JPFSM: Regular Article

Age-related changes in myostatin expression in rat skeletal muscles

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Received: December 13, 2017 / Accepted: January 16, 2018

Abstract Recent evidence suggests that myostatin, a negative regulator of skeletal muscle growth, may play a key role in age-related muscle loss (sarcopenia). However, there is still no unified view on the changes in myostatin protein expression in skeletal muscle with aging. Therefore, this study was to investigate the age-associated changes in the protein expression of myostatin in both slow and fast rat skeletal muscles. Slow soleus and fast plantaris (PL), extensor digitorum longus (EDL), and tibialis anterior (TA) muscles were dissected from male Wistar strain rats at different ages (7 weeks, and 1 and 2 years). A significant loss of muscle mass and myofibrillar protein content was observed between 1 year and 2 years of age in both slow soleus and fast PL, EDL, and TA muscles. Aging also resulted in a shift of myosin heavy chain expression toward slower isoforms in both slow and three fast skeletal muscles, although this isoform shift was already noted at 1 year of age. In contrast to these age-related changes, western blot analysis showed significantly higher expression of myostatin in fast PL, EDL, and TA, not in slow soleus, muscles in the 2-year-old group compared to the 7-week-old and 1-year-old groups. These results suggest that an age-associated increase in myostatin expression in fast skeletal muscle plays a key role in the onset and/or progression of sarcopenia, characterized by selective atrophy of fast type II muscle fibers.

Keywords : sarcopenia, aging, myostatin, myosin heavy chain, slow and fast skeletal muscle

Introduction

Skeletal muscle has greater plasticity to adapt various physiological stimuli. It has been well-known that increased mechanical loading leads to muscle hypertrophy through stimulation of protein synthesis, whereas physical inactivity induces muscle atrophy through stimulation of protein degradation and/or suppression of protein synthesis^{1,2}. Aging is also associated with loss of skeletal muscle mass and strength with a shift towards slower muscle fiber types, commonly called sarcopenia³. The onset and progression of sarcopenia leads to not only reduced physical activity and quality of life, but also increased health-care expenditure⁴. Therefore, exploring the mechanism and countermeasure for sarcopenia is an urgent issue in our society.

Growing evidence indicates that an aging-related increase of myostatin expression was observed in rat, cattle, and human skeletal muscles⁵⁻⁷. Myostatin, also called

growth differentiation factor-8, is a member of the transforming growth factor superfamily, and acts as a negative regulator of skeletal muscle mass by inhibiting protein synthesis and proliferation and differentiation of muscle satellite cells^{8,9}, as well as activating proteolytic pathways¹⁰. Myostatin overexpression causes skeletal muscle atrophy in mammals^{11,12}. In contrast, mutation or genetic ablation of the myostatin gene induces hypertrophy of mammalian skeletal muscle¹³⁻¹⁵. Furthermore, it was reported that myostatin inhibition increases skeletal muscle mass and strength in both adult and aged animals¹⁶⁻¹⁹, suggesting that myostatin could be a powerful therapeutic target for sarcopenia. However, there is still no unified view on the age-related changes in myostatin protein expression in skeletal muscle. For example, Kawada et al. reported that myostatin content, in both slow soleus and fast gastrocnemius and plantaris (PL) muscles, was stable between 11 and 92 weeks of age in mice²⁰.

Therefore, the present study was performed to clarify the changes in myostatin protein expression with aging in both slow and fast skeletal muscles of rats.

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Materials and Methods

Animals. All experimental procedures were conducted in accordance with the Physiological Society of Japan *Guide for the Care and Use of Laboratory Animals*. This study was also approved by the Committee on Animal Care and Use in Yamaguchi University (approval ID: 174). Male Wistar rats, aged 7 weeks, 1 year, and 2 years, were used in the present study ($n = 10/\text{age-group}$). The rats were housed in an animal room ($24 \pm 1^\circ\text{C}$ and 50–60% humidity) with a 12:12 h light-dark cycle. A standard solid chow and water were supplied *ad libitum*. Following the body weight measurement, the animals were killed by Somnopentyl (Kyoritsu Seiyaku) overdoses. Soleus, plantaris (PL), extensor digitorum longus (EDL), and tibiars anterior (TA) muscles were quickly removed, weighed, and frozen rapidly in liquid nitrogen. The muscles were then stored at -80°C until analysis.

