Acute antibody-directed myostatin inhibition attenuates disuse muscle atrophy and weakness in mice

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Murphy KT, Cobani V, Ryall JG, Ibebunjo C, Lynch GS. Acute antibody-directed myostatin inhibition attenuates disuse muscle atrophy and weakness in mice. J Appl Physiol 110: 1065–1072, 2011. First published January 26, 2011; doi:10.1152/japplphysiol.01183.2010.—Counteracting the atrophy of skeletal muscle associated with disuse has significant implications for minimizing the wasting and weakness in plaster casting, joint immobilization, and other forms of limb unloading, with relevance to orthopedics, sports medicine, and plastic and reconstructive surgery. We tested the hypothesis that antibody-directed myostatin inhibition would attenuate the loss of muscle mass and functional capacity in mice during 14 or 21 days of unilateral hindlimb casting. Twelve-week-old C57BL/10 mice were subjected to unilateral hindlimb plaster casting or served as controls. Mice received subcutaneous injections of saline or a mouse chimera of anti-human myostatin antibody (PF-354, 10 mg/kg; n = 6–9) on days 0 and 7 and were tested for muscle function on day 14, or were treated on days 0, 7, and 14 and tested for muscle function on day 21. Hindlimb casting reduced muscle mass, fiber size, and function of isolated soleus and extensor digitorum longus (EDL) muscles (P < 0.05). PF-354 attenuated the loss of muscle mass, fiber size, and function with greater effects after 14 days than after 21 days of casting, when wasting and weakness had plateaued (P < 0.05). Antibody-directed myostatin inhibition therefore attenuated the atrophy and loss of functional capacity in muscles from mice subjected to unilateral hindlimb casting with reductions in muscle size and strength being most apparent during the first 14 days of disuse. These findings highlight the therapeutic potential of antibody-directed myostatin inhibition for disuse atrophy especially within the first 2 wk of disuse.

MUSCLE DISUSE OR UNLOADING as a consequence of plaster cast immobilization, enforced bed rest, or the long-term zero-gravity conditions of spaceflight is associated with skeletal muscle atrophy. Independent of the cause, disuse atrophy impairs muscle function severely (4, 34), with unilateral lower limb suspension reducing muscle strength in men by ~1.1% per day during the first 2 wk and by ~0.7% per day during the third week (9). The magnitude of the muscle weakness with disuse atrophy is fiber type dependent, with type I fibers exhibiting greater impairments in strength than type II fibers (33). Physical rehabilitation is the preferred, and in many cases, the only treatment available (34), but this approach is limited by the patient's ability to perform exercise and is rarely associated with a complete restoration of muscle function. Pharmacological interventions are needed that can prevent or reverse muscle wasting and weakness and potentially complement physical rehabilitation programs for patients with disuse atrophy.

Myostatin, otherwise known as GDF-8, is a member of the transforming growth factor-β (TGF-β) superfamily of proteins and is a negative regulator of skeletal muscle growth and development. Experimental overexpression of myostatin induces skeletal muscle atrophy (2, 25, 39), whereas inhibition of myostatin via spontaneous mutations in the myostatin gene causes muscle hypertrophy (18, 38). Several studies have highlighted the therapeutic potential of myostatin inhibition for enhancing skeletal muscle mass and function in conditions associated with muscle wasting and weakness, including the muscular dystrophies (5, 27, 35), sarcopenia (26, 31, 32), and cancer cachexia (3, 37). However, despite these promising results and the numerous findings of elevated myostatin expression in muscles of patients with chronic disuse atrophy (29) or unilateral limb casting (15), and in rodents exposed to hindlimb unloading (1, 7, 16, 36) or spaceflight (20), only two studies have investigated how deletion of myostatin influenced muscle wasting and weakness during disuse atrophy. Both studies employed 7 days of hindlimb suspension and, surprisingly, found that myostatin null mice (Mstn−/−) exhibited a similar or greater loss of muscle mass than wild-type mice (17, 24). However, it is difficult to interpret these findings since transgenic mice have a life-long inhibition of myostatin and because neither of these studies assessed muscle function, which is more severely affected by disuse atrophy than just muscle mass (8).

