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Long Term Adrenal Insufficiency Induces Skeletal Muscle Atrophy and Increases the Serum Levels of Active Form Myostatin in Rat Serum

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ABSTRACT—Skeletal muscle wasting is a common symptom in the adrenal insufficiency such as Addison's disease. Although it has been suspected that several cytokines and/or growth factors are responsible for the manifestation of the symptom, the precise mechanisms underlying the phenomenon have so far been poorly understood. Myostatin is predominantly expressed in skeletal muscles and involved in the regulation of skeletal muscle mass. Recently, several reports indicated that myostatin is secreted into the circulation and the increased levels of circulating myostatin is associated with the induction of skeletal muscle wasting in adult animals. We, therefore, hypothesized that the increased levels of circulating myostatin may account for the development of skeletal muscle wasting in adrenal insufficiency. To test the validity of this hypothesis, we compared the serum levels of myostatin in normal with those in bilaterally adrenalectomized (ADX) rats, a model of Addison's disease, by Western blot analysis. The active form of myostatin (13 kDa) was barely detectable in the sera collected either 1 month or 2 month after adrenalectomy, but present at conspicuously detectable levels in those obtained 3 month after the operation, while the total amounts of myostatin proteins (sum of the precursor and the active forms) remained constant at all the time points examined post-operatively. These results are consistent with the hypothesis that the increased serum levels of active form of myostatin protein, induced yet unknown post-translational control mechanisms may be responsible, at least in part, for the muscle wasting associated with the adrenal insufficiency syndromes.

Key words: adrenalectomy, skeletal muscle, atrophy, serum, myostatin

INTRODUCTION

Skeletal muscle wasting is a common symptom in chronic pathological conditions such as cancer and aging, and causes a general weakening of physiological capabilities and threatens quality of life. Several cytokines and/or growth factors have been shown to have roles in muscle wasting. Patients with Addison's disease (adrenocortical insufficiency) mainly caused by autoimmune attack show commonly symptoms of muscle weakness and fatigue with the most pronounced impairment associated with the proximal limb muscle (Betterle *et al.*, 2002, Kong and Jeffcoate, 1999). Since the rats after bilateral adrenalectomy have been shown to possess reduced muscle mass (Long *et al.*, 2003), they have been proved to be a useful model to elucidate the mechanism that leads to skeletal muscle atrophy in the patients of Addison's disease.

Myostatin, also known as GDF-8 (Growth/Differentiation Factor-8), is a member of TGF-β superfamily and negatively regulates skeletal muscle mass (McPherron *et al.*, 1997). Myostatin is synthesized as the 375-amino acid precursor form protein and following cleavage at the processing site ($R^{263}SRR^{266}$), the C-terminal domain obtains activity as the 110-amino acid active form protein (McPherron *et al.*, 1997). Disruption of myostatin gene in mice and cattle causes a dramatic and widespread increase in skeletal muscle mass

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resulting from both muscle hypertrophy and hyperplasia (McPherron *et al.*, 1997, McPherron and Lee, 1997, Kambadur *et al.*, 1997). Moreover, when postnatal inactivation of myostatin is induced by the conditional knockout techniques, muscle hypertrophy similar to that observed in the animals congenitally lacking the myostatin gene either by mutation or by the artificial knockout of the gene, could be observed, indicating that myostatin may act also in the postnatal animals (Grobet *et al.*, 2003).

Recently, several groups of investigators reported that myostatin is secreted into the circulation (Gonzalez-Cadavid *et al.*, 1998; Hosoyama *et al.*, 2002; Zimmers *et al.*, 2002; Hill *et al.*, 2002), and circulating levels of myostatin are increased in HIV-infected men and elderly persons with weight loss and muscle wasting symptoms (Gonzalez-Cadavid *et al.*, 1998, Yarasheski *et al.*, 2002), suggesting the possible association between the increased levels of myostatin in circulation and the muscle atrophy. Indeed, systemic administration of myostatin was shown to induce cachexia-like symptoms with severe weight loss and decreased muscle mass accompanied by skeletal muscle fiber atrophy (Zimmers *et al.*, 2002).

The results obtained in the past studies mentioned above lead us to hypothesize that increased levels of circulating myostatin may also contribute to muscle wasting in adrenal insufficiency such as Addison's disease. As an initial approach to test the validity of this hypothesis, we examined, by means of Western blot analysis, the serum levels of myostatin protein in the bilaterally adrenalectomized (ADX) rats that exhibited the symptoms of pathological atrophy of skeletal muscle fibers.