Sample preparation. A portion of each muscle sample (40–80 mg) was minced and homogenized in 10 volumes of ice-cold isolation buffer (50 mM Tris·HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, and 1% Triton X-100) containing 1% phosphatase inhibitors (Phosphatase Inhibitor Cocktail; Nacalai Tesque, 07574-61) and protease inhibitors (Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts; Nacalai Tesque, 03969-21). Homogenates were centrifuged at 1,500 g for 15 min at 4°C and the supernatant was collected as the cytoplasmic protein-rich fraction for subsequent western blot analysis. The protein concentration of the supernatant was measured by using a protein assay kit I (Bio-Rad, 5000001JA).

The remaining portions of each muscle sample were minced and homogenized in 20 volumes of ice-cold homogenized solution (250 mM Sucrose, 50 mM KCl, 5 mM EGTA, 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mM Tris·HCl, pH 6.8). Following the centrifugation at 1,000 g for 10 min at 4°C , the supernatant was isolated as a soluble protein-rich fraction. Myofibrillar protein-rich fraction was also isolated from the remaining pellets according to the method of Solaro et al.²¹⁾. The soluble and myofibrillar protein contents were determined by the biuret method, and then the rate of myofibrillar protein per whole protein (soluble + myofibrillar) was calculated.

Western blot. The cytoplasmic protein-rich extract of each muscle was solubilized in SDS sample buffer (30% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 62.5% mM Tris·HCl, pH 6.8, and 0.05% bromophenol blue) at 2 mg protein/ml and heated at 60°C for 10 min. Equal amounts of protein (40 μg protein/lane) were subjected 15% SDS-PAGE, and then the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) using a mini-transblot cell (Bio-Rad) at 100 V for 1 h at 8°C . Following the transfer, the membranes

were blocked with Tween Tris-buffered saline (TTBS; 150 mM NaCl, 20 mM Tris·HCl, pH 7.5, and 0.05% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature. After serial washes with TTBS, the membranes were reacted overnight at 8°C with anti-myostatin antibody (1:100; R&D Systems, AF788) diluted in the blocking buffer. The membranes were then reacted with horseradish peroxidase-conjugated anti-goat IgG antibody (1:10,000; Sigma, A5420) diluted in the blocking buffer for 2 h at room temperature. After several washes with TTBS, protein bands were visualized by ECL plus reagents (Amersham Biosciences) using the ATTO light capture system (ATTO). Quantification of band intensities was performed using CS Analyzer 2.0 software (ATTO). The expression level of myostatin is expressed as a percentage of the value of a 7-week-old rat.

Electrophoretic analysis of myosin heavy chain (MyHC) isoforms

The myofibrillar protein-rich extract of each muscle was diluted to 0.5 mg protein/ml by SDS sample buffer and incubated at 60°C for 10 min. The composition of MyHC isoforms was determined according to the method of Sugiura and Murakami²²⁾ with slight modifications. Briefly, myofibrillar protein samples (5 μg protein/lane) were loaded onto 3.5% polyacrylamide stacking gel containing 35% glycerol and 8% polyacrylamide/33.3% glycerol running gel, and then electrophoresed at constant voltage of 150 V for 16.5 h at 8°C . After the electrophoresis, the gels were stained with coomassie brilliant blue to visualize the protein bands of each MyHC isoform. This method enabled the separation of four adult (MyHC I, IIa, IIb, and IIx) MyHC isoforms (Fig. 1). The protein bands were captured by ATTO light capture system and quantified using CS Analyzer 2.0 software. The relative proportions of MyHC isoforms were then calculated.

