

## Dexamethasone and Corticosterone Induce Similar, but not Identical, Muscle Wasting Responses in Cultured L6 and C2C12 Myotubes

Michael Menconi,<sup>1</sup> Patricia Gonnella,<sup>1</sup> Victoria Petkova,<sup>2</sup>  
Stewart Lecker,<sup>2</sup> and Per-Olof Hasselgren<sup>1\*</sup>

<sup>1</sup>Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

<sup>2</sup>Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

### ABSTRACT

Dexamethasone-treated L6 (a rat cell line) and C2C12 (a mouse cell line) myotubes are frequently used as *in vitro* models of muscle wasting. We compared the effects of different concentrations of dexamethasone and corticosterone (the naturally occurring glucocorticoid in rodents) on protein breakdown rates, myotube size, and atrogin-1 and MuRF1 mRNA levels in the two cell lines. In addition, the expression of the glucocorticoid receptor (GR) and its role in glucocorticoid-induced metabolic changes were determined. Treatment with dexamethasone or corticosterone resulted in dose-dependent increases in protein degradation rates in both L6 and C2C12 myotubes accompanied by 25–30% reduction of myotube diameter. The same treatments increased atrogin-1 mRNA levels in L6 and C2C12 myotubes but, surprisingly, upregulated the expression of MuRF1 in L6 myotubes only. Both cell types expressed the GR and treatment with dexamethasone or corticosterone downregulated total cellular GR levels while increasing nuclear translocation of the GR in both L6 and C2C12 myotubes. The GR antagonist RU38486 inhibited the dexamethasone- and corticosterone-induced increases in atrogin-1 and MuRF1 expression in L6 myotubes but not in C2C12 myotubes. Interestingly, RU38486 exerted agonist effects in the C2C12, but not in the L6 myotubes. The present results suggest that muscle wasting-related responses to dexamethasone and corticosterone are similar, but not identical, in L6 and C2C12 myotubes. Most notably, the regulation by glucocorticoids of MuRF1 and the role of the GR may be different in the two cell lines. These differences need to be taken into account when cultured myotubes are used in future studies to further explore mechanisms of muscle wasting. *J. Cell. Biochem.* 105: 353–364, 2008. © 2008 Wiley-Liss, Inc.

**KEY WORDS:** MUSCLE WASTING; GLUCOCORTICIDS; MYOTUBES

Muscle wasting, mainly reflecting increased myofibrillar protein breakdown, is commonly seen in patients with various catabolic conditions, including severe injury [Pereira et al., 2005], sepsis [Tiao et al., 1997; Klaude et al., 2007], and cancer [Williams et al., 1999; Acharyya and Guttridge, 2007]. Glucocorticoids are important mediators of muscle proteolysis and were found in previous studies to upregulate ubiquitin-proteasome-dependent protein degradation in skeletal muscle [Hasselgren, 1999; Menconi et al., 2007]. In addition, muscle wasting induced by sepsis or burn injury was prevented by treatment with the glucocorticoid receptor antagonist RU38486 [Fang et al., 1995; Tiao et al., 1996].

Glucocorticoid-treated cultured myotubes have been used in multiple previous studies as an *in vitro* model of muscle wasting [Hong and Forsberg, 1995; Wang et al., 1998; Thompson et al., 1999;

Du et al., 2000; Sacheck et al., 2004; Stitt et al., 2004; Evenson et al., 2005; Yang et al., 2005a; Marinovic et al., 2006]. In most of those studies, rat L6 or mouse C2C12 myotubes were treated with dexamethasone, resulting in increased protein degradation and upregulated expression of several genes in the ubiquitin-proteasome proteolytic pathway [Wang et al., 1998; Thompson et al., 1999; Du et al., 2000; Sacheck et al., 2004; Stitt et al., 2004; Evenson et al., 2005; Latres et al., 2005; Marinovic et al., 2006; Sultan et al., 2006]. Because these and other metabolic changes induced by dexamethasone resemble changes seen in atrophying muscle in experimental animals [Tiao et al., 1994, 1996; Wray et al., 2003] and patients [Tiao et al., 1997; Williams et al., 1999; Acharyya and Guttridge, 2007; Klaude et al., 2007], dexamethasone-treated myotubes have been used to define mechanisms of muscle wasting.

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\*Correspondence to: Dr. Per-Olof Hasselgren, MD, PhD, Department of Surgery, Beth Israel Deaconess Medical Center, 330 Brookline Avenue ST919, Boston, MA 02215.

E-mail: phasselg@bidmc.harvard.edu

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Despite the frequent use of dexamethasone-treated myotubes in muscle wasting research, there are a number of important questions that have not been addressed regarding the validity of this experimental model. First, it is not known if the effects are specific for the artificial glucocorticoid dexamethasone or whether similar changes can be induced by corticosterone, the naturally occurring glucocorticoid in rodents. Second, the concentrations of dexamethasone used in previous studies varied substantially (10,000-fold) from 10 nM to 100  $\mu$ M [Du et al., 2000; Stitt et al., 2004; Latres et al., 2005; Marinovic et al., 2006]. Considering the approximately 25-fold higher potency of dexamethasone compared with corticosterone [Orth et al., 1992], the dexamethasone concentrations used in many previous studies were supraphysiologic and it is not known if L6 and C2C12 myotubes respond similarly to different concentrations of dexamethasone, including concentrations reflecting physiological glucocorticoid concentrations. Finally, it is not known if similar mechanisms of muscle wasting are activated by glucocorticoids in L6 and C2C12 myotubes. This is particularly important because recent studies suggest that the expression of the glucocorticoid receptor (GR) may be different in L6 and C2C12 myotubes [Sultan et al., 2006] and different responses of gene regulation were reported in the two cell lines after treatment with dexamethasone [Tortorella and Pilch, 2002].

In the present study, we compared the effects of different concentrations of dexamethasone and corticosterone on protein degradation and the expression of the muscle wasting-associated ubiquitin ligases atrogin-1 and MuRF1 in L6 and C2C12 myotubes. Results suggest that both dexamethasone and corticosterone induce similar changes in protein degradation and atrogin-1 mRNA levels in the two cell lines. In contrast, the regulation of MuRF1 expression by the glucocorticoids as well as the role of the glucocorticoid receptor (GR) in dexamethasone- and corticosterone-induced changes were different in L6 and C2C12 myotubes.

## MATERIALS AND METHODS

### MATERIALS

Corticosterone, dexamethasone, mifepristone (RU38486), and fetal bovine serum (FBS), were from Sigma-Aldrich (St. Louis, MO). L-[ring-3,5-<sup>3</sup>H]-tyrosine (40–60 Ci/mmol), Western Lightning<sup>TM</sup> Chemiluminescence Reagent Kit for enhanced chemiluminescence detection, and Solvable<sup>TM</sup> tissue solubilizer were purchased from PerkinElmer Life Sciences (Boston, MA). The NE-PER<sup>®</sup> Nuclear and Cytoplasmic Extraction Kit was purchased from Pierce Biotechnology (Rockford, IL). Immun-Blot PVDF Membrane used for Western blotting and 10% Ready Tris-HCl Mini-gels were purchased from Bio-Rad Laboratories (Hercules, CA). Primers and probes for quantitative real-time PCR were custom-synthesized by Biosearch Technologies, Inc. (Novato, CA). TaqMan One Step PCR Master Mix Reagents Kits were purchased from Applied Biosystems (Foster City, CA). Kodak X-Omat blue film was from Eastman Kodak (Rochester, NY). Dulbecco's modified Eagle's medium (DMEM) was purchased from Mediatech, Inc. (Herndon, VA). Penicillin/streptomycin and trypsin were from Invitrogen Corporation (Carlsbad, CA). All tissue culture plastic ware was purchased from Corning (Corning, NY). All chemicals were reagent-grade.

