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Original Article

Glutamine prevents myostatin hyperexpression and protein hypercatabolism induced in C2C12 myotubes by Tumor Necrosis Factor-α

Andrea Bonetto, Fabio Penna, Valerio G. Minero, Patrizia Reffo, Domiziana Costamagna, Gabriella Bonelli, Francesco M. Baccino, Paola Costelli

Department of Experimental Medicine and Oncology, University of Torino, Italy

Corresponding author: Paola Costelli

Department of Experimental Medicine and Oncology

Corso Raffaello, 30

10125 Torino, Italy

phone: +39-0116707062, fax: +39-0116707753

e-mail: paola.costelli@unito.it

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Abstract

Depletion of skeletal muscle protein mainly results from enhanced protein breakdown, caused by activation of proteolytic systems such as the Ca2+-dependent and the ATP-ubiquitin-dependent ones. In the last few years, enhanced expression and bioactivity of myostatin have been reported in several pathologies characterized by marked skeletal muscle depletion. More recently, high myostatin levels have been associated to glucocorticoid-induced hypercatabolism.

The search for therapeutical strategies aimed at preventing/correcting protein hypercatabolism has been directed to inhibit humoral mediators known for their pro-catabolic action, such as TNFα.

The present study has been aimed to investigate the involvement of TNFα in the regulation of both myostatin expression and intracellular protein catabolism, and the possibility to interfere with such modulations by means of amino acid supplementation. For this purpose, C2C12 myotubes exposed to TNFα in the presence or in the absence of amino acid (glutamine or leucine) supplementation have been used.

Myotube treatment with TNFα leads to both hyperexpression of the muscle specific ubiquitin ligase atrogin-1, and enhanced activity of the Ca^{2+}-dependent proteolytic system. These changes are associated with increased myostatin expression. Glutamine supplementation effectively prevents TNFα-induced muscle protein loss and restores normal myostatin levels.

The results shown in the present study indicate a direct involvement of TNFα in the onset of myotube protein loss and in the perturbation of myostatin-dependent signaling. In addition, the protective effect exerted by glutamine suggests that amino acid supplementation could represent a possible strategy to improve muscle mass.

Introduction

Skeletal muscle is the most abundant tissue and the major protein reservoir in the human body. Muscle mass usually results from a dynamic balance between protein synthesis and degradation (Goll et al., 1989; Sandri et al., 2008). Indeed, alterations between anabolic and catabolic pathways may lead to either muscle hypertrophy or atrophy, respectively (Guttridge, 2004; Ventadour and Attaix, 2006). The latter, in particular, is peculiar to conditions such as disuse (e.g. immobilization, muscle unloading, denervation), aging, starvation, and disease states (among which muscle dystrophies, diabetes, and cancer) usually characterized by the occurrence of 'cachexia' (reviewed in Jackman and Kandarian, 2004; Sandri, 2008).

Depletion of skeletal muscle protein may result from reduced rates of protein synthesis, enhanced rates of proteolysis, or both, although increased protein breakdown, mainly caused by activation of proteolytic systems such as the Ca^{2+}-dependent and the ATP-ubiquitin-dependent ones is believed to play a crucial role (reviewed in Ventadour and Attaix, 2006). In this regard, a subset of genes, known as 'atrogenes', have been found up-regulated in different models of muscle atrophy. These include two muscle-specific ubiquitin ligases, namely atrogin-1 and MuRF-1, which are associated with protein degradation through the ubiquitin-proteasome system (Bodine et al., 2001; Gomes et al., 2001). Recently, a role for the acidic vacuolar proteolysis has emerged (Capel et al., 2009).

The search for strategies aimed at preventing/correcting protein hypercatabolism has significantly increased in the last years. In this regard, previous studies have shown that amino acid supplementation counteracts muscle wasting, although the underlying mechanisms remain unclear. Indeed, glutamine administration has been reported to prevent muscle protein breakdown in septic rats (Hickson et al., 1995). Similarly, leucine supplementation to both animals and humans has been shown to stimulate muscle protein synthesis and to modulate the activity of proteins involved in the control of mRNA translation, thus resulting in suppressed skeletal muscle proteolysis (Nair et al., 1992; Louard et al., 1995; Sugawara et al., 2008). Finally, glucocorticoid-induced muscle wasting is prevented in dexamethasone-treated rats fed a glutamine-enriched diet (Ma et al., 2003; Salehian et al., 2006).

Most of the therapeutical strategies proposed in the literature have been directed to inhibit humoral mediators known for their pro-catabolic action, such as TNFα. Indeed, increased nitrogen and amino acid efflux from skeletal muscle and loss of body protein have been shown in mice administered TNFα (Hoshino et al., 1991; Buck and Chojkier, 1996; Lang et al., 2002), in animals expressing a TNFα transgene, as well as in conditions characterized by elevated endogenous TNFα, i.e. experimental sepsis (Ahmad et al., 1994) or cancer (Costelli et al., 1993). Most of these effects can be counteracted by treatment with anti-TNFα antibodies, or by either knocking-down the cytokine receptors or overexpressing soluble receptor isoforms (reviewed in Argilés and Lopez-Soriano, 1999).

In the last few years, significant progress has been made in identifying signaling pathways potentially contributing to muscle atrophy, with particular reference to that activated by a TGFβ family member known as myostatin. This is synthesized as an inactive precursor protein, that, after proteolytic cleavage, can dimerize to give the active peptide (McPherron et al., 1997; Lee and McPherron, 2001). Once secreted, myostatin circulates as an inactive latent and highly conserved dimer bound to the propeptide or to other myostatin-binding proteins, such as follistatin (Lee and McPherron, 2001).

Following activation myostatin binds with high affinity the activin type IIB receptor (ActRIIB), that in turn recruits a type I receptor (ALK-4 or ALK-5), to form a heteromeric complex (Lee and McPherron, 2001; Rebbapragada et al., 2003). Subsequently, recruitment and phosphorylation of signal transducers Smads 2 and 3, and their interaction with co-Smad 4, result in the translocation of this complex to the nucleus, where it regulates the transcription of myostatin target genes (Zhu et al., 2004).

