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# TNF-ALPHA AND CORTISONE IMPAIR PEPTIDE CHAIN INITIATION BY ALTERING THE AVAILABILITY OF INITIATION FACTOR EIF-4E

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## 1. ABSTRACT

The effect of chronic tumor necrosis factor-α (TNF-α) and cortisone treatment on the concentration of translational initiation factor eIF-4E in rat skeletal muscle was evaluated. Crude muscle extracts from control and experimental groups (TNF-α s.c. @ 50 μg/kg body wt each day for 5 days or cortisone s.c. @ 100 mg/kg body wt for 5 days) were used to purify eIF-4E by immunoprecipitation and polyacrylamide gel electrophoresis (PAGE) followed by Western blot analysis. Quantification of eIF-4E was done by densitometry. Both TNF-α and cortisone induced a marked decline in the concentration of eIF-4E in rat skeletal muscle. There was no difference in the ratio of phosphorylated to unphosphorylated eIF-4E after TNF-α treatment. These findings suggest that both TNF-α and cortisone inhibit peptide chain initiation in skeletal muscle cells by decreasing the expression of eIF-4E.

## 2. INTRODUCTION

Sepsis, trauma and burn injury cause anorexia, fever and a marked decline in lean body mass due to a significant impairment in muscle protein balance. This negative nitrogen balance is due to both reduction in protein synthesis (1-3) and accelerated protein breakdown (2, 4). Both hormones (e.g. glucocorticoids) and cytokines such as TNF-α have been implicated. Several studies have reported reduced protein synthesis during excess of glucocorticoids (5-9) and TNF-α (2, 10-13). The inhibitory effect of TNF-α on muscle protein synthesis has been reported to be mediated through glucocorticoids (13, 14).

Overexpression of TNF-α in cells induces intense metabolic changes including inhibition of cell proliferation and induction of an antiviral state. These metabolic alterations necessitate changes in both transcription and translation. Though considerable information has been accumulated regarding TNF-α's actions on transcriptional events, very little is known about the role of TNF-α in the regulation of translation. In rat skeletal muscles, chronic TNF-α inhibits global protein synthesis as well as synthesis of specific myofibrillar proteins such as heavy & light chain myosin, and actin (9, 13, 15). This inhibition of protein synthesis is associated with a marked impairment in peptide-chain initiation as indicated by an accumulation of free ribosomal subunits and disaggregation of polysomes (16). Sepsis causes an inhibition of peptide-chain initiation which is due to decreased expression of eIF-2B (17) and is reversed by amrinone, a TNF-α release inhibitor (18). Chicheportiche and Vassalli (19) observed that TNF reduced the synthesis of cell surface protein F*cg* RIIB in macrophages. This decrease in cell surface protein was accompanied by a marked increase in its mRNA, which did not result from an increased transcription but from increased mRNA stability. It appears that the increase in mRNA was due to an alteration of the coupling between translation and degradation of mRNA known as cotranslation. The mechanisms involved in the TNF-mediated inhibition of peptide-chain initiation are poorly understood. The initiation of mRNA translation is a complicated multi-step process involving over a dozen eukaryotic initiation factors (eIFs). However, two steps have been identified to be subject to physiological regulation: i) binding of initiator met-$tRNA_i$ to the 40S ribosomal subunit to form 43S preinitiation complex, mediated by eIF-2, and ii) the binding of the 43S to the 5' end of mRNA, mediated by eIF-4. One of the eIF-4 family members, eIF-4E, makes a complex with eIF-4G and then binds to the $m^7$GTP cap at the 5' end of mRNA. The

activity of eIF-4E can be regulated at multiple levels (20). First, the binding of eIF-4E to the $m^7$GTP cap at the 5'-end can be regulated via phosphorylation/dephosphorylation of eIF-4E at SER-209 (21-23). Both p38 mitogen-activated protein (MAP) kinase and Erk signaling pathways are involved in phosphorylation of eIF-4E (24, 25). Phosphorylation of eIF-4E increases it affinity for $m^7$GTP cap and decreases its association with 4E-BP1 (PHAS-I), an acid and heat stable translational regulator (26). The initiation of the peptide-chain can be inhibited by the sequestering of eIF-4E by 4E-BP1 as 4E-BP1·eIF-4E complex. Phosphorylation of 4E-BP1 reduces its affinity for eIF-4E and phosphorylation of eIF-4E increases its affinity for $m^7$GTP cap of mRNA. In this study, we investigated the effect of chronic excess of TNF- α and glucocorticoids (cortisone) on the expression and phosphorylation of eIF-4E in rat skeletal muscle.

