Myostatin-deficient mice lose more skeletal muscle mass than wild-type controls during hindlimb suspension

Christopher D. McMahon,1 Ljiljana Popovic,1,2 Jenny M. Oldham,1 Ferenc Jeanplong,1 Heather K. Smith,3 Ravi Kambadur,1 Mridula Sharma,1 Linda Maxwell,2 and James J. Bass1

1Functional Muscle Genomics, AgResearch Limited, Ruakura Agricultural Centre, Hamilton; and Departments of 2Sport and Exercise Science and 3Pathology, University of Auckland, Auckland, New Zealand

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Although the absence of functional myostatin results in increased muscularity during embryonic and postnatal development, the role of myostatin in regulating adult muscle mass is not clear. Expression of myostatin is increased during a variety of muscle atrophy conditions associated with reduced load-bearing activity. For example, expression of myostatin is increased in muscles of patients confined to bed rest before surgery (21) and in muscles of rats after 17 days of microgravity in spaceflight (13), and is either transiently or persistently increased during loss of muscle mass from unloaded muscles of rodents subjected to hindlimb suspension (HS; see Refs. 2 and 28). Despite recent evidence that systemic overexpression of myostatin induces atrophy of muscle, it remains unclear whether this is a direct action on degradation of myofibers or because of decreased myogenesis (30).

Growth of skeletal muscle, both hypertrophy and regeneration, requires incorporation of muscle precursor cells from a reserve pool of self-renewing satellite cells, the fate of which is regulated by myogenic regulatory factors (MRFs; see Ref. 9). Specifically, MyoD and Myf-5 regulate proliferation, and myogenin and MRF4 regulate differentiation of myoblasts (9, 11, 17, 19). Given that myogenesis is reduced and myonuclei are lost when weight-bearing activity is reduced, we hypothesized that mice lacking the myostatin gene [Mstn(−/−)] would not lose as much muscle mass as wild-type controls during unloading of muscle induced by HS.

To test this hypothesis, we subjected wild-type (C57) and Mstn(−/−) mice to 7 days of HS to induce atrophy of skeletal muscle and measured changes in body and muscle mass and expression of myostatin protein forms in wild-type mice. In addition, we measured protein expression of MyoD, Myf-5, and myogenin in wild-type and Mstn(−/−) mice as markers of myogenic status.

METHODS

Animals. Twenty-four male wild-type (C57) and Mstn(−/−) mice (16–18 wk of age) were obtained from the...
Small Animal Colony at the Ruakura Agricultural Centre. Generation of Mstn−/− mice has been described previously (15). We obtained a breeding pair of these mice as a gift from S.-J. Lee. At AgResearch, Mstn−/− mice were bred from offspring of C57BL/6J founders that were backcrossed four to five times into the 129/SvJ strain. Mice were genotyped, and the colony is maintained as homozygous null [Mstn−/−].

Experimental design. Mice were pair matched by body mass within genotype and then assigned for use as either ground-based controls or HS (n = 6/group). Treatments, controls or HS, were continued for 7 days, after which time mice were killed via CO₂ asphyxiation followed by cervical dislocation. At death, the biceps femoris, quadriceps femoris, soleus, and extensor digitorum longus (EDL) muscles were excised, weighed, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent analysis.

HS. The procedures for HS and cage design were based on those described by Grindeland et al. (7) and Park and Schultz (20). Briefly, mice subjected to HS had their hindlimbs elevated off the cage floor by their tail. A nylon cord was taped to the base of each mouse’s tail, and the other end was attached to a swivel that could slide on a bar mounted on rollers on the cage ceiling. Each HS mouse could move freely, using its forelimbs, over the entire floor area of the cage. Food and water were available ad libitum, and the room was maintained at 25°C with a 14:10-h light-dark cycle. Mice were allowed to acclimate to their cages for 1 wk before experiments commenced.

The Ruakura Animal Ethics Committee approved this study.

Western blot analyses. The soleus and EDL muscles were too small to provide adequate tissue for analysis. Therefore, Western blots were only performed on biceps femoris and quadriceps femoris muscles. Lysis buffer (1 ml PBS, pH 7.2) with 0.05% IGEPAL detergent (Sigma Chemical, St. Louis, MO) and an enzyme inhibitor (Complete; Roche Diagnostics NZ, Auckland, New Zealand) was added to 100 mg of biceps femoris or quadriceps femoris from each animal. Samples were homogenized on ice and then centrifuged at 11,000 g for 10 min. Supernatant was recovered, mixed with Laemmli loading buffer (12), boiled for 5 min, and then stored at −20°C until analysis. The protein concentration of the supernatant was determined using the bicinchoninic acid assay (Sigma Chemical).