Myostatin is hyperexpressed in several conditions associated with muscle atrophy (Gonzalez-Cadavid et al., 1998; Zimmers et al., 2005; Holzbaur et al., 2006). In particular, we have recently reported that muscle myostatin signaling is enhanced in two different experimental models of cancer cachexia (Costelli et al., 2008; Bonetto et al., 2009). This up-regulation seems to depend, partially at least, on TNFα. Indeed, administration of pentoxifylline, an inhibitor of TNFα synthesis, improves skeletal muscle wasting in tumor bearers and restores the myostatin/follistatin ratio (Costelli et al., 2008).

The present study has been aimed to investigate whether TNFα could be directly involved in the regulation of myostatin expression and whether amino acid supplementation could both prevent TNFα-induced hypercatabolism and myostatin up-regulation. For this purpose, C2C12 myotubes exposed to TNFα in the presence or in the absence of an excess of amino acid (glutamine or leucine) have been used as an *in vitro* model system. Both the state of activation of the myostatin signaling pathway and the modulations of protein hypercatabolism have been assessed.

Materials and Methods

Cell culture

Murine C2C12 skeletal myoblasts (ATCC, Manassas, VA, USA) were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mg/ml sodium pyruvate, 2 mM L-glutamine (all reagents were supplied by Sigma-Aldrich, Milan, Italy), and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were regularly checked for mycoplasma contamination by staining with the DNA specific fluorochrome 4'-6-diamidine-2-phenylindole dihydrochloride (DAPI; Boehringer, Mannheim, Germany). For the experiments, cells were seeded at 35000/cm^2 to obtain full confluence 24 h later. Differentiation to myotubes was induced by shifting confluent cultures to DMEM supplemented with 2% horse serum (DM). The medium was changed every 2nd day, and within 5 days most of the cells were fused to form myotubes. On day 6, the cultures were exposed to 100 ng/ml TNFα (Immunological Sciences, Rome, Italy), and cells collected after 24 h. Both TNFα-treated and untreated cultures were photographed under phase contrast microscopy, and monolayers were stored at -80°C for further analysis.

In a different set of experiments, C2C12 cells were induced to differentiate to myotubes (see above) in DM enriched in either glutamine (8 mM; Salehian et al., 2006) or leucine (2 mM; Du et al., 2007). The medium was changed every 2nd day, maintaining glutamine or leucine excess. At day 6 of differentiation, myotubes were treated with TNFα (100 ng/ml) for 24h (if not otherwise specified). These experimental conditions allowed to evaluate whether excess amino acid could affect both TNFα-induced protein hypercatabolism and myostatin bioactivity.

Morphological analyses

To evaluate nuclear morphology, cells were grown on chamber slides (Nalge Nunc International, Naperville, IL, USA) and treated as described above. Monolayers were then washed with PBS, fixed in 95% ethanol, stained with the DAPI fluorochrome (10 ng/ml, dissolved in methanol), mounted in Mowiol (Calbiochem, La Jolla, Ca, USA), and viewed in an epiilluminated fluorescence microscope (Dialux 20, Leitz, Germany).

Alternatively, in order to assess myosin heavy-chain expression and localization, after complete differentiation myotubes were washed in PBS, fixed in acetone-methanol solution (1:1) for 20 minutes at -20°C and then probed with the primary mouse monoclonal antibody (Clone MY32; Sigma-Aldrich, Milan, Italy). Detection was performed using a Cy3-conjugated mouse IgG secondary antibody (GE Healthcare, Milano, Italy). Nuclei were stained with the DAPI fluorochrome and the images captured in an epiilluminated fluorescence microscope (Axiovert 35, Zeiss, Germany).

Calpain enzymatic activity

Calpain activity in C2C12 myotubes was determined by evaluating the cleavage of a specific fluorogenic substrate (see Beyette et al., 1998; Ruiz-Vela et al., 1999). Fresh cells were lised in 50 mM TRIS-HCl pH 7.6, 150 mM NaCl, 10 mM NaH_2PO_4, 10mM Na_2HPO_4, 1% (v/v) Nonidet P-40, 0.4 mM Na_3VO_4, and then centrifuged at 13000 g for 15 min at 4 °C. The supernatant was collected and protein concentration determined by the method of Lowry et al. (1951).

Aliquots of 50 μg protein were then incubated for 60 min at 37 °C in the presence of the a specific fluorogenic substrate (Suc-Leu-Tyr-AMC, Calbiochem, La Jolla, CA, USA). The incubation buffer for the evaluation of calpain activity was 25 mM HEPES pH 7.5, 0.1% CHAPS, 10% sucrose, 10 mM dithiothreitol, 0.1 mg/ml ovalbumin.

Fluorescence was read with a spectrofluorometer (380 nm excitation-460 nm emission; Perkin-Elmer, Norwalk, CT, USA). The activity, expressed as nkatal/mg protein (1 katal = 1 mol substrate hydrolyzed/sec) was calculated by using free AMC as working standard.

Reverse transcription-PCR

Total RNA was obtained using the TriPure reagent (Roche, Indianapolis, IN, USA) following the instructions provided by the manufacturer. RNA concentration was determined spectrophotometrically (SmartSpec 3000, Bio-Rad, Hercules, CA, USA) and its purity ensured by evaluating the 260/280 nm ratio. RNA integrity was checked by electrophoresis on 1.2% agarose gel, containing morpholino propane-sulfonic acid (MOPS) 0.02 M and 18% formaldehyde.

Atrogin-1 mRNA levels were determined by semiquantitative reverse-transcription polymerase chain reaction using the kit 'Ready-to-Go RT-PCR Beads' (GE Healthcare, Milano, Italy). Following manufacturer's protocol, 0.5 μg total RNA and 400 nM mixture of each couple of primers were added to a RT-PCR reaction mixture containing ~2.0 units *Taq* DNA-polymerase, 10 mM Tris-HCl pH 9.0, 60 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase, ribonuclease inhibitor and stabilizers to reach a final volume of 50μl in each reaction tube. Table 1 shows primers sequences and accession numbers for mouse atrogin-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), that were obtained according to published sequences (Invitrogen, Milano, Italy).