The aim was to determine whether antibody-directed myostatin inhibition could counteract the atrophy and loss of skeletal muscle function from mice during the disuse caused by unilateral hindlimb casting. Since the magnitude of disuse atrophy and the expression of myostatin are fiber type dependent (7), we examined responses in both the soleus (predominantly comprised of type I fibers; slow twitch) and extensor digitorum longus muscles (EDL, predominantly comprised of type II fibers; fast twitch). The efficacy of myostatin inhibition for preserving muscle mass and function was examined after 14 days of disuse, the time when the loss of muscle strength is greatest, and also after 21 days of disuse, the time when further losses of muscle strength occur more slowly (9). We tested the hypothesis that antibody-directed myostatin inhibition would attenuate the loss of mass and functional capacity in fast and slow muscles of mice during 14 or 21 days of unilateral hindlimb casting.

MATERIALS AND METHODS

Experimental animals. All experiments were approved by the Animal Experimental Ethics Committee of The University of Melbourne and conducted in accordance with the Australian code of
practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (Australia). Twelve-week-old male C57BL/10ScSn mice were allocated randomly into either a hindlimb-casted or control group. For unilateral hindlimb casting, mice were anesthetized with an intraperitoneal (ip) injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) such that they were unresponsive to tactile stimuli. The left hindlimb was shaved and wrapped in plaster (Vet-lite bandage, DLC, Hoppers Crossing, Victoria, Australia) with the foot positioned in plantar flexion to induce maximal atrophy of the lower hindlimb muscles (13). The contralateral limb exhibits steady growth during hindlimb immobilization and was therefore not used as a control (19). Mice received subcutaneous injections of a mouse chimera of anti-human myostatin antibody (PF-354, Pfizer Global Research and Development, Groton, CT; 10 mg·kg⁻¹·wk, n = 6–9), or an equivalent volume of saline (0.1 ml/10 g body mass, n = 6–9), on days 0 and 7 and were tested for muscle function on day 14, or were treated on days 0, 7, and 14 and were tested for muscle function on day 21. Separate cohorts of mice were used for the 14-day and 21-day studies. The dose of PF-354 was based on previous dose-response data obtained in experiments that caused maximal muscle hypertrophy and that have been described previously in detail (26, 27). All mice were obtained from the Animal Resources Centre (Canning Vale, Western Australia) and housed in the Biological Research Facility at The University of Melbourne under a 12:12-h light-dark cycle. Water was available ad libitum, and standard laboratory chow was provided, changed and monitored daily.

Assessment of in vitro contractile properties. At the completion of the 14- or 21-day treatment period, mice were anesthetized with sodium pentobarbitone (Nembutal, 60 mg/kg, Sigma-Aldrich, Castle Hill, NSW, Australia) via intraperitoneal injection. Plaster was removed from the casted limb using a modified pair of pliers (Sidchrome, Just Tools, South Melbourne, Victoria, Australia). The methods for assessment of the contractile properties of mouse soleus and EDL muscle in vitro have been described in detail previously (11, 14). Briefly, optimal muscle length (L₀) was determined from the micro-manipulation of muscle length to produce maximum isometric twitch force (Fₜ). Maximum isometric tetanic force (Fₜₒ) was recorded from the plateau of the frequency-force relationship (10–150 Hz for 200 ms with 2 min rest between stimulations). Optimum fiber length (Lₘ) was determined by multiplying L₀ by the Lₘ/L₀ ratio for the soleus and EDL muscles of mice, which were 0.71 and 0.44, respectively (6).

