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The effect of glutamine on prevention of glucocorticoid-induced skeletal muscle atrophy is associated with myostatin suppression

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Abstract

Excess glucocorticoids (GCs) cause muscle atrophy. Glucocorticoid-induced muscle atrophy is associated with increased intramuscular myostatin expression. Myostatin is a negative regulator of skeletal muscle mass. Glutamine prevents GC-induced muscle atrophy. We hypothesized that glutamine effect on reversal of GC-induced muscle atrophy is mediated in part by suppression of myostatin. We administered daily to male Sprague-Dawley rats dexamethasone, dexamethasone plus glutamine, saline or saline plus glutamine, all pair-fed. Animals were killed on day 5. Body weight and weights of gastrocnemius muscles were measured. Myostatin expression was measured by Northern and Western blots, and was compared with glyceraldehyde-3-phosphate dehydrogenase. Myoblast C2C12 cells were exposed to dexamethasone, or dexamethasone and glutamine, and their myostatin messenger RNA and protein expression compared with glyceraldehyde-3-phosphate dehydrogenase. Myostatin promoter activity was measured by luciferase activity of transfected C2C12 cells, grown in medium including dexamethasone, or dexamethasone plus glutamine. Rats that received dexamethasone showed significant body and muscle weight loss accompanied by an increase in intramuscular myostatin expression, compared with their saline-treated controls. Pair-fed rats given dexamethasone plus glutamine had significantly less reduction in body and muscle weights and lower myostatin expression when compared with those treated with dexamethasone alone. In C2C12 myoblast cells, addition of glutamine to dexamethasone prevented the hyperexpression of myostatin induced by dexamethasone. Myostatin promoter activity increased in cells exposed to dexamethasone, but this increase was partially blocked by addition of the glutamine. Administration of glutamine partially prevents GC-induced myostatin expression and muscle atrophy, providing a potential mechanism for the prevention of muscle atrophy induced by glucocorticoids.

1. Introduction

Glucocorticoid (GC) treatment causes rapid muscle atrophy in animals and humans leading to serious clinical side effects of wasting and debilitation [1]. This GC-induced atrophy of skeletal muscles primarily affects the type II muscle fibers (fast twitch) [2]. Glucocorticoids have catabolic and antianabolic effects on the major contractile muscle proteins including myosin heavy chain (MHC) [2]. Glucocorticoid catabolic effects in increasing protein breakdown have been shown in part to be mediated by activation of ubiquitin proteasome pathways, which induces increased synthesis of proteosomal proteins and the cellular machinery for protein degradation [3,4]. However, this is only a part of the complex regulation of muscle metabolism by GCs because they also inhibit the rate of skeletal muscle protein synthesis, growth, and repair [5]. The mechanism for this antianabolic effect of GC is not completely understood, but it is consistent with the up-regulation by GC of a negative myogenic regulatory protein, myostatin [6], which inhibits proliferation of muscle satellite precursor cells [7,8].

Glutamine is a conditional essential amino acid during catabolic states [9]. Glutamine and alanyl-glutamine dipeptide are able to prevent GC-induced muscle atrophy in rats compared with alanine and alanyl-alanine, respectively [10,11]. In humans, during acute and severe illnesses, the daily need for glutamine increases by several-fold and makes it an indispensable amino-acid to meet the requirement of acute critical illnesses [12,13]. Recent meta-analysis of published data have shown that treatment with glutamine decreases mortality, morbidity, and hospital stay in severely ill patients [12], conditions known to be associated with endogenous hypercortisolemia.

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Myostatin protein is a negative regulator of muscle mass expressed predominantly in type II muscle fibers (fast twitch) and secreted in the blood [14,15], which directly inhibits muscle precursor myoblast cell proliferation [7,8] and differentiation [16,17]. The myostatin gene promoter contains several GC response elements (GREs), making myostatin a potential target of GC regulation [18]. We have previously shown that administration to rats of the potent GC, dexamethasone, dose dependently induces up to a 7-fold increase in intramuscular myostatin messenger RNA (mRNA) and up to 3- to 4-fold increase in myostatin protein expression [6]. RU486, a nonspecific GC receptor inhibitor, inhibits this dexamethasone-induced myostatin overexpression and muscle atrophy [6]. After dexamethasone administration, a decrease of MHC type II protein expression, with a concomitant muscle mass and weight loss, is associated with this increase of myostatin expression [6]. In addition, we have shown that myostatin protein is induced by dexamethasone in C2C12 myocytes and is localized perinuclearly in myotubes with myosin heavy chain type II [19]. The up-regulation of myostatin by GCs is consistent with, and may directly contribute to inhibition of MHC expression observed during skeletal muscle atrophy.

The mechanism of glutamine counteracting GC-induced muscle atrophy is unknown. In this study, we examine the hypothesis that glutamine's effect in prevention of GC-induced muscle atrophy is in part mediated through down-regulation of myostatin expression, specifically by blocking GC induction of myostatin. We have studied the effects of glutamine on GC induction of myostatin protein and mRNA expression in rat muscles, in the well-studied C2C12 myoblast cell line, and using a myostatin-luciferase promoter plasmid construct.

