General and Comparative Endocrinology 186 (2013) 9–15

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Myostatin induces atrophy of trout myotubes through inhibiting the TORC1 signaling and promoting Ubiquitin–Proteasome and Autophagy-Lysosome degradative pathways

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ARTICLE INFO

Article history:
Received 10 October 2012
Revised 4 February 2013
Accepted 14 February 2013
Available online 28 February 2013

Keywords:
Myostatin
IGF1
Myotubes
Protein synthesis
Proteolysis
Fish

ABSTRACT

Myostatin (MSTN) is well known as a potent inhibitor of muscle growth in mammals and has been shown to both inhibit the growth promoting TORC1 signaling pathway and promote Ubiquitin–Proteasomal and Autophagy-Lysosomal degradative routes. In contrast, in non-mammalian species, despite high structural conservation of MSTN sequence, functional conservation is only assumed. Here, we show that treatment of cultured trout myotubes with human recombinant MSTN (huMSTN) resulted in a significant decrease of their diameter by up to 20%, validating the use of heterologous huMSTN in our in vitro model to monitor the processes by which this growth factor promotes muscle wasting in fish. Accordingly, huMSTN stimulation prevented the full activation by IGF1 of the TORC1 signaling pathway, as revealed by the analysis of the phosphorylation status of 4E-BP1. Moreover, the levels of the proteasome-dependent protein Atrogin1 exhibited an increase in huMSTN treated cells. Likewise, we observed a stimulatory effect of huMSTN treatment on the levels of LC3-II, the more reliable marker of the Autophagy-Lysosomal degradative system. Overall, these results show for the first time in a piscine species the effect of MSTN on several atrophic and hypertrophic pathways and support a functional conservation of this growth factor between lower and higher vertebrates.

1. Introduction

The control of muscle mass is determined by a dynamic balance of anabolic and catabolic processes. Muscle hypertrophy is characterized by an increase of both the diameter and the total protein content of fibers and occurs as a result of an enhanced rate of protein synthesis. In contrast, muscle atrophy induced by a decrease in activity and load or by catabolic loss of muscle mass is characterized by a decrease of the size of pre-existing muscle fibers, resulting from a dramatic increase of protein degradation.

In the last decade, myostatin (MSTN), a member of the TGFβ superfamily, has emerged as a key factor in muscle growth regulation. The importance of MSTN in muscle growth comes from the phenotype of MSTN-deficient cattle (natural mutation or deletion) called double-muscled bovines, like the Belgium Blue breed. In these bovines, muscle overgrowth is due to both hyperplasia (increased number of muscle fibers) and hypertrophy (increased size of individual muscle fibers) (McPherron and Lee, 1997). Others studies in mouse (Lee and McPherron, 1999) or in human (Schuelke et al., 2004) confirmed that an inhibition of *mstn* expression induce muscle hypertrophy. Accordingly, several studies reports that ectopic expression of MSTN induces muscle atrophy (Durieux et al., 2007).

The efforts of several laboratories have shed new insight into how MSTN induces muscle atrophy in mammals. Earlier work showed that MSTN inhibited the proliferation and the differentiation of satellite cells (Langley et al., 2002; Rios et al., 2001; Thomas et al., 2000). These adult stem cells are resident of skeletal muscle fibers and are responsible for postnatal muscle cell growth and new muscle protein production (Moss and Leblond, 1971), supporting the relevance of satellite cell contribution to the induction of muscle hypertrophy consecutive to *mstn* loss. However, contradictory observations suggest that the muscle hypertrophic phenotype of transgenic *mstn* null mice involves little or no input from satellite cells (Amthor et al., 2009). More recently, Lokireddy et al. (2011b) demonstrated that MSTN promotes the wasting of human myotube cultures by promoting Ubiquitin–Proteasome pathway-mediated loss of sarcomeric proteins through a FoxO1-dependent mechanism. Likewise, MSTN has been shown to stimulate autophagosome formation as well as the expression of several autophagy-related genes in C2C12 muscle cells (Lee et al., 2011).

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0016-6480/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2013.02.008

There is also substantial evidence to suggest that MSTN could be connected with pathways regulating hypertrophy. Indeed, hypertrophied skeletal muscle of MSTN-deficient mice showed a higher activation status of the TORC1 signaling pathway, a known regulator of muscle hypertrophy (Lipina et al., 2010; Morissette et al., 2009). Transfection of a dominant negative MSTN receptor into adult skeletal muscle leads to an increase of muscle mass associated with increased phosphorylation of S6, a downstream effectors of the TORC1 pathway (Sartori et al., 2009). In contrast, down-regulation of the TORC1 signaling was seen in muscles after *mstn* gene electrotransfer (Amirouche et al., 2009). Furthermore, in vitro studies have demonstrated that modulation of TORC1 pathway is important for myostatin anti-hypertrophic effects (McFarlane et al., 2006; Morissette et al., 2009; Rodriguez et al., 2011; Trendelenburg et al., 2009).

