

Chapter 3: Results

1. Myostatin gene deletion prevents glucocorticoid-induced muscle atrophy

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Collaborations:

L Combaret and D Attaix performed the measurements of proteasomal activities according to their methodology previously described (Combaret L 2005).

L Grobet has kindly provided a couple of Mstn KO mice from which we developed the breeding colony in our own facilities. These Mstn KO mice harbor a constitutive deletion of the third Mstn exon as previously described (Grobet L 2003).

Authors contributions:

H Gilson, O Schakman and JP Thissen designed research.

H Gilson, O Schakman and P Lause performed research.

H Gilson and JP Thissen analyzed data.

H Gilson, JM Ketelslegers and JP Thissen wrote the paper.

Abstract

Glucocorticoids mediate muscle atrophy in many catabolic states. Myostatin expression, a negative regulator of muscle growth, is increased by glucocorticoids and myostatin overexpression is associated with lower muscle mass. This suggests that myostatin is required for the catabolic effects of glucocorticoids. We therefore investigated whether myostatin gene disruption could prevent muscle atrophy caused by glucocorticoids. Male myostatin knock-out (KO) and wild-type (WT) mice were subjected to dexamethasone treatment (1mg/kg.day for 10 days or 5mg/kg.day for 4 days). In WT mice, daily administration of low dose dexamethasone for 10 days resulted in muscle atrophy (tibialis anterior: -15%; gastrocnemius: -13%; $p < 0.01$) due to 15% decrease in the muscle fiber cross-sectional area (CSA) (1621 ± 31 vs $1918 \pm 64 \mu\text{m}^2$, $p < 0.01$). In KO mice, there was no reduction of muscle mass nor fiber CSA after dexamethasone treatment. Muscle atrophy after 4 days of high dose dexamethasone was associated with increased mRNA of enzymes involved in proteolytic pathways (Atrogin-1, MuRF1, and Cathepsin L) and increased chymotrypsin-like proteasomal activity. In contrast, the mRNA of these enzymes and the proteasomal activity were not significantly affected by dexamethasone in KO mice. Muscle IGF-I mRNA was paradoxically decreased in KO mice (-35%, $p < 0.05$); this was associated with a potentially compensatory increase of IGF-II expression in both saline and dexamethasone-treated KO mice (two fold, $p < 0.01$). In conclusion, our results show that myostatin deletion prevents muscle atrophy in glucocorticoid-treated mice, by blunting the glucocorticoid-induced enhanced proteolysis, and suggest an important role of myostatin in muscle atrophy caused by glucocorticoids.

Introduction

Catabolic conditions are associated with hypercortisolism which in turn plays a major role in skeletal muscle atrophy (1,2). Indeed, administration of high doses of glucocorticoids induces muscle atrophy, mainly due to the stimulation of muscle proteolysis (3,4). This glucocorticoid-induced protein degradation is mainly mediated by the activation of the ubiquitin-proteasome and lysosomal pathways. In particular, the muscle specific E3-ligases Atrogin-1 and MuRF1, and the lysosomal enzyme Cathepsin L, are highly stimulated by glucocorticoids (1,5,6). Furthermore, the inhibition of glucocorticoid action by RU486, an antagonist of the glucocorticoid receptor, markedly reduces the loss of muscle mass encountered in several catabolic states (2,7).

Myostatin, a member of the transforming growth factor- β family, is an important negative regulator of skeletal muscle mass (8). Indeed, targeted disruption of myostatin gene expression in mice leads to dramatic increases in skeletal muscle mass due to muscle fiber hyperplasia and/or hypertrophy (8,9). In addition, natural inactivating mutations of the myostatin gene in cattle are associated with a “double muscling” phenotype (10-12). Conversely, transgenic mice which overexpress myostatin selectively in skeletal muscle have lower muscle mass (13). All together, these observations indicate that muscle myostatin regulates negatively the skeletal muscle mass.

Dexamethasone-induced muscle atrophy is associated with a dose-dependent marked induction of muscle myostatin mRNA and protein expression (14). Increased muscle myostatin expression has been also reported in several models of muscle atrophy such as immobilization, microgravity and burn injuries, where glucocorticoids play a major role (15-17). These observations indirectly suggest that myostatin may play a crucial role in glucocorticoid-induced muscle loss. However it is not yet directly established whether the presence of myostatin is required for the expression of the glucocorticoid-induced catabolic effects. Moreover, the potential role of myostatin in the glucocorticoid activation of the components of the ubiquitin-proteasome and lysosomal proteolytic pathways is still unknown. We therefore investigated whether myostatin gene disruption could prevent muscle atrophy caused by glucocorticoids, and whether this protective effect may result from downregulation of the ubiquitin-proteasome and lysosomal pathways.

Materials and methods

Animals

Eight-week-old male myostatin KO FVB mice harboring a constitutive deletion of the third myostatin exon (n=28 KO mice, Grobet L 2003) and 8-week-old wild-type male FVB mice (n=28 WT mice) provided by Janvier Breeding (Le-Genest-Saint-Isle, France) were used to assess the role of myostatin in the muscle atrophy caused by glucocorticoids. The animals were housed individually under controlled conditions of lighting (12-h light, 12-h dark cycle) and temperature ($22 \pm 2^{\circ}\text{C}$). All animals were allowed to free access to chow and water. Body weight was measured daily. The study was conducted in accordance with the directives of the institutional animal care and use Committee of the University of Louvain.

Experimental design

After one week adaptation to environment and diet, KO as well as WT mice were randomly allocated to one of four treatment groups. Two groups were injected with dexamethasone, while the two others were injected with saline. The saline groups received a daily subcutaneous injection of saline solution (vehicle, 0.9% NaCl). The glucocorticoid-treated groups received a daily subcutaneous injection of saline solution of dexamethasone (Aacidexam[®], Organon, Oss, The Netherlands), either at 1mg/kg/d (low dose) or at 5mg/kg/d (high dose). Mice injected with the high and low dose and their corresponding control groups were sacrificed by decapitation respectively after 4 and 10 days of dexamethasone treatment. For biochemical analysis, gastrocnemius muscles were removed, weighted, deep frozen in liquid nitrogen and stored at -80°C until further analysis. For histological analysis, Tibialis anterior (TA) muscles were dissected, weighted and a transversal slice of 0.5 cm thickness was made in the middle belly of the muscle. This slice was embedded in Tissue Tek (Sakura, Zoeterwoude, The Netherlands), frozen in precooled isopentane and stored at -80°C until further analyzed. The morphological analyses and protein measurements were performed on samples collected at day 10 and the biochemical analyses were obtained from muscles collected at day 4.

