Heat shock protects L6 myotubes from catabolic effects of dexamethasone and prevents downregulation of NF-κB

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Luo, Guangju, Xiaoyan Sun, Eric Hungness, and Per-Olof Hasselgren. Heat shock protects L6 myotubes from catabolic effects of dexamethasone and prevents downregulation of NF-κB. Am J Physiol Regulatory Integrative Comp Physiol 281: R1193–R1200, 2001.—Glucocorticoids are the most important mediator of muscle cachexia in various catabolic conditions. Recent studies suggest that the transcription factor NF-κB acts as a suppressor of genes in the ubiquitin-proteasome proteolytic pathway and that glucocorticoids increase muscle proteolysis by downregulating NF-κB activity. The heat shock (stress) response, characterized by the induction of heat shock proteins, confers a protective effect against a variety of harmful stimuli. In the present study, we tested the hypothesis that the heat shock response protects muscle cells from the catabolic effects of dexamethasone and prevents downregulation of NF-κB. Cultured L6 myotubes were subjected to heat shock (43°C for 1 h) followed by recovery at 37°C for 1 h. Thereafter, cells were treated for 6 h with 1 μM dexamethasone, during which period protein degradation was measured as release of TCA-soluble radioactivity from proteins that had been prelabeled with [3H]tyrosine. Heat shock resulted in increased protein and mRNA levels for heat shock protein 70. The increase in protein degradation induced by dexamethasone was prevented in cells expressing the heat shock response. In the same cells, dexamethasone-induced downregulation of NF-κB DNA binding activity was blocked. The present results suggest that the heat shock response may protect muscle cells from the catabolic effects of dexamethasone and that this effect of heat shock may be related to inhibited downregulation of NF-κB activity.

heat shock protein 70; muscle cachexia; nuclear factor-κB; proteolysis

MUSCLE CACHEXIA IS A PROMINENT metabolic response to a number of different disease states, including cancer (32, 40), diabetes (19), uremia (1), injury (8), sepsis (13, 34, 35), and acquired immunodeficiency syndrome (21). Muscle breakdown in these conditions is mainly caused by increased protein breakdown; in particular, ubiquitin-proteasome-dependent myofibrillar protein breakdown (13). Although the pathogenesis of muscle protein degradation is probably multifactorial, there is evidence that glucocorticoids are the most important factor in regulating muscle protein breakdown in different catabolic conditions (14). For example, sepsis and injury-induced muscle proteolysis can be blocked by a glucocorticoid receptor antagonist (7, 12), and muscle protein breakdown can be induced by treating normal animals (33) or humans (5) with glucocorticoids. We and others reported previously that treatment of cultured myotubes with dexamethasone resulted in increased protein degradation and that this effect of dexamethasone was associated with upregulated activity and expression of the ubiquitin-proteasome pathway (6, 17, 37), lending further support to the important role of glucocorticoids in the development of muscle cachexia.

A recent report by Du et al. (6) provided insight into the molecular regulation of glucocorticoid-induced muscle protein degradation. In that study, treatment of cultured L6 myotubes with dexamethasone stimulated proteolysis, increased proteasome C3 subunit gene transcription, and downregulated nuclear factor (NF)-κB DNA binding activity. Additional experiments in which myotubes were transfected with a proteasome subunit promoter plasmid suggested that NF-κB is a repressor of certain proteasome subunit genes in muscle cells and that glucocorticoids may stimulate protein degradation by opposing this suppressor activity.

Muscle cachexia is important from a clinical standpoint for a number of reasons. Continuous muscle protein breakdown results in muscle wasting and fatigue that prevent or delay ambulation of patients with sepsis or severe injury and increase the risk for thromboembolic complications. When respiratory muscles are affected (31), pulmonary complications may occur and there may be a need for prolonged ventilatory support. In cancer patients, almost one-third of deaths has been estimated to be related to muscle cachexia (38), and there is evidence that the response to chemotherapy is impaired in patients with cachexia (36). Thus means to prevent or reduce muscle cachexia may have important clinical implications.

In recent years, accumulating evidence suggests that the heat shock (stress) response confers a protective effect against a variety of harmful factors, including hyperthermia, oxidants, and inflammation (4, 41). Al-
though the exact mechanisms by which the stress response exerts its protective effect are not completely understood, it is generally believed that heat shock proteins act as “chaperones” to damaged proteins and prevent aggregation of these proteins, thus preventing further cell injury (24). One of the most widely studied inducible heat shock proteins is the 70-kDa heat shock protein 70 (HSP70), the induction of which is commonly used to monitor the heat shock response.