Fig. 1 Representative separation pattern of myosin heavy chain (MyHC) isoforms in soleus, plantaris (PL), extensor digitorum longus (EDL), and tibiars anterior (TA) muscles at 7 weeks and 1 and 2 years of age. MyHC IIa, IIb, IIx and I were separated in order of increasing electrophoretic mobility by SDS-PAGE.

Statistical analysis. All data are presented as means $\pm$ standard error (SE). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. $P < 0.05$ was considered significant.

Results

Body and muscle weights. Table 1 shows changes in body weight and absolute and relative weights of soleus, PL, EDL, and TA muscles during aging. The body weight significantly increased between 7-weeks and 1-year of age ($P < 0.05$), and almost stable thereafter. The absolute weight of slow soleus and fast PL, EDL, and TA muscles was also significantly higher at 1 and 2 years compared to 7-week animals ($P < 0.05$), whereas the relative weight of these slow and fast muscles was almost stable between 7-weeks and 1-year of age. However, both absolute and relative weights in all skeletal muscles were significantly lower in the 2-year than 1-year group ($P < 0.05$).

Myofibrillar protein content. Compared to 7-week and 1-year groups, myofibrillar protein content-to-total protein content ratio in soleus and PL muscles was significantly lower at 2 years (Fig. 2, $P < 0.05$). Moreover, the relative myofibrillar protein content in EDL and TA muscles was also less in 2-year-old than in 7-week-old animals ($P < 0.05$).

MyHC isoforms. Fig. 1 and Table 2 illustrate separation

of MyHC isoforms by gel electrophoresis and percent composition of each MyHC isoform in soleus, PL, EDL, and TA muscles, respectively. In the slow soleus muscle, the percentage of MyHC I was significantly higher at 1 and 2 years than at 7 weeks ($P < 0.05$), whereas the percentage of MyHC IIa was significantly less at 1 and 2 years than at 7 weeks ($P < 0.05$). In the fast PL, EDL, and TA muscles, there were no significant changes in the percentage of MyHC I and IIa during aging. However, the MyHC IId/x content in the three fast muscles was significantly greater at 1 and 2 years compared to the 7-week-old animals ($P < 0.05$). The opposite age-related change in the MyHC IIb content in these fast muscles was also observed ($P < 0.05$).

Myostatin expression. There were no significant changes in the protein expression of myostatin in slow soleus muscle between the 7-week-old and 2-year-old groups (Fig. 3). In contrast, myostatin expression in PL, EDL, and TA muscles was significantly higher in the 1-year compared to the 2-year-old group ($P < 0.05$). Myostatin levels in these fast muscles also tended to be greater at 7 weeks than at 2 years, based on a comparison of the two groups ($P > 0.05$), but reached significance only in the PL muscle ($P < 0.05$).

Discussion

Muscle mass and myostatin expression. It has been well established that aging leads to a loss of muscle mass in

Table 1. Changes in body and skeletal muscle weights during aging

	7 weeks	1 year	2 years
Body weight, g	216.0 $\pm$ 1.5	639.4 $\pm$ 15.3*	626.5 $\pm$ 21.8*
Muscle weight, mg			
Soleus	81.2 $\pm$ 1.9	217.1 $\pm$ 9.6*	166.6 $\pm$ 21.8*†
PL	204.7 $\pm$ 3.7	665.8 $\pm$ 20.0*	361.5 $\pm$ 27.1*†
EDL	97.1 $\pm$ 2.2	282.5 $\pm$ 10.5*	206.2 $\pm$ 15.9*†
TA	365.4 $\pm$ 9.9	1137.0 $\pm$ 28.6*	629.8 $\pm$ 54.5*†
Relative muscle weight, mg/kg			
Soleus	375.9 $\pm$ 7.9	337.0 $\pm$ 14.5	268.5 $\pm$ 19.8*†
PL	947.5 $\pm$ 14.6	929.8 $\pm$ 28.3	581.2 $\pm$ 45.8*†
EDL	449.4 $\pm$ 8.1	394.5 $\pm$ 15.5	331.6 $\pm$ 28.0*†
TA	1691.8 $\pm$ 44.8	1587.8 $\pm$ 39.9	1014.0 $\pm$ 94.5*†

Values are means $\pm$ SE. $n = 10$ per group. PL, plantaris; EDL, extensor digitorum longus; TA, tibialis anterior.