### CELL CULTURES

Rat L6 and mouse C2C12 skeletal muscle cells, both originally developed by Yaffe [1968] and Yaffe and Saxel [1977], were purchased from the American Type Culture Collection (Manassas, VA). Both myogenic cell lines have been extensively used in muscle wasting research. L6 and C2C12 cells were grown and maintained in high-glucose DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin in a 10% CO<sub>2</sub> humidified atmosphere at 37°C. Since the extent of myoblast fusion into myotubes declines with increasing passage, cells were used at low passage (between passages 2 and 4) for all experiments to maintain the differentiation potential of the cultures. Cells grown to approximately 80% confluence in T75 culture flasks were trypsinized and seeded into 10 cm culture dishes or 6 or 12-well culture plates for experiments. The cells were grown in the presence of 10% FBS until they reached about 90% confluence at which time the medium was replaced with DMEM containing 2% FBS for the induction of differentiation into myotubes. During this time, the myoblasts fused to form elongated, multi-nucleated myotubes. After 7 days, when greater than 90% of the cells had fused into myotubes, the cultures were treated with 10  $\mu$ M cytosine arabinoside for 3 days to remove any dividing myoblasts. L6 and C2C12 myotube cultures were then treated with different concentrations of dexamethasone, corticosterone, or RU38486 dissolved in 0.1% ethanol for 24 h (except when myotube diameters were measured and treatment was extended to 48 h) as described in Results Section. Control myotubes were treated with solvent (0.1% ethanol).

### MEASUREMENT OF MYOTUBE DIAMETER

Myotube cultures were photographed under a phase contrast microscope at 100 $\times$  magnification after 24 and 48 h treatment. The diameters were measured in a total of 60 myotubes from at least 10 random fields using ImageJ software (NIH, Frederick, MD). The measurements were conducted in a "blinded" fashion on coded pictures with the investigator being unaware of the group (control, dexamethasone- or corticosterone-treated myotubes) from which the cultures originated. Results were expressed as percent of the diameter in the control group.

### MEASUREMENT OF PROTEIN DEGRADATION

Rates of protein degradation were determined by measuring the release of TCA-soluble radioactivity from proteins prelabeled with [<sup>3</sup>H]-tyrosine as described previously (9,10). After completing differentiation, myotubes were labeled with 1.0  $\mu$ Ci/ml of L-[3,5-<sup>3</sup>H]-tyrosine for 48 h in DMEM containing 2% FBS. Cells were then treated for 24 h with glucocorticoids and RU38486 in DMEM containing 2 mM unlabeled tyrosine. After treatment, the culture medium was transferred into a microcentrifuge tube containing 100  $\mu$ l of bovine serum albumin (10 mg/ml) and TCA was added to a final concentration of 10% (w/v). Samples were incubated at 4°C for 1 h followed by centrifugation for 5 min. The supernatant was used for determination of TCA-soluble radioactivity. The protein precipitates were dissolved with a tissue solubilizer (Solvable<sup>TM</sup>). Cell monolayers were washed with ice-cold PBS and solubilized with 0.5 M NaOH containing 0.1% Triton X-100. Radioactivity in the cell monolayer and TCA soluble and insoluble fractions were measured

using a Packard TRI-CARB 1600 TR liquid scintillation analyzer. Protein degradation was expressed as the percentage protein degraded over the 24 h period and was calculated as 100 times the TCA-soluble radioactivity in the medium divided by the TCA-soluble plus the TCA-insoluble radioactivity in the medium plus the cell layer (i.e., myotube) radioactivity [Hong and Forsberg, 1995; Wang et al., 1998].

### MEASUREMENT OF PROTEIN SYNTHESIS

In order to determine whether the effect of glucocorticoids on protein degradation resulted from a generalized effect on protein turnover or was specific for protein degradation, the effect of glucocorticoids on protein synthesis was examined. Rates of protein synthesis were determined by measuring the incorporation of TCA insoluble radioactivity into cellular proteins pre-labeled with [<sup>3</sup>H]-tyrosine, as described previously [Wang et al., 1998]. Myotubes were treated for 48 h with glucocorticoids. During the last hour of incubation, myotubes were incubated with 2.0  $\mu$ Ci/ml of L-[3,5-<sup>3</sup>H]-tyrosine in DMEM (2% FBS) containing 200 mM unlabeled tyrosine. After incubation, the medium was removed and the cell layers were harvested in 10% TCA. The TCA-precipitated proteins were washed several times and then solubilized with 0.1 N NaOH containing 1% Triton X-100. Radioactivity and protein levels were measured in aliquots from the solubilized proteins, as described above and below, respectively, and protein synthesis was expressed as cpm/mg protein.

### PREPARATION OF TOTAL CELL LYSATES AND NUCLEAR EXTRACTS

Total cell lysates were prepared by harvesting the myotubes directly in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Nonidet P-40) containing Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN). After scraping the lysates into eppendorf tubes, the samples were briefly sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, Model 100) followed by centrifugation at 14,000g for 10 min at 4°C. Nuclear extracts were prepared from differentiated myotubes using the NE-PER<sup>®</sup> Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions. Concentrations of soluble proteins in the supernatants of the nuclear extracts and total cell lysates were determined by using the BCA Protein Assay Reagent Kit with bovine serum albumin as standard. Cell lysates were stored at -80°C until analyzed.

### WESTERN BLOTTING

Western blotting was performed to determine protein levels of GR in myotubes. Aliquots containing 50  $\mu$ g of cellular protein, prepared as described above, were boiled in Laemmli sample buffer containing 2-mercaptoethanol. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 10% Tris-HCl gels and then transferred electrophoretically at 2,000 mA for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol) onto Immun-Blot PVDF membranes at 4°C. The membranes were blocked with TTBS buffer (50 mM Tris-HCl, 130 mM NaCl and 0.05% Tween-20, pH 7.4) containing 5% nonfat

dry milk for 1 h at room temperature. The membranes were then incubated overnight with a 1:1,000 dilution of GR antibody (rabbit polyclonal antibody raised against amino acids 121-420 of human GR; Santa Cruz Biotechnology, Santa Cruz, CA). This antibody detects both the GR $\alpha$  and  $\beta$  isoforms of rat and mouse GR (Santa Cruz Biotechnology, technical notes). Following incubation with the primary antibody, the membranes were washed with TTBS 3 times and incubated for 1 h at room temperature with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at a 1:10,000 dilution. Following an additional three washes with TTBS, immunoreactive protein bands were visualized by using the Western Lightning<sup>™</sup> Kit for enhanced chemiluminescence followed by exposure to Kodak X-Omat blue film. The identity of the bands on the Western blots was confirmed by including precision molecular weight standards during electrophoresis. Blots were stripped using Restore<sup>™</sup> Western Blot Stripping Buffer according to the manufacturer's instructions and reprobed with  $\alpha$ -tubulin antibody to confirm equal loading.