The influence of the heat shock response on glucocorticoid-induced muscle cachexia is not known. In the present study, we tested the hypothesis that induction of the heat shock response would inhibit the catabolic effect of dexamethasone and prevent the downregulation of NF-κB activity in dexamethasone-treated muscle cells.

MATERIALS AND METHODS

Cell culture. L6 rat skeletal muscle cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown and maintained as described in detail previously (37) and were used between passages 2 and 8. Cells were seeded in 24-well culture plates (2.5 × 10^4 cells/well) or 10-cm dishes (2.5 × 10^5 cells/dish). Experiments were performed when ∼90% of the cells had formed myotubes.

The L6 muscle cell line was originally developed by Yaffe (44) from trypsin-suspended thigh muscle cells of newborn rats. During culture, the cells differentiate into multinucleated fibers that become cross-striated and develop contractility, thus resembling mature muscle cells. L6 muscle cells were used in previous studies examining the regulation of protein turnover, and results from those experiments suggest that the response in the myotubes to various treatments, including treatment with dexamethasone, calcium, and hormones, is similar to the response seen in vivo or in incubated intact muscles (6, 9–11, 17, 37). Thus, although results obtained in vitro in cultured cells always need to be interpreted with caution, regulatory mechanisms of protein turn-over in L6 myotubes are likely to reflect mechanisms in muscle cells in vivo.

Protein degradation. Protein degradation was measured by determining the release of TCA-soluble radioactivity from proteins that had been labeled for 48 h with l-[3,5-3H]tyrosine (New England Nuclear, Boston, MA) as described previously (17, 37). Release of TCA-soluble radioactivity was measured over a 6-h period, and the rate of protein degradation (%/6 h) was equal to 100 times the TCA-soluble radioactivity fluid for 4 h in a buffer consisting of 50% formamide, 7% SDS, 50 mM sodium phosphate (pH 7.0), 2% blocking reagent (Boehringer Mannheim), 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), and 0.1% Na-lauroylsarcosine. Hybridization was carried out overnight at 56°C in the same buffer with 25 ng/ml DIG-labeled cDNA probe. After hybridization, the membranes were washed twice in 2 × SSC, 0.1% SDS for 5 min at room temperature and twice in 0.1 SSC, 0.1% SDS for 15 min at 65°C. Chemiluminescence was detected by using 4-DIG alkaline phosphatase-conjugated Fab fragment (37.5 mU/ml; Boehringer Mannheim) and the substrate CDP-Star (0.25 mM; Boehringer Mannheim). The membranes were then exposed to X-ray film (X-Omat, Eastman-Kodak, Rochester, NY) and quantitated by densitometry.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was used to determine HSP70 mRNA levels. In these experiments, myotubes were cultured in 10-cm dishes to increase the amount of tissue. RNA was extracted and dot blot hybridization was carried out as described previously in detail from this laboratory (39, 40). cDNA probes for HSP70 and GAPDH were identified by using 4-DIG alkaline phosphatase-conjugated anti-DIG antibody at a dilution of 1:1,000 for 2 h. After washing three times in TBS containing 0.1% Tween 20, a secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) was applied at a dilution of 1:2,000 for 1 h. The blots were washed three times in TBS containing 0.1% Tween 20 and were then incubated in enhanced chemiluminescence reagent (Amer- sham, Buckinghamshire, UK) and exposed to radiographic film (Eastman Kodak, Rochester, NY) and quantitated by densitometry.
were treated with 1 mM dexamethasone for 1 h and then returned to 37°C. Cell viability was assessed by determining trypan blue exclusion or release of LDH into the medium. High temperature (50°C for 2 h) resulted in a substantial loss of cell viability; this effect of high temperature was prevented in cells that had been subjected to heat shock (Fig. 4).

In other cell types, the heat shock response conferred protection against the noxious effects of high temperature (41 or 43°C) (41). To test whether heat shock can induce a similar thermostolerance in muscle cells, myotubes were subjected to hyperthermia of 50°C for 2 h, whereafter cell viability was assessed by determining trypan blue exclusion or release of LDH into the medium. High temperature (50°C for 2 h) resulted in a substantial loss of cell viability; this effect of high temperature was prevented in cells that had been subjected to heat shock (Fig. 4).