2. Methods

2.1. Animal care and experimental treatments

Male Sprague-Dawley rats, 10 to 12 weeks of age, with initial body weights ranging from 250 to 420 g, were purchased from Harlan Laboratories (Indianapolis, IN). Each animal was housed individually in controlled environmental conditions (temperature, 22°C; 12-hour light-dark cycles of period starting at 06:00 AM) and provided standard laboratory rodent chow and water. All experimental procedures on animals were approved by the Institutional Animal Care and Use Committee of the Charles R. Drew University of Medicine and were in accordance with National Institutes of Health guidelines for humane treatment of laboratory animals.

2.2. In vivo study

We examined the effects of glutamine (2 g/100 g body weight) and dexamethasone treatment at 600 μg/kg body weight, on skeletal muscle weight, and intramuscular myostatin protein and mRNA expression. Dexamethasone was used because, similar to other fluorinated steroids, it causes more severe muscle atrophy compared with hydrocortisone and other nonfluorinated GCs. Sixteen adult male rats were divided into 4 weight-matched groups by matching equally in each category their equally weighted counterpart rats. Group 1 received intraperitoneal injections of 1 mL of dexamethasone (Sigma, St. Louis, MO) dissolved in saline (0.85% NaCl) at a daily dose of 600 μg/kg body weight. This dose was selected because administration of dexamethasone at 600 μg/kg body weight and higher has been shown previously to cause maximum muscle atrophy [20,21] and also a 7-fold increase in expression of myostatin mRNA [6]. Group 2, a control group, received daily intraperitoneal injection of 1 mL of saline (vehicle, 0.85% NaCl). The 5-day treatment period was chosen because, in our previous study, we observed that the rapid decline of muscle mass ensues in the first 5 days of dexamethasone administration compared with 10-day treatment [6]. Each control animal had a pair-mate (matched by their body weights) in the corresponding group receiving dexamethasone treatment. All dexamethasone-treated animals were allowed free access to food and water. Dexamethasone treatment has been reported to decrease food intake [20,6]. It is not known whether food intake can independently affect myostatin expression in animals; therefore, each control animal was pair-fed the same amount of food as was consumed by its dexamethasone-treated, paired mate during the previous day. The study group 3 received dexamethasone (600 μg/kg body weight intraperitoneally) and glutamine (2 g/100 g body weight per day) was provided to their drinking water, of which about half was consumed. They were also allowed to consume the same amount of food as the dexamethasone group 1. In previous studies in rats the dose of glutamine used was 4.32 mmol (604 mg per rat) per day intravenously [10,11]. This dose has been demonstrated to prevent at least 50% muscle atrophy induced by GCs. Group 4 consisted of 4 rats weight-matched with group 1, but they received only glutamine in their drinking water at the dose of 2 g/100 g body weight per day and the same amount of food as the dexamethasone group. The reason to choose the glutamine control group is to eliminate the hypothetical possibility of glutamine alone affecting myostatin expression. The average daily glutamine intake of rats was 1 g/100 g body weight per day.

2.3. Muscle collection

After each experimental protocol, animals were killed by carbon dioxide asphyxiation. The muscles of the gastrocnemius and flexor digitorum superficialis (referred to as G/FDS) complex from both legs were excised together, weighed on an analytical balance, and quickly frozen with liquid nitrogen.

2.4. Northern blot analysis

Total RNA was extracted from the G/FDS muscle complex by homogenization with Trizol (Invitrogen-Gibco, Rockville, MD) following the instructions provided by the

manufacturer. The extracted RNA pellets were dissolved in distilled water pre-autoclaved with diethyl pyrocarbonate. After RNA concentrations were determined by absorption at 260 nm, RNA samples were stored at −80°C until use. Twenty-five micrograms of heat-denatured total RNA for each muscle was electrophoresed (4 V/cm for 4 hours) on a 0.8% agarose denaturing gel prepared in MOPS (40 mmol/L 3-[*N*-morpholino]-2-hydroxy propanesulfonic acid, 10 mmol/L sodium acetate, 1 mmol/L EDTA, all from Sigma) and 6.6% formaldehyde (Sigma) to determine the relative abundance of myostatin and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. The RNA was transferred by Northern blotting to a Hybond-XL nylon membrane (Amersham, Buckinghamshire, UK) by overnight capillary action in 20× SSC (3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.0). RNA bound membranes were treated with ultraviolet cross-linking (UV Stratalinker 1800, Stratagene, La Jolla, CA) at 70 000 μJ, as recommended by the manufacturer, to covalently bind RNA. Northern blot membranes were first prehybridized in hybridization buffer (5× Denhardt's, 6× SSC, 2.5% sodium dodecyl sulfate [SDS], and 100 μg/mL denatured sonicated salmon sperm DNA, 5 mL/100 cm^2 filters) in hybridization bottles at 60°C for at least 2 hours. DNA probes were radiolabeled by random priming with [α^{32}P]dCTP (ICN) using High Prime kit (Roche) following the manufacturer's instruction. For myostatin mRNA detection, a rat myostatin complementary DNA (cDNA) fragment of 748 base pairs (bp) (corresponding to nucleotides 178-925) was used as the probe. For GAPDH mRNA detection, a rat GAPDH cDNA fragment of 1.2 kilobases (kb) was used as the probe.