*†Significantly different from 7 weeks and 1 year groups, respectively ($P < 0.05$).

Fig. 2 Age-related changes in myofibrillar protein content-to-total protein content ratio in soleus, PL, EDL, and TA muscles. Values are means $\pm$ SE. $n = 10$ per group. *†Significant difference between 7 weeks and 1 year groups, respectively ($P < 0.05$).

Table 2. Changes in myosin heavy chain (MyHC) isoforms in skeletal muscles during aging

Muscle	MyHC	7 weeks	1 year	2 years
Soleus	I (%)	84.7 $\pm$ 2.1	98.8 $\pm$ 0.8*	96.1 $\pm$ 1.9*
	Ila (%)	15.3 $\pm$ 2.1	2.0 $\pm$ 0.8*	3.8 $\pm$ 1.9*
PL	I (%)	3.6 $\pm$ 0.5	4.0 $\pm$ 0.6	6.3 $\pm$ 1.1
	Ila (%)	10.8 $\pm$ 2.0	14.9 $\pm$ 1.5	16.3 $\pm$ 2.3
	IId/x (%)	33.9 $\pm$ 1.0	63.6 $\pm$ 2.2*	60.3 $\pm$ 2.2*
	Iib (%)	51.7 $\pm$ 1.3	17.5 $\pm$ 2.5*	17.1 $\pm$ 2.1*
EDL	I (%)	1.6 $\pm$ 0.2	1.2 $\pm$ 0.2	1.3 $\pm$ 0.1
	Ila (%)	8.6 $\pm$ 2.1	8.8 $\pm$ 0.9	10.1 $\pm$ 0.3
	IId/x (%)	25.9 $\pm$ 1.3	56.6 $\pm$ 2.8*	61.9 $\pm$ 0.9*
	Iib (%)	63.9 $\pm$ 1.0	33.4 $\pm$ 3.5*	26.7 $\pm$ 1.1*
TA	I (%)	1.5 $\pm$ 0.7	1.7 $\pm$ 0.3	2.3 $\pm$ 0.4
	Ila (%)	7.9 $\pm$ 0.8	9.1 $\pm$ 0.8	13.0 $\pm$ 2.6
	IId/x (%)	21.2 $\pm$ 0.9	45.8 $\pm$ 2.0*	55.2 $\pm$ 1.3*
	Iib (%)	69.4 $\pm$ 1.2	43.4 $\pm$ 2.5*	29.4 $\pm$ 3.3*

Values are means $\pm$ SE. $n = 10$ per group. See Table 1 for the abbreviations. *Significantly different from 7 weeks group ($P < 0.05$).

both slow and fast skeletal muscles²³⁻²⁵), which is mainly caused by selective atrophy of fast muscle fibers³). In agreement with these findings, the present study showed a significant reduction of muscle mass and myofibrillar protein content with aging, especially between 1 and 2 years, in both slow soleus and fast PL, EDL, and TA muscles (Table 1 and Fig. 2).

In these fast muscles, the opposite age-associated changes in myostatin protein expression were observed in this study (Fig. 3), although Kawada et al. showed no significant change in the expression level of myostatin with aging between 11 and 92 weeks in fast gastrocnemius and plantaris muscles in mice²⁰). Our results support the results from Baumann et al., who reported that myostatin

Fig. 3 Changes in the mean level of myostatin in soleus, PL, EDL, and TA muscles during aging. Values are means $\pm$ SE. $n = 10$ per group. *†Significant difference between 7 weeks and 1 year groups, respectively ($P < 0.05$).