Although the results reported here of upregulated HSP70 levels and protection of the myotubes against the catabolic effects of dexamethasone and the noxious effects of high temperature suggest that HSP70 may be involved in the mechanisms of cell protection, additional experiments were performed to further test that notion. In those experiments, we examined the correlation between HSP70 levels and the protective effects by testing the influence of dexamethasone and high temperature (50°C) at a time point when the HSP70 levels had declined. A time course with regards to
HSP70 levels after subjecting the myotubes to 43°C for 1 h was first established. Results from that experiment showed that HSP70 levels were increased for a relatively long period of time after the 1-h treatment at 43°C. Thus HSP70 levels remained elevated 4 days after heat shock and did not approach control levels until after 7 days (Fig. 5).

Cells that had been subjected to hyperthermia 7 days earlier were then treated with 1 μM dexamethasone for 6 h and protein degradation was measured. No

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Cells that had been subjected to hyperthermia 7 days earlier were then treated with 1 μM dexamethasone for 6 h and protein degradation was measured. No
protective effect of the heat shock response on dexamethasone-induced proteolysis was seen in these cells (Fig. 6). Likewise, there was no protective effect of the heat shock response in these cells with regards to the noxious effects of high temperature (Fig. 7). These results suggest that there is a temporal relationship between HSP70 levels and the protective effects in the myotubes, lending support to the concept that HSP70 may at least, in part, account for the protective effects of the heat shock response noticed in this study.

Because a recent study suggested that downregulated NF-κB activity may be an important mechanism by which dexamethasone stimulates protein degradation in cultured myotubes (6), we next examined the effect of the heat shock response on NF-κB activity in dexamethasone-treated myotubes. Treatment of the cells with 1 μM dexamethasone for 6 h resulted in reduced NF-κB DNA binding activity (Fig. 8), similar to a recent report by Du et al. (6). Addition of an excess amount of cold competitor to the reaction, but not of a mutant competitor, deleted the NF-κB band, confirming the specificity of the EMSA. The most common form of NF-κB in other cell types is a p50/p65 heterodimer (2). Supershift analysis suggested that NF-κB contained p50 and p65 subunits in the L6 myotubes as well (Fig. 8).

When cells had been subjected to hyperthermia 1 h before treatment with dexamethasone, the downregulation of NF-κB activity was blocked (Fig. 9). Because
NF-κB is retained in its inactive form in the cytoplasm by its inhibitory protein IκBα, cytoplasmic levels of IκBα were measured. Treatment of the myotubes with dexamethasone resulted in increased levels of IκBα, providing a potential mechanism for the dexamethasone-induced downregulation of NF-κB activity. The effect of dexamethasone on IκBα levels was blocked in cells expressing the heat shock response (Fig. 9).

**DISCUSSION**

In the present study, induction of the heat shock response protected cultured myotubes from the catabolic effect of dexamethasone and induced thermotolerance in the same cells. To our knowledge, this is the first report of inhibited glucocorticoid-induced muscle protein breakdown by the heat shock response. The observation is significant because glucocorticoids are an important mediator of muscle protein breakdown in various catabolic conditions, including cancer (32, 40), sepsis (33), injury (7), and renal failure (1), and methods to reduce the catabolic effect of glucocorticoids may have important clinical implications.

The present finding of increased protein degradation in cultured muscle cells after treatment with dexamethasone is in line with previous reports from our (37) and other laboratories (6, 17). In a recent study, we found that treatment of cultured L6 myotubes with dexamethasone resulted in increased energy-proteasome-dependent protein degradation and upregulated gene expression of ubiquitin and the proteasome subunit C3 (37). These effects of dexamethasone are similar to the effects of sepsis, injury, and other catabolic conditions in skeletal muscle, providing support for the use of dexamethasone-treated myotubes as a model of muscle cachexia.

Although muscle cachexia during sepsis and other catabolic conditions is caused by a combination of increased protein degradation and reduced protein synthesis, there is evidence that the increase in protein breakdown is the most important component of muscle cachexia (3, 15, 16). In recent experiments, treatment of cultured myotubes with dexamethasone stimulated protein degradation but did not influence protein synthesis (37). Consequently, the present study focused on the effect of heat shock on protein breakdown in dexamethasone-treated myotubes.