Muscle protein extraction

Briefly, 100 mg of whole gastrocnemius muscle, previously pestled in liquid nitrogen, were homogenized with Ultraturrax (IKA-Labortechnik, Staufen, Germany) in 1 ml ice-cold lysis buffer (50 mM Tris/HCl [pH7.5], 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 1 mM

phenylmethylsulfonylfluoride, 10 µg/mL aprotinin, 10 µg/ml leupeptin, 10 mM β-glycero-phosphate, 1 mM KH₂PO₄, 1 mM vanadate, 50 mM NaF, 10 mM NaPPi). Homogenates were centrifuged 10 min at 10 000 rpm (Sorvall SS-34 rotor) to pellet myofibrillar proteins. Myofibrillar proteins, resuspended in 8 M urea/50 mM Tris/HCl, pH 7.5, were stored at – 80°C as well as the supernatant containing the soluble proteins. Myofibrillar and soluble muscle protein concentrations were determined using Bradford's protein assay (Bio-Rad, Munich, Germany).

Peptidase activities of the 20S proteasome

Proteolytic activities of the 20S proteasome were measured on individual gastrocnemius muscles collected after four days of treatment with dexamethasone 5mg/kg.day, as previously described (18). 100 mg of skeletal muscles from WT and KO mice treated or not with dexamethasone were homogenized in ice-cold buffer (pH 7.5) containing 50 mM Tris, 250 mM sucrose, 10 mM ATP, 5 mM MgCl₂, 1 mM DTT, protease inhibitors (10 µg/ml of antipain, aprotinin, leupeptin and pepstatin A, and 20 µM PMSF). The proteasomes were isolated by three sequential centrifugations. The final pellet was resuspended in buffer containing 50 mM Tris (pH 7.5), 5 mM MgCl₂ and 20 % glycerol. The protein content of the proteasome preparation was determined according to Lowry et al. (1951). Peptidase activities of the proteasome were determined by measuring the hydrolysis of the fluorogenic substrates Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY) and Boc-Leu-Arg-Arg-7-amido-4-methylcoumarin (LRR). These substrates are preferentially hydrolysed by the chymotrypsin-like and the trypsin-like peptidase activities of the proteasome, respectively. To measure peptidase activity, 15 µl of the proteasome extract was added to 30 µl of preincubation medium containing 50 mM Tris (pH 8.0), 12.5 mM MgCl₂, 1.5 mM DTT, 0.012 U/µl apyrase and +/- 66.6 µM MG132, and pre-incubated 15 min at room temperature to allow complete inhibition of the proteasome. Then, 30 µl of the reaction buffer containing 50 mM Tris (pH 8.0), 10 mM MgCl₂, 1 mM DTT, 0.008 U/µl apyrase and 750 µM LLVY or 2 mM LRR was added to the 45 µl of the preincubation reaction so that final concentrations were as follows: 300 µM LLVY-AMC or 800 µM LRR-AMC and 40 µM MG132. The peptidase activity was determined by measuring the accumulation of the fluorogenic cleavage product (methylcoumaryl-amide or MCA) using a luminescence spectrometer FLX800 (Biotek). Fluorescence was measured continuously during 45 min at 380 nm (MCA) excitation wavelength, and 440 nm (MCA) emission wavelength. The difference between arbitrary fluorescence units recorded with or without 40 µM of the proteasome inhibitor

MG132 (Affiniti) in the reaction medium was calculated, and the final data were corrected by the amount of protein in the sample. The time course for the accumulation of MCA after hydrolysis of the substrate was analysed by linear regression to calculate peptidase activities, e.g. the slopes of best fit of accumulated MCA versus time. Different kinetics were performed to measure individually the two proteasome peptidase activities.

RNA extraction and analysis by real time quantitative (RTQ)-PCR

Total RNA was isolated from the gastrocnemius muscle using TRIzol reagent as described by the manufacturer. Recovery was 1 µg/mg gastrocnemius. Reverse transcription using 1 µg of total RNA was done as previously described (19). Specific primers were determined using the Primer Express software (Applied Biosystems, Foster City, CA, USA). Accession number for the sequences and primers used were IGF-I: NM184052 (GCTATGGCTCCAGCATTCG - TCCGGAAGCAACACTCATCC), IGF-II: NM031511 (GTCGATGTTGGTGCTTCTCATCT - CGGTCCGAACAGACAAACTGAA), myostatin: AY204900 (GGCTTGACTGCGATGAG - ATATAGCATATTAATGGGAGACAT), Atrogin-1: AF441120 (AGCGACCTCAGCAGTTACTGC - CTTCTGGAATCCAGGATGGC), MuRF1: AF294790 (TGTCTGGAGGTCGTTTCCG - ATGCCGGTCCATGATCACTT), Cathepsin L: NM009807 (CTCATGAAGGCTGTGGCGA - AACTGGAGAGACGGATGGCTT), FOXO3a: NM019740 (TCGTCTCTGAACTCCTTGCGT - TGGAGTGTCTGGTTGCCGT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): AF106860 (TGCACCACCAACTGCTTA - GGATGCAGGGATGATGTTC) used as reporter gene. Primers were tested in order to avoid primers dimers, self-priming formation or unspecific amplification. Primers were designed to have standardized optimal PCR conditions.