A radiolabeled probe was boiled for 5 minutes before being added to the prehybridized membranes at 60°C. After incubation overnight at 60°C, the membranes were washed (in SSC, 0.1% SDS at 60°C) to the desired stringency by reducing the SSC concentration. The membranes were then exposed to Kodak Biomax films for 2 to 5 days at −80°C to visualize bands corresponding to myostatin and GAPDH transcripts. Concentrations of myostatin and GAPDH mRNA were quantified by densitometry scanning (Flour-S Multiimager, Bio-Rad, Hercules, CA). The myostatin mRNA band intensity was normalized by dividing by the optical density of the GAPDH band, relative to the control assigned as 100%, using the densitometric software package, Quantiscan version 1.5.

2.5. Western blot analysis

Protein was extracted from the G/FDS complex (or cell cultures) using a denaturing-reducing lysis buffer containing 1% SDS, Tris-HCl, and 5% β-mercaptoethanol with protease inhibitors. Samples (40 μg of each) were heat denatured (95°C for 5 minutes) and electrophoretically separated using 12% Tris-glycine polyacrylamide gels (ReadyGel, Bio-Rad). The protein gels were then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham); the proteins were visualized using ponceau S staining and immunodetected using the previously described procedure with the myostatin polyclonal antibody "B" [21] and an antirabbit immunoglobulin G secondary antibody linked to horseradish peroxidase. Blots were developed with an enhanced chemiluminescent substrate for horseradish peroxidase (ECL, Amersham, RPN 2106) and exposed to film (ECL Hyperfilm, Amersham). The membranes were stripped by incubation in stripping buffer (100 mmol/L 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mmol/L Tris-HCl pH 6.7) at 50°C for 30 minutes and then reprobed with a rat monoclonal antibody for GAPDH to normalize the protein sample loading. The concentrations of myostatin and GAPDH proteins were quantified by densitometry scanning of the films (Flour-S Multiimager, Bio-Rad). The quantity of myostatin protein was normalized by dividing its 30-kd band intensity by the optical density of the corresponding 40-kd GAPDH band, using the densitometric software package, Quantiscan version 1.5.

2.6. Cell culture

C2C12 (mouse) skeletal muscle myoblast cell line was obtained from the American Type Culture Collection (no. CRL 1772). The cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (Invitrogen-Gibco), antibiotic-antimycotic, and containing 2 mmol/L glutamine (growth medium). Once cells became confluent, myoblasts could be harvested. Alternatively, after growing to 100% confluence, cells were further cultured in DMEM containing 5% horse serum (Invitrogen-Gibco) for 4 days to induce differentiation of myotubes.

2.7. Cell culture transfection

Cell culture transfection was done as reported previously [6,18], using the pMK1 (pMst-luc) plasmid construct having 3322 bp of the human myostatin promoter fused just downstream from the +1 mRNA initiation site with the luciferase reporter gene in vector pGL3. This myostatin promoter contains 4 tat-GRE elements plus 2 GRE elements for GR binding at −277/287 and −607/−621 [18]. Plasmid DNA for pMst-luc was prepared from bacterial cultures (Endo-free Maxikit, Qiagen, Valencia, CA) and also a small amount of the control pRL-TK *Renilla*/luciferase plasmid were co-transfected into myoblasts and cultured in 24-well plates. Transfected myoblasts were cultured to 70% confluence, to study promoter activity, whereas myotubes were cultured to 90% confluence. Transfection was carried out using lipofectamine, but cells were washed with serum-free medium before the DNA-lipofectamine mixture was added. Plasmids containing the specific promoter construct (0.3 μg per well) and the pRL-TK (5 ng per well) were first mixed with lipofectamine in 300 μL of serum-free medium (DMEM) and then incubated with the cells. After incubation for 4 hours at 37°C, the transfected cells were fed with 300 mL of medium containing 20% fetal calf serum and incubated at 37°C for a further 20 hours. The transfection

efficiency in our experiments was between 50% and 60% as assessed with the pCMV-EGFP plasmid and fluorescent microscopy. At this stage, the medium was replaced with the various treatment media. The treatments included addition of dexamethasone at 100 nmol/L (our previous study showed maximum expression of myostatin at 100 nmol/L dexamethasone) and increasing doses of glutamine from the 2 mmol/L glutamine control to 4, 6, 8, and 10 mmol/L. The normal cell culture medium had 2 mmol/L glutamine, which served as a control. The medium was then changed every other day for a period of 96 hours with fresh growth medium containing 10% FCS (5% horse serum for myotubes), with their respective added hormonal treatments. Cell extracts, harvested in Passive Lysis Buffer (Promega, Madison, WI), were assayed 48 to 96 hours after transfection using a dual-luciferase detection kit, according to the instructions provided by the manufacturer (Promega). Promoter activity was measured using an analytic luminometer TD-20/20 (Turner Designs, Sunnyvale, CA).