Protein (20 μg) from each muscle sample was loaded and separated in a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. Gels were stained with Coomassie brilliant blue, and membranes were stained with Ponceau S to verify transfer of protein. Membranes were incubated overnight with rabbit anti-myostatin antibody (1:3,000; see Ref. 23), rabbit anti-MyoD antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Myf-5 antibody (1:2,000; Santa Cruz Biotechnology), or rabbit anti-myogenin antibody (1:1,000; Santa Cruz Biotechnology). Membranes were then washed in 0.05 M Tris-buffered saline with 0.05% Tween 20 (TBST, pH 7.6), incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Sigma Chemical) at 1:10,000 for 2 h, and washed again in TBST. Bound HRP activity was detected with enhanced chemiluminescence, and then blots were exposed to film, after which the relative optical densities were determined using a densitometer (GS 800; Bio-Rad Laboratories, Auckland, New Zealand) and Quantity One software (Bio-Rad Laboratories). The membranes were then stripped (0.2 M Tris, pH 7.6, 2% SDS, and 0.05 M β-mercaptoethanol at 50°C for 30 min) and then exposed to rabbit anti-actin (Sigma Chemical) at a dilution of 1:10,000 and developed as above to assess uniformity of loading.

Statistical analysis. Data were subjected to ANOVA with factors of treatment (HS or controls), genotype [Mstn−/− or wild type], and their interaction included in the model. In addition, initial body mass was used as a covariate within each genotype. Data are presented as means along with the pooled or standard SE.

RESULTS

Effect of HS on body mass of wild-type and Mstn−/− mice. Mstn−/− mice had 28% more body mass (P < 0.001) than controls before HS. After 7 days of HS, body mass of wild-type mice was 8% less than their ground-based controls (P < 0.01), and body mass of Mstn−/− mice was 13% less than their ground-based controls (P < 0.001). Contrary to our expectations, Mstn−/− mice lost relatively more body mass than wild-type mice (P < 0.05) during HS, although they remained heavier (20%) than wild-type mice after HS (P < 0.001; Fig. 1).

Effect of HS on wet mass of hindlimb muscles in wild-type and Mstn−/− mice. After HS, biceps femoris, quadriceps femoris, and soleus muscles of wild-type mice were lighter, but not significantly so, compared with the ground-based controls (Figs. 2). In contrast, the wet mass of biceps femoris, quadriceps femoris, and EDL of Mstn−/− mice were lighter (P < 0.01) after HS than their ground-based controls. Furthermore, Mstn−/− mice lost more muscle mass from the quadriceps femoris (17% vs. 11%, P < 0.001) and EDL (33% vs. 0%, P < 0.001) than did the wild-type mice after HS. Although there was no treatment by genotype interaction on soleus muscles, the main effect of treatment alone [wild-type and Mstn−/− combined] was a decrease in wet muscle mass (P < 0.05).

Fig. 1. Mean ± SE body mass of wild-type (WT) and myostatin gene-deficient [Mstn−/−] mice subjected to 7 days of hindlimb suspension (HS) or serving as ground-based controls (C). ***P < 0.01 and ****P < 0.001, differences between treatment groups (within genotype).
Effect of HS on protein expression of myostatin in muscles of wild-type mice. Three immunoreactive bands identified on Western blots from wild-type mice corresponded to the precursor (52 kDa), latency-associated peptide (LAP; 40 kDa), and mature (26 kDa) forms of myostatin (Fig. 3). With HS there was no difference in expression of the precursor, LAP, or processed forms of myostatin in biceps femoris or quadriceps femoris compared with ground-based controls (Table 1).