## 3. MATERIALS AND METHODS

### 3.1. Animals

Male Wistar rats weighing 80-100 g were purchased from Charles River Laboratories, Wilmington, Massachusetts, U.S.A. and maintained in a controlled temperature room on a 12 h/12 h light-dark cycle for at least 3 days before the start of each experiment. They were provided with food and water *ad libitum*.

### 3.2. TNF treatment

Human recombinant TNF-α (Peprotech, Rocky Hill, New Jersey, U.S.A.) was dissolved in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin. Controls received s.c. injections of equal volumes of PBS containing albumin. TNF-α was administered at 50 μg/kg body wt each day for 5 days. This dosage has been shown to inhibit protein synthesis in rat aortic smooth muscle (9).

### 3.3. Cortisone treatment

Cortisone acetate (Sigma Chemical, St. Louis, Missouri, U.S.A.) was dissolved in ethanol immediately prior to use. Controls received s.c. injections of equal volumes of ethanol. Cortisone acetate was administered s.c. at 100 mg/kg body wt each day for 5 days.

### 3.4. Preparation of muscle extracts

Animals were sacrificed and their hind limb muscles immediately removed and flash frozen in liquid nitrogen. Muscles were pulverized into a fine powder using a mortar and pestle while keeping the tissue in liquid nitrogen. Pulverized muscles (0.5 g) were homogenized in 7 volumes (3.5 ml) of cold buffer A (pH 7.4, 20 mM HEPES, 2.0 mM EGTA, 50mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β-glycerophosphate, 1.0 mM DTT, 0.1 mM PMSF, 1.0 mM benzamidine, 0.5 mM sodium vanadate) using a polytron homogenizer. Muscle homogenates were centrifuged (10,000g at 4°C) for 10 minutes and supernatants stored at -80°C.

### 3.5. Immunoprecipitation of eIF-4E

500 μl of 10,000g supernatant was added to a 1.5 ml microcentrifuge tube which contained 175 μl PBS, 12.5 μl Trition X-100 and 75 μl primary antibody. The primary antibody used was a mouse monoclonal $IgG_1$ antibody raised against full length eIF-4E of human origin (Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.). This antibody reacts with eIF-4E of mouse, rat, human and porcine origins. Tubes were incubated overnight in an orbital rocker at 4°C. Magnetic anti-mouse IgG Biomag beads (Qiagen, Valencia, California, U.S.A.) were used to isolate eIF-4E bound to the primary antibody. Prior to use, beads were washed 3 times in low salt buffer (LSB) [pH 7.4, 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% β-mercaptoethanol]. The beads were re-suspended in LSB + 0.1% nonfat dry milk and added to each microcentrifuge tube and incubated in an orbital rocker for 1 h at 4°C. The beads were then recaptured using a magnetic rack, carefully aspirated, and washed twice with 0.5 ml of LSB. A final wash was performed using high salt buffer [pH 7.4, 50 mM TrisHCl, 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.04% β-mercaptoethanol]. The beads were re-suspended in 100 μl of sample buffer [62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.025% bromophenol blue]. The beads were then boiled for 5 minutes to elute the eIF-4E, centrifuged at 13,000 g for 3 minutes and supernatant stored at -80°C for electrophoresis and Western blot analysis.

### 3.6. Electrophoresis and Western blot analysis

The supernatants containing immunoprecipitated eIF-4E were subjected to SDS-PAGE using a Protean II gel system and precast 10-20% polyacrylamide gradient gels (BioRad Laboratories, Hercules, California, U.S.A.). Gels were run in duplicate; one gel was stained with Coomassie blue stain while the second gel was used for Western blot analysis as follows. Protein bands were transferred overnight at 30V current to a 0.45 μm PVDF membrane in Towbin's transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3]. Membranes were then blocked with 3% non-fat dry milk/tris buffer saline (TBS) and incubated at 37°C in a primary antibody buffer containing a 1:300 titre of eIF-4E antibody (Santa Cruz Biotechnology) in TBS. This was followed by a second incubation in a secondary binding conjugate solution containing a 1:1500 titre of anti-mouse IgG with an AP conjugate (BioRad). Color development was achieved using an AP conjugate color development kit (BioRad). Western blot analysis and SDS-PAGE gels were scanned and analyzed using BioRad's GS-800 calibrated densitomer.

