Glucocorticoids Enhance Muscle Proteolysis through a Myostatin-Dependent Pathway at the Early Stage

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Abstract

Myostatin, a member of the TGF-β superfamily of secreted proteins, is expressed primarily in skeletal muscle. It negatively regulates muscle mass and is associated with glucocorticoid-induced muscle atrophy. However, it remains unclear whether myostatin is involved in glucocorticoid-induced muscle protein turnover. The aim of the present study was to investigate the role of myostatin in protein metabolism during dexamethasone (DEX) treatment. Protein synthesis rates and the expression of the genes for myostatin, ubiquitin-proteasome atrogin-1, MuRF1, FoxO1/3a and mTOR/p70S6K were determined. The results show that DEX decreased (P<0.05) protein synthesis rates while increasing the abundance of myostatin. DEX increased (P<0.05) the level of phospho-FoxO1/3a (Thr 24/32) and the expression of MuRF1. In contrast, DEX treatment had no detectable effect on atrogin-1 protein levels (P>0.05). The phosphorylation levels of mTOR and p70S6K were decreased by DEX treatment (P<0.05). Follistatin treatment inhibited the DEX-induced increase in myostatin (P<0.05) and the activation of phosphor-FoxO1/3a (Thr 24/32) (P<0.05) and MuRF1 (P<0.05). Follistatin treatment had no influence on the protein synthesis rate or on the phosphorylation levels of mTOR (Ser 2448) and p70S6K (Thr 389) (P> 0.05). In conclusion, the present study suggests that the myostatin signalling pathway is associated with glucocorticoid-induced muscle protein catabolism at the beginning of exposure. Myostatin is not a main pathway associated with the suppression of muscle protein synthesis by glucocorticoids.

Introduction

Stress can cause muscle wasting and atrophy [1, 2]. When animals suffer from severe stress, excessive glucocorticoids in the circulation cause muscle loss by enhancing proteolysis and impeding protein synthesis [3, 4, 5]. The increased protein breakdown during stress is mainly caused by the activation of proteolytic systems, such as the Ca2+-dependent and the ATP-ubiquitin-dependent systems [6]. In particular, the ubiquitin-proteasome system seems to play a major role in the catabolic action of glucocorticoids [7]. Two important muscle-specific
ubiquitin E3 ligases, muscle-specific RING finger protein 1 (MuRF1) and atrogin-1, have been shown to be related to a variety of atrophic conditions.

Myogenic differentiation is a complex and well-coordinated process that is regulated by growth factors, principally myostatin and insulin-like growth factor-1 (IGF-I). Myostatin is a member of the transforming growth factor β (TGF-β) family and acts as a negative regulator of skeletal muscle growth. Myostatin is essential for the negative regulation of skeletal muscle growth [8]. A mutation in the coding region of myostatin results in a double-muscling phenotype in Belgian blue and Piedmontese cattle [9, 10]. The mutation of myostatin is associated with muscle hypertrophy in humans as well [11]. A blockade of endogenous myostatin results in an adverse increase in muscle mass and size and a decrease in muscle degeneration in an X chromosome-linked muscular dystrophy (mdx) mouse-model of Duchenne muscular dystrophy [12]. Knockout of the myostatin gene induces a significant increase in muscle mass in mice after developmental muscle growth has ceased [13]. As a negative regulator of myogenesis, myostatin is involved in the control of myoblast proliferation. In C2C12 muscle cells, recombinant myostatin proteins inhibit cell proliferation, DNA synthesis, and protein synthesis, suggesting that myostatin may control muscle mass by inhibiting muscle growth or regeneration [14]. Myostatin inhibits myoblast differentiation through an interaction with Smad 3 [15]. The negative effect of myostatin on muscle growth is counteracted by the positive effect of insulin-like growth factor-1 (IGF-I), which promotes protein synthesis via activation of the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway [16]. The IGF-I/PI3K/Akt pathway may inhibit myostatin signalling during myoblast differentiation [17].

Myostatin has been implicated in several forms of muscle wasting, including the severe cachexia observed as a result of conditions such as AIDS and liver cirrhosis. Myostatin increases the activity of the ubiquitin-proteasome system and negatively regulates the activity of the Akt pathway, thereby inducing muscle atrophy [18]. Myostatin results in an increased activation of atrogin-1 and MuRF1 [19, 20]. Moreover, the overexpression of myostatin contributes to muscle atrophy under several conditions, particularly in the presence of glucocorticoids [2, 21, 22]. Myostatin has been shown to reverse the IGF-I/PI3K/AKT hypertrophy pathway by inhibiting AKT phosphorylation, thereby increasing the levels of active FoxO1 and allowing for an increased expression of atrophy-related genes [19]. Hence, we hypothesized that myostatin is involved in the muscle atrophy induced by glucocorticoids.