MATERIALS AND METHODS

Animals and preparation of tissue samples

In all the experiments presently described, Wistar-Imamichi male rats (2 to 5 months old) were used. They were housed in plastic cages under regulated temperature (23°C) and humidity (50%) with daily illumination cycle of 14 h light and 10 h dark. Food and water were provided *ad libitum*. Bilateral adrenalectomy was performed when the rats were 2 months old, and thereafter they were provided with 0.85% aqueous solution of NaCl instead of water for drinking. The animals were killed by decapitation, and blood samples and *gastrocnemius* muscles were collected. The *gastrocnemius* muscle from each leg was snap-frozen in isopentane bath cooled by liquid nitrogen and stored at –80°C until use. The blood samples were centrifuged at 1,200 g for 15 min at 4°C, and the sera were collected and stored at –20°C until use. Body weight and skeletal muscle (*gastrocnemus*) wet weight were measured at all time points examined. Serum glucose concentrations were measured using the glucose electrode method (Antsense II, Sankyo, Tokyo, Japan). Frozen tissue sections (5-μm in thickness) prepared from the ADX rats and their age-mached control were stained with hematoxylin-eosin (HE), and muscle fiber diameter was measured. All experiments using rats were carried out according to the Guideline for the Care and the Use of Laboratory Animals, the University of Tokyo.

Ion-exchange chromatography

Rat serum (1 ml) was dialyzed against 20 mM Tris buffer (pH7.4) at 4°C overnight. After filteration through a filter disk (pore size, 0.22 μm), the serum was applied onto an anion exchange column (0.7×2.5 cm) (HiTrap Q HP, Amersham Bioscience, Uppsala, Sweden), and eluted with 5 ml each of 20 mM Tris buffer (pH7.4) containing NaCl at concentrations of 0.01, 0.02, 0.03, 0.05, 0.1, 0.2, 0.3 0.5 and 1 M, in a stepwise manner. Each eluate and flow-through fraction were collected and dialyzed against 20 mM Tris buffer (pH7.4) at 4°C overnight.

Western blotting for myostatin

The frozen sample of skeletal muscle (*gastrocnemus*) was thawed on ice and homogenized in the buffer (125 mM Tris-HCl (pH 6.8), 10% β-mercaptoethanol, 4% SDS, 10% sucrose, 0.5 mM PMSF), followed by centrifugation at 15,000 g for 10 min at 4°C. The supernatant was saved and used as the sample designated as skeletal muscle (SKM) sample in the following for the subsequent analyses. Bromophenolblue (BPB) (0.004%) was added to each SKM sample after the determination of protein concentration. Serum samples partially fractionated by the anion-exchange chromatography as described in the previous section were diluted with an equal volume of 2 x sample buffer described by Laemmli (1970). Protein concentrations of the SKM and serum samples were measured using the Protein Assay Reagent (Bio-Rad, CA, USA) based on the dye-binding method of Bradford (1976). An aliquot of the SKM or serum sample containing 50 μg of protein was boiled for 5 min and the sediment was removed by centrifugation. The clear supernatant was applied to 15% polyacrylamide-SDS gel for the electrophoretic analysis. Following electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, MA, USA). The membrane was soaked in 5% skim milk dissolved in phosphate-buffered saline (PBS) (pH 7.4) at room temperature for 1 hour, and then incubated with goat anti-mouse GDF8 polyclonal antibody (R&D Systems, MN, USA) diluted with PBS (1:500) at 4°C overnight; the anti-GDF8 polyclonal antibody recognizes the C-terminal region of myostatin protein. After washing with PBS (5 min × 3), the membrane was incubated with biotin-labeled anti-goat IgG (Chemicon, CA, USA) diluted (1:4,000) with PBS containing skim milk (8%) at room temperature for 1 hour. After incubation with the horseradish peroxidase (HRP)-labeled streptavidin-biotin complex (Vector Lab, CA, USA) at room temperature for 30 min, the membrane was washed with PBS (5 min × 3) and the signal was detected with ECL Western Blotting Detection Kit (Amersham Bioscience, Uppsala, Sweden) according to manufacturer's protocol. ECL signals were visualized by exposing the membrane to X-ray film. Quantification of myostatin protein on each lane was done by scanning the X-ray film by an image scanner (model GT-9700F, EPSON, Nagano, Japan), and the intensities of signal were digitally expressed as pixel units.