Effect of HS on protein expression of MRFs. Representative Western blots are shown for MyoD, Myf-5, and myogenin, along with actin used as a loading control, in quadriceps femoris muscle for each of the treatments (Fig. 4). There was a greater abundance of MRFs in biceps femoris of control Mstn(−/−) compared with control wild-type mice. In wild-type mice, HS induced an increase in protein expression of all MRFs in biceps femoris and of MyoD and Myf-5 in quadriceps femoris. In contrast, the abundance of all MRFs was numerically lower in all HS vs. control Mstn(−/−) mice. This reduction in MRFs after HS reached significance with myogenin in quadriceps femoris (P < 0.05), and the abundance of Myf-5 tended to decrease (P > 0.1) in biceps femoris (Figs. 5 and 6).

DISCUSSION

Myostatin inhibits myogenesis and may promote atrophy of adult skeletal muscle by inhibiting proliferation of satellite cells (26). Activation of satellite cells is reduced during HS and in other studies has been associated with increased expression of myostatin (2, 28). Therefore, we speculated that Mstn(−/−) mice should not lose, or at least should not lose as much, muscle mass as wild-type mice during HS. However, in the present study, we observed that Mstn(−/−) mice lost more, rather than less, body and muscle mass than wild-type controls after 7 days of HS. These data are

Table 1. Effect of hindlimb suspension

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<td>BF</td>
<td>81 83 7.7</td>
<td>127 139 23.8</td>
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<td>QF</td>
<td>71 75 3.0</td>
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Data are mean densitometric units of Western blots ± pooled SE. LAP, latency associated peptide. Effect of hindlimb suspension (HS) compared with ground-based controls (C) on protein expression of myostatin in biceps femoris (BF) and quadriceps femoris (QF) muscles of wild-type mice.
contrary to our hypothesis and suggest that the absence of myostatin accelerates inactivity-induced atrophy in skeletal muscles.

A growing number of reports show increased expression of myostatin in skeletal muscle during atrophy states. The most convincing of these shows that systemic overexpression of myostatin induces wasting of muscle in mice (30). Others report that increased expression of myostatin is associated with atrophy of skeletal muscles in rodents subjected to HS (2, 28), in rats after periods of microgravity in space (13), and in patients confined to bed rest (21, 29). In the current study, we have observed that HS-induced atrophy of quadriceps and biceps femoris muscles was not associated with increased protein expression of myostatin. This lack of association between expression of myostatin and atrophy of muscle is not new. For example, Carlson et al. (2) found that expression of myostatin mRNA was increased at day 1 of HS but was not different from ground-based controls at days 3 or 7 of HS despite progressive atrophy of hindlimb muscles. Furthermore, if rats were released from HS and exercised on a treadmill for 30 min each day, wasting of muscle was prevented, whereas the expression of myostatin remained elevated (28). Because expression of myostatin did not correlate with the extent of body and muscle mass loss, those authors suggested that myostatin does not induce atrophy of muscle, a view diametrically opposed to that held by Zimmers et al. (30).

We did not perform Western blot analyses on the soleus or EDL muscles because there was not enough mass to detect protein abundance in individual muscles.

The size of the myostatin protein forms detected by Western blot remains controversial. Myostatin is a 375-amino-acid peptide belonging to the transforming growth factor-β family and, as such, is suggested to be proteolytically cleaved at a specific RSRR sequence (amino acids 263–266) and secreted as an NH2-terminal LAP that is noncovalently attached to the processed and biologically active COOH-terminal mature peptide. The theoretical sizes of the precursor, LAP, and mature peptides are 40, 26, and 12.5 kDa, respectively (6, 14, 25, 26). These latter sizes are consistent with those detected in the current study.

Proliferation of myogenic cells is associated with increased expression of MyoD and Myf-5, whereas differentiation is associated with increased expression of myogenin and MRF4 (17, 24). In the current study, we measured three of these MRFs and observed that they were generally more abundant in control Mstn(−/−) mice.

Fig. 4. Representative Western blot of MyoD, Myf-5, and myogenin in quadriceps femoris muscles of control and HS WT and Mstn(−/−) mice. Uniformity of loading was assessed by immunoblotting for actin.