RTQ-PCR was carried out using following cycle parameters: 10 min at 95°C, followed by 40 cycles of 1 min at 60°C and 15 sec at 95°C. For each gene, RTQ-PCR was conducted in duplicate with 25 µl reaction volume of 1 ng of cDNA. To ensure the quality of the measurements, each plate included for each gene a negative control and a positive control. Results were expressed using the comparative cycle threshold (Ct) method as described in the User Bulletin #2 (Applied Biosystem). Briefly, the ΔC_t values were calculated in every sample for each gene of interest as followed: $C_{t_{\text{gene of interest}}} - C_{t_{\text{reporter gene}}}$ with GAPDH as the reporter gene. The calculation of the relative changes in the expression level of one specific gene ($\Delta\Delta C_t$) was performed by subtraction of the ΔC_t from the control group to the

corresponding ΔCt from the treated groups (19,20) The values and ranges given in different figures were determined as followed: $2^{-\Delta\Delta Ct}$ with $\Delta\Delta Ct + SEM$ and $\Delta\Delta Ct - SEM$, where SEM is the standard error of mean of the $\Delta\Delta Ct$ value (User Bulletin #2, Applied Biosystem).

Histological analysis of muscle

Cryostat serial sections (10 μm thick) were obtained and mounted onto glass slides. The sections were fixed 2 min with acetone for Hematoxylin-Eosin staining. Muscle and fiber cross-sectional area (CSA) were measured with a microscope (Leitz Wetzlar) coupled to an image analyzer system (Kontron image analysis division MOP-Videoplan, Eching, Germany). To evaluate muscle fiber CSA, 200 fibers were randomly chosen and counted per muscle.

Statistical analysis

Results are presented as means $\pm$ standard error of the mean (SEM). Interaction between the glucocorticoids and the absence of myostatin was assessed by using two-way ANOVA followed by a Bonferroni post-Test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA, USA). Fiber cross-sectional area distribution statistical analysis was performed using χ^2 Pearson test. Statistical significance was set at $P < 0.05$.

Results

Animal weight

Baseline body weights were 6% higher in KO than in WT mice ($p<0.01$). Body weight remained stable over ten days in saline-injected WT and KO mice (fig.1). Dexamethasone administration, at the low dose, caused a progressive decline in body weight of WT mice which was already significant ($p<0.05$) at day 1. At the end of the ten days of dexamethasone treatment, body weight decreased by 5% versus baseline ($p<0.05$). In contrast, no detectable decrease in body weight was observed in dexamethasone-injected KO mice after 10 days. The high dose of dexamethasone decreased the body weight in both groups, the effect being slightly more pronounced in WT than in KO mice (-5.3%, $p<0.01$ and -2.7 %, $p<0.05$ after 4 days respectively, interaction genotype * treatment: $p=0.08$)

Figure 1

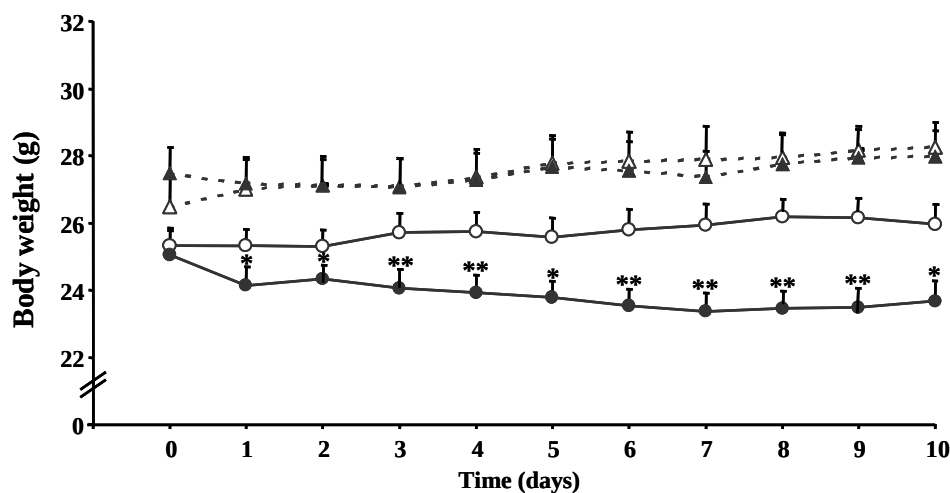


Fig. 1. Deletion of myostatin gene prevents the body weight loss due to low dose dexamethasone treatment. Evolution of body weight of KO (dotted line) and WT (solid line) mice, treated for 10 days with dexamethasone (1mg/kg.d, black points) or saline (white points). Results are expressed as mean $\pm$ SEM. *, $p<0.05$ and **, $p<0.01$ vs day 0.

Muscle weight

Deletion of myostatin in mice led to excessive muscle growth, as illustrated by a large increase in skeletal muscle mass (tibialis anterior: +62%, $p<0.001$; gastrocnemius: +47%, $p<0.001$; soleus: +41%, $p<0.001$ vs. WT mice) (fig.2).

After 10-d dexamethasone treatment, the tibialis anterior and gastrocnemius muscle weights were significantly lower in WT mice injected with the low dose of dexamethasone compared to their controls injected with saline (-15% and -13% respectively, $p<0.01$). Soleus muscle weight was not significantly affected by dexamethasone although the same tendency was observed (-11%, $p>0.05$). By contrast, in the KO mice, no reduction of muscle mass was observed after the low dose dexamethasone treatment (tibialis anterior: +2%, $p>0.05$; gastrocnemius: -3%, $p>0.05$; soleus: +12%, $p>0.05$). The effects of 4-d treatment with the high dose of dexamethasone were similar to those observed after 10-d treatment with the low dose (WT mice: tibialis anterior: -13%, $p<0.01$; gastrocnemius: -14%, $p<0.01$; soleus: -11%, $p>0.05$ and KO mice: tibialis anterior: +5%, $p>0.05$; gastrocnemius: -3%, $p>0.05$; soleus: +4% $p>0.05$) (fig.3). These data suggest that KO mice are protected against the muscle loss induced by dexamethasone.