RT-PCR

Total RNA was collected from the *gastrocnemius* muscle tissues (approximately 250 mg) with TRIzol reagent (Invitrogen, CA, USA). Three micrograms of the total RNA were reverse transcribed into cDNA using SuperScript II (Invitrogen, CA, USA) in a reaction volume of 20 μl according to the manufacturer' s protocol. Three microlitters of reverse transcribed products (out of 20 μl) were used as the template for semi-quantitative RT-PCR. The primer set for myostatin used consisted with the forward primer, 5'-TTT CAC TTG GCA TTACTC AAA AG-3' and the reverse primer, 5'-ACA GTG TTT GTG CAA ATC CTGAGA-3' (GenBank accession no. U84005, position 28–631). PCR amplification was performed using αTaq polymerase (Bionex, Seoul, Korea) under the following conditions: 27 PCR cycles, each cycle involving 1 min at 94°C for denaturing, 1 min at 50°C for annealing, and 1 min at 72°C for extension. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control; the primer set for G3PDH was purchased from Maxim Bitech, Inc., CA, USA. PCR amplification for G3PDH was performed under the following conditions: 24 PCR cycles each

Table 1. Body weight and skeletal muscle weight after adrenalectomy.

		1 month	2 month	3 month
Body weight (g)	Ctl	352.1±24.1	425.5±13.1	624.7±25.9
	ADX	321.8±16.4	439.0±39.0	517.3±68.6*
Muscle weight (g) (*Gastrocnemius*)	Ctl	1.79±0.10	2.26±0.04	3.09±0.15
	ADX	1.72±0.06	2.35±0.14	2.48±0.23*
Blood Glucose (mg/dl)	Ctl	117.3±6.2	117.3±2.6	119.7±8.4
	ADX	94.7±13.1*	110.3±3.3*	85.2±7.6**

Values are expressed as means±SEM. Ctl; sham-operated age-matched control. n=3 rats per point.
* $P<0.05$, $P<0.01$ vs Ctl.

cycle involving 1 min at 94°C for denaturing, 1 min at 50°C for annealing, and 1 min at 72°C for extension. The appropriate number of PCR cycles was determined so that it is well within a range where the number of cycles and the intensity of the ethidium bromide-stained PCR product on agarose gel are linearly related.

Extraction and detection of myosin heavy chains (MHCs)

MHC isoform was detected by Western blotting with anti-slow (NOQ 7.5.4) and fast MHC (MY-32) monoclonal antibody (Sigma, MO, USA). Myosin proteins were extracted form *gastrocnemius* muscle of the ADX rats of 3 month. Briefly, *gastrocnemius* muscle were homogenized in the pre-extraction buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 0.5 mM PMSF), followed by centrifugation at 20,000 g for 15 min at 4°C. The pellet was dissolved in the solution A (15 mM Tris-HCl, pH 7.5, 0.6 M KCl, 10 mM DTT, 10 mM, 2 mM $MgCl_2$, 0.5 mM PMSF). After centrifugation at 550 g for 3 min at 4°C, the supernatant was collected and added with five volumes of 2 mM $MgSO_4$. The supernatant was centrifuged at 10,000 g for 10 min at 4°C, and the pellet was dissolved in the solution B (40 mM $Na_4P_2O_7$, pH 8.8, 1 mM EDTA, 0.5 mM PMSF). Myosin proteins were mixed with an equal volume of glycerol and stored at −20°C until use. Followed by measurement of protein concentrations by the Protein Assay Reagent (Bio-Rad, CA, USA), 1 μg of myosin proteins diluted with 2 × sample buffer (Laemmli *et al.*, 1970) was separated by 10% polyacrylamide gel including 0.25% glycerol at 4°C overnight and transfered to PVDF membrane. The membrane was blocked by 8% skim milk dissolved in PBS containing 0.1% Tween 20 (PBS-T) at room temperature for 1 hour, and the membrane was incubated with each monoclonal antibody diluted with PBS-T (1:500) at room temperature for 2 hours. Following washing with PBS-T (5 min×3), the membrane was incubated with anti-mouse IgG conjugated with HRP (Jackson Immunoresearch Laboratory, ME, USA) diluted with 8% skim milk dissolved in PBS (1:10,000) at room temperature for 1 hour. Membrane was washed with PBS-T (5 min×3), and then the signal was detected using ECL method as described before.

Statistical analysis

Results were expressed as mean±SEM. Student's *t*-test was used to estimate differences between age-matched control rats and adrenalectomized rats for all parameter tested, e.g. body weight, muscle weight and blood glucose concentration (Table 1). The statistical analysis used to test the differences in the effects of adrenalectomy on myostatin expression in the serum was one-way ANOVA. When a significant interaction was identified ($P<0.05$), statistical significance levels less than 0.05 were followed up with *post hoc* comparison test using Bonferroni/Dunn correction.