In the present study, the role of myostatin on the protein synthesis of C2C12 cells in the presence of dexamethasone (DEX), a synthetic glucocorticoid, was investigated in vitro. The protein synthesis rate was estimated using a nonradioactive method by labelling the newly synthesized polypeptides with low concentrations of puromycin and subsequently detecting these proteins with an anti-puromycin antibody [23, 24]. The involvement of the ubiquitin-proteasome and mTOR/p70S6K signalling pathways were also evaluated in this study.

**Materials and Methods**

**Cell culture**

Murine C2C12 skeletal myoblasts (CCTCC, China) were seeded in 6-well plates and cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone, China) supplemented with 10% FBS (Gibco, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Solarbio, China) and maintained at 37°C in a humidified atmosphere with 5% CO₂. The medium was changed every two days. At 80% confluence, the cells were incubated with DMEM containing 2% horse serum (Gibco, USA) to induce myotube formation. After 84 h of differentiation, the cultures were shifted to serum-free DMEM for 12 hours and the C2C12 cells were prepared for treatment with agents as described below.
DEX treatment

The C2C12 cells were incubated in DMEM supplemented with 100 μM dexamethasone (DEX; Cisen, China) for different time periods (12 h, 24 h, 36 h, 48 h) [25]. The level of myostatin expression was estimated using real-time PCR and western blot methods.

Follistatin treatment

To investigate the role of myostatin in glucocorticoid-induced catabolism, follistatin, a powerful antagonist of myostatin, was used to inhibit the influence of myostatin [26]. The C2C12 cells were randomly subjected to the following treatments: DEX (100 μM), follistatin (800 ng/ml; Sigma, USA), DEX (100 μM) plus follistatin (800 ng/mL), and control (no treatment). After a 36-hour treatment, the cultures were supplemented with puromycin (1 μM; Solarbio, China) for another 30 min, and the cells were then harvested for further analysis.

Measurement of protein synthesis rate

The protein synthesis rate was determined using a nonradioactive method [23]. The newly synthesized polypeptides were labelled with low concentrations of puromycin, and these proteins were subsequently detected with an anti-puromycin antibody. The accumulation of puromycin-conjugated peptides into nascent peptide chains reflects the rate of protein synthesis [23, 24].

RNA extraction and quantitative real-time PCR analyses

RNA was extracted using TRIzol (Invitrogen, USA). The RNA concentration was measured by spectrophotometry (Eppendorf, Germany), and the purity of the RNA was verified by calculating the ratio between the absorbance values at 260 and 280 nm (A260/280 ≈1.75–2.01). Total RNA (1 μg) was used for first-strand cDNA synthesis with the Prime Script™ RT reagent kit for RT-PCR (TaKaRa, China) according to the manufacturer’s instructions. Then, real-time PCR was performed using SYBR Premix Ex Taq™ (TaKaRa, China). Following the manufacturer’s protocol, the resulting cDNA was amplified in a 20-μL PCR reaction system containing 0.2 μmol/L of each specific primer (Sangon, China) and of the SYBR Green master mix. An ABI 7500 PCR machine (Applied Biosystems; Thermo, USA) was used for the amplification of cDNA, and primers were designed with Primer 6.0 software. The primers were as follows:

- mouse myostatin: forward, CAGGAGAAGATGGGCTGAATCC and reverse, AAGCCCAAA GTCTCTCGG; atrogin-1: forward, GCTGGATTGGAAGAAGAT and reverse, GAGAA TGTGGCAGTGTTTTG; MuRF1: forward, CTGGAGGTCGGTTTCCGTC and reverse, TCG GTGGGCTGGCTTTTCGTGC; FoxO3: forward, CCCTAACCCAGCAGACTGT and reverse, GGAAACAAACACAAGACGC; FoxO1: forward, GAGTGGATGTTGAAGAGCGT and reverse, GGAGACAGATTGTTGGCGAAT; IGF-I: forward, ATGTCTTGCAATTCAGGTGTG and reverse, TCTTGGCATGCGTGTTG; and β-actin: forward, ACCACACCTTC TACAATGAG and reverse, ACGACCAGGGCATAAC. As a control, β-actin primers were used in a duplexed reaction, and all of the mRNA values were normalised to the β-actin values.