Following excision of the soleus and EDL muscles for analysis of contractile properties, the tibialis anterior (TA), plantaris, gastrocnemius, quadriceps muscles, and the heart were carefully excised and trimmed of tendon and any adhering nonmuscle tissue. Muscles were blotted once on filter paper and weighed on an analytical balance. Mice were killed as a consequence of heart excision while anesthetized. At the conclusion of the contractile measurements in vitro, the soleus and EDL muscles were removed from the organ bath, blotted once on filter paper, and weighed on an analytical balance. They were then mounted in a bath containing Tyrode solution gassed with 95% O₂ and 5% CO₂ and at 37°C. Two metallic transducers were fixed to both ends of the muscle and to the floor of the organ bath, respectively. The length of the muscle was adjusted to 80% of L₀ until a steady resting tension was achieved.

Real-time RT-PCR analyses. Total RNA was extracted from 10–20 mg of EDL muscles from mice in the 14-day study using a commercial available kit, according to the manufacturers’ instructions (PureLink RNA Mini Kit, Invitrogen). RNA concentration was determined spectrophotometrically at 260 nm, and the samples were stored at −80°C. RNA was transcribed into cDNA using the Invitrogen SuperScript VILO cDNA Synthesis Kit, and the resulting cDNA was stored at −20°C for subsequent analysis. Real-time RT-PCR was performed as described previously (26). The forward and reverse primer sequences used were: myostatin, 5′-GGTACAAGGTATACGGGATCGCAGATCC-3′ and 5′-CTTACAATCATGCTCCTGCAA-3′; Smad3, 5′-GGCGTTTGTGTTCAAGCCT-3′ and 5′-AGGCCAGCAGGGACTTCTAA-3′; Atrogin-1, 5′-GTGGTTCAGCAGCCAAGAG-3′ and 5′-TGGCAGAGGACACGCTATG-3′; and MuRF-1, 5′-AGGTGTACGCGAAGAACGATG-3′ and 5′-CCCTCTTGTCTTGGTGC-3′, respectively. The content of single-stranded DNA (ssDNA) in each sample was determined using the Quanti-IT OliGreen ssDNA Assay Kit (Molecular Probes, Eugene, OR), as described previously (26). Gene expression was quantified by normalizing the logarithmic cycle threshold (Cₚ) value (2⁻ˣCₚ) to the cDNA content of each sample to obtain the expression 2⁻ˣCₚ cDNA content (ng/ml).

Statistical analyses. All values are expressed as means ± SE, unless stated otherwise. Groups were compared using a two-way ANOVA (casting, treatment), where appropriate. Bonferroni’s post hoc test was used to determine significant differences between individual groups. The level of significance was set at P < 0.05 for all comparisons. Muscle fiber CSA was not normally distributed (Anderson-Darling Normality test), and so data for soleus and EDL muscle CSA were presented as 95% confidence intervals of the median. Differences were considered significant when no overlap existed between the 95% confidence interval of the median (30).

RESULTS

Myostatin inhibition prevents loss of muscle mass in mice during 14 days but not 21 days of disuse. After 14 days of disuse, there was a main effect for reduced mass of the TA (P < 0.001), EDL (P < 0.001), soleus (P < 0.001), plantaris (P < 0.03), gastrocnemius (P < 0.001), and quadriceps muscles compared with muscles from uncasted mice (P < 0.001, Fig. 1). PF-354 treatment induced a main effect for enhanced mass of the TA, EDL, and quadriceps muscles (all P < 0.05, Fig. 1). The greater mass of the quadriceps muscles of PF-354-treated mice was retained after normalizing to body mass (P < 0.01, Table 1). There was no difference between groups with respect to body mass at the start or end of treatment (Table 1), the percentage change in body mass (Table 1), absolute heart mass, or heart mass normalized to body mass between groups (data not shown).

After 21 days of disuse, there was a main effect for reduced mass of the TA (P < 0.001), EDL (P < 0.001), soleus (P < 0.001), and gastrocnemius muscles (P < 0.01) compared with muscles from uncasted mice (Fig. 1). However, there was no significant effect of PF-354 treatment on the absolute mass (Fig. 1) or the mass normalized for body mass of any muscle examined (Table 2). Body mass at the start and end of treatment, the percentage change in body mass (Table 2), absolute heart mass, and heart mass normalized to body mass were not different between groups (data not shown).