Retrotranscription was performed at 42°C for 30 min, and before entering the cycling protocol the samples were denatured (2 min, 95°C). Amplification conditions are reported in Table 1. Both positive and negative controls were performed. PCR products (atrogin-1: 75 bp; GAPDH: 177 bp) were electrophoresed on 2% agarose gels and

visualized with ethidium bromide. A 100 bp-standard DNA ladder (Fermentas, Burlington, ON, Canada) was used to estimate the length of each PCR product. Quantification was performed by densitometric analysis. The results were normalized according to GAPDH gene expression. Comparisons among groups were made in the linear phase of amplification.

Western blotting

C2C12 myotubes (~$1.5x10^6$ cells) were homogenized in 80 mM TRIS-HCl, pH 6.8 (containing 1 mM DTT, 70 mM SDS, and 1 mM glycerol), kept on ice for 30 min, centrifuged at 15000 x g for 10 min at 4°C, and the supernatant collected. Protein concentration was assayed according to Lowry et al. (1951) using BSA as working standard. Equal amounts of protein (30 µg) were heat-denaturated in sample-loading buffer (50 mM TRIS-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved on a SDS-PAGE (12% polyacrilamide, 0.1% SDS) and transferred for 2h to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Protein transfer was checked by Ponceau S staining. The filters were then blocked with TRIS-buffered saline (TBS) containing 0.05% Tween and 5% non-fat dry milk and incubated overnight with a polyclonal anti-myostatin antibody (1:1000; Società Italiana Chimici, Roma, Italy), specific for the ~30 kDa processed myostatin, raised against a synthetic peptide (aa 133-148) representing a portion of human GDF-8 encoded within exon 3 (LocusLink ID 2660); a goat polyclonal anti-follistatin antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against a peptide mapping within an internal region of follistatin of human origin (~36 kDa); a mouse monoclonal anti-calpain-1 antibody (1:1000; VWR International, Milan, Italy), recognizing the ~80 kDa active form of calpain-1. Antibody against atrogin-1 (~41 kDa) and MyHC (~220 kDa) were from ECMbiosciences (Versailles, KY, USA), and Sigma (St. Louis, MO, USA), respectively.

Goat anti-rabbit, goat anti-mouse (Bio-Rad, Hercules, CA, USA) or rabbit-anti-goat (Millipore, Vimodrone, MI, Italy) peroxidase-conjugated IgG were used as secondary antibodies.

The filters were then stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, containing 100 mM 2-mercaptoethanol and 2% SDS for 30 min at 50° C, and reprobed with a mouse polyclonal antibody directed against tubulin (~50 kDa; Sigma, St. Louis, MO, USA) to normalize sample loading. The membrane-bound immune complexes were detected by enhanced chemiluminescence (Santa Cruz Biotechnology, USA) on a photon-sensitive film (Hyperfilm ECL; GE Healthcare, Milano, Italy). Band quantification was performed by densitometric analysis with a specific software (TotalLab, NonLinear Dynamics, Newcastle upon Tyne, UK).

Electrophoretic-mobility-shift-assay (EMSA)

To prepare nuclear extracts (Blough et al., 1999) C2C12 myotubes (~$1.5x10^6$ cells) were homogenized in ice cold 10 mM HEPES, pH 7.5, containing 10 mM $MgCl_2$, 5mM KCl, 0.1 mM EDTA pH 8.0, 0.1% Triton X-100, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM DTT, 2 µg/ml aprotinin, 2 µg/ml leupeptin. Samples were then centrifuged (5 min, 3000g), pellets resuspended in ice cold 20 mM HEPES, pH 7.9, containing 25% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, pH 8.0, 0.2 mM PMSF, 0.5 mM DTT, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and incubated on ice for 30 min. Cell debris were removed by centrifugation (5 min, 3000g) and the supernatant collected and stored at -80°C. SMAD and NF-κB oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Promega (Milan, Italy), respectively (Table 2). Oligonucleotide labeling and binding reactions were performed by using the reagent supplied in the Gel Shift Assay System (Promega, Milano, Italy). Binding reaction mixtures, containing nuclear proteins (10 µg) and Gel Shift Binding Buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 0.05 µg/µl poly(dI-dC)·poly(dI-dC), 4% glycerol), were incubated at room temperature for 10 min in the presence of 0.035 pmol ^{32}P-ATP end-labeled double-stranded oligonucleotide. At the end of the incubation, samples were electrophoresed in 0.5x Tris-borate-EDTA (TBE) buffer at 350 V for 40 min on a 4% nondenaturing acrylamide gel. The gel was dried for 45 min and exposed overnight or longer to a film for autoradiography (GE Healthcare, Milano, Italy) at -80°C with intensifying screens. Specificity of the bands was confirmed by adding an excess amount of aspecific oligonucleotide (1.75 pmol) to a control sample. HeLa cell nuclear extract was used as positive control (Promega, Milano, Italy).

Data analysis and presentation

All results were expressed as mean ± SD. Significance of the differences was evaluated by analysis of variance (ANOVA) followed by Tukey's test.

Results

During differentiation, C2C12 murine myoblasts shift from a fusiform or star-shaped morphology to elongated confluent cells, eventually originating long, multinucleated myotubes (Burattini et al., 2004). Treatment with TNFα exerted a cyotoxic effect (Fig. 1A, Fig. S), and reduced the expression of fast-type myosin heavy-chain in differentiated myotubes (Li et al., 1998; Li and Reid, 2000; Fig. 1A). As expected, the DNA-binding activity of NF-κB, a well-known mediator of TNFα action, also significantly increased following TNFα challenge (Fernandez-Celemin et al., 2002; Fig. 1B). Confirming previous observations (Li et al., 2005), TNFα significantly increased the mRNA levels of the muscle-specific ubiquitin ligase atrogin-1 after 2, 4, and 6h (Fig. 1C), but not after 24h treatment (C = 0.59±0.067, TNFα = 0.65±0.016; data expressed as arbitrary densitometric units, n =3 for each group). The Ca^{2+}-dependent proteolytic system also appeared activated, as suggested by increased levels of the ~80 kDa calpain subunit (active calpain) in myotubes after exposure to TNFα for 2, 4, 6, and 24h (Fig. 2).