Figure 2

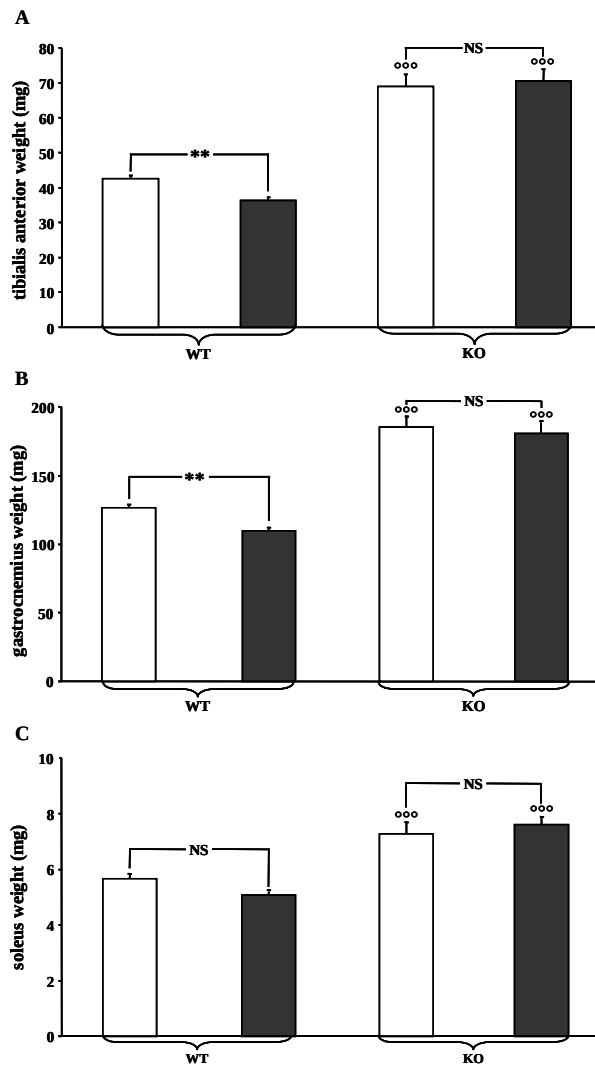


Fig. 2. Deletion of myostatin gene prevents the muscle atrophy caused by dexamethasone treatment. WT and KO mice were injected for 10 days with dexamethasone (1mg/kg.d, black box) or saline (white box). Tibialis anterior (A), gastrocnemius (B) and soleus (C) masses are expressed as mean $\pm$ SEM. **, p<0.01 and ooo, p<0.001 vs WT mice injected with saline.

Figure 3

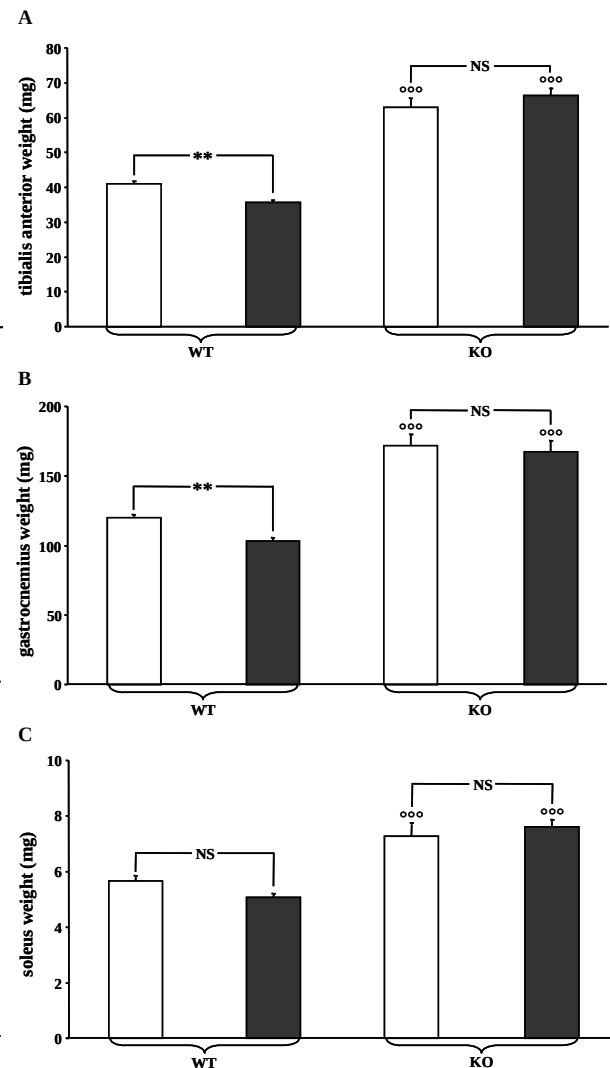


Fig. 3. Deletion of myostatin gene prevents the muscle atrophy caused by dexamethasone treatment. WT and KO mice were injected for 4 days with dexamethasone (5mg/kg.d, black box) or saline (white box). Tibialis anterior (A), gastrocnemius (B) and soleus (C) masses are expressed as mean $\pm$ SEM. **, p<0.01 and ooo, p<0.001 vs WT mice injected with saline.

Muscle fiber cross sectional area (CSA)

The histomorphological analysis of the tibialis anterior (TA) showed that the increased muscle mass observed in the KO mice results from hyperplasia but no effect on fiber size (hypertrophy) could be detected. Indeed the total muscle area was largely increased in the KO mice compared to WT (10.57 ± 0.54 vs 6.40 ± 0.3 mm², $p < 0.001$), while the muscle fiber cross sectional area (CSA) was similar in the two groups (KO: 1878 ± 74 vs WT: 1918 ± 64 μm², $p > 0.05$). As showed in figure 4A, the muscle loss induced by dexamethasone in the WT mice was due to a decrease of 15% of the mean muscle fiber CSA (1621 ± 31 vs 1918 ± 64 μm², $p < 0.01$). By contrast, dexamethasone treatment did not reduce the muscle fiber CSA in the KO mice (1898 ± 34 vs 1878 ± 74 μm², $p > 0.05$). We also compared the fiber size distribution of the TA in WT and KO mice injected with dexamethasone or saline. In the WT mice, dexamethasone clearly increased the proportion of fibers of small size ($p < 0.001$) (fig.4B). This was in contrast with the observation that in KO mice fiber size distribution was unaffected by dexamethasone ($p > 0.05$) (fig.4C). These results confirm the fact that the invalidation of the myostatin gene prevents the muscle atrophy caused by the dexamethasone treatment.