Protein preparation and western blot analyses

C2C12 myotubes were homogenised in 0.2 mL of lysis buffer (Beyotime, China) and kept on ice during the trial procedure. The homogenate was centrifuged at 12,000 g for 5 min at 4°C, and the supernatant was collected. Protein concentration was assayed using a BCA assay kit (Beyotime, China) according to the manufacturer’s protocol. Aliquots of 18 μg of protein were separated with 7.5–10% SDS polyacrylamide gels (Bio-Rad, Richmond, 246 CA) according to
the method described by Laemmli (1970) [27], and the proteins were then transferred onto a PVDF membrane (Millipore, USA) at 200 mA for 2 h in a Tris-glycine buffer with 20% anhydrous ethanol at 4°C. Membranes were blocked with western blocking buffer (Beyotime, China) for 1 h at room temperature. The membranes were then probed with primary antibodies at 4°C with gentle shaking overnight. The primary antibodies used were anti-myostatin, anti-MuRF1, anti-atrogin-1, anti-phospho-FoxO1/3a (Thr24/32) (Abcam, UK), anti-FoxO1, anti-mTOR, anti-phospho-mTOR (Cell Signalling Technologies, USA), anti-mouse puromycin (Kerafast, USA), and anti-β-actin (Beyotime, China). After being washed, the membranes were incubated with horseradish peroxidase-linked anti-rabbit, anti-mouse, or anti-rat secondary antibodies for 4 h at 4°C. Membranes were then visualized by exposure to Hyperfilm ECL (Beyotime, China). Films were scanned, and specific bands were quantified using ImageJ 1.43 software (National Institutes of Health, USA). The band intensity was normalised to the β-actin band in the same sample.

Statistical analysis
The main effect of each treatment on protein metabolism was evaluated using a one-way ANOVA performed with the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute, USA). Multiple comparisons between the means were conducted using Duncan’s honestly significant difference test. The means were considered to be significantly different at $P < 0.05$.

Results
Effect of DEX treatment
The protein synthesis rate was significantly decreased by DEX treatment ($P < 0.05$, Fig 1). However, the level of myostatin protein was significantly increased ($P < 0.05$) by DEX treatment at 36 h, while there was no significant change at 24 and 48 h ($P > 0.05$, Fig 2A). The level of myostatin mRNA was significantly upregulated by DEX after a 24 h treatment ($P < 0.05$) but not after 36 h ($P > 0.05$, Fig 2B).

The effect of DEX on the ubiquitin-proteasome pathway was investigated. DEX treatment significantly decreased the level of the protein FoxO1 ($P < 0.05$, Fig 3A) but increased the phosphor-FoxO1/3a (Thr24/32) level ($P < 0.05$, Fig 3B). DEX upregulated the transcription of FoxO1 mRNA ($P < 0.05$), but it had no influence on the mRNA level of FoxO3 ($P > 0.05$, Fig 3C).

The two downstream proteins of FoxO1, MuRF1 and atrogin-1, were then measured. DEX treatment dramatically increased the expression of MuRF1 at both the protein ($P < 0.05$, Fig 3D) and mRNA levels ($P < 0.05$, Fig 3E). In contrast, DEX treatment significantly upregulated ($P < 0.05$, Fig 3F) the mRNA level of atrogin-1 but had no detectable effect on the protein level of atrogin-1 ($P > 0.05$, Fig 3G).

We then investigated the effect of DEX treatment on the mTOR signalling pathway and on IGF-I expression. The results showed that the levels of phosphorylated mTOR and p70S6K proteins were both significantly decreased by DEX treatment ($P < 0.05$, Fig 4A and 4B). In addition, the mRNA level of IGF-I was downregulated by DEX treatment ($P < 0.05$, Fig 4C).

Effect of myostatin blockage with follistatin on DEX-induced effects
To explore the effect of myostatin on protein metabolism, follistatin was used to inhibit myostatin in C2C12 cells. Follistatin treatment inhibited the DEX-induced increase of myostatin ($P < 0.05$, Fig 5). There was no significant difference between the control and follistatin treatments ($P > 0.05$).
Follistatin significantly inhibited the activation of phosphor-FoxO1/3a (Thr 24/32) (P < 0.05, Fig 6A) and MuRF1 (P < 0.05, Fig 6C) caused by DEX. However, follistatin had no influence on the DEX-induced increases in total FoxO1 (P > 0.05, Fig 6B) and atrogin-1 (P > 0.05, Fig 6D).

**Fig 1. The inhibitory effect of dexamethasone (DEX) on protein synthesis.** After DEX (100 μM) treatment for 36 h, C2C12 cells are cultured with puromycin (1 μM) for extra 30 min and then the protein-synthesis rate was assayed by western blot. The values are presented as the means ± SEM (n = 6). a,b Means with different letters differ significantly (P < 0.05).

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Compared with synthesis in the control, follistatin had no significant influence on the protein synthesis rate ($P > 0.05$, Fig 7A) or on p-mTOR (Ser 2448) ($P > 0.05$, Fig 7B) and p-p70S6K (Thr 389) ($P > 0.05$, Fig 7C) levels. Follistatin did not restore the suppressing effect of DEX on the protein synthesis rate or on the phosphorylation of mTOR and p70S6K ($P > 0.05$).