2.8. In vitro study of myostatin in C2C12 cells

Cell cultures of the C2C12 cell line were grown as described above and then treated with different media in triplicate wells: (1) a control group with no glutamine and no dexamethasone added to culture medium (growth medium) with 5% horse serum (containing 2 mmol/L glutamine by manufacture); (2) 10 nmol/L dexamethasone and 2 mmol/L glutamine (no extra glutamine added to the medium); (3) 100 nmol/L dexamethasone and 2 mmol/L glutamine (no extra glutamine added to the medium); (4) 100 nmol/L dexamethasone and 6 mmol/L glutamine; (5) 6 mmol/L glutamine; and also (6-9) 100 nmol/L dexamethasone and 8 mmol/L or 10 mmol/L glutamine, including controls with 8 or 10 mmol/L glutamine alone. After growth for 4 days, cells were harvested for extraction of total RNA and protein, for analysis by Northern and Western blots, respectively. The study was repeated 3 times to confirm the accuracy of data. Because there were no significant differences between the 6, 8, and 10 mmol/L glutamine groups in terms of myostatin expression, data on 6 mmol/L glutamine were presented in this article (Fig. 3).

2.9. Analysis of endogenous myostatin by reverse transcriptase–polymerase chain reaction

Total RNA was extracted from the cultured C2C12 myoblasts using TriZol reagent following the manufacturer's suggested procedure (Invitrogen-Gibco). Each sample was DNAse-treated, and cDNA was synthesized by reverse transcription of 2 μg of total RNA using an oligo-dT primer (RNA polymerase chain reaction [PCR] kit, Perkin-Elmer, Shelton, CT). Multiplex PCR amplification was carried out on a 2-μL aliquot of the resultant cDNA, diluted 1:100 in water. We used 2 primer pairs: rat myostatin (911 bp) 74-94/985-967 on AF019624 (RMst-For: ATG AGG ACA GTG AGA GAG AGG, Rev: GCA CAA GAT GAG TAT GCG G) and the housekeeping gene GAPDH (152 bp) 606-626/758-738 on BC023196 (R-GAP-For: ATC ACT GCC ACC CAG AAG ACT, Rev: CAT GCC AGT GAG CTT CCC GTT). Thermocycling conditions were denaturation at 94°C for 2 minutes, amplification (94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 50 seconds) for 35 cycles, and a final elongation at 72°C for 10 minutes. Polymerase chain reaction DNA products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed.

2.10. Statistical analysis

All data are reported as mean ± SEM. All statistical tests are performed using the Jandel SigmaStat statistical software package (San Rafael, CA). $P < .05$ is taken as the level of statistical significance. For in vivo study, the key outcome variables were body weight, weights of G/FDS muscle complex, myostatin protein, and mRNA concentrations corrected by GAPDH protein and mRNA. All tests passed the normality distribution. Treatment effects were analyzed by using a 2-way analysis of variance model. Comparisons between groups are performed by using the analysis of variance. χ^2 Test was used when appropriate. For in vitro study, the data from this experiment were analyzed by using a 2-way analysis of variance model, with factors for treatment (saline or dexamethasone) and treatment duration (5 days).

3. Results

3.1. Glutamine blocked GC-induced rat muscle atrophy

Treatment of rats with dexamethasone was associated with a significant decrease in body weight, whereas addition of glutamine in those weight-matched, pair-fed rats partially prevented 50% of weight loss induced by dexamethasone ($P < .01$, comparison between control and dexamethasone; $P < .05$ between dexamethasone plus glutamine compared with control groups and dexamethasone alone). The mean masses of G/FDS muscles were significantly lower in dexamethasone-treated rats compared with pair-fed rats. However, glutamine prevented by 50% the muscle mass loss ($P < .01$, comparison between control and dexamethasone; $P < .05$ between dexamethasone plus glutamine group compared with control groups and dexamethasone alone). Weight-matched and pair-fed control rats and glutamine-fed control rats had statistically similar body and muscle weight at day 5 of their treatment (Fig. 1A). To assess the relative loss of muscle per body weight, we examined the percentage of G/FDS muscle as a fraction of body weight (Fig. 1B). In pair-fed control, the rats' G/FDS muscle represented in average 1.36% of body weight. In dexamethasone-treated rats the G/FDS represented 1.22% of body weight, which is an 11% decrease in fractional muscle weight ($P < .05$ compared with the 3 other groups). In dexamethasone plus glutamine–treated rats the proportion of G/FDS to body weight was restored to 1.32% (not significant compared with pair-fed control and glutamine-