In order to understand if TNFα may interfere with myostatin expression and activity in muscle cells, myostatin signaling was investigated in C2C12 myotubes exposed to the cytokine for 24h. While follistatin was not modified by TNFα, myostatin abundance was significantly increased (Fig. 3A). Consistently, the DNA-binding activity of SMAD was increased compared to controls (Fig. 3B).

To assess whether amino acid supplementation of C2C12 myotubes could both reduce the hypercatabolic state and prevent muscle myostatin up-regulation induced by TNFα, C2C12 myoblasts have been completely differentiated in glutamine or leucine enriched medium. No difference in cell morphology could be observed once differentiation was completed in the presence of glutamine or leucine excess. When TNFα is added to glutamine supplemented cultures, myotube reduction in size appears less marked than in the absence of glutamine (data not shown).

The increase of calpain enzymatic activity after 24h TNFα treatment was significantly reversed by glutamine (Fig. 4), though not by leucine supplementation (TNFα = $1.02x10^{-3}$ ± $1.03x10^{-4}$, TNFα+Gln = $6.94x10^{-4}$ ± $1.73x10^{-4}$, data expressed as nkatal/mg, n=3 for each group). The conspicuous hyperexpression of atrogin-1 mRNA observed after 6h TNFα treatment (see Fig. 1C) was paralleled by a modestly increased protein abundance, that was reduced, although without reaching significance, by glutamine supplementation (Fig. 5). By contrast, atrogin-1 expression was not modified by leucine treatment (TNFα = 2.45±0.04, TNFα+Leu = 2.00±0.29, data expressed as arbitrary densitometric units, n = 3 for each group). Finally, glutamine supplementation also appeared to prevent the reduction of myosin-heavy chain induced by 6h TNFα treatment (Fig. 5).

Previous studies showed that both glucocorticoid-induced muscle wasting and myostatin up-regulation decreased when dexamethasone-treated rats were fed a glutamine-enriched diet (Ma et al., 2003; Salehian et al., 2006). In this regard, we investigated whether TNFα-induced hyperexpression of myostatin could be counteracted by glutamine supplementation. The data reported in Fig. 6 show that TNFα-dependent myostatin up-regulation was significantly reduced in excess glutamine-differentiated myotubes, while high glutamine alone did not significantly affect myostatin basal levels (C = 0.67 ± 0.31, Gln = 0.87 ± 0.32, data expressed as arbitrary densitometric units, n = 3 for each group).

Discussion

The present study shows that hyperactivation of both the myostatin-dependent signaling and the intracellular proteolytic machinery induced in C2C12 myotubes by TNFα exposure can be reversed by glutamine supplementation.

The occurrence of enhanced protein breakdown in TNFα-treated C2C12 myotubes is demonstrated by the increased calpain enzymatic activity and is suggested by the early overexpression of atrogin-1. This is conceivable also in view of both the reduced myosin heavy-chain levels and the increased NF-κB DNA-binding activity (see Li et al., 1998; Li and Reid, 2000; Fernandez-Celemin et al., 2002; Cai et al., 2004). The enhancement of atrogin-1 expression induced by TNFα, both in myocyte cultures and in the skeletal muscle, has been already reported by previous studies (Li et al., 2003, 2005; Frost et al., 2007). In the last few years, evidence has been provided that atrogin-1 expression is regulated by the IGF-1/Akt/FoxO axis. However, although TNFα treatment of C2C12 myotubes results in significantly decreased expression of IGF-1 mRNA (C=0.54±0.06, TNFα 24h=0.37±0.02, p=0.016, n=3, arbitrary densitometric units), the levels of phosphorylated (active) Akt remain comparable to control values (C=1.55±0.51, TNFα 24h=1.27±0.32, n=3, arbitrary densitometric units). These results suggest that, at least in the model system used in the present study, atrogin-1 hyperexpression does not rely on down-regulation of the Akt/FoxO signaling. This is in line with a previous report showing that IGF-1 supplementation of TNFα-treated myotubes is unable to restore normal atrogin-1 levels (Moylan et al., 2008), and also with the observation that muscle wasting and atrogin-1 hyperexpression in experimental cancer cachexia are not associated with down-regulation of the IGF-1 signaling pathway, despite reduced IGF-1 levels (Costelli et al., 2006; Penna et al., 2010). By contrast, the MAPK/ERK pathway seems to play a role in the hyperexpression of atrogin-1, both in TNFα-treated C2C12 myotubes and in the skeletal muscle of tumor-bearing rats (Penna et al., submitted for publication).

As far as we know, this is the first study reporting that the Ca^{2+}-dependent proteolytic system is activated by TNFα in C2C12 myotubes. This result is consistent with a previous report showing that the increased calpain enzymatic activity in the skeletal muscle of tumor-bearing rats can be effectively prevented by treatment with pentoxifylline, an inhibitor of TNFα synthesis (Costelli et al., 2002). Quite recently calpain inhibition has been reported to reduce TNFα-induced neutrophil recruitment and activation (Wiemer et al., 2010). Consistently, LPS-induced myocardial alterations can be prevented by over-expression of calpastatin, the physiological inhibitor of calpain, by transfection of calpain-1 siRNA, or by various pharmacological calpain inhibitors (Li et al., 2009). On the whole, these observations suggest that inhibition of calpain may be used as an approach to limit TNFα-induced inflammatory responses.

While the involvement of cytokines in the onset of muscle protein hypercatabolism is largely recognized (reviewed in Costelli and Baccino, 2003; Argilés et al., 2009), little is known about the specific targets on which these mediators impinge to activate the intracellular proteolytic systems.