**Discussion**

In the present study, the role of myostatin in glucocorticoid-induced protein catabolism was investigated. DEX suppressed the protein synthesis rate and induced proteolysis. The inhibition of myostatin by follistatin attenuated the DEX-induced proteolysis by initiating the ubiquitin-proteasome system. These results suggest that myostatin is associated with the glucocorticoid-induced muscle protein catabolic effect rather than with the suppression of protein synthesis.

**Myostatin is involved in glucocorticoids (GCs)-induced muscle protein catabolism**

In mammals, the GC-induced catabolic effect and muscle atrophy have been well studied [28]. Similar to the effects in mammals, GCs result in suppressed muscle development in chickens [29, 30]. In line with previous studies, the results of the present study show that the rate of protein synthesis was decreased by DEX treatment.

Myostatin is a member of the transforming growth factor β (TGF-β) family and is essential for the negative regulation of skeletal muscle growth [8]. Hence, we tested if myostatin was associated with the DEX-induced catabolic effect on skeletal muscle. In line with previous studies, myostatin levels were significantly reduced by DEX treatment (Fig 2A).

![Fig 2. Effect of dexamethasone (DEX) on myostatin expression.](image-url)

Myostatin protein levels (A) and mRNA levels (B) in C2C12 cells treated with dexamethasone (DEX, 100 μM) for 12 h, 24 h, 36 h and 48 h. The values are presented as the means ± SEM (n = 6). Means with different letters differ significantly ($P < 0.05$).

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studies [31, 32], glucocorticoids upregulated both the mRNA level and protein level of myostatin. The present study showed that the protein level of myostatin was increased by DEX, suggesting that myostatin is associated with the catabolic effect induced by GCs. DEX-induced

![Fig 3](image1.png)

Fig 3. Effect of dexamethasone (DEX) treatment on the expression of ubiquitin-proteasome-related factors involved with protein catabolism. The total FoxO1 protein (A), phospho-FoxO1/3a (Thr 24/32) (B), FoxO1 and FoxO3 mRNA levels (C), MuRF1 protein (D), MuRF1 mRNA (E), atrogin-1 protein (F), and atrogin-1 mRNA (G) in C2C12 cells treated with DEX (100 μM) for 36 h. The values are presented as the means ± SEM (n = 6). a,b Means with different letters differ significantly (P < 0.05).

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![Fig 4](image2.png)

Fig 4. Effect of dexamethasone (DEX, 100 μM for 36 h) treatment on mTOR, p70S6K, and IGF-I expression in C2C12 cells. p-mTOR protein levels (A), p-p70S6K (B), and IGF-I mRNA level (C). The values are presented as the means ± SEM (n = 6). a,b Means with different letters differ significantly (P < 0.05).

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upregulation of myostatin mRNA was partially attributed to the binding of glucocorticoid receptor to glucocorticoid-binding element motifs along myostatin promoter [22]. In this study, the upregulated myostatin mRNA was observed at 24 h while myostatin protein at 36 h after DEX treatment indicated that the discrepancy between myostatin mRNA and protein
levels. In C2C12 myoblast cells, addition of glutamine fully abolished the DEX-induced hyperexpression of myostatin at mRNA level rather than at protein level [21]. The posttranscriptional mechanism plays an important role in the regulation of myostatin [32]. Hence, the result implies that DEX could regulate myostatin expression at both transcriptional and posttranscriptional levels.

In vivo, myostatin mRNA and protein could be upregulated by dexamethasone at Day 5 and restored to normal level at Day 10 in a 10-day time course [31]. Recently, another research group reported that in vivo myostatin expression was upregulated by DEX at only Day 5 during 10-days treatment [33]. In line with the previous studies in vivo, the present in vitro result further demonstrated that dexamethasone induced upregulation of myostatin was time dependent. The result may suggest that myostatin play a role in glucocorticoid-induced muscle atrophy at the beginning of exposure.

![Fig 6. Effect of follistatin on the expression of ubiquitin-proteasome-related factors in C2C12 cells treated with dexamethasone (DEX, 100 μM for 36 h). The effect of DEX (100 μM) supplementation on C2C12 cells in the presence of follistatin (800 ng/ml) for 36 h on phospho-FoxO1/3a (Thr 24/32) (A), total FoxO1 (B), MuRF1 (C) and atrogin-1 (D) protein levels. The values are presented as the means ± SEM (n = 6). a,b Means with different letters differ significantly (P < 0.05). doi:10.1371/journal.pone.0156225.g006](image)