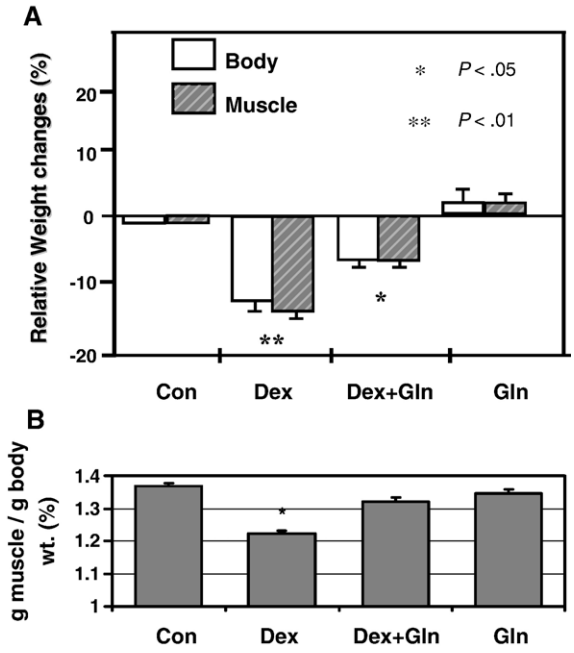

Fig. 1. Body and G/FDS weight changes. A, Glutamine partially prevents dexamethasone-induced body weight loss and inhibits G/FDS complex muscle mass reduction. Animals were weight-matched and pair-fed. *P < .05, significance for the Dex + Gln group compared with the Dex and control groups; **P < .01, significance between the Dex and control groups. B, The ratio of G/FDS muscle complex per body weight expressed in percentage (for doses, see Methods). Con indicates pair-fed control rats; Dex, rats treated with dexamethasone; Dex + Gln, rats treated with dexamethasone plus glutamine; Gln, glutamine and pair-fed for 5 days. *P < .05, significance between the Dex group compared with other groups.

fed rats). In the pair-fed glutamine-fed rats, the proportion of G/FDS complex was 1.34% of body weight of the animal. In summary, the treatment with dexamethasone significantly decreased the fractional muscle weight compared with the control, but this was abrogated by glutamine administration. Glutamine alone had no effect on fractional muscle weight of G/FDS (Fig. 1B).

3.2. Glutamine partially prevented GC induction of myostatin expression in vivo

Myostatin mRNA expression in skeletal muscle from rats, indicated by the intensity of the 2.9-kb transcript seen by Northern blot analysis, was significantly higher in dexamethasone-treated animals than corresponding pair-fed and glutamine-fed control animals (P < .01) (Fig. 2A). The relative myostatin mRNA expression compared with GAPDH expression in G/FDS muscle of rats that received dexamethasone was 410% ± 23% higher than in their pair-fed counterpart. However, glutamine decreased the dexamethasone-induced myostatin expression to near its baseline expression (P < .001).

The myostatin protein expression, as measured by the intensity of the 30-kd immunoreactive band seen by Western blot analysis, was higher in dexamethasone-treated rats than in their corresponding pair-fed and glutamine control animals (Fig. 2B, upper and lower panels). This confirmed data from our previous study, in which increased intramuscular myostatin mRNA and protein expression was observed in rat G/FDS muscles after treatment with GCs [6]. Addition of glutamine to dexamethasone treatment partially