In the last few years myostatin/GDF-8 has been proposed as a negative regulator of skeletal muscle mass. Loss of function mutations of myostatin have been detected in sheeps and cattles characterized by the so-called 'double-muscle' phenotype, and adult mice in which the myostatin gene has been disrupted showed a marked muscle hypertrophy compared to control littermates (McPherron et al., 1997; Clop et al., 2006). Myostatin has been found overexpressed in various conditions characterized by muscle atrophy, such as ageing, disuse, sepsis, amyotrophic lateral sclerosis, dystrophy and cancer (Lecker et al., 1999; Bogdanovich et al., 2002; Zimmers et al., 2002; Holzbaur et al., 2006; Costelli et al., 2008; Liu et al., 2008; Bonetto et al., 2009).

We demonstrated that in rats bearing the AH-130 hepatoma muscle wasting and perturbations of the myostatin signaling pathway are both partially prevented by pharmacological inhibition of TNFα synthesis (Costelli et al., 2002, 2008). The present study shows that TNFα treatment of C2C12 myotubes causes changes in the myostatin pathway comparable to those observed in the AH-130 hosts: both myostatin expression and bioactivity are increased by exposure to the cytokine. Enhanced myostatin expression in TNFα-treated myotubes has also been recently reported (Lenk et al., 2009). The role played by TNFα in mediating myotube atrophy, likely through enhancement of myostatin expression, is further supported by the observation that follistatin hyperexpressing myotubes are resistant to the catabolic effects of the cytokine (Penna et al., submitted for publication).

The occurrence of a direct correlation between myostatin and protein metabolism has not yet been demonstrated. McFarlane et al. (2006) show that muscle depletion induced in mice by myostatin hyperexpression is associated with upregulation of atrogin-1. On the other side, glucocorticoid administration to mice knocked-out for myostatin does not result in increased levels of both atrogin-1 or MuRF1 (Gilson et al., 2007). Consistenly with these reports, the results shown in the present study further support the hypothesis that a link likely occurs between protein degradation machinery and myostatin-dependent signaling pathway.

Muscle protein wasting represents a prominent complication in the management of patients affected by chronic pathologies, often resulting in increased morbidity and mortality rates, less benefit from therapies, and worsening of the quality of life. From this point of view the search for appropriate therapeutical strategies should be given a high priority. In this regard, amino acid supplementation is long known for its effectiveness in both improving muscle mass and preventing protein depletion (Dillon et al., 2009). Indeed, a group of amino acids in the diet, normally considered as non-essential, has been suggested to become essential in several disease states. This is the case of glutamine, glutamate,

arginine, ornithine, citrulline and proline, that are metabolically related and fall within a common biosynthetic family tree derived from α-ketoglutarate (Robinson et al., 1999; Baracos, 2001).

Prolonged amino acid supplementation has been reported to decrease skeletal muscle protein breakdown via selective inhibition of both lysosomal and Ca^{2+}-dependent proteolysis in young rats, as well as proteasome activities in adult rats (Capel et al., 2008). Moreover, administration of an amino acid mixture (containing, among others, leucine, valine, methionine and cysteine; 8 g/day for 16 months) effectively reduces circulating TNFα levels and increases plasmatic IGF-1, contributing to significantly improve muscle mass in geriatric patients with sarcopenia (Solerte et al., 2008). Quite recently, glutamine administration to LPS-treated mice has been demonstrated to preserve muscle force without modifying circulating TNFα, suggesting that glutamine target(s) is/are downstream of cytokine production (Meador and Huey, 2009). Finally, Salehian et al. (2006) reported that in glucocorticoid-treated animals both muscle wasting and myostatin hyperexpression can be prevented by administration of glutamine. Consistently, enrichment of culture medium with this amino acid results in restoration of normal myostatin expression in dexamethasone-treated C2C12 myotubes (Salehian et al., 2006).

The present study shows that glutamine supplementation restores physiological levels of myostatin expression in TNFα-treated C2C12 myotubes. Of interest, myostatin modulations are associated with normalization of both Ca^{2+}-dependent proteolysis and atrogin-1 expression, further supporting the occurrence of a relationship between myostatin and intracellular proteolysis. The mechanism by which glutamine can exert such effecs, however, remains to be elucidated.

Our results show that leucine supplementation does not interfere with the modulations of proteolytic systems induced by TNFα. This is in contrast with recent works reporting that branched-chain amino acids, and especially leucine, suppress atrogin-1 mRNA expression via mTOR pathway in C2C12 myocytes (Talvas et al., 2006; Du et al., 2007; Herningtyas et al., 2008). The different experimental design can account for the discrepancy with the data reported in the present study. Indeed, in the studies reported above, myotubes were initially deprived of amino acids, thus likely inducing a complex form of cell death with hallmarks of both apoptosis and autophagy, and soon later exposed to a medium containing leucine, which is a well known inhibitor of the autophagic process (Martinet et al., 2005).

In conclusion, the present study indicates that TNFα plays a role in increasing both myostatin expression and bioactivity, thus supporting the hypothesis that this cytokine could exert its pro-catabolic action via this pathway as well. This view, however, is questioned by previous observations performed on rats bearing the AH-130 hepatoma, where prevention of muscle wasting and myostatin up-regulation by pentoxifylline is not associated with restoration of normal atrogin-1 mRNA levels (Costelli et al., 2008). In this regard, further studies are needed to clarify whether

TNFα-induced modulations of the myostatin pathway follow rather than precede the activation of muscle protein breakdown. In addition, the results shown in the present study improve the knowledge about the mechanisms involved in the beneficial effect exerted by glutamine on muscle wasting, suggesting the myostatin signaling pathway as a possible target of nutritional interventions based on amino acid supplementation.

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Figure Captions

Figure 1 Effects of TNF-α treatment (24h) on C2C12 myotubes.

(A) Morphological analysis. Phase contrast microscopy (panels a, e) and DAPI staining (panels b, f); MyHC expression (panels c, g). Merged pictures are represented in panels d and h. Bars= 100µm.