![Fig 7. Effect of follistatin on protein synthesis and activation of mTOR/p70S6K pathway in C2C12 cells treated with dexamethasone (DEX, 100 μM for 36 h). The changes in protein synthesis rate (A) and phospho-mTOR (Ser 2448) (B) and phospho-p70S6K (C) levels in C2C12 cells after treatment with DEX (100 μM) and follistatin (800 ng/ml) for 36 h. The values are presented as the means ± SEM (n = 6). a,b Means with different letters differ significantly (P < 0.05). doi:10.1371/journal.pone.0156225.g007](image)
Skeletal muscle atrophy is mediated through the activation of FoxO1 [34]. FoxO1 plays an important role in the regulation of atrogin-1 and MuRF1 expression in glucocorticoid-induced muscle atrophy [7]. In human skeletal muscle tissue, the regulation of Akt and its downstream FoxO1 signalling pathway are both associated with the processes of skeletal muscle atrophy [35]. The proximal promoter of the FoxO1 gene contains multiple functional glucocorticoid response elements (GREs) and FoxO1 gene expression is regulated by binding of the glucocorticoid receptor to GREs [36]. We therefore measured the expression of FoxO1/3a and muscle atrophy-related genes, such as atrogin-1 and MuRF1. In line with previous study, FoxO1 mRNA was upregulated by DEX. DEX treatment regulated FoxO1 gene expression not only at the mRNA level but also at the protein level [36, 37]. The reduced phosphorylation is an important mechanism of FoxO1 activation [38]. In the present study, the p-FoxO1/3a was increased by DEX treatment, suggesting that the suppression of FoxO1 pathway, in line with previous study [28]. However, the upregulated MuRF1 and antrogin-1 mRNA and MuRF1 protein by DEX indicated the activated muscle atrophy related genes, which agrees with previous results [28, 36, 37]. Recently, it was proved that the upregulation of MuRF1 in response to DEX might be mediated through the Kruppe-like factor 15 (KLF15) transcription factor, which is an Akt/FoxO-independent pathway [39]. Moreover, the transcription factor peroxisome proliferator-activated receptor β/δ (PPARβ/δ) upregulates muscle FoxO1 expression and activity with a downstream upregulation of atrogin-1 and MuRF1 expression during glucocorticoid [37]. Hence, the present implies that glucocorticoid could upregualte muscle atrophy related gene expression without the activation of FoxO1.

We thus hypothesized that myostatin may be involved in the GC-induced expression of MuRF1. Follistatin, a physiological inhibitor of myostatin, was used to reduce the effect of myostatin by binding to myostatin and acting as an effective myostatin inhibitor [40, 41, 42]. The suppression of protein expression caused by myostatin was eliminated by follistatin, suggesting that the effect of DEX was impeded by myostatin. Furthermore, the decrease in MuRF1 caused by follistatin further demonstrates that the GC-induced catabolic effect on muscle protein is at least partially via a myostatin-dependent pathway. Myostatin has been reported to be involved in stress-induced muscle atrophy. Acute daily psychological-stress-induced atrophic gene expression and the loss of muscle mass appear to be myostatin-dependent [2]. DEX-induced muscle loss is mediated, at least in part, by the upregulation of myostatin expression through a glucocorticoid receptor-mediated pathway [31]. The DEX-induced upregulation of myostatin gene expression was partly attributable to the binding of the glucocorticoid receptor to the glucocorticoid response element motifs in the myostatin promoter region [22]. Moreover, the increased level of phosphorylated FoxO1/3a caused by DEX was restored to normal by follistatin. The role of myostatin in the activation of Akt/FoxO1 needs to be investigated further.

Myostatin is not responsible for the glucocorticoid-induced suppression of protein synthesis

High doses of GCs decrease the rate of protein accumulation by both increasing the rate of degradation and decreasing the rate of synthesis [4]. In rats, dexamethasone induces a significant decrease in protein synthesis in fast-twitch glycolytic and oxidative glycolytic muscles [43]. In line with previous studies, DEX decreased the protein synthesis rate in C2C12 cells. The inhibitory effect of GCs on muscle protein synthesis is thought to result mainly from the inhibition of the mTOR/p70S6K pathway [44]. The Akt/mTOR pathway plays a role in the regulation of skeletal muscle hypertrophy [45]. Hence, we further investigated the effect of DEX on the mTOR pathway. The significant decreases in the phosphorylation level of mTOR

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and its downstream protein, S6K, indicate that the blockage of the mTOR/p70S6K pathway is involved in the DEX-induced suppression of protein synthesis. The activation of the Akt/mTOR pathway and its downstream targets, p70S6K and PHAS-1/4E-BP1, is essential in regulating skeletal muscle fibre size \[46, 47, 48\]. These results demonstrate that glucocorticoids could reduce the synthesis of proteins via the inhibitory effects of glucocorticoids on mTOR/p70S6K signalling.