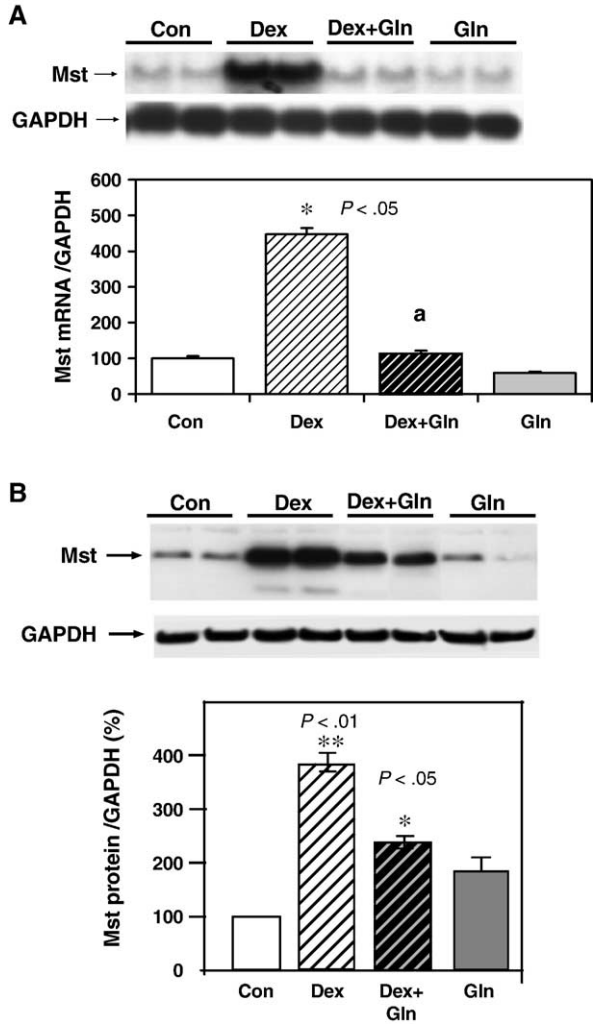

Fig. 2. Glutamine inhibits dexamethasone-induced myostatin expression in rat skeletal muscle. G/FDS muscle complexes were harvested from control pair-fed rats (Con), dexamethasone-treated pair-fed rats (Dex), dexamethasone plus glutamine pair-fed rats (Dex + Gln), and glutamine and pair-fed rats (Gln), for extraction of total RNA and protein (see Methods). A, The effect of glutamine administration on myostatin mRNA, showing a representative Northern blot (upper panel) of the 2.8-kb myostatin mRNA bands, and also the 1-kb GAPDH mRNA bands for the same RNA samples. The bar graph (lower panel) represents the relative intensity of mRNA levels of myostatin expression normalized to their respective GAPDH bands, and averaged with all samples in a group, with the control group as 100%. * P < .001, significance between dexamethasone and other groups; [a]P < .05 between Dex + Gln compared with the Dex group. B, The effect of glutamine administration on myostatin protein expression, showing a representative Western blot (upper panel) of the 30-kd myostatin protein bands, and also the 40-kd GAPDH bands for the same samples. The bar graph (lower panel) represents the relative intensity of levels of myostatin immunoreactive protein normalized to their respective GAPDH bands, and averaged with all samples in a group, with the control group as 100%. *P < .05, significance for the Dex + Gln group compared with the Dex group and the saline control group; **P < .01, significance between the Dex group and the control groups.

inhibited the increased intramuscular expression of myostatin mRNA and protein by 50% ($P < .05$) (Fig. 2B). However, glutamine alone had nonsignificant variable stimulatory effect on myostatin protein expression (Fig. 2B). The size of the myostatin immunoreactive band detected in this study is consistent with our previously reported 30-kd molecular weight.

3.3. Glutamine blocked GC-induced myostatin protein and mRNA expression in C2C12 cells

To test whether glutamine also has a similar inhibitory effect on myostatin expression in cell cultures, we used the C2C12 cell line, a well-studied model for myoblast differentiation into myotubes. Cells were grown 4 days in media with 10 and 100 nmol/L dexamethasone, 6 mmol/L glutamine, or both dexamethasone plus glutamine treatments vs control (2 mmol/L glutamine) during myotube differentiation (see Methods). Cultures were harvested for extraction of total RNA and proteins for analysis of myostatin expression by reverse transcriptase–PCR and Western immunoblotting, respectively (Fig. 3). Myostatin mRNA (Fig. 3A) expression was highly stimulated when cells were treated with dexamethasone in a dose-dependent manner. The addition of 6 mmol/L and more up to 10 mmol/L glutamine (data on glutamine of >6 mmol/L not shown) suppressed the hyperexpression of myostatin mRNA induced by dexamethasone and myostatin protein (Fig. 3A). The effect of glutamine, in suppression of overexpression of myostatin mRNA induced by dexamethasone in C2C12 cells, was similar to RU486 [6], a nonspecific GC receptor antagonist. The relative change of myostatin protein expression compared with GAPDH in C2C12 cells treated with dexamethasone plus 6 mmol/L glutamine vs dexamethasone alone was statistically significant (210 ± 20 compared with 420 ± 20 respectively, $P < .05$). However, control cells exposed to 6 mmol/L glutamine alone did affect the level of myostatin protein expression compared with a control culture medium with 2 mmol/L glutamine (Fig. 3B). When increasing concentrations of alanine were added to culture medium in the presence or absence of dexamethasone, no significant change in myostatin expression was observed (data not shown).

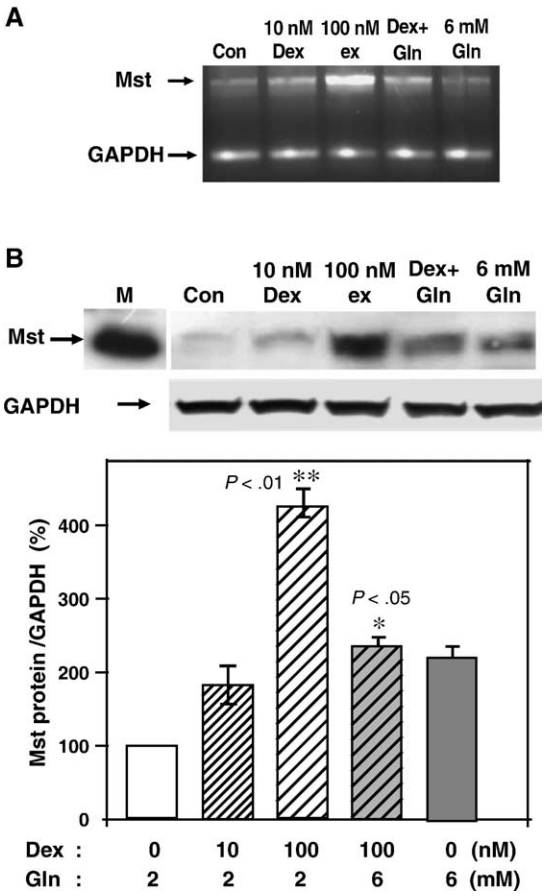

Fig. 3. The effect of glutamine on suppression of myostatin mRNA and protein expression induced by dexamethasone in C2C12 cells. A, Myostatin mRNA expression measured by semiquantitative reverse transcriptase–PCR and gel electrophoresis of myostatin 911-bp PCR products vs 152-bp GAPDH PCR products (see Methods). B, Myostatin protein expression in C2C12 cells, detected by Western blot (upper panel), compared with GAPDH. M indicates expression of myostatin in muscle; Con, C2C12 cells treated with control culture medium containing 2 mmol/L glutamine; Dex, 10 and 100 nmol/L dexamethasone-treated cells; Dex + Gln, 100 nmol/L dexamethasone + 6 mmol/L glutamine–treated cells; Gln, 6 mmol/L glutamine–treated C2C12 cells. The bar graph (lower panel) represents the relative intensity of levels of myostatin immunoreactive protein expression normalized to their respective GAPDH bands, and averaged with all samples in a group, with the control group as 100%. *$P < .05$, significance for the Dex + Gln group compared with the Dex group and the 2 mmol/L Gln control group; **$P < .01$, significance between the Dex group and the control groups.