### MEASUREMENT OF MRNA LEVELS

Atrogin-1, MuRF1, and GR mRNA levels were determined by real-time PCR, as described in detail recently [Wei et al., 2005]. Primers for atrogin-1, MuRF1, and GR were designed for rat (L6 myotubes) and mouse (C2C12 myotubes) using Primer Express 1.5 (Applied Biosystems). Cellular RNA was extracted from cultured myotubes grown in six-well plates by the acid guanidinium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987] using TRI REAGENT<sup>®</sup> (Molecular Research Center, Inc., Cincinnati, OH). Multiplex real-time PCR was performed for quantitation of mRNA expression with simultaneous amplification of 18S RNA as endogenous controls to normalize the mRNA concentrations. TaqMan analysis using the One Step PCR Master Mix Reagents Kit and subsequent calculations were performed with an ABI PRISM<sup>®</sup> 7700 Sequence Detection System (Applied Biosystems). For each sample, 100 ng of total RNA was subjected to real-time PCR (in duplicate) according to the protocol provided by the manufacturer. The sequences of the forward and reverse primer oligonucleotides, and the double-labeled TaqMan oligonucleotide probes for the mRNA's were as follows, respectively: rat MuRF1; 5'-CGA CTC CTG CCG AGT GAC C-3', 5'-GCG TCA AAC TTG TGG CTC AG-3', and 5'-AGG AAA ACA GCC ACC AGG TGA AGG AGG-3'; rat Atrogin-1; 5'-CTT TCA ACA GAC TGG ACT TCT CGA-3', 5'-CAG CTC CAA CAG CCT TAC TAC GT-3', and 5'-TCG CAT CCT GGA TTC CAG AAG ATT CAA C-3'; mouse MuRF1; 5'-TGT CTG GAG GTC GTT TCC G-3', 5'-TGC CGG TCC ATG ATC ACT T-3', and 5'-TGC CCC TCG TGC CGC CAT-3'; rat GR; 5'-GAA TGA CTT GGG CTA CCC ACA-3', 5'-GAA GCC GAA AGT CTG TTT CCC-3', and 5'-CAG GGC CAA CTT GGC CTT TCC TCT-3'; mouse GR; 5'-ACA GCA ACG GGA CCA CCT C-3', 5'-AAT GGC ATC CCG AAG CTT C-3', and 5'-CAA ACT CTG CCT GGT GTG CTC CGA-3'. To measure mouse atrogin-1 mRNA levels, rat atrogin-1 primers were used, as these primers were validated to cross-react with mouse (C2C12) RNA (V. Petkova, unpublished work). Three or four individual wells were used for each experimental group. mRNA levels in control myotubes were arbitrarily set to 1.0.

## STATISTICAL ANALYSIS

Results are presented as means  $\pm$  SEM. Statistical analysis was performed by ANOVA followed by Tukey's or Student–Newman–Keul's test, as appropriate.  $P < 0.05$  was considered statistically significant. Most experiments were repeated at least three times to provide evidence of reproducibility.

## RESULTS AND DISCUSSION

In several previous reports, treatment of cultured L6 or C2C12 myotubes with dexamethasone was used as an in vitro model of muscle atrophy. The concentration of dexamethasone varied substantially in those studies with effects of dexamethasone induced by concentrations ranging from 10 to 50 nM [Thompson et al., 1999; Du et al., 2000; Marinovic et al., 2006] to 100  $\mu$ M [Stitt et al., 2004; Latres et al., 2005]. No comparison of the effects of different concentrations of dexamethasone on protein degradation

in the two cell lines has been reported previously. Here, we compared protein breakdown rates in L6 and C2C12 myotubes treated with different concentrations of dexamethasone (Fig. 1A,B). Basal protein breakdown rates were similar (approximately 20–22%/24 h) in the two cell lines. Also, the sensitivity and responsiveness to dexamethasone were similar in L6 and C2C12 myotubes with a significant increase in protein degradation induced by 10 nM dexamethasone and a maximal effect (approximately 15–20%) noticed for 100 nM dexamethasone. The results are similar to previous reports in which treatment with dexamethasone stimulated protein degradation in both L6 [Wang et al., 1998; Evenson et al., 2005; Li et al., 2005] and C2C12 myotubes [Thompson et al., 1999; Sacheck et al., 2004; Stitt et al., 2004; Latres et al., 2005].

The present observations are important because they suggest that protein degradation is regulated by dexamethasone to a similar extent in the two cell lines and that dexamethasone can induce proteolysis at concentrations corresponding to expected physiological corticosterone levels. Serum concentrations of corticosterone

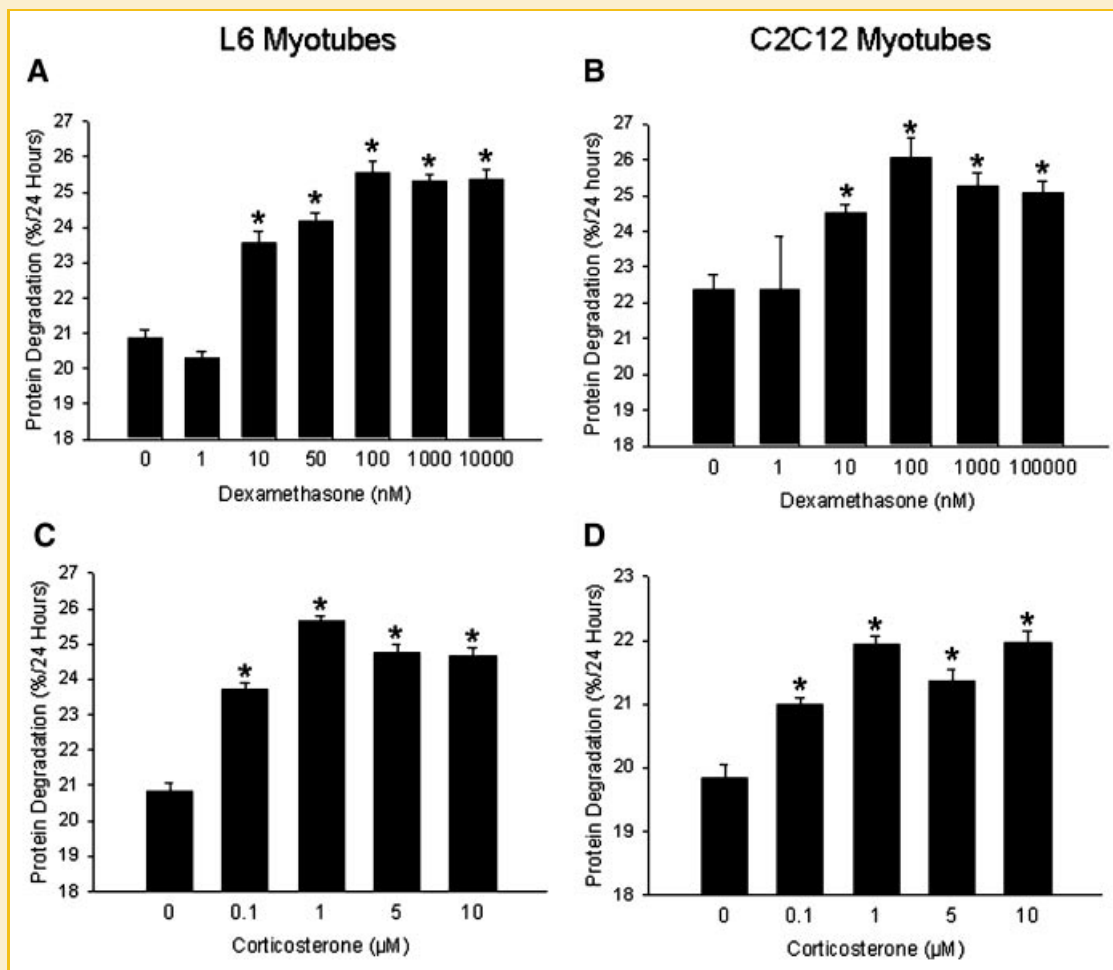


Fig. 1. Glucocorticoids increase protein degradation in cultured L6 and C2C12 myotubes in a dose-dependent manner. Proteins in differentiated myotubes were prelabeled for 48 h with [ $^3$ H]-tyrosine and the myotubes were then treated for 24 h with different concentrations of dexamethasone (A,B) or corticosterone (C,D). Control myotubes were treated with solvent (0.1% ethanol). Protein degradation was determined by measuring release of TCA-soluble radioactivity into the culture medium as described in Materials and Methods Section. Results are means  $\pm$  SEM with  $n = 6$  per group. \* $P < 0.05$  versus control (0 dexamethasone or corticosterone).