Figure 4

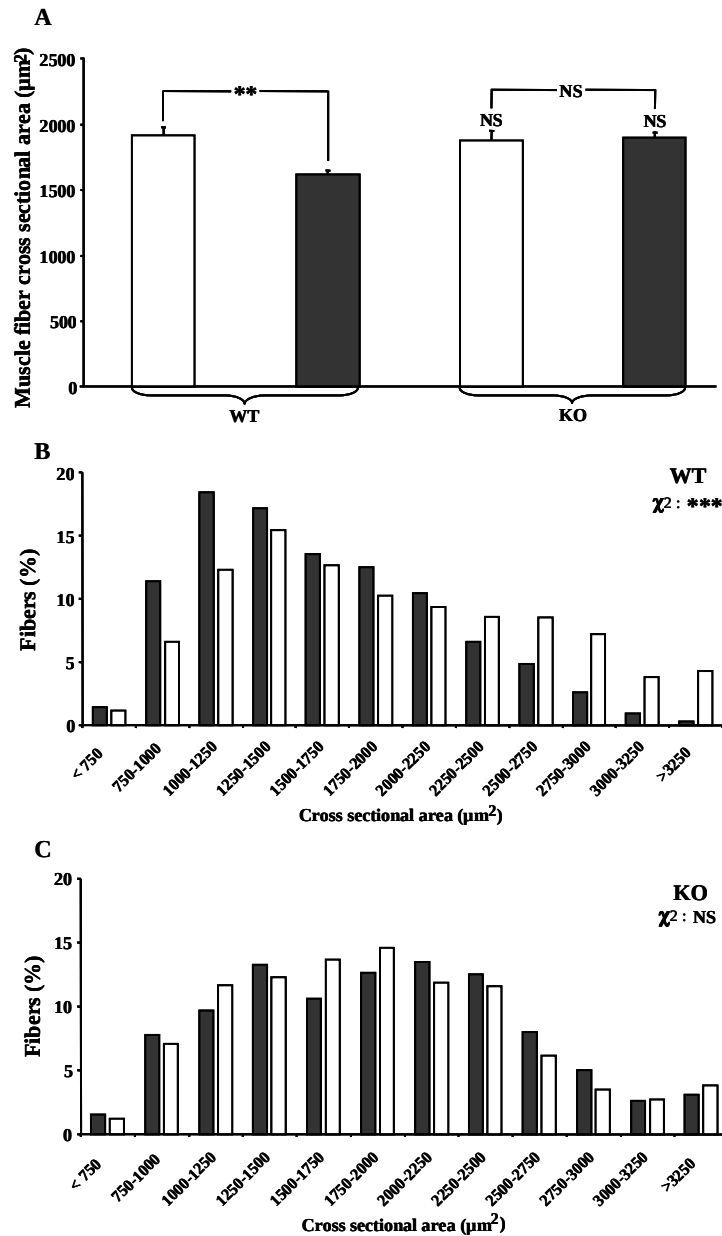


Fig. 4. Deletion of myostatin gene prevents the muscle fiber atrophy induced by dexamethasone treatment. The fiber cross sectional area (CSA) (A) was measured on tibialis anterior after 10 days of treatment with dexamethasone (1mg/kg.d, black box) or saline (white box). Results are expressed as mean $\pm$ SEM. **, $p < 0.01$ vs WT injected with saline. In WT mice (B), the proportion of large fibers was smaller in dexamethasone-treated mice (1mg/kg.d, black box) than in saline group (white box). By contrast, in KO mice (C), the fiber CSA was similar in dexamethasone- and saline-treated groups. Statistical analysis was performed using χ^2 Pearson test. ***, $p < 0.001$.

Myofibrillar and soluble muscle proteins

Like the muscle weight, the myofibrillar and soluble protein contents were significantly greater in the KO mice compared to the WT animals (myofibrillar: 16.3 ± 0.6 vs 11.6 ± 0.3 mg/muscle, $p < 0.001$; soluble: 14.2 ± 1.2 vs 9.0 ± 0.5 mg/muscle, $p < 0.001$) (fig.5A). The myofibrillar content was significantly decreased with low dose dexamethasone in the WT mice (8.9 ± 0.2 vs 11.6 ± 0.3 mg/muscle, $p < 0.01$) but not in the KO (16.1 ± 0.6 vs 16.3 ± 0.6 mg/muscle, $p > 0.05$). The muscle soluble protein content did not decrease significantly with dexamethasone in any of the two groups (WT: 7.5 ± 0.4 vs 9.0 ± 0.5 mg/muscle, $p > 0.05$ and KO: 13.3 ± 1.2 vs 14.2 ± 1.2 mg/muscle, $p > 0.05$).

Proteolysis related gene expression

Glucocorticoids are known to increase the protein degradation in muscle by the activation of the ubiquitin-proteasome and lysosomal proteolytic systems. We therefore investigated in WT and KO mice the expression of Atrogin-1, MuRF1 and Cathepsin L enzymes involved respectively in activation of the ubiquitin-proteasome and lysosomal systems. The mRNA levels of these enzymes were equivalent in saline-injected WT and KO mice, showing that myostatin is not a major regulator of their expression in baseline conditions (fig.5B). Dexamethasone increased the expression of Atrogin-1 (+67%, $p < 0.05$), MuRF1: (+89%, $p < 0.01$) and Cathepsin L (+42%, $p < 0.01$), in the gastrocnemius muscle of WT mice. In contrast, the mRNA of these enzymes was not significantly affected by dexamethasone in KO mice (Atrogin-1: -14%, $p > 0.05$; MuRF1: +36%, $p > 0.05$; Cathepsin L: -1%, $p > 0.05$). As the expression of Atrogin-1 and Cathepsin L is upregulated by the transcription factor FOXO3a, we also evaluated changes in its gene expression in the four experimental groups. Dexamethasone increased significantly the FOXO3a expression in the WT animals (+96%, $p < 0.01$) but not significantly in the KO mice (+39%, $p > 0.05$). All together, these data suggest that a lack of response to dexamethasone treatment in several pathways involved in muscle proteolysis is closely associated to absence of myostatin.