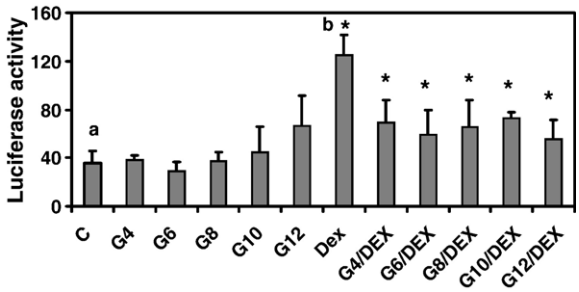

Fig. 4. Myostatin promoter activity in C2C12 cells. Cells were transfected with pMst-luc plasmid (Methods) and grown 4 days in medium with 2 mmol/L glutamine as a control (C) or with increasing concentration of glutamine (G4, 4 mmol/L; G6, 6 mmol/L; G8, 8 mmol/L; G10, 10 mmol/L; and G12, 12 mmol/L) added to culture medium (D, dexamethasone 100 nmol/L), and dexamethasone 100 nmol/L with increasing concentrations of glutamine (G[n]/DEX). Cell lysates were assayed for luciferase enzyme as a measure of myostatin promoter activity. *$P < .05$ in myostatin promoter activity of cells treated with dexamethasone alone compared with dexamethasone plus glutamine at different concentrations; $^{a,b}P < .05$, statistically significant difference between control and dexamethasone-treated cells.

3.4. Glucocorticoid activation of myostatin promoter blocked by glutamine

We determined whether transcription from the myostatin promoter is regulated by glutamine in C2C12 cells, by

transfection of the myoblasts with a plasmid pMst-luc, having 3.3 kb of the human myostatin promoter, which contains several GREs, fused with luciferase. After cell growth in media containing various concentrations of dexamethasone, glutamine, dexamethasone plus glutamine, or control growth medium for 4 days, cell lysates were assayed for luciferase enzyme, as a measure of myostatin promoter activity (Fig. 4). C2C12 cells exhibited approximately a 3-fold increase in myostatin promoter activity (from 35 to 125) when they were exposed to dexamethasone as we showed previously [18]. This hyperexpression was abolished partially, and not dose dependently, by almost 50% by addition of glutamine (70% with addition of 4 mmol/L, 59 with 6 mmol/L, and 65 with 8 mmol/L glutamine cotreated with 100 nmol/L dexamethasone). Increasing concentrations of glutamine alone (>6 mmol/L) caused an increase in myostatin promoter activity, but this effect was not statistically significant relative to the control.

4. Discussion

To investigate the effects of glutamine in suppression of myostatin hyperexpression induced by dexamethasone and its linkage to muscle atrophy, we administered glutamine to rats treated with dexamethasone and compared them to 2 sets of pair-fed and pair-fed, glutamine-administered, weight-matched control rats. We observed for the first-time partial inhibitory effects of glutamine on myostatin mRNA and protein hyperexpression, and, in addition, we confirm the coordinate effects in blocking the GC-induced atrophy of body mass and muscle loss, as described previously [10,11]. In this study, we confirmed these in vivo observations with an in vitro study showing that addition of glutamine in culture medium inhibits dexamethasone-induced hyperexpression of myostatin mRNA, protein, and myostatin promoter activation in C2C12 cells, a well-recognized model for myoblast differentiation into muscle myotubes. This effect on C2C12 cells was observed with glutamine, but not alanine (data not shown).

Myostatin belongs to a growth and differentiation factor superfamily and is a negative regulator of muscle growth [14,15]. Disrupted myostatin gene expression, either by gene targeting in mice or because of naturally occurring mutations in cattle, is associated with increased skeletal muscle mass, resulting from muscle fiber hyperplasia as well as hypertrophy [8,14,15,22]. Conversely, it has been shown that increased serum myostatin concentrations were associated with a loss of skeletal muscle mass in patients with the AIDS wasting syndrome [23]. Furthermore, increases in myostatin expression were found to be associated with loss of skeletal muscle after GC administration [6], in experimental animals after exposure to microgravity during space flight [24], and hindlimb suspension associated with the involvement of type II fast twitch muscle fibers [25]. An increased expression of myostatin mRNA and protein in an animal model of thermal injury has been also described, which was repressed by RU486 [26], implying its GC-dependent nature.