Myostatin inhibition during disuse partially preserves force production of soleus muscles. Soleus muscles from mice subjected to 14 days of disuse had a main effect for higher peak twitch force (Pₜ, P < 0.05), a prolonged time to peak tension (TPT, P < 0.05) and one-half relaxation time (1/2RT, P < 0.01), and a smaller cross-sectional area compared with muscles from uncasted mice (CSA, P < 0.001, Supplemental Table
S1, available with the online version of this article). PF-354 treatment had no effect on twitch characteristics of the soleus muscle in either casted or uncasted mice (Supplemental Table S1). The frequency-force relationship revealed that casted muscles from saline-treated mice had lower tetanic forces at higher (≥70 Hz) stimulation frequencies than uncasted muscles from saline-treated mice (P < 0.05, Fig. 2A). However, there was a main effect for higher tetanic forces in PF-354-treated mice than saline-treated mice (P < 0.001, Fig. 2A). There was also a main effect for lower peak tetanic force production (Po) in muscles from casted mice compared with uncasted mice (P < 0.001), which was not altered with PF-354 treatment (Fig. 2C). There was also no difference between groups when tetanic forces were normalized to muscle CSA (sPo, Fig. 2D).

After 21 days of disuse there was a main effect for lower CSA of the soleus muscle compared with uncasted muscles (P < 0.001), but other characteristics were not significantly different. There was also a main effect for lower peak tetanic force production (Pono) in muscles from casted mice compared with uncasted mice (P < 0.001), which was not altered with PF-354 treatment (Fig. 2C). There was also no difference between groups when tetanic forces were normalized to muscle CSA (sPono, Fig. 2D).

There was a main effect for lower peak tetanic force production (Pono) in muscles from casted mice compared with uncasted mice (P < 0.001), which was not altered with PF-354 treatment (Fig. 2C). There was also no difference between groups when tetanic forces were normalized to muscle CSA (sPono, Fig. 2D).

Table 1. Selected morphological parameters from control and limb-casted mice treated for 14 days with saline or myostatin inhibitory antibody (PF-354)

<table>
<thead>
<tr>
<th>Morphological Parameter</th>
<th>Saline</th>
<th>PF-354</th>
<th>Casted</th>
<th>Casted + PF-354</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass at treatment start, g</td>
<td>27.0 ± 1.1</td>
<td>26.3 ± 1.1</td>
<td>25.6 ± 0.7</td>
<td>26.6 ± 0.5</td>
</tr>
<tr>
<td>Body mass at treatment end, g</td>
<td>25.8 ± 0.8</td>
<td>27.7 ± 0.8</td>
<td>26.3 ± 0.8</td>
<td>28.5 ± 0.5</td>
</tr>
<tr>
<td>%Change body mass from initial</td>
<td>2.5 ± 2.6</td>
<td>5.6 ± 2.2</td>
<td>2.8 ± 1.9</td>
<td>7.1 ± 2.0</td>
</tr>
<tr>
<td>TA/body mass, mg/g</td>
<td>1.59 ± 0.07</td>
<td>1.71 ± 0.06</td>
<td>1.24 ± 0.04†</td>
<td>1.18 ± 0.18†</td>
</tr>
<tr>
<td>EDL/body mass, mg/g</td>
<td>0.32 ± 0.01</td>
<td>0.35 ± 0.01</td>
<td>0.23 ± 0.03†</td>
<td>0.27 ± 0.01†</td>
</tr>
<tr>
<td>Soleus/body mass, mg/g</td>
<td>0.26 ± 0.02</td>
<td>0.27 ± 0.01</td>
<td>0.19 ± 0.01†</td>
<td>0.19 ± 0.01†</td>
</tr>
<tr>
<td>Plantaris/body mass, mg/g</td>
<td>0.58 ± 0.04</td>
<td>0.60 ± 0.03</td>
<td>0.45 ± 0.05†</td>
<td>0.51 ± 0.05†</td>
</tr>
<tr>
<td>Gastroc/body mass, mg/g</td>
<td>4.44 ± 0.14</td>
<td>3.95 ± 0.57</td>
<td>4.65 ± 0.38</td>
<td>4.66 ± 0.17</td>
</tr>
<tr>
<td>Quad/body mass, mg/g</td>
<td>7.98 ± 0.25</td>
<td>8.50 ± 0.21†</td>
<td>6.10 ± 0.17†</td>
<td>7.00 ± 0.15†</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 6-9. TA, tibialis anterior; EDL, extensor digitorum longus; Gastroc, gastrocnemius; Quad, quadriceps. *P < 0.01 vs. saline treated (treatment main effect). †P < 0.05 vs. uncasted controls (casting main effect).
different from controls (Supplemental Table S1). The frequency-force relationship revealed that muscles from casted mice had lower tetanic forces at most stimulation frequencies examined (30–150 Hz) compared with uncasted mice ($P < 0.01$, Fig. 2B), and muscles from PF-354-treated mice had a main effect for higher tetanic forces compared with saline-treated mice ($P < 0.001$, Fig. 2B). Muscles from casted mice had a main effect for lower peak tetanic forces compared with uncasted mice ($P < 0.001$), but this was not altered significantly with PF-354 treatment (Fig. 2C). There was also no difference in sPo between groups (Fig. 2D).