(B) State of activation of NF-κB in C2C12 myotubes exposed to TNFα for 24h. EMSA analysis (representative pattern) indicating the DNA-binding activity of NF-αB; § positive control (HeLa cells), *control sample incubated with excess cold oligonucleotide (specific competition).

(C) RT-PCR representative pattern for atrogin-1 gene expression in C2C12 myotubes, controls (C) and treated with TNF-α for 2, 4, and 6h.

Figure 2 Calpain expression in TNF-α-treated C2C12 myotubes.

Calpain (~80kDa active subunit) protein levels and representative western blotting in C2C12 myotubes exposed to TNF-α for 2, 4, 6, 24h. Data (means ± SD; n=3) are expressed as percentage of controls (100%). Significance of the differences: *$p<0.05$, **$p<0.01$ *vs.* controls.

Figure 3 Myostatin and follistatin expression (A), and SMAD DNA-binding activity (B) in C2C12 myotubes treated with TNF-α for 24h.

Representative western blotting for myostatin and follistatin levels, and EMSA for SMAD DNA-binding activity. Data (means ± SD; n=3, from 3 independent experiments) expressed as percentage of controls; § positive control (HeLa cells), *control sample incubated in the presence of an excess cold oligonucleotide (specific competition). Significance of the differences: *$p<0.05$ vs. controls.

Figure 4 Calpain enzymatic activity in C2C12 cells differentiated in the presence of excess glutamine (Gln), treated or not with TNF-α for 24h.

Data (means ± SD; n=3, from 3 independent experiments) are expressed as percentage of controls (100%). Significance of the differences: *p<0.05, **p<0.01 *vs.* controls; §p<0.05 *vs.* TNF

Figure 5 Atrogin-1 and MyHC protein levels in C2C12 cells differentiated in the presence of glutamine supplementation, treated or not with TNF-α for 6 to 24h.

Data (means ± SD; n=3, from 3 independent experiments) are expressed as percentage of controls (100%).

Figure 6 Myostatin protein levels in C2C12 cells differentiated in the presence of excess glutamine (Gln), treated or untreated with TNF-α for 24h.

Data (means ± SD; n=3, from 3 independent experiments) are expressed as percentage of controls (100%). Significance of the differences: *p<0.05 *vs.* controls; §p<0.05 *vs.* TNF-α.

Supplementary figure (S) Representative pattern of annexin V-positive C2C12 myoblasts and myotubes exposed to TNFα (100 ng/ml, 24 h).

The occurrence of cell death was determined by analysing phosphatidylserine exposure on the cell membrane. Binding to Annexin V-FITC in non-fixed cells indicates the amount of accessible phosphatidylserine. Being both necrotic and apoptotic cells annexin V-positive, they must be discriminated by simultaneously evaluating the exclusion of a vital dye, in this case propidium iodide (PI). A commercially available kit was used for the analysis (Medical System, Bender). Double fluorescence (green for annexin V, red for PI) was acquired and analysed by the CELLQUEST software (Becton & Dickinson, Mountain View, CA, USA). For each cytogram: low-right panel indicates apoptotic (annexin-V positive, PI negative) cells, up-right panel shows necrotic (annexin-V and PI positive) cells.

Figure 1

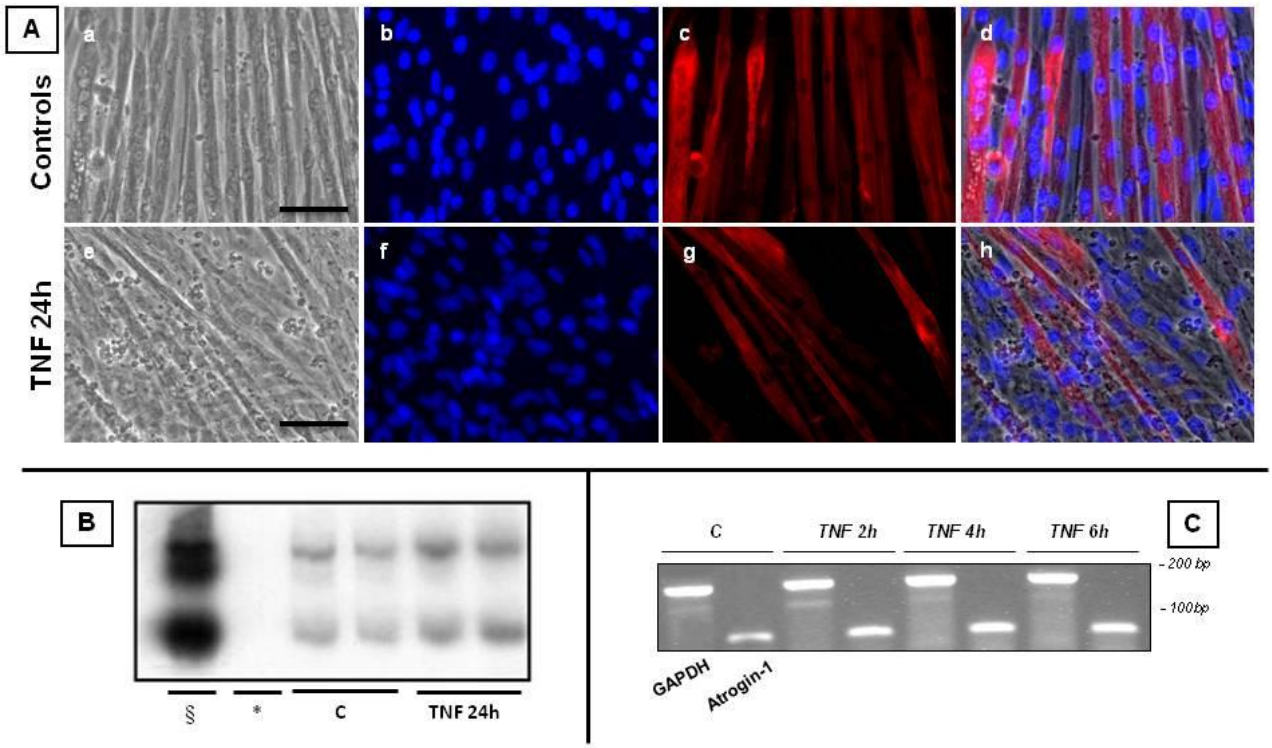

A
Controls
TNF 24h
a
b
c
d
e
f
g
h
B
§
*
C
TNF 24h
C
C
TNF 2h
TNF 4h
TNF 6h
- 200 bp
- 100bp
GAPDH
Atrogin-1