RESULTS

Myostatin in adult rat serum

Attempts were made to detect myostatin protein in the serum of young adult rats (4 months old) by Western blot-

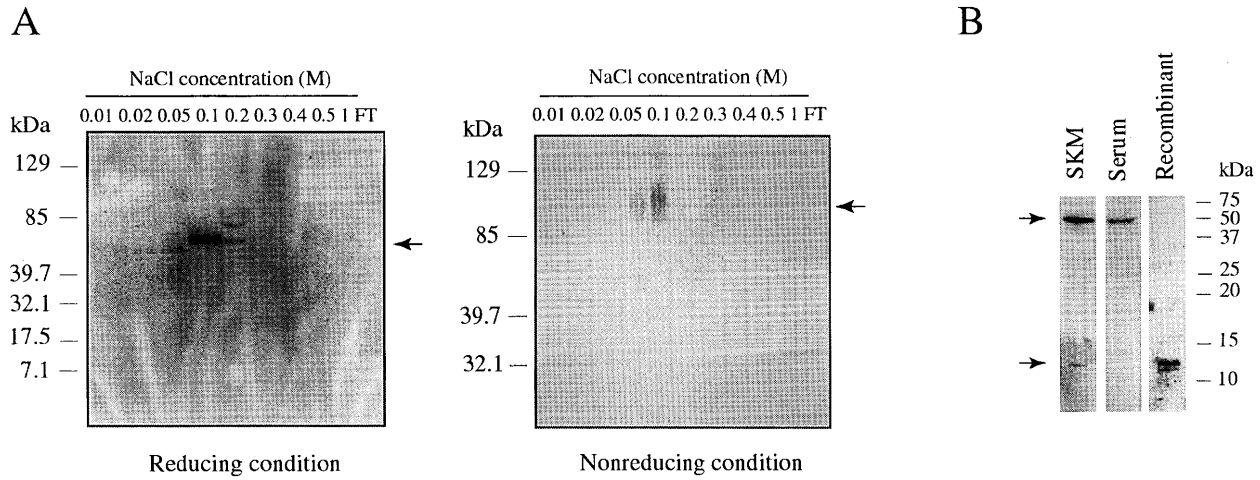

Fig. 1. Detection of myostatin protein in skeletal muscle (SKM) and serum (Serum) of adult male rats by Western blotting. (A): Adult rat serum was purified by ion-exchange chromatography and myostatin protein was detected by Western blotting in reducing (left panel) and non-reducing (right panel) condition. Arrows indicate myostatin immunoreactive bands corresponding to approximately 50 kDa and 100 kDa, respectively. (B): Recombinant mouse GDF8 (Recombinant) (R&D systems, MN, USA) was used as positive control for the active form. Arrows indicate immunoreactive bands corresponding to the precursor (50 kDa) and the active form (13 kDa) myostatin.

ting. Following fractionation of serum proteins by anion-exchange chromatography as described in Materials and Methods, aliquots of each eluate were subjected to electrophoresis and reacted with the anti-myostatin antibody. The immunoreactive bands were detected in the 0.1 M NaCl fraction under either reducing (about 50 kDa) or non-reducing (about 100 kDa) conditions (Fig. 1A). In the skeletal muscles, about 50 kDa and 13 kDa immunoreactive bands were detected under the reducing condition. Since the antibody against myostatin used in the present study recognizes the C-terminus of myostatin protein, these two bands would correspond to the precursor and the active form of myostatin protein, respectively (Fig. 1B). On the other hand, only the precursor form was detected in the sera from normal rats (Fig. 1B).

Characteristics of ADX rats

Two-month old male rats were adrenalectomized and after 1, 2 and 3 months, their skeletal muscles and sera were collected. The glucose concentrations in the sera, wet weight of the muscles and the fiber diameters were measured and recorded. Glucose concentrations in the sera were significantly lower in the ADX rats than in the sham-operated age-matched control at all time points examined, indicating the completeness of adrenalectomy (Table 1). Wet weight of skeletal muscles, as represented by *gastrocnemius* muscle in the ADX rats was significantly lower than that in the age-matched control at the end of 3rd postoperative month (Table 1). To examine whether the observed decrease in the mass of skeletal muscle in ADX rats 3 months after the operation, may be attributed to the atrophy of muscle fibers, diameters of the fibers were measured as described in Materials and Methods.

The fiber diameters in the *gastrocnemius* muscle of the ADX rats measured at the end of 3 months after the operation were smaller than those in the sham-operated controls (Fig. 2A), accounting for the observed reduction in skeletal muscle mass (Table 1). At the same time, expression level of the slow type MHC isoform (MHC I) increased in the *gastrocnemius* muscle, while the levels of MHC IIb expression decreased, suggesting that a change in the fiber type composition in the *gastrocnemius* muscle had been induced in the ADX rats (Fig. 2B).