In a recent elegant study, Du et al. (6) characterized some of the molecular mechanisms of dexamethasone-induced protein degradation in cultured myotubes. Results from these experiments provided evidence that the transcription factor NF-κB is a suppressor of the proteasome subunit C3 gene and that glucocorticoids stimulate C3 subunit expression (and muscle protein degradation) by downregulating NF-κB activity. Inhibition of the dexamethasone-induced downregulation of NF-κB may therefore be a potential mechanism by which the heat shock response protects the cells from the effects of dexamethasone. The finding in the present study that NF-κB binding activity was not reduced by dexamethasone in myotubes that had been subjected to heat shock lends support to this concept. In addition, the present results suggest that the effects of the heat shock response and dexamethasone on NF-κB binding activity were mediated by changes in IκBα levels.

Although the exact mechanisms by which the heat shock response exerts a protective effect are not fully understood, production of heat shock proteins is central to the stress response (4, 24, 41). In the present study, we measured HSP70 levels to monitor the induction of the heat shock response. HSP70 is one of the best characterized inducible heat shock proteins, and its induction has been used as a “marker” of the heat shock response in different tissues and cell types. It is important to point out that elevated HSP70 levels do not necessarily mean that the protective effects of heat shock were conveyed by this specific protein under the present experimental conditions but could have been conferred by other heat shock protein(s) as well. The temporal relationship between HSP70 levels and the protective effects of the heat shock suggests (but does not prove) that HSP70 was at least, in part, responsible for the protective effects noticed here. More direct evidence for a protective effect of HSP70 was reported in other cell types including cardiomyocytes and enterocytes (18, 25, 27).

We and others reported previously that hyperthermia stimulates protein degradation in incubated muscles and cultured myotubes (23). The present result of downregulated protein degradation in dexamethasone-treated myotubes after induction of the heat shock response, therefore, may seem contradictory to earlier studies. It should be noted, however, that in the present study, cells that were subjected to heat shock for 1 h were allowed to recover at 37°C for 1 h before they were treated with dexamethasone. This model differs from previous experiments in which we found that protein degradation was increased in myotubes that were subjected to hyperthermia continuously for 6 h (23). In the present study, the heat shock inhibited the response to dexamethasone but did not alter basal protein degradation rates.

In several previous studies, the heat shock response downregulated NF-κB activity in different tissues during inflammation and in stimulated cells (30). This differs from the present study in which the heat shock prevented the inhibition of NF-κB activity induced by dexamethasone. Of interest was the finding that heat shock itself did not reduce NF-κB activity in the present study. One explanation for this may be that NF-κB activity was determined 7 h after the heat shock in the present study (1-h recovery at 37°C and 6-h incubation with or without dexamethasone at 37°C) and the influence of a limited period of hyperthermia on basal IκBα levels and NF-κB activity may be of shorter duration.

Induction of the heat shock response in skeletal muscle was reported after physical exercise in several previous reports (20, 28), but the physiological significance of this response to exercise is somewhat unclear. Two recent reports examined the potential role of the...
heat shock response in the regulation of muscle protein turnover. In one of those studies, treatment of cultured L8 myotubes with glutamine potentiated the induction of HSP70 caused by hyperthermia, and it was speculated that the stimulation of protein synthesis and inhibition of protein degradation by glutamine were related to the stress response (45). In another study, induction of the heat shock response by subjecting rats to 60 min of hyperthermia resulted in increased expression of HSP72 (the inducible form of HSP70) in soleus muscles and blunted the catabolic effect of hindlimb unweighting (26). Potential mechanisms of stress response-induced prevention of muscle cachexia were not explored in that study, but it was speculated that the heat shock response blocked the inhibition of muscle protein synthesis and the increase in protein degradation caused by extremity unweighting.

Although the present results are novel and may have clinical implications, a number of limitations needs to be kept in mind when the results are interpreted. First, the observations were made in vitro and it will be important in future experiments to determine the influence of the stress response on glucocorticoid-induced muscle proteolysis in vivo. Second, cells were pretreated with heat shock before treatment with dexamethasone, and an important question from a clinical standpoint is whether subjecting muscle cells to the stress response after the induction of protein degradation by dexamethasone will reduce proteolysis. Finally, although a mechanism of heat shock-induced proteolysis of the myotubes from the catabolic effect of glucocorticoids was provided in the present study (i.e., inhibition of the dexamethasone-induced increase in IkB levels and decrease in NF-kB activity), it remains to be determined which specific heat shock protein(s) is responsible for the protective effect. Despite the limitations, however, the present study is important because it provides the first support to the concept that the heat shock (stress) response may downregulate glucocorticoid-mediated muscle cachexia.

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