation of PHAS-I and p70S6k. However, those animals were fed ad libitum before death, and a significant fraction of PHAS-I (eIF4E-BP1) and p70S6k was in a highly phosphorylated state in the absence of DEX, a pattern similar to that seen in the current work in the control animals given insulin or amino acids. The action of DEX in that study was to increase the nonphosphorylated fraction of PHAS-I and p70S6k. It remains possible that DEX treatment of those ad libitum-fed animals caused reversion of the pattern of PHAS-I and p70S6k phosphorylation to a state similar to that seen in overnight-fasted animals in the current study. Shah et al. also demonstrated that the expected effect of decreasing the phosphorylation of PHAS-I to increase its association with eIF4E and decrease the amount of 4E associated with p70S6k is indeed observed.

The observation in the current study that DEX did not affect the basal phosphorylation of PHAS-I or p70S6k may in part explain the failure of previous investigations to detect an effect of glucocorticoids on protein synthesis in vivo (1, 5, 11, 17, 26). All of these studies were done in the postabsorptive state i.e., in the absence of an acute stimulus to protein synthesis. Alternatively, because the latter studies were done in humans, a species difference in glucocorticoid action remains a possibility.

In a recent study, Dardevet et al. (4) concluded that, in adult rats, the catabolic effect of glucocorticoids was secondary to an effect to stimulate protein degradation, whereas in aged rats, the effect was due to an inhibition of protein synthesis. These measurements were made in animals that were not stimulated with either insulin or nutrients before measurement of protein turnover. Subsequently, the same group observed that glucocorticoid treatment blocked the ability of both insulin and insulin-like growth factor I to stimulate protein synthesis in incubated epitrochlearis muscle from adult rats (3). These findings are consistent with both the decrease in the in vivo rate of protein synthesis and the decreased ability of insulin and amino acids to phosphorylate PHAS-I and p70S6k seen in the current study.

If the increase in the total PHAS-I seen in the DEX-treated animals is considered, a technical concern would be whether this might result from the antibody being more active against the unphosphorylated form of PHAS-I and thus giving a higher overall signal in DEX-treated rats. Against this concern is the fact that the difference was observed even when comparing the basal samples from the DEX and control animals where the distribution of protein between the various phosphorylated forms of protein was not different. Also against this, as seen in Fig. 6, is the fact that, in animals treated with insulin or amino acids, there was no difference in the total integrated amount of protein identified in the immunoblot before and after treatment with insulin or amino acids, even though the phosphorylation state of the protein changed considerably. The increased amount of PHAS-I in a setting where its phosphorylation is blocked by DEX would be expected to result in more eIF4E bound to PHAS-I by simple mass action, leaving less 4E available to bind eIF4G.

The question naturally arises as to precisely where the glucocorticoids are acting to inhibit the phosphorylation of PHAS-I and p70S6k. Several days of high-dose glucocorticoid treatment of adult rats are reported to decrease muscle content of insulin receptor substrate-1 (8, 24) and its association with phosphatidylinositol 3-kinase (8). The latter changes are not seen in insulin-deficient rats, suggesting that they may be secondary to the hyperinsulinemia seen during glucocorticoid treatment. It is of interest that the effects of insulin and amino acids to phosphorylate PHAS-I and p70S6k are each blocked by DEX. However, amino acids act to increase the net phosphorylation of PHAS-I and p70S6k by acting at a site quite distal to the early events in insulin signaling that are reported to be blocked by DEX (8, 24). This suggests either that DEX acts at several sites in the kinase cascade that leads to the phosphorylation of PHAS-I and p70S6k or that the inhibition of the early events in the insulin signaling cascade are not related to the effect of DEX on mRNA translation.

It is important to note that our data, although showing an effect of DEX on insulin- and amino acid-stimulated protein synthesis, do not at all discount the suggestion that glucocorticoids have a major catabolic action to accelerate protein degradation; indeed, as noted previously, this is the most consistently observed action of glucocorticoids in humans. In the rat, an effect on degradation is reported in several studies (13, 18), but it is seen less consistently than the inhibition of synthesis. In these studies, protein degradation is typically measured in vitro by use of one or another of perfused muscle preparations that tend to be highly catabolic, even in the absence of glucocorticoids. This might potentially obscure further increases. Recent studies have suggested that glucocorticoids are involved in the increases in ubiquitin-mediated proteolysis seen in rat models of acidosis and diabetes (19, 20, 30).

In conclusion, the current results provide further insight into the mechanism by which glucocorticoids produce muscle wasting. In particular, our findings suggest that these agents are able to specifically block the activation by either insulin or amino acids of two proteins that regulate mRNA translation, PHAS-I and p70S6k. This action, combined with an action to increase the relative amount of unphosphorylated PHAS-I in the muscle, may explain the effect of DEX to blunt the stimulatory effect of insulin and amino acids on protein synthesis.

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REFERENCES