Myostatin gene (*MSTN*) and protein expression in skeletal muscle of ADX rats

It has been thought that the production of myostatin protein is associated with skeletal muscle atrophy, since *MSTN* mRNA expression levels increases under some atrophy-inducing conditions, such as hindlimb unloading (Carlson *et al.*, 1999; Wehling *et al.*, 2000), HIV-infection (Gonzalez-Cadavid *et al.*, 1998) and microgravity environment (Lalani *et al.*, 2000). We therefore examined whether the levels of *MSTN* mRNA expression also increased in the atrophied *gastrocnemius* muscles of ADX rats. Our results showed that the expression levels of *MSTN* in the skeletal

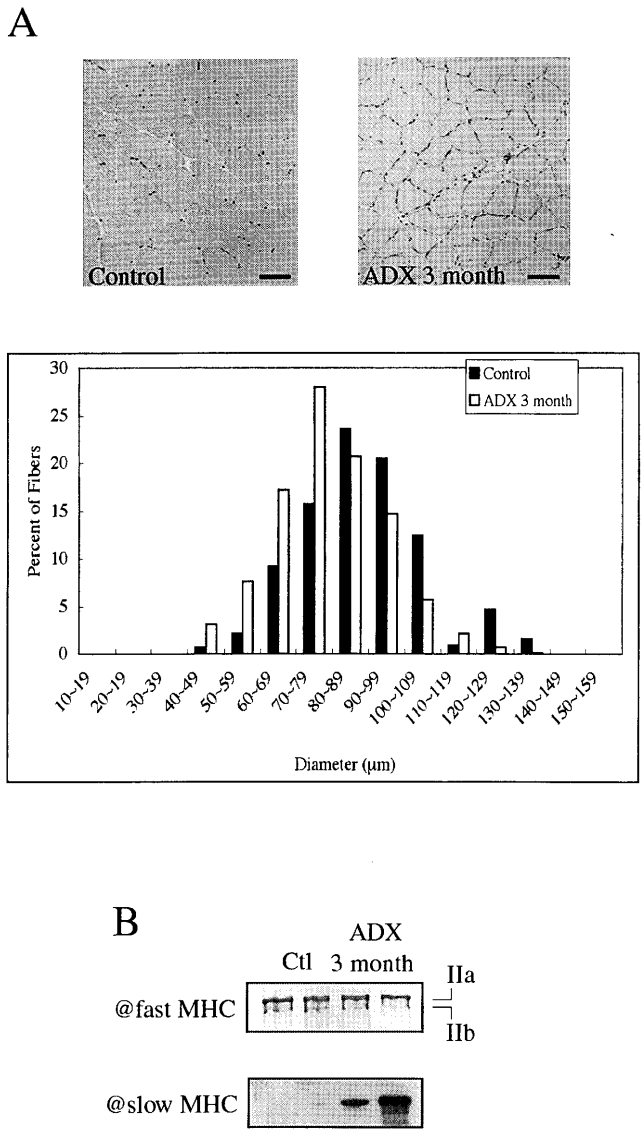

Fig. 2. Characteristics of adrenalectomized (ADX) rats. (A): Muscle fiber size distribution in *gastrocnemius* muscle of Sham-operated age-matched control (black bars, n=702) and ADX 3 month (white bars, n=829). Upper panel shows photomicrographs of *gastrocnemius* muscle, frozen and stained with Hematoxylin and Eosin. Scale bar=100 μm. (B): Fiber type specific myosin heavy chain (MHC) isoforms in *gastrocnemius* muscle of ADX 3 month.

muscles had not been altered during the long term adrenalectomy (Fig. 3A), indicating that the loss of adrenocortical hormones does not affect *MSTN* gene expression. On the other hand at the protein level, however, the amount of active form myostatin was gradually decreased after adrenalectomy, and it was significantly lower at 3 months after the operation compared with the amount at pre-operation (P<0.05; Fig. 3B).

Serum myostatin level in ADX rats

To examine if the atrophy of skeletal muscle fibers seen in the ADX rats was accompanied by the changes in the serum myostatin level, Western blot analysis of the sera from