### 3.7. Phosphorylation of eIF-4E

eIF-4E was separated by isoelectric focusing (IEF) using a Mini-Protean III apparatus (BioRad) into two predominant forms with pI values of 5.9 and 6.3. Several lines of evidence suggest that these represent unphosphorylated and phosphorylated forms of the protein; the phosphorylation site is Ser-209 (21). Samples were loaded onto a precast isoelectric gel (pH 3-10) and subjected to a current of 100V for 60 minutes, 250V for 60 minutes and 500V for 30 minutes. Gels were stained using IEF stain (Coomassie brilliant blue R-250 and Crocein scarlet) for 45 minutes. Destaining was performed for 2-3

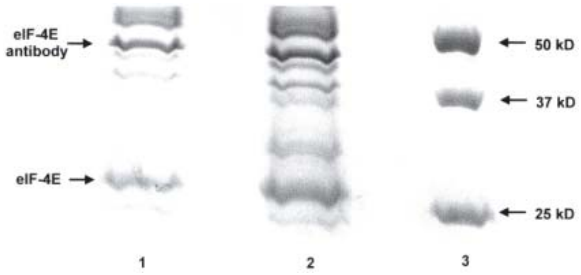


**Figure 1**. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10-20% gradient) of eIF-4E immunoprecipitated from hind limb muscles from control (lane 2) and TNF-α treated rats at 50 μg/kg body wt for 5 days (lane 1). Prestained precision proteins were electrophoresed as standards (lane 3).

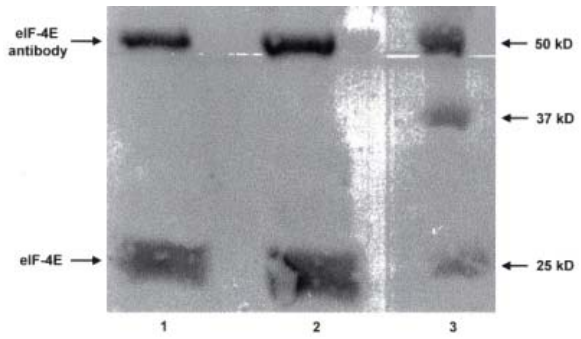


**Figure 2**. Western blot analysis of eIF-4E immunoprecipitated from hind limb muscles from control (lane 2) and TNF-α treated rats at 50 μg/kg body wt for 5 days (lane 1). Prestained precision proteins were transferred as standards (lane 3).

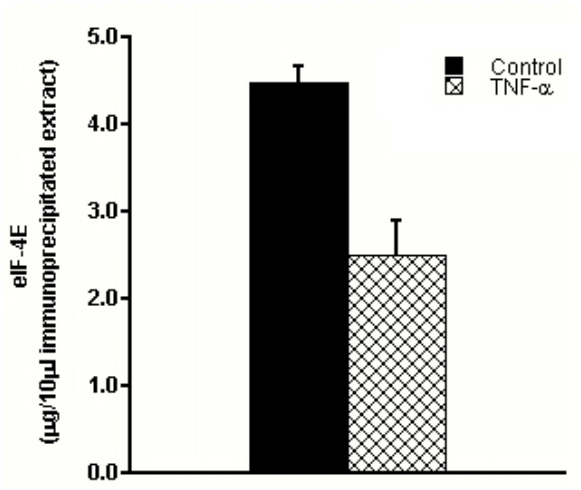


**Figure 3**. Effect of TNF-α treatment at 50 μg/kg body wt for 5 days on eIF-4E availability in hind limb muscles of rat. Quantification of eIF-4E was done by immunoprecipitation, SDS-PAGE followed by desitometry.

h and gels were scanned using the GS-800 calibrated densitometer.

### 3.8. Statistical Analysis

The results were shown as mean ± SEM. Differences between means were analyzed by Student's t-test for paired and unpaired observations.

## 4. RESULTS AND DISCUSSION

Both acute and chronic TNF-α and glucocorticoids (cortisone) excess has been shown to inhibit total protein synthesis in rat skeletal muscles of different fiber composition (9, 13). TNF-α inhibits synthesis of specific myofibrillar proteins in rat skeletal muscle (15). The inhibitory effect of TNF-α on muscle protein synthesis is caused by a decreased translation efficiency, specifically due to an impairment of peptide chain initiation (15, 16). The translation of mRNA into protein occurs in three stages: initiation, elongation and termination. Translation initiation is regulated by over a dozen eukaryotic initiation factors. Of these, eIF-2 and eIF-4 play a critical role in the regulation of translation. eIF-2 regulates the binding of initiator met-$tRNA_i$ to the 40S ribosomal subunit to form 43S preinitiation complex. The binding of the 43S to the 5' end of mRNA is mediated by eIF-4. One of the eIF-4 family members, eIF-4E, makes a complex with eIF-4G and then binds to the $m^7$GTP cap at the 5' end of mRNA. This study presents findings on the effect of chronic excess of TNF-α and glucocorticoids (cortisone) on the expression and phosphorylation of eIF-4E in rat skeletal muscle.