In contrast, in non-mammalian species, despite high structural conservation of MSTN sequence (Garikipati et al., 2006), functional conservation is only assumed. This is particularly true for the fish due to the presence of several myostatin paralogs; two (*mstn1* and *mstn2*) in most species (Kerr et al., 2005) and four (*mstn1a*, *mstn1b*, *mstn2a* and *mstn2b*) in salmonids (Garikipati et al., 2007, 2006). In rainbow trout, three functional *mstn* genes (*mstn1a*, *mstn1b* and *mstn2a*) are described while *mstn2b* gene is a pseudogene because of a stop codon in its open reading frame (Garikipati et al., 2007). Several attempts have been made to elucidate the function of MSTN in fish but the results are somewhat divergent between species and even within species. Injection of morpholinos in zebrafish embryos increases the size of somites (Amali et al., 2004). The overexpression of the prodomain of MSTN (a negative regulator of the active protein) fails to increase the muscle mass of zebrafish and induces a moderate increase (10%) of the fiber number (Xu et al., 2003). Similarly, the overexpression of a dominant negative form of MSTN does not have any strong consequences for medaka muscle mass (Sawatari et al., 2010). However, a recent study in zebrafish carrying a stable heritable *mstn* knockdown genotype, reports a "double-muscled" phenotype (Lee et al., 2009). In trout, the overexpression of follistatin, an inhibitor of the TGF-beta family member, induces an increase of muscle mass (Medeiros et al., 2009), but it remains to be established whether the effect is due to solely MSTN inhibition or to another TGF-beta inhibition. The only available functional in vitro data in this species focused on the role of MSTN on myosatellite cell proliferation and differentiation (Garikipati and Rodgers, 2012a,b; Seiliez et al., 2012), but report conflicting results as regard the effect of this growth factor on the differentiation of myogenic cells. Surprisingly, no data is available in any fish species on the effect of MSTN on the main functions involved in the control of muscle protein turnover, namely the TORC1 growth promoting signaling pathway and the Ubiquitin–Proteasomal as well as the Autophagy-Lysosomal proteolytic pathways. Therefore, in order to gain deeper insight into the function of MSTN in fish, we aimed at determining whether MSTN controls these cellular functions, using an in vitro model of cultured trout myotubes.

2. Materials and methods

2.1. Animals

Juvenile immature rainbow trout (~5 g) were maintained in our own experimental facilities (INRA, Donzacq, France) at 18 °C under natural photoperiods (12 h/12 h). All experiments were carried out in accordance with legislation governing the ethical treatment of animals, and investigators were certified by the French Government to carry out animal experiments.

2.2. Myosatellite cell isolation and culture

Myotubes were carried out as follows: for each culture, 30–60 animals, each weighing approximately 5 g, were killed by a blow to the head and then immersed for 30 s in 70% ethanol to sterilize external surfaces. Cells were isolated, pooled, and cultured following previously described protocols (Gabillard et al., 2010; Seiliez et al., 2012). Briefly, after removal of the skin, dorsal white muscle was isolated under sterile conditions and collected in Dulbecco's modified Eagle's medium (DMEM) containing 9 mM $NaHCO_3$, 20 mM HEPES, 15% horse serum, and antibiotic–antimycotic cocktail [100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 g/ml fungizone (Sigma, A5955)] at pH 7.4. After mechanical dissociation of the muscle in small pieces, the tissue was enzymatically digested with a 0.2% collagenase (Sigma, C-9891) solution in DMEM for 1 h at 18 °C and gentle shaking. The suspension was centrifuged (300*g* for 5 min at 15 °C) and the resulting pellet was subjected to two rounds of enzymatic digestion with a 0.1% trypsin solution in DMEM for 20 min at 18 °C with gentle agitation. After each round of trypsinization the suspension was centrifuged and the supernatant was diluted in two volumes of cold DMEM supplemented with 15% horse serum (Sigma, H1270) and the same antibiotic–antimycotic cocktail mentioned above. After two washes with DMEM, the cellular suspension was filtered through 100- and 40-μm nylon filters. All experiments were conducted three times with cells seeded at a density of $1.5–2 \times 10^6$ per well, in 12- or 24-wells plastic plates (Nunc, 140675). Plates and coverslips were previously treated with poly-L-lysine (Sigma, P6282) and laminin (Sigma, L2020) to facilitate satellite cell adhesion. Cells were incubated at 18 °C, the optimal temperature for culture of trout origin, in a proliferating medium containing F10 (Sigma, N6635), 9 mM $NaHCO_3$, 20 mM HEPES, 10% fetal bovine serum, and antibiotic–antimycotic cocktail under an air atmosphere until day 4. Then, the cells were cultivated in a differentiating medium composed of DMEM (Sigma, D7777), 9 mM $NaHCO_3$, 20 mM HEPES, 2% fetal bovine serum and antibiotic–antimycotic cocktail until obtaining differentiated myotubes (at day 7). The medium was renewed every 2 days and observations of morphology were regularly made to control the state of the cells.

2.3. Treatment conditions

On the day of the experiment, cells (myotubes, as verified by visual microscopy) were incubated in a differentiation medium containing or not 4 nM of human MSTN (#CYT-418, ProSpec) and/or 100 nM of salmon/trout IGF1 (WU100 GroPep) in presence or not of 100 nM of Bafilomycin A_1 (B1793 Sigma), as specified in figure legends. After 2 h, 4 h and 24 h of incubation, the medium was removed, the wells were washed with ice-cold PBS and the cells were used for immunofluorescence or western blot analysis. Each experiment was performed three times.

2.4. Myotube diameter analysis by immunofluorescence

Cells on glass coverslips were briefly washed two times by PBS and fixed 30 min with paraformaldehyde 4%. For permeabilization, cells were incubated 3 min in 0.2% TritonX100/PBS. After three washes, cells were saturated for 1 h with 3% BSA, 0.1% Tween20 in PBS (PBST). Cells were incubated 3 h with the primary antibody anti-myosin (#MF20; Hybridoma Bank) diluted in blocking buffer (3%BSA/PBST). The secondary antibody (Invitrogene, #A11001) were diluted in PBST and applied for 1 h. Cells were mounted with Mowiol 4-88 (#475904, Calbiochem) containing DAPI (0.5 μg/ml). Cells were photographed using a Canon digital camera coupled to a Canon 90i microscope. Four wells (4 images/well) per treatment conditions were analyzed using an image analysis software (Visilog