Amirouche et al (2009) found that myostatin overexpression suppressed muscle protein synthesis by downregulating Akt/mTOR pathway \[49\]. We then investigated the role of myostatin in the reduction of protein synthesis by DEX. Although follistatin restored DEX-induced upregulation of myostatin (Fig 5), the inhibited protein synthesis or suppressed phosphorylation of mTOR and p70S6K induced by DEX were not eliminated by follistatin treatment, suggesting that myostatin was not a major factor contributing to the retarded protein synthesis induced by DEX.

Moreover, follistatin, besides of function as an inhibitor of myostatin, could mediate muscle growth independently of myostatin-driven mechanisms \[50, 51\]. Follistatin mediates in vivo muscle growth via Akt/mTOR/S6K signaling pathway \[51\]. Follistatin-induced muscle hypertrophy requires the activation of insulin/IGF-I pathway by either insulin or IGF-I \[52\]. In contrast, follistatin fails to stimulate muscle growth when both insulin and IGF-I are deficient \[52\]. In the present study, C2C12 were cultured with serum-free media to avoid the possible interference of serum. The absence of both insulin and IGF-I in the cultural media should be a reason of the unaffected mTOR/p70S6K in follostatin treatment. Therefore, the role of myostatin and follistatin in glucocorticoid-induced muscle development and growth remains to be elucidated with gene interfering technique in future \[53\].

In conclusion, the present study suggests that the myostatin signalling pathway is associated with glucocorticoid-induced muscle protein catabolism at the beginning of exposure and that myostatin may not a main pathway in the suppression of muscle protein synthesis by glucocorticoids.

Author Contributions
Conceived and designed the experiments: HL. Performed the experiments: RW. Analyzed the data: RW JZ. Contributed reagents/materials/analysis tools: HJ XW. Wrote the paper: RW HL.