The exact mechanism of myostatin action is unknown. However, previous work has demonstrated that recombinant myostatin protein inhibits C2C12 myoblast proliferation [7], blocks DNA replication in the cycle from G_1 to S phase [8], and impairs protein synthesis in vitro [7], suggestive of its antianabolic effect. Paradoxically, myostatin cDNA overexpression enhances the survival of differentiating C2C12 myocytes while inhibiting myoblast cell proliferation and differentiation [17,27]. Myostatin also rapidly activates the extracellular signal-regulated kinase 1/2 cascade in C2C12 myoblasts in differentiating cells [28] and down-regulates MyoD, a key skeletal muscle transcription factor [29,30]. Our recent studies have demonstrated that transient overexpression of myostatin by dexamethasone is associated with dramatic body weight and skeletal muscle loss [6]. The loss of muscle mass was also seen in a transgenic mouse model overexpressing myostatin generated in our laboratory [31] and in adult mice injected with CHO cells engineered to overexpress myostatin protein [32].

Pharmacologic doses of GC cause rapid muscle atrophy in rats [2-4,33]. Glucocorticoids activate on one hand the ubiquitin proteasome pathway as a catabolic mediator [3,4], but on the other hand cause induction of myostatin [6] possibly as an antianabolic factor. The combination of catabolic and antianabolic processes explains this rapid and massive muscle atrophy. Pharmacologic doses of GCs cause depletion of muscle glutamine content, decrease in serum glutamine levels, and decrease in MHC synthesis rate [10,11]. This rapid muscle atrophy in rats has been considered as a model of acute muscle loss similar to that in acute illnesses [34]. Interestingly, glutamine infusion in rats restores the decline of MHC synthesis rate by 50% [10,11]. The glutamine concentration necessary to counteract the rapid decline of the muscle mass is several folds higher than the daily glutamine intake both in rats [10-12] treated with GCs and in humans during acute critical illnesses [39].

Glucocorticoids and endotoxinemia also cause an increase of glutamine synthase expression and activity [35-38], possibly for gluconeogenesis [33]. It has also been shown that glutamine prevents GC [11,35] and GC-mediated, endotoxinemia-induced [36] glutamine synthase activity in rats as well.

In humans, during GC therapy and critical illnesses, glutamine becomes a “conditional essential amino acid” [39]; the daily need of glutamine increases by 3- to 5-fold. In addition, glutamine mediates several essential physiologic responses, when provided sufficiently in acute critical illnesses [40]. Muscle glutamine flux increases to a similar extent in volunteers after treatment with GCs and in patients with burn [41]. During acute critical illnesses and postoperatively, a decline in serum and intramuscular glutamine levels has been observed [39,41], and this depletion of glutamine presents as a negative prognostic value for

survival. Glutamine replacement decreases mortality and hospital stay in critically ill patients in medical intensive care units [42-46] and in patients with severe burns [41]. All those conditions are known to be associated with hypercortisolemia. Whether the protective effect of glutamine in terms of survival in critical illnesses is directly related to muscle mass preservation, or other multifactorial effects of glutamine on various aspects of cell metabolism, is yet to be clarified. The effect of glutamine in muscle atrophy associated with Cushing's disease is another area of interest in which no study is yet available.

The myostatin promoter region contains at least 4 tat-GRE elements and 2 GRE elements for GC protein binding, located within 3.3 kb of DNA upstream from the myostatin gene. Transcription from this myostatin promoter is induced by 2- to 3-fold by dexamethasone in transfected C2C12 mouse myoblasts [18], but this effect is inhibited by approximately 50% during parallel treatment with 4 mmol/L and more of glutamine. Although this observation of myostatin transcriptional regulation was made using the human myostatin promoter in mouse muscle cells as a model system, it is substantially consistent with the rat in vivo experiments, in which glutamine partially blocks GC induction of myostatin mRNA and protein expression. Our observations are consistent with the suggestion that glutamine may be able to alter GC actions at the promoter region of the myostatin gene. This could abrogate GC-induced muscle loss, by inhibiting the antianabolic function of myostatin, although the catabolic effects of GCs remain unabated. It is not known whether glutamine inhibits GC effects on myostatin expression through regulating the direct binding of GC to the myostatin promoter GRE or by other means. For instance, there may be a signal transducing mechanism acting on a specific promoter region of the myostatin gene with an inhibitory regulatory role similar to the glutamine synthase silencer element [47]. Alternatively, one can speculate that myostatin expression may be negatively regulated by intracellular glutamine concentration. However, further study using myostatin-null mice will be necessary to determine whether glutamine-associated muscle sparing and blocking of GC-induced myostatin expression is a causal relationship, or a parallel co-regulatory association.

It is noteworthy that excess glutamine in our experiment had no significant additional effect on body weight, muscle mass, and mRNA expression. In addition, the myostatin promoter activity was increased by addition of glutamine of >8 mmol/L in cell culture medium, but this effect was not statistically significant. Those confounding effects might be due to the small sample size. However, glutamine alone caused a variable increase in expression of myostatin protein in vivo and in vitro. Although the mechanism for increased myostatin protein levels is not known, it could be that high glutamine increases the processing or stability of myostatin. Access to free food does not influence myostatin expression compared with food restriction [6].

In summary, the glutamine-dependent muscle sparing in GC-induced muscle atrophy is associated with the diminished expression of myostatin, a negative regulator of muscle mass, which is a GC-inducible gene in skeletal muscle. This association suggests that glutamine may function as a potential inhibitor of the atrophic effect of GCs on muscle through inhibiting myostatin.

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