Fig. 5. Mean ± SE optical densities of MyoD (A), Myf-5 (B), and myogenin (C) in biceps femoris muscles of control and HS WT and Mstn(−/−) mice. †P < 0.1, *P < 0.05, and **P < 0.01, differences between indicated groups.
compared with wild-type mice, which is consistent with increased incorporation of satellite cells to attain and maintain their greater mass of muscle. In support, we have recently observed that there is both increased incorporation of committed satellite cells into muscle after 3 days (1, 3). However, decreased muscle mass observed in longer-term HS studies is associated with reduced activation of satellite cells, as measured by the incorporation of bromodeoxyuridine, a reduced number of myonuclei, and loss of myofibrils (3, 18, 22, 27). Therefore, increased abundance of MRFs in wild-type mice may be a consequence of the relatively short duration of HS in the current study and may reflect an early increased incorporation of activated satellite cells. Interestingly, protein expression of MRFs tended to decrease in Mstn(−/−) mice subjected to HS. This suggests that there was reduced incorporation of satellite cells in skeletal muscles of Mstn(−/−) mice subjected to HS and is consistent with the greater loss of muscle mass observed. It also suggests that the increased myogenesis occurring in the absence of myostatin cannot proceed without a minimum of normal weight-bearing muscle activity.

The amount of muscle atrophy is dependent on the composition of myosin heavy chain fibers in each muscle. These fibers fall into the following two broad categories: type I (slow twitch) and type II (fast twitch). In general, muscle composed predominantly of type I fibers are more susceptible, whereas those composed predominantly of type II fibers are more resistant, to atrophy during HS (2, 27). In particular, the soleus, a load-bearing muscle that is composed almost entirely of types I and Ila fibers, is very susceptible to HS-induced atrophy, while the EDL, which is composed of IIXd and Iib fibers and is nonload bearing, is very resistant to HS-induced atrophy (2, 5, 27). Our data are consistent with this relationship between fiber type and unloading-induced atrophy, because a similar amount of mass was lost from the soleus muscles of wild-type and Mstn(−/−) mice. Moreover, there is very little myostatin expressed in the soleus muscle, and the mass of this muscle did not differ significantly between genotypes, which further supports the lack of difference in loss of muscle between wild-type and Mstn(−/−) mice subjected to HS. There was no significant loss of mass in biceps femoris, quadriceps femoris, and EDL muscles of wild-type mice subjected to HS. However, there was a significant loss of mass from biceps femoris, quadriceps femoris, and EDL of Mstn(−/−) mice compared with their ground-based controls and a greater amount compared with that of wild-type mice. This suggests either that the absence of myostatin renders Mstn(−/−) mice more susceptible to unloading-induced atrophy or that there is a change or difference before HS in the distribution of fiber type in Mstn(−/−) mice. The biceps femoris and quadriceps femoris muscles are largely composed of mixed type II fibers and very few type I fibers. In fact, the white portion of the quadriceps muscle is composed only of type Iib fibers (2). In this regard, it could be suggested that Mstn(−/−) mice would have a higher abundance of type Iib fibers because of the positive relationship between expression of myostatin and abundance of of muscle atrophy. Increased expression of MRFs has been observed previously after HS and was associated with an initial increase in incorporation of committed satellite cells into muscle after 3 days (1, 3). However, decreased muscle mass observed in longer-term HS studies is associated with reduced activation of satellite cells, as measured by the incorporation of bromodeoxyuridine, a reduced number of myonuclei, and loss of myofibrils (3, 18, 22, 27). Therefore, increased abundance of MRFs in wild-type mice may be a consequence of the relatively short duration of HS in the current study and may reflect an early increased incorporation of activated satellite cells. Interestingly, protein expression of MRFs tended to decrease in Mstn(−/−) mice subjected to HS. This suggests that there was reduced incorporation of satellite cells in skeletal muscles of Mstn(−/−) mice subjected to HS and is consistent with the greater loss of muscle mass observed. It also suggests that the increased myogenesis occurring in the absence of myostatin cannot proceed without a minimum of normal weight-bearing muscle activity.
type IIB fibers (2). Therefore, this could suggest that greater muscle wasting in Mstn(−/−) mice is attributed to a preferential loss of type IIB fibers. This would explain the greater loss in muscles rich in type IIB fibers that we observed in the current study. However, we have not yet characterized the fiber-type composition in hindlimb muscles of Mstn(−/−) mice to confirm this possibility.

In summary, the absence of myostatin in Mstn(−/−) mice results in increased loss of body and muscle mass with HS relative to wild-type controls in a number of hindlimb muscles. These data are contrary to our hypothesis, and we conclude that the absence of myostatin does not attenuate the loss of body and muscle mass during HS. Instead, our data suggest that Mstn(−/−) mice are more susceptible than wild-type mice to HS-induced atrophy of skeletal muscle.

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