Myostatin inhibition during disuse partially preserves force production in EDL muscles. Mice subjected to 14 days of disuse had a main effect for a prolonged TPT ($P < 0.05$) and 1/2RT ($P < 0.001$), and a smaller CSA of the EDL muscle assessed in vitro compared with uncasted mice ($P < 0.001$, Supplemental Table S2, available with the online version of this article). There was a main effect for a higher CSA with PF-354 treatment compared with saline treatment ($P < 0.001$, Supplemental Table S2). There was no effect of PF-354 treatment on the twitch characteristics of the EDL muscle (Supplemental Table S2). The frequency-force relationship revealed that muscles from casted mice produced lower tetanic forces than uncasted mice ($P < 0.01$), but this was not altered with PF-354 treatment (Fig. 3B). There was a main effect for lower maximum force in muscles from casted mice compared with uncasted mice ($P < 0.05$), which was not altered with PF-354 treatment, and sPo was similarly not different between groups (Fig. 3D).

After 21 days of disuse, there was a main effect for a prolonged TPT ($P < 0.05$) and 1/2RT ($P < 0.05$) and a smaller EDL muscle CSA compared with muscles from uncasted mice ($P < 0.01$, Supplemental Table S2). There was no effect of PF-354 treatment on the twitch characteristics of the EDL muscle (Supplemental Table S2). The frequency-force relationship revealed that muscles from casted mice produced lower tetanic forces than uncasted mice ($P < 0.01$), but this was not altered with PF-354 treatment (Fig. 3B). There was a main effect for lower maximum force in muscles from casted mice compared with uncasted mice ($P < 0.05$), which was not altered with PF-354 treatment, and sPo was similarly not different between groups (Fig. 3D).

Table 2. Selected morphological parameters from control and limb casted mice treated for 21 days with saline or myostatin inhibitory antibody (PF-354)