in rats and mice vary from 0.06 to 0.40  $\mu\text{M}$  under normal conditions and from 0.9 to 3.0  $\mu\text{M}$  during stress [Raina and Jeejeebhoy, 1998; Voisin et al., 1998; Faggioni et al., 2000; Fischer et al., 2001]. Since dexamethasone is an artificial glucocorticoid with a potency approximately 25 times greater than that of the naturally occurring corticosterone [Orth et al., 1992], concentrations up to about 120 nM are in the physiological range. Hence, dexamethasone concentrations used in many previous studies corresponded to supraphysiological concentrations of corticosterone, at least when comparing with normal serum concentrations of corticosterone.

It should be noted, however, that comparing in vitro concentrations of glucocorticoids with physiological circulating concentrations needs to be done with caution. First, although normal ranges of corticosterone serum concentrations in rats have been established, it is not known what the corresponding muscle tissue concentrations are. Second, the in vitro experiments in the present study were conducted in the presence of serum (2% FBS), which contains albumin that is known to bind steroids [Milgrom, 1990], so even the free hormone concentration directly acting upon the cultured myotubes is not precisely known.

The influence of different concentrations of corticosterone, including concentrations in the range of normal rat and mouse serum levels, on protein degradation in cultured L6 and C2C12 myotubes is not known. Here, we found that treatment of the two cell lines with corticosterone at concentrations well within the range of estimated physiological concentrations resulted in increased protein degradation (Fig. 1C,D). A significant increase in protein degradation was noticed at a corticosterone concentration of 0.1  $\mu\text{M}$  in both cell lines consistent with a similar sensitivity to corticosterone in L6 and C2C12 myotubes. The maximal effect was seen at a corticosterone concentration of 1  $\mu\text{M}$  and was somewhat greater in the L6 myotubes (approximately 20% increase in protein degradation) than in the C2C12 myotubes (approximately 10% increase in protein degradation). Taken together, the results shown in Figure 1 suggest that glucocorticoid-induced protein degradation in cultured L6 and C2C12 myotubes is not specific for the artificial glucocorticoid dexamethasone but can be induced by the naturally occurring corticosterone as well, even at concentrations corresponding to normal serum levels of the hormone.

We next examined whether the increase in protein degradation caused by dexamethasone and corticosterone was associated with reduced size of the myotubes. Treatment of the cultured muscle cells with glucocorticoids did not significantly affect myotube diameter after 24 h but decreased the diameter of L6 and C2C12 myotubes by approximately 25% and 30%, respectively, after treatment for 48 h (Fig. 2A,B). These results suggest that the increase in protein degradation caused by dexamethasone and corticosterone results in myotube atrophy.

In order to determine whether reduced protein synthesis may also have contributed to the reduction in myotube size, the response to glucocorticoids of protein synthesis was examined. In L6 cells, dexamethasone induced a slight but statistically significant decrease in protein synthesis, while corticosterone did not alter protein synthesis rates (Fig. 3A), in C2C12 cells, glucocorticoids had no effect on protein synthesis (Fig. 3B). Although reduced muscle protein synthesis after treatment with glucocorticoids has been reported in

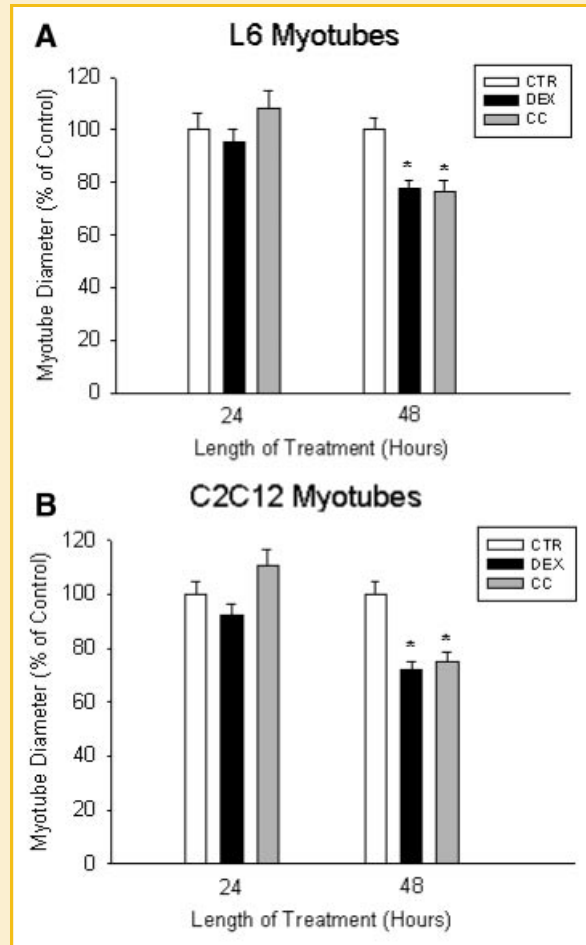


Fig. 2. Treatment of cultured L6 and C2C12 myotubes with glucocorticoids results in myotube atrophy. Differentiated L6 (A) and C2C12 (B) myotubes were treated with vehicle (0.1% ethanol), 1  $\mu\text{M}$  dexamethasone (DEX), or 1  $\mu\text{M}$  corticosterone (CC) for 24 and 48 h. Myotube diameters from randomly selected fields were quantified using an image analysis program as described in Materials and Methods Section. Diameters were expressed as percent of control. Results are means  $\pm$  SEM with  $n = 60$  per group. \* $P < 0.05$  versus control (CTR).

several studies [Schakman et al., 2008], variable and apparently conflicting results have also been reported. For example, studies in L6 myotubes and myoblasts demonstrated that dexamethasone either increased or decreased protein synthesis depending upon the concentration of hormone used [Orzechowski et al., 2003], or the length of time of treatment [Roeder et al., 1986]. Other studies showed that short-term treatment (hours) with dexamethasone reduced protein synthesis in C2C12 myotubes [Desler et al., 1996] and L6 myoblasts [Shah et al., 2000]. Thus, the effect of dexamethasone on protein synthesis appears to depend upon the concentration of glucocorticoid used, the length of time of treatment, and the differentiation state of the cells (myoblasts vs. myotubes). Regardless, the results in the present study suggest that the increase in protein degradation induced by glucocorticoids is the primary cause of myotube atrophy, at least under the present experimental conditions.