Figure 2

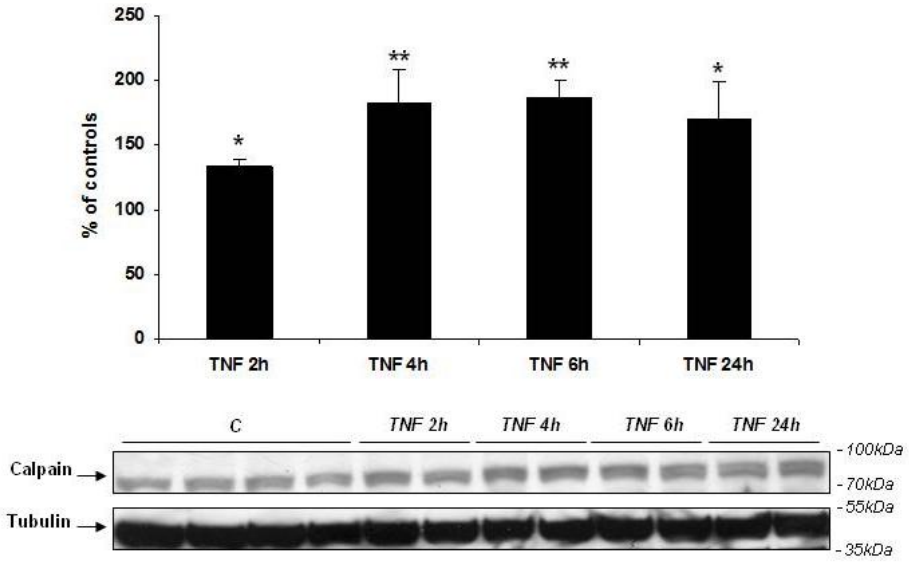

250
200
150
100
50
0
% of controls
*
**
**
*
TNF 2h
TNF 4h
TNF 6h
TNF 24h
C
TNF 2h
TNF 4h
TNF 6h
TNF 24h
Calpain
Tubulin
100kDa
70kDa
55kDa
35kDa

Figure 3

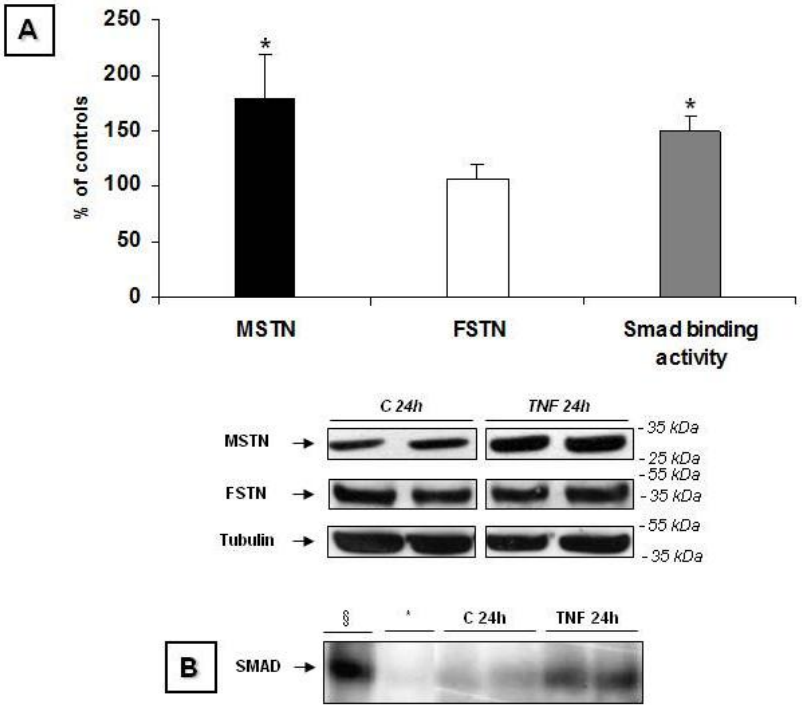

A
250
200
150
100
50
0
% of controls
*
*
MSTN
FSTN
Smad binding activity
C 24h
TNF 24h
MSTN
FSTN
Tubulin
35 kDa
25 kDa
55 kDa
35 kDa
55 kDa
35 kDa
B
SMAD
§
*
C 24h
TNF 24h