References
1. Salehian B, Kejriwal K. 1999. Glucocorticoid-induced muscle atrophy: mechanisms and therapeutic strategies. Endocr Pract 5, 277–81. PMID: 15251668
2. Allen DL, McCall GE, Loh AS, Madden MC, Mehan RS. 2010. Acute daily psychological stress causes increased atrophic gene expression and myostatin-dependent muscle atrophy. Am J Physiol Regul Integr Comp Physiol 299, R889–98. doi: 10.1152/ajpregu.00296.2010 PMID: 20592178
3. Goldberg AL, Tischler M, DeMartino G, Griffin G. 1980. Hormonal regulation of protein degradation and synthesis in skeletal muscle. Fed Proc. 39, 31–6. PMID: 7351242
4. Tomas FM, Murray AJ, Jones LM. 1984. Interactive effects of insulin and corticosterone on myofibrillar protein turnover in rats as determined by N tau-methylhistidine excretion. Biochem J 220, 469–73. PMID: 6378188
5. Löfberg E, Gutierrez A, Wernerman J, Anderstam B, Mitch WE, Price SR, et al. 2002. Effects of high doses of glucocorticoids on free amino acids, ribosomes and protein turnover in human muscle. Eur J Clin Invest 32, 345–53. PMID: 12027875
6. Ventadour S, Attaix D. 2006. Mechanisms of skeletal muscle atrophy. Curr Opin Rheumatol 18, 631–5. PMID: 17053511
7. Schakman O, Gilson H, Thissen JP. 2008. Mechanisms of glucocorticoid-induced myopathy. J Endocrinol 197, 1–10. doi: 10.1677/JOE-07-0606 PMID: 18372227
8. McPherron AC, Lawler AM, Lee SJ. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387, 83–90. PMID: 9139826
9. Kambadur R, Sharma M, Smith TP, Bass JJ. 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res 7, 910–6. PMID: 9314496
10. McPherron AC, Lee SJ. 1997. Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci U S A 94, 12457–61. PMID: 9356471
11. Schuelke M, Wagner KR, Stolz LE, Hübner C, Riebel T, Kömen W, et al. 2004. Myostatin mutation
12. McPherron AC, Lee SJ. 1997. Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci U S A 94, 12457–61. PMID: 9356471
13. Welle S, Bhatt K, Pinkert CA, Tawil R, Thornton CA. 2007. Muscle growth after postdevelopmental myostatin gene knockout. Nature 420, 418–21. PMID: 12459784
14. Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH Jr, et al. 2001. Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. Am J Physiol Endocrinol Metab 280, E221–8. PMID: 11158924
15. Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. 2002. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. J Biol Chem. 277, 49831–40. PMID: 12244043
16. Glass DJ. 2010. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. Curr Top Microbiol Immunol 346, 267–78. doi: 10.1007/82_2010_78 PMID: 20593312
17. Retamales A, Zuloaga R, Valenzuela CA, Gallardo-Escarate C, Molina A, Vaidés JA. 2015. Insulin-like growth factor-1 suppresses the myostatin signaling pathway during myogenic differentiation. Biochem Biophys Res Commun 464, 596–602. doi: 10.1016/j.bbrc.2015.07.018 PMID: 26151859
18. Rodriguez J, Vernus B, Cheih I, Cassar-Malek I, Gabillard JC, Hadj Sassi A, et al. 2014. Myostatin and the skeletal muscle mass and myostatin signaling pathways. Cell Mol Life Sci 71, 4361–71. doi: 10.1007/s00018-014-1689-x PMID: 25080109
19. McFarlane C, Plummer E, Thomas M, Hennébry A, Ashby M, Ling N, et al. 2006. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO-dependent mechanism. J Cell Physiol 209, 501–14. PMID: 16883577
20. Lokireddy S, Moully V, Butler-Browne G, Gluckman PD, Sharma M, Kambadur R, et al. 2011. Myostatin promotes the wasting of human myoblast cultures through promoting ubiquitin-proteasome pathway-mediated loss of sarcomeric proteins. Am J Physiol Cell Physiol 301, C1316–24. doi: 10.1152/ajpcell.00114.2011 PMID: 21900687
21. Salehian B, Mahabadi V, Bilas J, Taylor WE, Ma K. 2006. The effect of glutamine on prevention of glucocorticoid-induced skeletal muscle atrophy is associated with myostatin suppression. Metabolism 55, 1239–47. PMID: 16919545
22. Qin J, Du R, Yang YO, Zhang HQ, Li Q, Liu L, et al. 2013. Dexamethasone-induced skeletal muscle atrophy was associated with upregulation of myostatin promoter activity. Res Vet Sci 94, 84–9. doi: 10.1016/jrxjs.2012.07.018 PMID: 22939086
23. Schmidt EK, Ciavarino G, Ceppi M, Pierre P. 2009. SluNSET, a nonradioactive method to monitor protein synthesis. Nat Methods 6, 275–8. doi: 10.1038/nmeth.1314 PMID: 19305406
24. Goodman CA, Mabrey DM, Frey JW, Miu MH, Schmidt EK, Pierre P, et al. 2011. Novel insights into the regulation of skeletal muscle protein synthesis as revealed by a new nonradioactive in vivo technique. FASEB J 25, 1028–39. doi: 10.1096/fj.10-168799 PMID: 21148113
25. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, et al. 2004. The IGF-I/Pi3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FoxO transcription factors. Mol Cell 14, 395–403. PMID: 15125842
26. Zhu J, Li Y, Lu A, Gharabiheh B, Ma J, Kobayashi T, et al. 2011. Follistatin improves skeletal muscle healing after injury and disease through an interaction with myostatin, angiogenesis, and fibrosis. Am J Pathol 179, 915–30. doi: 10.1016/j.ajpath.2011.04.008 PMID: 21689628
27. Laemmli UK. 1970. SDS-polyacrylamide gel electrophoresis of protein. Nature 2, 680–685.
28. Umeki D, Ohnuki Y, Mototani Y, Shiozawa K, Suita K, Fujita T, et al. 2015. Protective effects of clenbuterol against dexamethasone-induced muscle atrophy and myosin heavy chain transition. PLoS One 10, e0128263. doi: 10.1371/journal.pone.0128263 PMID: 26053620
29. Yuan L, Lin H, Jiang KJ, Jiao HC, Song ZG. 2008. Corticosterone administration and high-energy feed results in enhanced fat accumulation and insulin resistance in broiler chickens. Br Poult Sci 49, 487–495. doi: 10.1080/00071660802251731 PMID: 18704796
30. Gao J, Lin H, Song ZG, Jiao HC. 2008. Corticosterone alters meat quality by changing pre-and post-slaughter muscle metabolism. Poult Sci 87, 1609–1617. doi: 10.3382/ps.2007-00007 PMID: 18648056

31. Ma K, Mallidis C, Bhasin S, Mahabadi V, Artaza J, Gonzalez-Cadavid N, et al. 2003. Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. Am J Physiol Endocrinol Metab 285, E363–71. PMID: 12721153

32. Allen DL, Loh AS. 2011. Posttranscriptional mechanisms involving microRNA-27a and b contribute to fast-specific and glucocorticoid-mediated myostatin expression in skeletal muscle. Am J Physiol Cell Physiol 300, C124–37. doi: 10.1152/ajpcell.00142.2010 PMID: 20980549

33. Macedo AG, Krug AL, Souza LM, Martinselli AM, Constantino PB, Zago AS, et al. 2016. Time-course changes of catabolic proteins following muscle atrophy induced by dexamethasone. Steroids 107, 30–6. doi: 10.1016/j.steroids.2015.12.016 PMID: 26730720

34. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, et al. 2004. FoxO transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 117, 399–412. PMID: 15109499

35. Léger B, Cartoni R, Praz M, Lamon S, Dériaz O, Crettenand A, et al. 2006. Akt signalling through GSK-3beta, mTOR and FoxO1 is involved in human skeletal muscle hypertrophy and atrophy. J Physiol 576, 923–33. PMID: 16916907

36. Qin W, Pan J, Qin Y, Lee DN, Bauman WA, Cardozo C. 2014. Identification of functional glucocorticoid response elements in the mouse FoxO1 promoter. Biochem Biophys Res Commun. 450, 979–83. doi: 10.1016/j.bbrc.2014.06.080 PMID: 24971545

37. Castillero E, Alamdari N, Aversa Z, Gurav A, Hasselgren PO. 2013. PPARδ/β regulates glucocorticoid-and sepsis-induced FoxO1 activation and muscle wasting. PLoS One. 8, e59726. doi: 10.1371/journal.pone.0059726 PMID: 23555761

38. Van Der Heide LP, Hoekman MF, Smitd MP. 2004. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J. 380, 297–309. PMID: 15005655

39. Shimizu N, Yoshikawa N, Ito N, Maruyama T, Suzuki Y, Takeda S, et al. 2011. Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. Cell Metab. 13, 170–82. doi: 10.1016/j.cmet.2011.01.001 PMID: 21284984

40. Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, et al. 2002. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem 277, 40735–41. PMID: 12194980

41. Tsuchida K. 2006. The role of myostatin and bone morphogenetic proteins in muscular disorders. Expert Opin Biol Ther 6, 147–54. PMID: 16436040

42. Tsuchida K, Sunada Y, Noji S, Murakami T, Uezumi A, Nakatani M. 2006. Inhibitors of the TGF-beta superfamily and their clinical applications. Mini Rev Med Chem 6, 1255–61. PMID: 17100637

43. Savary I, Debras E, Dardevet D, Soret C, Capitan P, Prugnaud J, et al. 1998. Effect of glucocorticoid excess on skeletal muscle and heart protein synthesis in adult and old rats. Br J Nutr 79, 297–304. PMID: 9577308

44. Schakman O, Kalista S, Barbé C, Loumaye A, Thissen JP. 2013. Glucocorticoid-induced skeletal muscle atrophy. Int J Biochem Cell Biol 45, 2163–72. doi: 10.1016/j.biocel.2013.05.036 PMID: 23806868

45. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, et al. 2001. Mediation of IGF-I-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 3, 1009–13. PMID: 11715022

46. Bodine SC, Stitt TN, Gonzalez M, Klene WO, Stover GL, Bauerlein R, et al. 2001. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 3, 1014–9. PMID: 11715023

47. Shah OJ, Anthony JC, Kimball SR, Jefferson LS. 2000. 4E-BP1 and S6K1: translational integration sites for nutritional and hormonal information in muscle. Am J Physiol Endocrinol Metab 279, E715–29. PMID: 11001751

48. Liu X, Powlas J, Shi Y, Oleksijew AX, Shoemaker AR, De Jong R, et al. 2004. Rapamycin inhibits Akt-mediated oncogenic transformation and tumor growth. Anticancer Res 24, 2697–704. PMID: 15517874

49. Amirouche A, Durieux AC, Banzet S, Koulmann N, Bonnefoy R, Mouret C, et al. 2009. Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. Endocrinology 150, 286–94. doi: 10.1210/en.2008-0959 PMID: 18801898

50. Lee SJ, Lee YS, Zimmers TA, Soleimani A, Matzuk MM, Tsuchida K, et al. 2010. Regulation of muscle mass by follistatin and activins. Mol Endocrinol. 24, 1998–2008. doi: 10.1210/me.2010-0127 PMID: 20810712
51. Winbanks CE, Weeks KL, Thomson RE, Sepulveda PV, Beyer C, Qian H, et al. 2012. Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. J Cell Biol. 197, 997–1008. doi:10.1083/jcb.201109091 PMID: 22711699

52. Barbé C, Kalista S, Lourmaye A, Ritvos O, Lause P, Ferracin B, et al. 2015. Role of IGF-I in follistatin-induced skeletal muscle hypertrophy. Am J Physiol Endocrinol Metab. 309, E557–67. doi:10.1152/ajpendo.00098.2015 PMID: 26219865

53. Mosler S, Relizani K, Mouisel E, Amthor H, Diel P. 2014. Combinatory effects of siRNA-induced myostatin inhibition and exercise on skeletal muscle homeostasis and body composition. Physiol Rep. 2: e00262. doi: 10.1002/phy2.262 PMID: 24760516