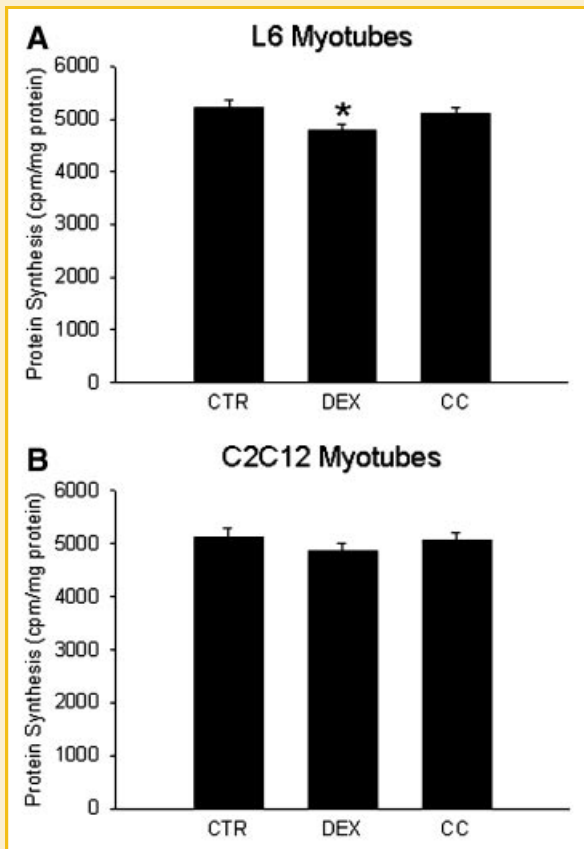


Fig. 3. Treatment of cultured L6 and C2C12 myotubes with glucocorticoids does not alter protein synthesis. L6 (A) and C2C12 (B) myotube proteins were prelabeled for 1 h with [ $^3$ H]-tyrosine following a 48 h treatment with 1  $\mu$ M dexamethasone (DEX) or 1  $\mu$ M corticosterone (CC). Control myotubes (CTR) were treated with solvent (0.1% ethanol). Protein synthesis was determined by measuring the incorporation of TCA-insoluble radioactivity into cellular proteins as described in Materials and Methods Section. Results are means  $\pm$  SEM with  $n = 4-8$  per group. \* $P < 0.05$  versus control.

Previous studies suggest that muscle proteolysis in various catabolic conditions, including sepsis [Tiao et al., 1994, 1996, 1997; Wray et al., 2003; Klaude et al., 2007], burn injury [Fang et al., 1995; Pereira et al., 2005], and cancer [Williams et al., 1999; Acharya and Guttridge, 2007], at least in part reflects increased ubiquitin-proteasome-dependent protein breakdown. Recent observations suggest that the ubiquitin ligases atrogen-1 and MuRF1 are particularly important for the regulation of the ubiquitin-proteasome system in atrophying muscle [Bodine et al., 2001; Gomes et al., 2001; Wray et al., 2003] and in dexamethasone-treated myotubes [Sacheck et al., 2004; Stitt et al., 2004; Evenson et al., 2005; Latres et al., 2005].

It is not known from previous studies whether atrogen-1 and MuRF1 are regulated similarly by dexamethasone and corticosterone in L6 and C2C12 myotubes. In the present study, treatment with dexamethasone (50 nM or 1  $\mu$ M) or corticosterone (0.1 or 1  $\mu$ M) resulted in an approximately 2- to 2.5-fold increase in atrogen-1 mRNA levels in L6 and C2C12 myotubes (Fig. 4A,B). Interestingly, the sensitivity to corticosterone with regards to atrogen-1 expression

was lower in C2C12 than in L6 myotubes. Thus, atrogen-1 mRNA levels were significantly increased by 0.1  $\mu$ M corticosterone in L6 myotubes whereas in the C2C12 myotubes, a corticosterone concentration of 10  $\mu$ M was required to induce an increase in atrogen-1 mRNA levels. Increased expression of atrogen-1 in dexamethasone-treated L6 [Evenson et al., 2005; Yang et al., 2005a] and C2C12 [Sacheck et al., 2004; Stitt et al., 2004; Latres et al., 2005] myotubes was observed in previous reports as well, supporting the important role of this ubiquitin ligase in glucocorticoid-regulated muscle wasting. The present findings expand previous observations by showing that corticosterone, like dexamethasone, increases the expression of atrogen-1 in cultured myotubes.

MuRF1 mRNA levels were increased two- to threefold by dexamethasone and corticosterone in L6 myotubes (Fig. 4C). In sharp contrast, MuRF1 expression was not influenced by the hormones in C2C12 myotubes (Fig. 4D). The unresponsiveness to both dexamethasone and corticosterone of MuRF1 expression in the C2C12 myotubes was observed in repeated experiments. This observation is supported by previous studies indicating that MuRF1 is not strongly regulated by glucocorticoids in C2C12 myotubes. For example, Stitt et al. [2004] reported that whereas treatment of cultured C2C12 myotubes for 24 h with different concentrations (1, 10, or 100  $\mu$ M) of dexamethasone resulted in a relatively robust upregulation of atrogen-1 mRNA levels, MuRF1 mRNA was not influenced by 1 or 10  $\mu$ M dexamethasone and was only slightly increased after treatment of the myotubes with 100  $\mu$ M dexamethasone. A similar relative unresponsiveness of MuRF1 to dexamethasone in C2C12 myotubes was reported by Sacheck et al. [2004]. In their study, treatment of cultured C2C12 myotubes with 400 ng/ml (1  $\mu$ M) of dexamethasone for 6 h resulted in a fivefold increase in atrogen-1 mRNA levels but only a twofold increase in MuRF1 mRNA levels. Notably, in the study by Sacheck et al. [2004], "dexamethasone" treatment consisted of a combined treatment with dexamethasone (400 ng/ml) and triiodothyronine (T3; 100 ng/ml) because other experiments in the same study indicated that the effects of dexamethasone were potentiated by T3. The combined treatment may be a reason why "dexamethasone" stimulated the expression of atrogen to a greater extent (5-fold) than the approximately 2- to 2.5-fold increase that was observed in the present and other studies [Sultan et al., 2006] and why MuRF1 mRNA levels were increased at all.

Interestingly, there is evidence to suggest that the down-regulation of atrogen-1 and MuRF1 by anabolic factors may also be differentially regulated in C2C12 myotubes. For example, in the report by Sacheck et al. [2004], treatment of C2C12 myotubes with insulin or IGF-1 for 6 h resulted in an approximately 70% decrease in atrogen-1 expression but did not reduce MuRF1 mRNA levels. The mechanism behind the unresponsiveness of MuRF1 to glucocorticoids (as well as insulin and IGF-1) in C2C12 myotubes is not known at present. Although the two cell lines studied in the present report originated from different species (L6 myotubes being a rat cell line and C2C12 myotubes being a mouse cell line) it is not likely that the unresponsiveness of MuRF1 in C2C12 noticed here and in other reports [Sacheck et al., 2004; Stitt et al., 2004] reflected the species difference because increased MuRF1 expression in skeletal muscle