Figure 5

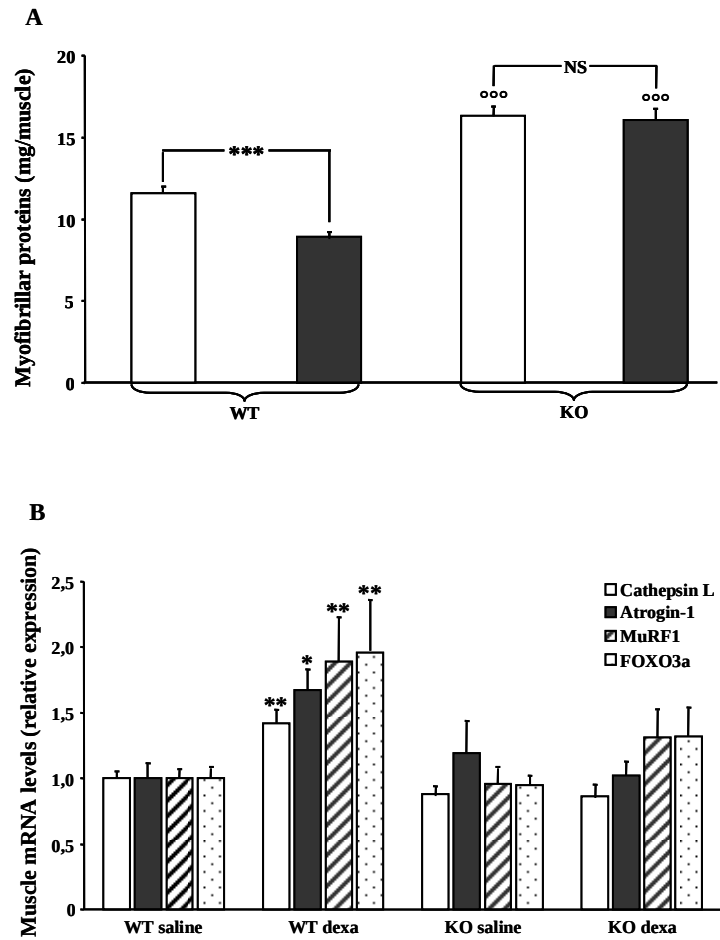


Fig. 5. (A) Deletion of Myostatin gene prevents the decrease in myofibrillar protein content caused by dexamethasone. The myofibrillar protein contents were determined in gastrocnemius after 10 days of treatment with dexamethasone (1mg/kg.d, black box) or saline (white box). Data are expressed as mean $\pm$ SEM. ***, $p < 0.001$ and $^{\circ\circ\circ}$, $p < 0.001$ vs WT injected with saline. (B) Deletion of Myostatin gene blunts the induction of muscle Cathepsin L, Atrogin-1, MuRF1 and FOXO3a mRNA caused by dexamethasone treatment. The mRNA levels were determined in gastrocnemius after 4 days of treatment with dexamethasone (5mg/kg.d) or saline. Results are expressed as mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ vs WT mice injected with saline.

Peptidase activities of the 20S proteasome

Proteolytic activities of the 20S proteasome were measured on individual gastrocnemius muscles collected after four days of treatment with dexamethasone (5mg/kg.day), according to the methodology previously described (18). Our results show that dexamethasone treatment increased the chymotrypsin-like activity of the 20S proteasome in WT mice (28.61 ± 1.62 vs 12.31 ± 1.21 Relative Fluorescence Units/min/ μ g protein, $p < 0.001$) (fig.6A). The baseline chymotrypsin-like activity was higher in KO than in WT mice (29.69 ± 2.67 vs 12.31 ± 1.21 RFU/min/ μ g, $p < 0.001$). Most importantly, this activity was not stimulated by glucocorticoids in KO mice (25.11 ± 2.11 vs 29.69 ± 2.67 RFU/min/ μ g, $p > 0.05$) supporting the concept that myostatin is mandatory to allow the glucocorticoid-induced stimulation of the proteasome. We also measured the trypsin-like activity of the 20S proteasome, but failed to show any difference between KO and WT mice after the dexamethasone treatment (WT: 17.86 ± 1.50 vs 17.91 ± 1.43 RFU/min/ μ g, $p > 0.05$; KO: 20.86 ± 1.13 vs 17.48 ± 2.05 RFU/min/ μ g, $p > 0.05$) (fig.6B). Combined with the data on Atrogin-1 and MuRF1, these results strongly suggest that myostatin gene deletion prevents the glucocorticoid-induced muscle atrophy partially at least by inhibition of the ubiquitin-proteasome system.