expression increased with aging in fast gastrocnemius muscle in rats. Similar observations were also demonstrated by McKay et al. in aged human vastus lateralis muscle⁷). It appears that these discrepancies may be attributed to the differences in animal species. In general, it has been demonstrated that a decline in the number and function of satellite cells, as well as an increase in the expression of proteolysis-related proteins, is observed in skeletal muscle during aging^{24,26}). Myostatin is a negative regulator of muscle growth that not only inhibits protein synthesis and proliferation and differentiation of muscle satellite cells^{8,9}), but also promotes proteolysis¹⁰). In fact, it was reported that increased myostatin levels in skeletal muscle are associated with age-related dysfunction of muscle satellite cells, whereas blockage of myostatin expression improves muscle mass and strength in aged animals¹⁶⁻¹⁹), partially due to suppression of proteolysis through ubiquitin-proteasome and apoptotic pathways^{18,19}). Thus, judging from the age-related changes in muscle mass and myostatin expression in the present study, our findings suggest that aging-related elevation of myostatin expression in fast skeletal muscle plays a key role in sarcopenia, characterized by selective atrophy of fast type II myofibers.

In the slow soleus muscle, however, myostatin expression was stable during aging even though aging-related muscle atrophy was noted in the present study (Table 1 and Figs. 2 and 3). It has been reported that there are fiber-type specific differences in protein expression and effect of myostatin between slow and fast muscles. For example, protein levels of myostatin were 2-fold and 2.5-fold higher in fast PL and EDL muscles compared to slow soleus muscle, respectively^{27,28}). Mendias et al. also showed the similar tendency between slow soleus and fast EDL muscles in the expression of myostatin receptor, activin type IIB²⁹), suggesting that the responsiveness

to myostatin could be less in slow than in fast skeletal muscles. Although we have no clear explanations for the stable expression of myostatin in the slow soleus muscle during aging at present, the aforementioned findings indicate that myostatin would not be a main factor in causing aging-associated atrophy of slow skeletal muscle.

It has been known that rat myostatin and growth differentiation factor-11 (GDF-11) proteins are highly homologous with 88% amino acid sequence identity in the mature region³⁰). According to the manufacturer's information, myostatin antibody used in the present study has approximately 30% cross-reactivity to GDF-11. De Domenico et al. recently reported that GDF-11 protein expression in quadriceps skeletal muscle was significantly higher in 18-month than in 3-month-old mice³¹). These findings suggest that the age-associated increase in skeletal muscle GDF-11 protein expression might have an impact on the results of myostatin protein expression in our study. However, we cannot confirm this possibility, because we have no credible way of evaluating only myostatin or GDF-11 protein by western blot analysis at present. Further studies are needed to clarify this issue.

MyHC isoforms and myostatin expression. In the present study, a significant slower phenotype shift of MyHC isoforms, such as from IIa to I in slow soleus and from IIb to IId/x in fast PL, EDL, and TA muscles, was observed between 7 weeks and 1 year of age. This result was consistent with the report by Sugiura et al., who indicated that this MyHC phenotype shift occurs in both slow soleus and fast EDL muscles between 10 weeks and 1 year during aging in rats²³). However, the expression level of myostatin was not significantly elevated in both slow and fast skeletal muscles until 2 years of age in this study. Girgenrath et al. reported that myostatin knockout mice exhibit a faster phenotype shift of MyHC isoforms

in both slow soleus and fast EDL muscles, whereas administration of myostatin inhibitor for 12 weeks in adult mice did not lead to similar changes in the composition of the MyHC isoform in fast EDL muscle³²). Therefore, an aging-associated phenotype shift of MyHC isoforms in both slow and fast skeletal muscles may be independent of the age-related change in myostatin expression.

Conclusions

In conclusion, the present study demonstrated that the protein expression of myostatin was increased in fast, but not in slow, skeletal muscles with aging. This finding suggests that myostatin may play a key role in the onset and progression of age-related sarcopenia, especially in fast skeletal muscle. Further studies are needed to clarify the mechanisms of increased myostatin expression in aged fast skeletal muscle.

Conflict of Interests

No conflict of interests, financial or otherwise, is declared by the authors.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research C (17500428, to T. Sugiura).

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