Figure 4

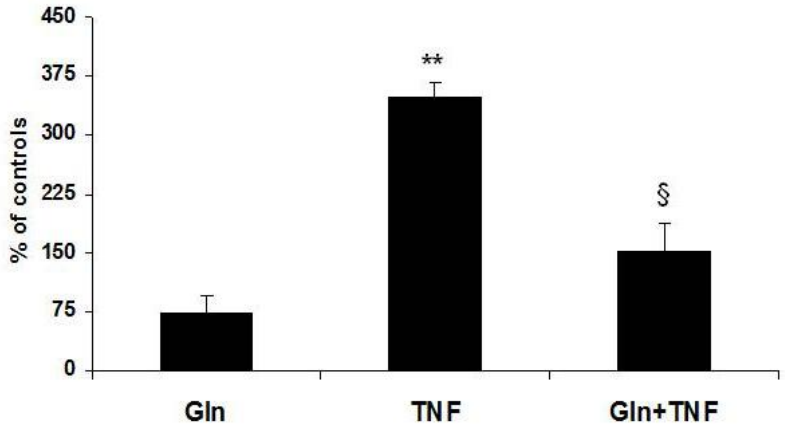


Figure 5

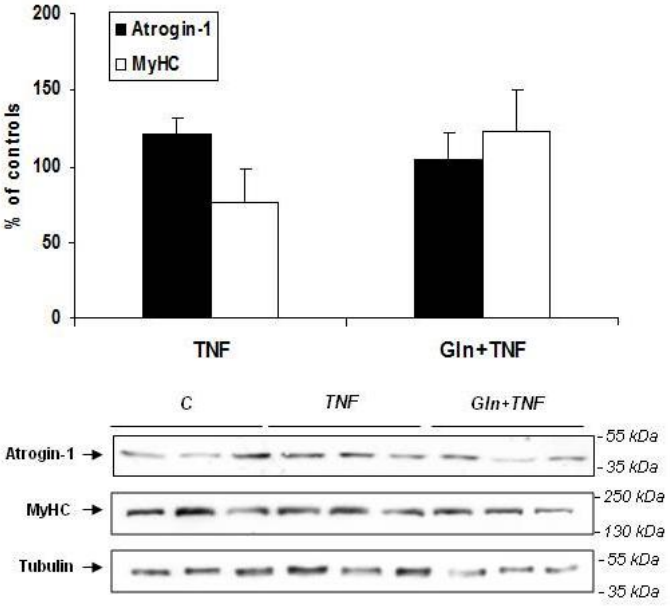

200
150
100
50
0
% of controls
Atrogin-1
MyHC
TNF
Gln+TNF
C
TNF
Gln+TNF
Atrogin-1
MyHC
Tubulin
55 kDa
35 kDa
250 kDa
130 kDa
55 kDa
35 kDa

Figure 6

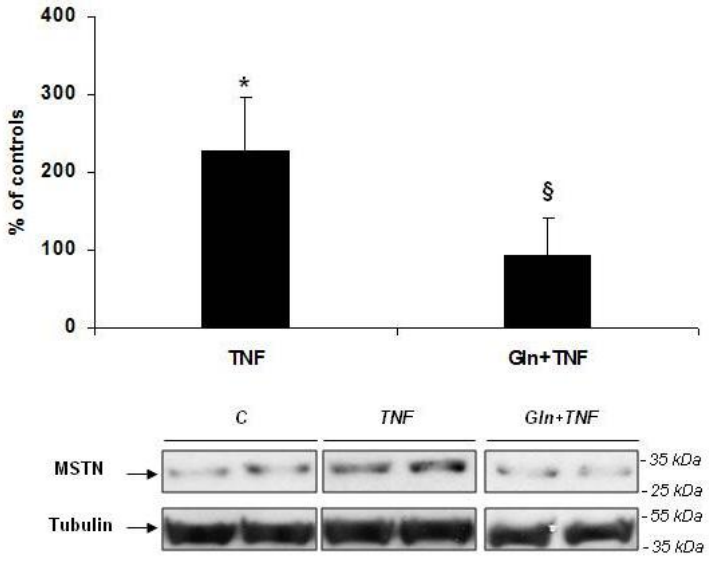
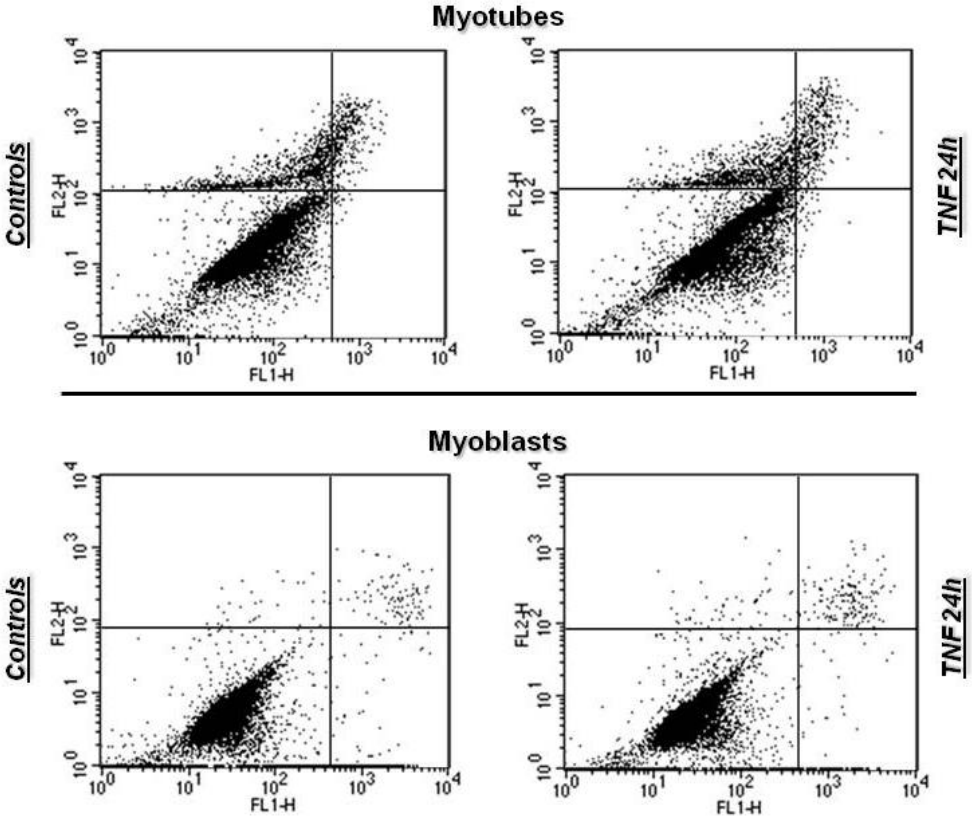

400
300
200
100
0
% of controls
*
§
TNF
Gln+TNF
C
TNF
Gln+TNF
MSTN
Tubulin
35 kDa
25 kDa
55 kDa
35 kDa

Figure S1


Myotubes
Controls
TNF 24h
FL2-H
FL1-H
Myoblasts
Controls
TNF 24h
FL2-H
FL1-H