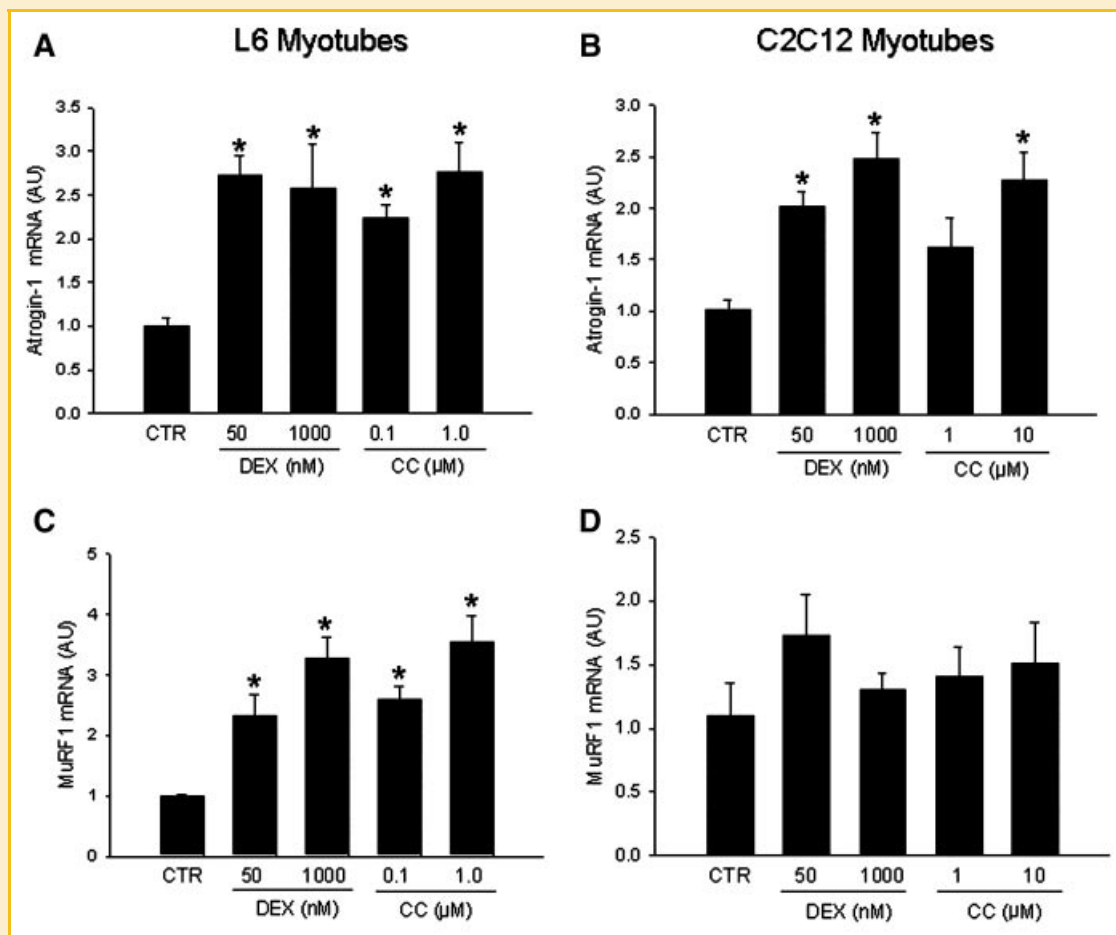


Fig. 4. Atrogin-1 and MuRF1 mRNA levels are differentially regulated by glucocorticoids in cultured L6 and C2C12 myotubes. Differentiated myotubes were treated for 24 h with different concentrations of dexamethasone (DEX) or corticosterone (CC) followed by determination of mRNA levels for atrogin-1 (A,B) or MuRF1 (C,D) by real-time PCR. mRNA levels in myotubes treated with solvent (0.1% ethanol; control) were arbitrarily set to 1.0. Results are means  $\pm$  SEM with  $n = 3$  or 4 per group. Similar results were seen in 3 repeated experiments. \* $P < 0.05$  versus control (CTR).

has been reported in various catabolic conditions in both rats [Wray et al., 2003; Lang et al., 2006] and mice [Bodine et al., 2001; Cai et al., 2004].

Another potential mechanism whereby glucocorticoids induce different responses in L6 and C2C12 myotubes may be different abundance of the GR in the two cell lines. In order to examine that possibility, we determined GR concentrations by Western blotting. C2C12 myotubes did not express lower levels of the GR but actually expressed higher concentrations of the hormone receptor (Fig. 5A). As expected, treatment with dexamethasone or corticosterone resulted in reduced levels of the GR in both cell lines, consistent with a negative feed-back effect [Vujcic et al., 2007]. Similar to previous reports in other cell types [Erdeljan et al., 2005], the glucocorticoid-induced downregulation of the GR protein levels was associated with reduced concentrations of GR mRNA (Fig. 5B), suggesting that glucocorticoids downregulate the expression of the GR at the transcriptional level.

Glucocorticoids bind to the GR in the cytoplasm followed by dimerization and translocation of the receptor into the nucleus. In the nucleus, the GR binds to glucocorticoid response elements of glucocorticoid-responsive genes, resulting in transcriptional

regulation [Heitzer et al., 2007]. In the present experiments, treatment of both L6 and C2C12 myotubes with dexamethasone or corticosterone resulted in increased nuclear translocation of the GR (Fig. 5C). Taken together, the results in Figure 5 suggest that a less pronounced response to glucocorticoids in C2C12 compared to L6 myotubes (with regards to MuRF1 expression and corticosterone-induced protein degradation) does not reflect a lower concentration of the GR or different regulation of the GR by glucocorticoids in C2C12 myotubes.

Importantly, in a recent study we found that treatment of cultured C2C12 myotubes with  $\text{TNF}\alpha$  or  $\text{IL-1}\beta$  activated the MuRF1 promoter [Cai et al., 2004] suggesting that MuRF1 may be sensitive to catabolic factors other than glucocorticoids in C2C12 myotubes. Indeed, additional experiments in the same study supported a model in which proinflammatory cytokines upregulate MuRF1 expression and cause muscle atrophy through an NF- $\kappa$ B-dependent mechanism. Thus, the present results and previous reports suggest that although both L6 and C2C12 myotubes can be used to examine glucocorticoid-induced protein degradation, there may be significant differences between the two cell lines with regards to the involvement of MuRF1 in the response to glucocorticoids.

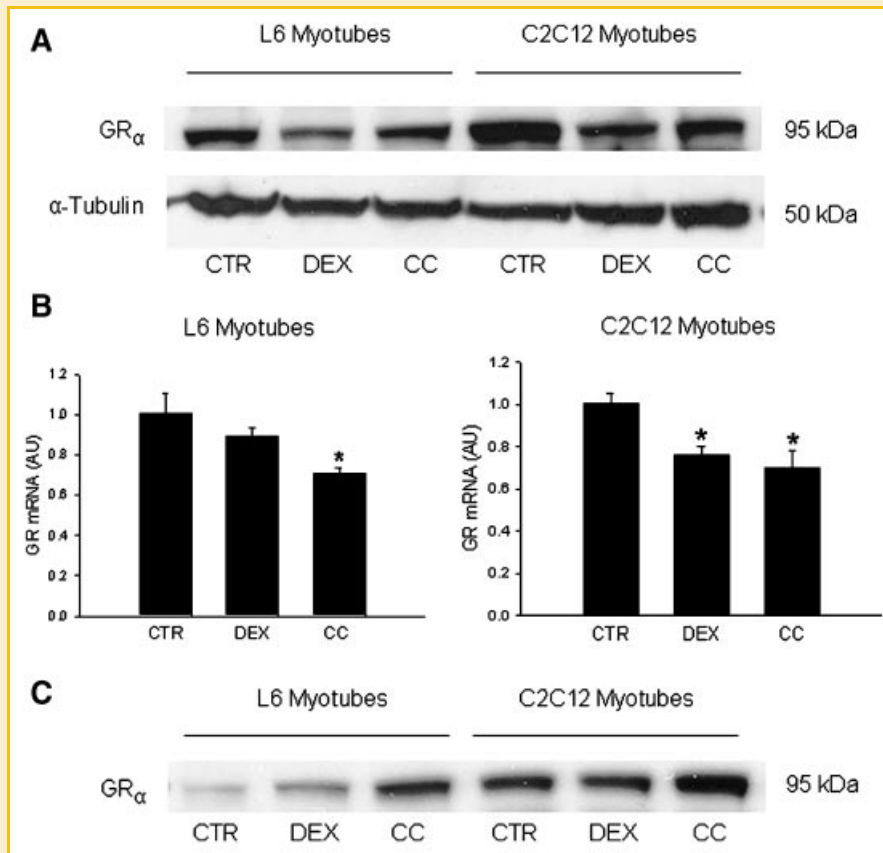


Fig. 5. The glucocorticoid receptor (GR) is expressed in cultured L6 and C2C12 myotubes. Differentiated myotubes were treated for 24 h with 1  $\mu$ M dexamethasone (DEX) or 1  $\mu$ M corticosterone (CC). Control myotubes (CTR) were treated with solvent (0.1% ethanol). A: GR protein levels were determined by Western blotting in total cell lysates showing a glucocorticoid-induced decrease in GR levels. The GR bands represent the GR $\alpha$  isoform with a molecular weight of 95 kDa.  $\alpha$ -Tubulin levels were determined as loading controls. B: GR mRNA levels were determined by real-time PCR as described in Materials and Methods Section. GR mRNA levels in control myotubes were arbitrarily set to 1.0. Results are means  $\pm$  SEM with  $n = 3$  per group. \* $P < 0.05$  versus control. C: GR protein levels in myotube nuclear extracts showing a glucocorticoid-induced translocation of GR from the cytoplasm into the nucleus.