Figure 6

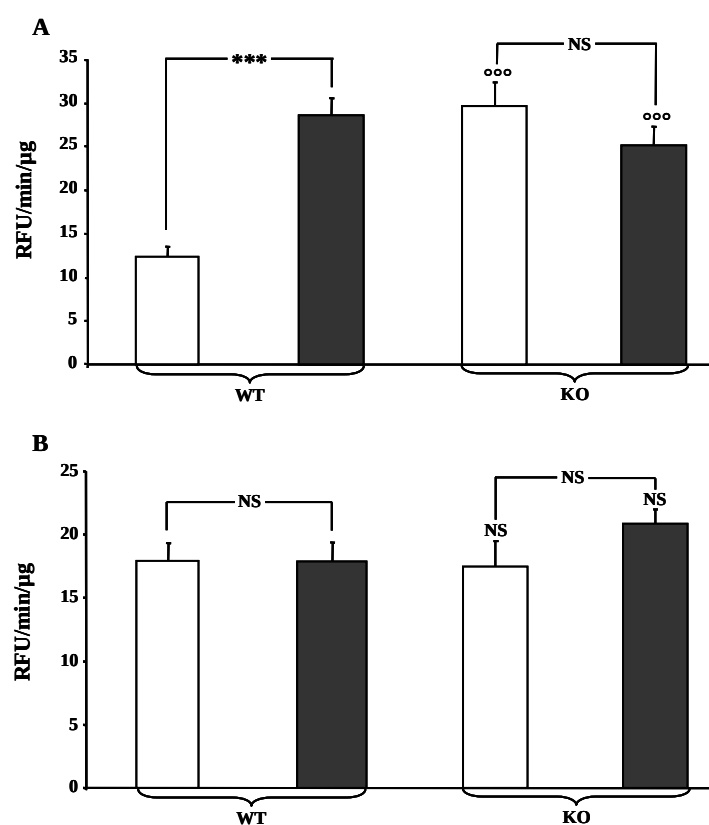


Fig.6. Deletion of myostatin gene prevents the increase of the chymotrypsin-like activity of the proteasome 20S induced by dexamethasone treatment (A). The trypsin-like activity of the proteasome 20S is not affected by dexamethasone treatment in WT and in KO mice (B). The proteasomal activities were measured in gastrocnemius after 4 days of injections with dexamethasone (5mg/kg.d, black box) or saline (white box). Results are expressed as mean $\pm$ SEM. ***, $p < 0.001$ and $^{\circ\circ\circ}$, $p < 0.001$ vs WT mice injected with saline.

Growth factors and myostatin gene expression

As IGF-I is known to possess an inhibitory effect on Atrogin-1, MuRF1 and Cathepsin L gene expression, we investigated the hypothesis that this growth factor could repress the glucocorticoid-induced activation of these enzymes in KO animals. As expected, dexamethasone reduced expression of IGF-I in WT mice (-53%, $p < 0.001$) (fig.7). Surprisingly IGF-I mRNA was lower in saline-injected KO mice than in WT animals (-35%, $p < 0.05$). Given the fact that IGF-II is also able to interact with the IGF-RI receptor, we monitored its expression in both groups. The muscle IGF-II expression was not affected by dexamethasone but strongly stimulated by the absence of myostatin (+104% in saline-treated and +79% in dexamethasone-treated KO mice respectively, $p < 0.01$).

As previously reported, muscle atrophy observed in WT mice treated with dexamethasone was associated with an increased expression of myostatin (+38%, $p < 0.05$ after 4 days). As expected, myostatin expression was not detected in KO mice.

Figure 7

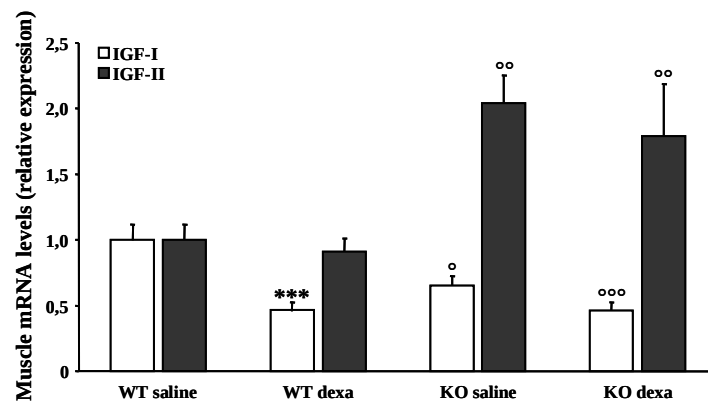


Fig. 7. Deletion of myostatin gene does not prevent the decrease of IGF-I mRNA (white box) induced by dexamethasone but causes an increase of IGF-II mRNA (black box) which is not affected by dexamethasone treatment. mRNA levels were measured in gastrocnemius after 4 days of injections with dexamethasone (5mg/kg.d) or saline. Results are expressed as mean $\pm$ SEM. ***, $p < 0.001$ and °, $p < 0.05$; °°, $p < 0.01$; °°°, $p < 0.001$ vs WT mice injected with saline.

Discussion

Our observations showed that myostatin gene deletion prevents the skeletal muscle atrophy caused by glucocorticoids. Thus, glucocorticoids administration decreased the muscle weight, the muscle fiber cross sectional area and the myofibrillar protein content in control WT mice, while these catabolic effects were not observed in myostatin KO mice. To our knowledge, this is the first report of the anti-atrophic effect of the absence of myostatin in a model of acquired muscle atrophy. The results suggest that muscle myostatin expression is a major player in the atrophic action of glucocorticoids towards muscle.

Increased proteolysis by the ubiquitin-proteasome and the lysosomal systems play a major role in glucocorticoid-induced muscle atrophy (1,5,6). Also recent evidence has been provided that the transcription factor FOXO stimulates the transcription of several components of these systems such as Atrogin-1, MuRF1 and Cathepsin L and causes muscle atrophy (21,22). Given the key role of myostatin in the protection of the muscle from the glucocorticoid-induced atrophy, we attempted to evaluate its possible effect as a down-regulator of these “atrogenes”. Anticipating early responses, we determined the muscle mRNA content of atrogenes at an early stage treatment (4 days treatment) in mice injected with a high dose of dexamethasone. In this model, we confirmed that glucocorticoids in WT mice increased muscle expression of Atrogin-1 and MuRF1, the two ubiquitin-ligases crucial for muscle atrophy (1,23) and Cathepsin L, a lysosomal enzyme considered as an early marker of muscle atrophy (6,24). Most importantly this activation of atrogenes was blunted in the myostatin-disrupted mice, pointing to a key role of this negative growth factor on these two proteolytic pathways. Very recently, using the *in vitro* approach, McFarlane *et al* showed that myostatin increased Atrogin mRNA in myotubes (25). This finding is in agreement with our data demonstrating that myostatin is required for the glucocorticoid-induced activation of this enzyme. The same authors (25) also showed that the effect of myostatin on Atrogin is mediated by the activation of FOXO1. In our experiment, we observed that the FOXO3a mRNA is strongly induced by glucocorticoids and that this activation is blunted in KO mice. This suggested that FOXO3a is also involved in the myostatin-mediated changes of Atrogin-1. Changes in FOXO3a phosphorylation could not be demonstrated given the very low expression of the protein in our model.