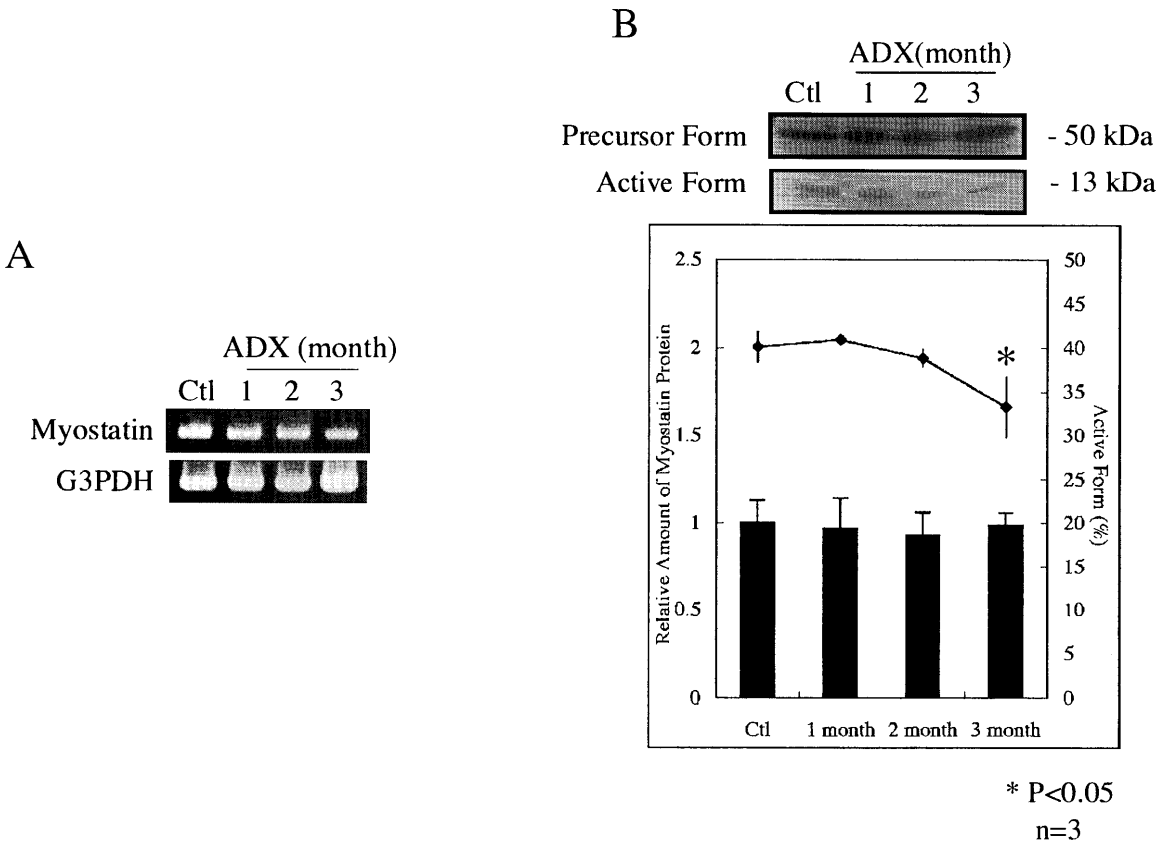

Fig. 3. Myostatin expression in skeletal muscle of adrenalectomized (ADX) rats. (A): Expression of myostatin gene in skeletal muscle of ADX rats. Total RNA was extracted from ADX and Normal (Ctl) rats and following synthesis of ss cDNA, semi-quantitative RT-PCR was carried out to measure myostatin mRNA expression. (B): Expression of myostatin protein in skeletal muscle of ADX rats. Myostatin protein was detected by Western blotting (upper panel). Graph indicates relative amount of myostatin protein (black bars) and % of the active form in total myostatin protein (lines). Values are mean±SE. *P<0.05. n=3.