Glucocorticoids typically exert their metabolic effects secondary to binding to the GR although other, GR-independent, mechanisms have been described as well [Sawart and Cabillic, 1985; Falkenstein et al., 2000; Borski et al., 2002]. The role of GR-binding in glucocorticoid-induced metabolic changes can be tested by using the GR antagonist RU38486 [Konagaya et al., 1986]. This drug also binds to and blocks the progesterone receptor [Philibert, 1984] but because skeletal muscle does not express the progesterone receptor [Perrot-Applanat et al., 1985], effects of RU38486 in skeletal muscle are usually considered to reflect GR inhibition [Konagaya et al., 1986]. RU38486 was used in several previous reports both from our [Hall-Angeras et al., 1991; Fang et al., 1995; Tiao et al., 1996] and other laboratories [Lang et al., 2006] to examine the role of glucocorticoids in muscle wasting.

In order to test the role of the GR in dexamethasone- and corticosterone-induced changes in protein degradation and ubiquitin ligase expression in the present study, we treated myotubes with RU38486. Treatment of L6 myotubes with 10  $\mu$ M RU38486 alone did not influence protein degradation but blunted the dexamethasone- and corticosterone-induced increase in protein degradation (Fig. 6A). In contrast to the finding in L6 myotubes, treatment of

C2C12 myotubes with RU38486 alone stimulated protein degradation (Fig. 6B). Although RU38486 usually does not exert an agonist effect after binding to the GR, agonist effects have been reported previously [Schulz et al., 2002; Yang et al., 2005b]. When dexamethasone-treated C2C12 myotubes were exposed to RU38486, the increased protein degradation was not reduced (third and fourth bar in Fig. 6B). The increase in protein degradation caused by RU38486 alone makes interpretation of this observation difficult and it is not known if the lack of inhibition by RU38486 reflected RU38486-induced increase in protein degradation or GR-independent effects of dexamethasone in C2C12 myotubes. The fact that RU38486 reduced protein degradation in corticosterone-treated C2C12 myotubes (last two bars in Fig. 6B), however, suggests that glucocorticoids induce protein degradation in C2C12 myotubes at least in part secondary to binding to the GR receptor, similar to the mechanism in glucocorticoid-treated L6 myotubes.

Similar to the effects on protein degradation, RU38486 alone did not influence the expression of atrogen-1 in L6 myotubes but upregulated atrogen-1 mRNA levels approximately 2.5-fold in C2C12 myotubes, almost identical to the effect of dexamethasone (Fig. 7A,B). Atrogen-1 mRNA levels in dexamethasone- and



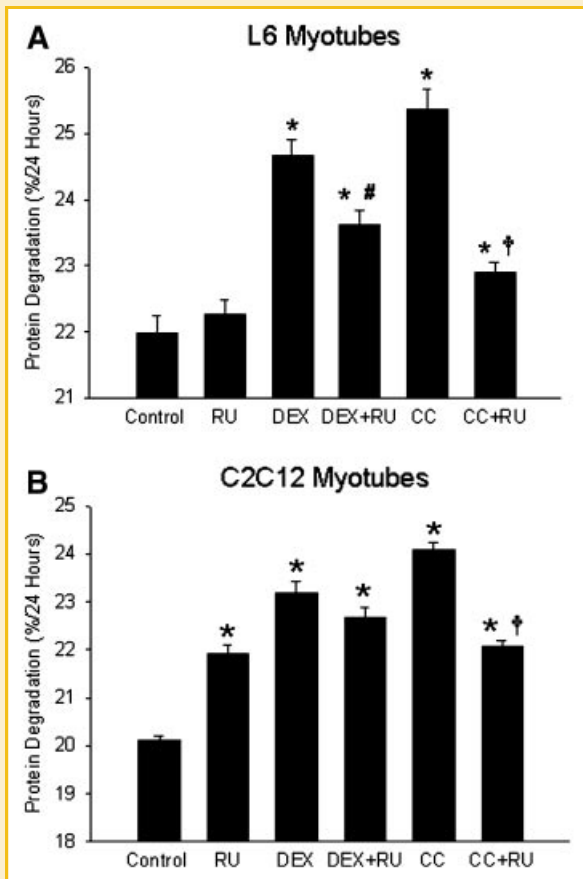


Fig. 6. Protein degradation is differentially regulated by the glucocorticoid receptor antagonist RU38486 in cultured L6 and C2C12 myotubes. Differentiated L6 (A) and C2C12 (B) myotubes were treated for 24 h with 1  $\mu$ M dexamethasone (DEX) or 1  $\mu$ M corticosterone (CC) in the absence or presence of 10  $\mu$ M RU38486 (RU). Other myotubes were treated with 10  $\mu$ M RU38486 alone. Control myotubes were treated with solvent (0.1% ethanol). Results are means  $\pm$  SEM with  $n = 6$  per group. \* $P < 0.05$  versus control; # $P < 0.05$  versus dexamethasone; † $P < 0.05$  versus corticosterone.

corticosterone-treated L6 myotubes were reduced by RU38486 but were not significantly influenced by the glucocorticoid receptor antagonist in glucocorticoid-treated C2C12 myotubes.

The effects of RU38486 on MuRF1 mRNA levels in control and glucocorticoid-treated myotubes are shown in Figure 7C,D. The regulation by RU38486 of MuRF1 expression in L6 myotubes (Fig. 7C) was similar to the regulation of atrogin-1 expression (see Fig. 7A). In C2C12 myotubes, RU38486 did not influence MuRF1 mRNA levels in control or glucocorticoid-treated C2C12 myotubes (Fig. 7D).

The reason why the effects of RU38486 were different in L6 and C2C12 myotubes is not known at present. An agonist effect of RU38486 combined with a relative inability to block glucocorticoid-induced changes (as noticed in the C2C12 myotubes in the present study) has been reported in other cell types as well [Schulz et al., 2002; Zhang et al., 2003] and may reflect the involvement of GR-independent signaling pathways induced by glucocorticoids [Sawart and Cabillic, 1985; Falkenstein et al., 2000; Borski et al., 2002]. Results from experiments in other cell types suggest that GR-

independent responses to glucocorticoid treatment may at least in part be mediated by membrane-associated receptors and activation of various intracellular signaling pathways [Lackner et al., 1998; Qiu et al., 2001].