Early studies have shown that myostatin inhibited protein synthesis (26,27). Up to now, an effect on proteolysis has never been proved. Examining the chymotrypsin-like activity of the proteasome, we observed a higher level in KO than in WT mice, suggesting some increase in protein degradation. Given the massive increase in muscle mass of KO mice, it could be speculated that this increased proteolysis might be overridden by accelerated protein synthesis. Such large changes in proteolysis and protein synthesis may indicate a turnover more characteristic of an extensive remodeling process than of normal growth. This hypothesis would require additional experimental demonstration. We further examined the effect of myostatin gene deletion on glucocorticoid-stimulated proteasomal activity. Induction of the chymotrypsin-like activity by glucocorticoids was clearly present in WT mice but was not observed in KO mice. Taken together, our observations showed that the presence of myostatin is mandatory for the induction of the components of the proteolytic pathways and proteasomal activity by glucocorticoids. This concept is directly supported by *in vitro* data showing that myostatin added directly to myotubes causes their atrophy as well as an induction of components of the proteolytic pathway (25). Some *in vivo* data also suggest that high myostatin levels in the circulation obtained by transplantation of CHO cells engineered *ex vivo* with myostatin cDNA (25,28) or in the muscle by overexpressing myostatin (13,25) cause some muscle atrophy. Glucocorticoid-induced muscle atrophy may be related to increased myostatin muscle expression, as we observed with others (14). An alternate explanation would be that glucocorticoids also act indirectly by relieving a suppressor of myostatin activity, such as follistatin.

The role of IGFs in the muscle hypertrophy observed in myostatin KO mice has not yet been explored, even though these growth factors possess anabolic and anti-catabolic effects (29). Indeed, persistent over-expression of IGF-I in mice results in skeletal muscle hypertrophy (30). Furthermore, localized IGF-I over-expression by gene electrotransfer *in vivo* prevents also muscle atrophy caused by glucocorticoids (31).

Unexpectedly we observed a slight reduction of muscle IGF-I mRNA levels in control KO versus control WT mice. This decrease in muscle IGF-I mRNA in myostatin KO mice has also been observed by another group using DNA microarray (32). However IGF-II mRNA expression was increased in the muscle of myostatin KO mice. This observation is in line of previous publications reporting by other technical approaches (immunohistochemistry and DNA microarray) (32,33), an increase in IGF-II mRNA in skeletal muscles of myostatin KO animals. Although this upregulation of IGF-II in myostatin KO might suggest a direct

negative effect of myostatin on this gene, no such evidence is yet available. Our observation of an inverse relationship between myostatin and IGF-II expression is in line with the work of Lalani *et al.* (15) showing that muscle atrophy induced in the rat by exposure to microgravity was associated with an upregulation of myostatin and a downregulation of IGF-II without change in IGF-I. The increased IGF-II expression in myostatin KO mice might contribute to the skeletal muscle hypertrophy of these animals. Indeed, it is possible that IGF-II acts through an interaction with the IGF-RI receptor (34). Moreover in a strain of pigs characterized by a nucleotide substitution in intron 3 of the IGF-II gene, muscle IGF-II mRNA levels are increased threefold, and this was associated with increased skeletal muscle mass (35). Persistent over-expression of IGF-II mRNA in dexamethasone-treated KO mice might therefore participate to the anti-atrophic effect of the myostatin deletion. It is not excluded that other signaling pathways such as Wnt, recently described to be activated in myostatin KO mice (32), participate to the dexamethasone resistance of these animals.

The results obtained in this experience contrast with those of Mc Mahon *et al.* who showed that myostatin KO mice lose more skeletal muscle mass than WT during hindlimb suspension (36). Some hypotheses can be raised to explain the absence of the protective effect of the myostatin deletion in this catabolic situation. First, while glucocorticoid-induced atrophy affects mainly fast-twitch muscle fibers (MCHIB) (37), where myostatin is abundantly expressed (38), muscle atrophy caused by unloading affects mainly slow-twitch muscle fibers (MCHI), where myostatin is less expressed (38). These slow-twitch fibers might be therefore less sensitive than the fast-twitch to the protective effects of the inhibition of myostatin, explaining the lack of protective response in the unloading model of muscle atrophy. Secondly, several data suggest that the glucocorticoid-induced muscle atrophy is mediated through the activation of FOXO (21). We showed that myostatin deletion blunted the FOXO3a gene expression in the dexamethasone-treated mice, consistent with an interaction between myostatin and FOXO regulation. In contrast, hindlimb suspension is thought to be related to the induction of the NFkB (39) and it could be hypothesized that this pathway is independent of myostatin expression.

Rodents treated with exogenous glucocorticoids may have reduced food intake. It is therefore possible that reduction in muscle weight caused by dexamethasone may be related partially to decreased food intake. To exclude this possibility, we assessed in a preliminary experiment the daily food intake in WT FVB 8 week-old male mice treated with dexamethasone 1

mg/kg.day for 10 days and could not find any reduction. However, because we did not measure food intake in animals exposed to higher doses of dexamethasone, we can not exclude a contribution of reduced food intake to muscle atrophy in animals treated with 5 mg/kg/day dexamethasone.

Our observation that myostatin deletion protects the muscle from dexamethasone-induced atrophy is obtained in animals in which the negative growth factor was deleted constitutively. It remains unknown, however, whether the inhibition of myostatin in postnatal life retains the potential to inhibit the glucocorticoid-induced muscle atrophy. An answer to this question is mandatory to justify the development of myostatin antagonists. Nevertheless, blockage of endogenous myostatin either by injections of blocking antibodies or by post-natal gene excision resulted in an increase in muscle mass and strength in normal (40,41) and mdx dystrophic mice. These observations suggest that postnatal inhibition of myostatin might also blunt the muscle atrophy caused by glucocorticoids.

In conclusion, we have demonstrated that invalidation of myostatin gene prevents the muscle atrophy caused by dexamethasone. As glucocorticoids are involved in muscle atrophy observed in many catabolic conditions (1), our data suggest that myostatin inhibition may be a very promising approach and a clinically relevant avenue for the preservation, attenuation, or reversal of disease-related muscle loss.

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