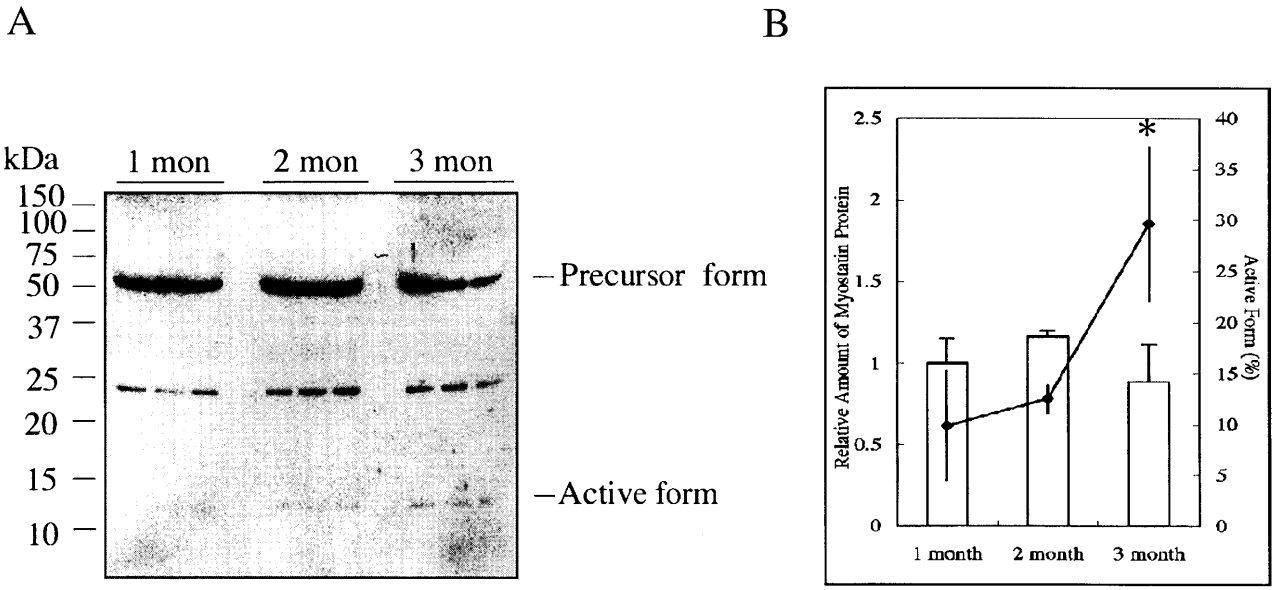

Fig. 4. Serum myostatin levels in adrenalectomized (ADX) rats. (A): Expression of myostatin protein in serum of ADX rats. (B): The open bars represent total myostatin, and lines show changes in the percentage of the active form myostatin (n=3). The active form myostatin at 3 month after adrenalectomy was significantly higher than those at earlier period. Values are mean±SE. *P<0.05.

ADX rats was performed. The acitve form myostatin (13 kDa) in the serum was detectable only at low levels either 1 month or 2 months after adrenalectomy. 3 month after operation, however, the serum obtained from ADX rats showed significantly higher content of myostatin active form than those at earlier periods ($P<0.05$; Fig. 4A and B). On the other hand, total amount of myostatin proteins (sum of precursor form and active form) remained at a constant level at all time points examined after adrenalectomy (Fig. 4B). A clear band around 25 kDa may be a nonspecific band, since a similar band was also detected in the serum of myostatin knock out mice (Zimmers *et al.*, 2002).

DISCUSSION

The present study demonstrated that long term adrenal insufficiency (3 months) caused the atrophy of skeletal muscle in the rat, which was due possibly to thinning of the muscle fibers, accompanied by the significant increase in the serum level of active form of myostatin compared to those in the normal rats. Since the finding by McPherron *et al.* (1997) of its exclusive expression in skeletal muscle and the studies showing its altered expression in regenerating muscles (Kirk *et al.*, 2000, Yamanouchi *et al.*, 2000, Mendler *et al.*, 2001) and its inhibiting activity on myoblast proliferation and differentiation (Thomas *et al.*, 2000, Taylor *et al.*, 2001, Rios *et al.*, 2002), myostatin has been believed to act locally in this tissue in a paracrine and/or autocrine manner. In addition to these modes of myostatin action, the subsequent study by Zimmers *et al.* (2002) that showed nude mice injected with myostatin-producing CHO cells exhibit systemic muscle loss and the fact that serum myostatin level is increased in HIV-infected men with a symptom of muscle wasting (Gonzalez-Cadavid *et al.*, 1998) suggested that myostatin could also affect on skeletal muscle in an endocrine manner. Therefore, it is plausible that skeletal muscle atrophy in the long term ADX rats was induced by the increased levels of active form myostatin in serum. Interestingly, adrenal insufficiency is often induced by immune deficiency and has been a well known complication of HIV infection (Klein *et al.*, 1983, Greene *et al.*, 1984), strongly suggesting the possibility that the increase of serum myostatin levels in HIV-infected men (Gonzalez-Cadavid *et al.*, 1998) is caused by the adrenal insufficiency and the same mechanisms might underlie muscle wasting in both HIV-infected persons and in Addisonians (Jakobi *et al.*, 2001)

Myostatin has a signal peptide motif for secretion in its N-terminal (McPherron and Lee, 1997), following secretion into extra-cellular space, it is thought to act through the activin type II receptor localized on cell surface (Lee and McPherron, 2001). Since the circulating myostatin is probably originated from skeletal muscle, the serum level of active form myostatin should mirror its amount secreted from the cells. However, in the present study, the amounts of active form myostatin in skeletal muscle were decreased after long term ADX while its levels in the serum were increased. Since the homogenate of muscle tissue would contain both intra-cellular (cytoplasmic) and extra-cellular secreted active form myostatin molecules, the amounts of myostatin in such a preparation determined by Western blot analysis might not necessarily reflect its exact amount secreted into extra-cellular space and this possibly accounts for the discrepancy in the level of active form myostatin between skeletal muscle and serum in the present study.