It should be noted that although results in the present study suggest that an agonist effect of RU38486 may be specific for the C2C12 myotubes, in recent studies we found that RU38486 had an agonist effect on the expression of the nuclear cofactor p300 and the transcription factor C/EBP $\beta$  in L6 myotubes and did not prevent the dexamethasone-induced increase in p300 and C/EBP $\beta$  levels [Yang et al., 2005a,b]. Thus, it is possible that different effects of glucocorticoids are mediated by different mechanisms in the same cell type as exemplified by the inability of RU38486 to prevent dexamethasone-induced p300 and C/EBP $\beta$  expression in L6 myotubes [Yang et al., 2005a,b] and inhibition of glucocorticoid-induced protein degradation and expression of atrogin-1 and MuRF1 by RU38486 in L6 myotubes in the present study.

Although the present results support the use of dexamethasone-treated L6 and C2C12 myotubes as in vitro models of muscle wasting, the study has several potential limitations that need to be considered. First, the effects of dexamethasone or corticosterone alone were determined. Even if glucocorticoids may be important regulators of muscle breakdown [Hasselgren, 1999; Menconi et al., 2007], there is evidence that muscle proteolysis in various catabolic conditions is regulated by multiple factors in addition to glucocorticoids, in particular the proinflammatory cytokines TNF $\alpha$  and IL-1 $\beta$  [Argiles et al., 2005]. The observation that MuRF1 was upregulated in C2C12 myotubes by TNF $\alpha$  and IL-1 $\beta$  in recent experiments [Cai et al., 2004] but not by glucocorticoids in the present study illustrates the importance of catabolic factors other than glucocorticoids in the development of muscle wasting. Interestingly, previous studies suggest that glucocorticoids and TNF interact at the cellular level to induce muscle wasting [Hall-Angeras et al., 1990]. Other studies suggest that most of the catabolic effects of TNF are secondary to TNF-induced release of glucocorticoids from the adrenal glands [Warren et al., 1988; Zamir et al., 1992], further supporting the use of myotubes treated with glucocorticoids as a model of muscle wasting.

An additional limitation of the present study is the fact that only L6 and C2C12 myotubes were studied. Although several other cell lines have been used to examine the regulation of muscle protein degradation, for example L8 myotubes [Hong and Forsberg, 1995], most previous muscle wasting-related studies, both from our and other laboratories, used cultured L6 or C2C12 myotubes. In addition, by using L6 (a rat cell line) and C2C12 (a mouse cell line) myotubes, potential species-specific differences in glucocorticoid-induced muscle wasting could be assessed.

Additionally, we determined total protein degradation in the present study, despite the fact that previous reports suggest that myofibrillar proteins are preferentially degraded in catabolic muscle [Tiao et al., 1994, 1996]. In previous reports, however, evidence was found that measurement of total protein degradation (determined as release of TCA-soluble radioactivity from myotube proteins pre-labeled with radioactive tyrosine or phenylalanine) in dexamethasone-treated myotubes reflected myofibrillar protein degradation [Wang et al., 1998; Thompson et al., 1999; Sacheck et al., 2004].

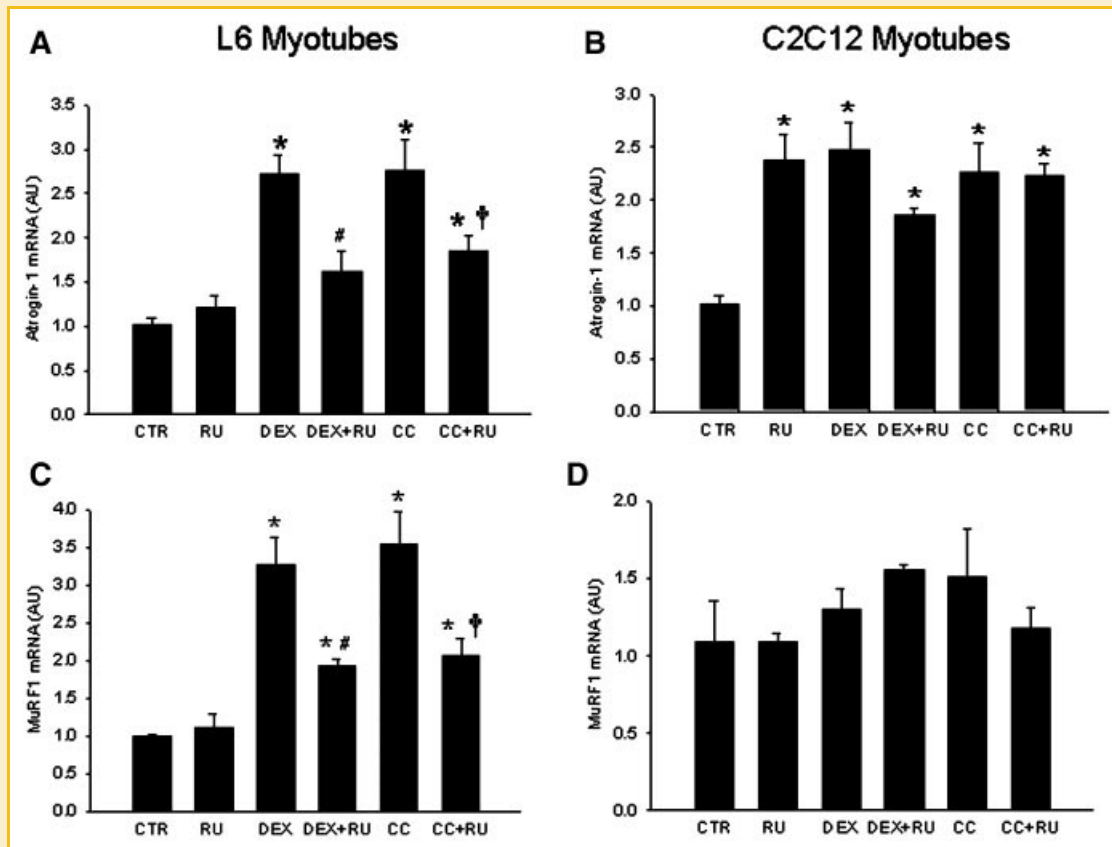


Fig. 7. Atrogin-1 and MuRF1 mRNA levels are differentially regulated by the glucocorticoid receptor antagonist RU38486 in cultured L6 and C2C12 myotubes. Differentiated myotubes were treated for 24 h with 1  $\mu$ M dexamethasone (DEX) or 1  $\mu$ M corticosterone (CC) in the absence or presence of 10  $\mu$ M RU38486 (RU). Other myotubes were treated with 10  $\mu$ M RU38486 alone. Control myotubes (CTR) were treated with solvent (0.1% ethanol). After treatments, mRNA levels for atrogin-1 (A,B) and MuRF1 (C,D) were determined by real-time PCR. Results are means  $\pm$  SEM with  $n = 3$  or 4 per group. \* $P < 0.05$  versus control; # $P < 0.05$  versus dexamethasone; † $P < 0.05$  versus corticosterone.

Finally, it is not known from the present study which cellular proteolytic pathway was stimulated by the glucocorticoids. Previous reports suggest, however, that treatment of cultured myotubes with dexamethasone stimulates both calcium-calpain- and ubiquitin-proteasome-dependent proteolysis [Hong and Forsberg, 1995; Wang et al., 1998], similar to the upregulated expression and activity of the calpain and ubiquitin-proteasome systems in vivo in various catabolic conditions [Tiao et al., 1994; Wray et al., 2003; Wei et al., 2005].

Although several muscle wasting-related responses to dexamethasone and corticosterone were similar in L6 and C2C12 myotubes in the present study, the effects of the glucocorticoids were not identical. Most notably, the regulation by glucocorticoids of MuRF1 and the role of the GR were different in L6 and C2C12 myotubes. These differences should be taken into account when cultured myotubes are used in future studies to further explore mechanisms of muscle wasting.

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