eIF-4E was immunoprecipitated from hind leg muscles of rats treated with TNF-α (@ 50 μg/kg body wt each day for 5 days), separated on PAGE (Figure 1) and identified by Western blot analysis (Figure 2). Quantification was done by densitometry (Figure 3). Chronic excess of TNF-α produced a significant decline in the availability of eIF-4E ($p<0.01$; $n=5$). These findings are in agreement with our previous observation that TNF-α inhibits translation in rat skeletal muscle by impairment of peptide chain initiation (15, 16). Lang *et al.* (27) did not find any significant difference in the availability of eIF-4E in gastrocnemius muscle after a 24 h infusion of TNF-α. However, eIF-4E was sequestered into an inactive complex through binding with the translational repressor 4E-BP1. The decline in eIF-4E availability noted in our study was produced after exposure to TNF-α for 5 days. It seems that during acute exposure to TNF-α cells respond by sequestering eIF-4E by 4E-BP1 whereas during chronic exposure to TNF-α there is a decline in the expression of eIF-4E. There was no difference in the ratio of phosphorylated versus unphosphorylated eIF-4E between control and TNF-α treated groups as determined by IEF (data not shown).

The effect of chronic excess of cortisone on eIF-4E expression was similar to that of TNF-α. There was a marked difference ($p<0.05$; $n=5$) in the skeletal muscle content of eIF-4E between the control group and the group treated with cortisone (@ 100 mg/kg body wt per day for 5

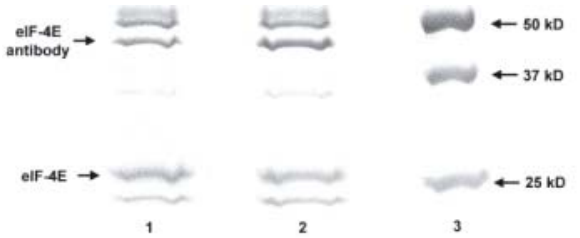


**Figure 4**. SDS-PAGE (10-20% gradient) of eIF-4E immunoprecipitated from hind limb muscles from control (lane 1) and cortisone treated rats at 100 mg/kg body wt for 5 days (lane 2). Prestained precision proteins were electrophoresed as standards (lane 3).

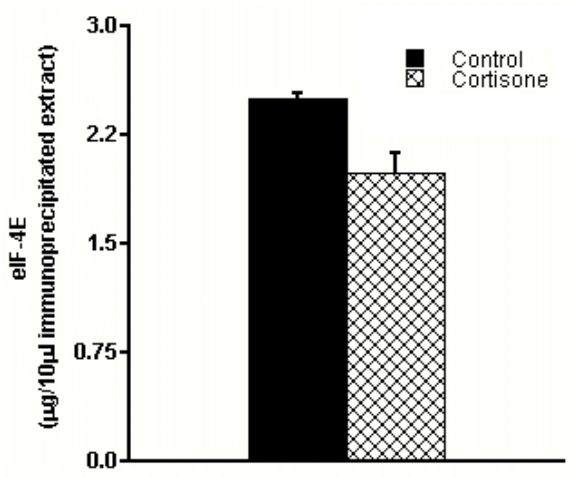


**Figure 5**. Effect of cortisone treatment at 100 mg/kg body wt for 5 days on eIF-4E availability in hind limb muscles of rat. Quantification of eIF-4E was done by immunoprecipitation, SDS-PAGE followed by desitometry.

days). Cortisone treatment induced a significant decrease in the expression of eIF-4E in hind limb muscles (Figure 4 and Figure 5). These findings suggest that chronic excess of both TNF-α and cortisone impair translation initiation by altering the availability of eIF-4E in skeletal muscle; these findings are consistent with our previous observation that both of these mediators inhibit global protein synthesis in skeletal muscle (13). Further elucidation of the effect of TNF-α and cortisone on the role of eIF-4E in translational regulation will be done by investigating the effect of these mediators on the interaction between eIF-4E and eIF-4G.

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