In the skeletal muscle of the ADX rats, the increase of type I MHC (slow) expression and the concomitant decrease of type IIb MHC (fast) expression were observed in the *gastrocnemius* muscle sampled 3 months after the operation, suggesting that a shift in fiber type composition from type II to type I fibers took place. Similar shift in the skeletal muscle fiber types had been shown to occur in atrophied skeletal muscles, *m. vastus lateralis* and *m. biceps brachii* of elderly men (Klitgaard *et al.*, 1990). On the other hand, the evidence for fiber type conversion in the opposite direction, i. e., from type I to type II , was reported in atrophied *m. vastus lateralis* muscles after short-term (11 days) space flight (Zhou *et al.*, 1995). The seeming discrepancy in the direction of fiber type shifts between these 2 cases, might be explained by the difference in the period of time the muscles are exposed to the particular physiological conditions, i. e., short-term exposure to the microgravity environment and chronic changes due to aging. Interestingly, it has been shown that the atrophy of muscle fibers is associated with the elimination of myonuclei from the fibers through an apoptotic mechanism (Allen *et al.*,1995, 1997, Vescovo *et al.*, 1998, 2000). Allen *et al.* (review, 1999) proposed that a shift in fiber type composition would take place after the loss of myonuclei. In this respect, it should be noted that systemic administration of myostatin increases expression of the pro-apoptotic gene, *bax*, in skeletal muscles (Zimmers *et al.*, 2002), and the transgenic mice with muscle-specific over-expression of *MSTN* gene show the atrophy of skeletal muscles accompanied by reduction in the number of myonuclei (Reisz-Porszasz *et al.*, 2003). These facts suggest that myostatin might primarily be responsible for the reduction of the number of myonuclei from muscle fibers. Taken together with the results obtained in the present study, it might be possible to infer that the increased levels of active form myostatin in serum, caused as a result of prolonged cortical insufficiency, induces the atrophy of skeletal muscles through the myonuclear apoptosis that had, *via* yet unknown mechanisms, resulted in the shift in fiber type composition.

It has been shown that the 5'-regulatory region of *MSTN* have multiple putative glucocorticoids response elements (GREs) (Ma *et al.*, 2001), and administration of synthetic glucocorticoid dexamethasone induces enhanced *MSTN* expression in skeletal muscles and/or muscle cells (Lang *et al.*, 2001, Artaza *et al.*, 2002, Ma *et al.*, 2003). Therefore, one would predict that the lack of glucocorticoids by adrenalectomy might result in a decrease of *MSTN* expression levels in skeletal muscles. However, this was not

the case as was shown in the present study, i. e., *MSTN* expression levels were not altered in the ADX rats. Thus, the present study suggets that glucocorticoids may be capable of acting as an enhancer of *MSTN* expression only under the condition where other factors maintain its expression at certain basal levels.

Myostatin protein is initially synthesized as a precursor form and subjected to proteolytic cleavage to generate active form protein. Although mechanisms of the proteolytic processing of myostatin is poorly understood, it has been conjectured that a proprotein convertase, furin, or metalloproteases may play a role in the procedures (McPherron *et al.*, 1997, Jin *et al.*, 2004, Rios *et al.*, 2004, Huet *et al.*, 2001, Wolfman *et al.*, 2003). In addition, recently published work demonstrated that several metalloproteases are present in serum and their expression levels are regulated in an adrenal-dependent manner (Kolomecki *et al.*, 2001). In the present study, we demonstrated that adrenalectomy induced the increased levels of active form myostatin in serum while the total concentration of myostatin protein in serum was unchanged. Therefore, it may be proposed that the long term adrenal insufficiency in ADX rats modified either the expression or the function of certain proteases in blood leading to the increase of the active form myostatin in serum.

Follistatin, an activin binding protein (Nakamura *et al.*, 1990), is also capable of binding to myostatin and is known to suppress myostatin activity (Zimmers *et al.*, 2002, Amthor *et al.*, 2004). Expression of follistatin is widely distributed in multiple tissues including skeletal muscle (Tuuri *et al.*, 1994). In our preliminary study concerning the involvement of follistatin in skeletal muscle atrophy observed in the long term ADX rats, follistatin mRNA levels in skeletal muscle were not changed after ADX (data not shown). However, this would not exclude the possibility that follistatin is involved in skeletal muscle atrophy in our experimental settings. In addition to its multiple tissue expression, follistatin is also shown to circulate in serum (Sugawara *et al.*, 1990). Therefore, it will be of interest to know whether the circulating levels of follistatin is altered in ADX rats and to examine if the circulating active form myostatin is being bound by follistatin.

In conclusion, our results indicate that in the rat, long term loss of adrenal hormones induces increase of the active form myostatin in serum and the atrophy of skeletal muscle fibers; the serum level of active form of myostatin appears more likely to be regulated post-translationally rather than at the transcription levels. These results strongly support the hypothesis that the increased serum level of myostatin plays a part in triggering the symptoms of muscle wasting in adrenal insufficiency such as Addison's disease. In turn, an appearance of the active form myostatin in seurm may be used as a biomarker to predict the onset of pathological skeletal muscle atrophy, since majority of the circulating myostatin molecules exists as the precursor form under normal physiological conditions.

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