<table>
<thead>
<tr>
<th>Morphological Parameter</th>
<th>Saline</th>
<th>PF-354</th>
<th>Casted</th>
<th>Casted + PF-354</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass at treatment start, g</td>
<td>24.7 ± 0.8</td>
<td>24.2 ± 0.8</td>
<td>23.6 ± 0.5</td>
<td>23.4 ± 0.8</td>
</tr>
<tr>
<td>Body mass at treatment end, g</td>
<td>25.8 ± 0.5</td>
<td>26.1 ± 0.8</td>
<td>24.9 ± 0.5</td>
<td>25.5 ± 0.6</td>
</tr>
<tr>
<td>%Change body mass from initial</td>
<td>4.7 ± 3.1</td>
<td>8.3 ± 3.6</td>
<td>5.6 ± 1.5</td>
<td>9.4 ± 2.0</td>
</tr>
<tr>
<td>TA/body mass, mg/g</td>
<td>1.60 ± 0.10</td>
<td>1.69 ± 0.06</td>
<td>1.23 ± 0.07†</td>
<td>1.16 ± 0.08†</td>
</tr>
<tr>
<td>EDL/body mass, mg/g</td>
<td>0.34 ± 0.03</td>
<td>0.32 ± 0.02</td>
<td>0.25 ± 0.01†</td>
<td>0.27 ± 0.01†</td>
</tr>
<tr>
<td>Soleus/body mass, mg/g</td>
<td>0.27 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td>0.16 ± 0.03†</td>
<td>0.17 ± 0.01†</td>
</tr>
<tr>
<td>Plantaris/body mass, mg/g</td>
<td>0.48 ± 0.10</td>
<td>0.61 ± 0.05</td>
<td>0.58 ± 0.04</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>Gastroc/body mass, mg/g</td>
<td>3.92 ± 0.33</td>
<td>4.41 ± 0.23</td>
<td>3.55 ± 0.11†</td>
<td>3.10 ± 0.24†</td>
</tr>
<tr>
<td>Quad/body mass, mg/g</td>
<td>7.06 ± 0.41</td>
<td>7.05 ± 0.47</td>
<td>6.42 ± 0.30</td>
<td>6.38 ± 0.17</td>
</tr>
</tbody>
</table>

Data are means ± SE; $n = 6–9$. †P < 0.01 vs. uncasted controls (casting main effect).

Fig. 2. Functional properties of soleus muscles from control and limb-casted mice treated for either 14 or 21 days with saline or myostatin inhibitory antibody (PF-354). Frequency-force relationship after 14 days treatment (A) or 21 days treatment (B), peak tetanic force ($P_o$, C), and specific (normalized) force (sPo) production (D). Separate cohorts of mice were used in the 14-day and 21-day studies. Data are means ± SE; $n = 6–9$. *P < 0.05 vs. saline-treated uncasted; †P < 0.01 vs. saline-treated (treatment main effect); ‡P < 0.01 vs. uncasted controls (casting main effect).
Mechanistic insights into the attenuation of disuse atrophy with myostatin inhibition in mice. Analysis of H and E stained sections of soleus (Fig. 4A) and EDL muscles (Fig. 4C) after 14 days of disuse revealed smaller fiber sizes and treatment with PF-354-enhanced muscle fiber size (Fig. 4, A and C). Median fiber CSA was 37% lower in the soleus muscles from casted mice compared with uncasted mice (P < 0.05), and PF-354 treatment increased the median fiber CSA in muscles from casted mice by 28% (P < 0.05, Fig. 4B). Surprisingly, median fiber CSA was 6% lower in soleus muscles of PF-354-treated...
uncasted mice compared with saline-treated uncasted mice ($P < 0.05$, Fig. 4B). Mean fiber area of the soleus muscles was 1,342, 1,307, 861, and 1,087 $\mu m^2$ for control, PF-354, casted, and casted + PF-354 groups, respectively. In the EDL muscle, median fiber CSA was 34% lower in casted mice compared with uncasted mice ($P < 0.05$), and PF-354 treatment increased the median fiber CSA by 41% ($P < 0.05$, Fig. 4D). In uncasted mice, PF-354 treatment reduced median EDL fiber CSA by 16% compared with saline treatment ($P < 0.05$, Fig. 4D). Mean fiber area of the EDL muscles was 1,719, 1,516, 1,187, and 1,709 $\mu m^2$ for control, PF-354, casted, and casted + PF-354 groups, respectively.

The effect of 14 days of disuse on the mRNA expression of the TGF-β signaling genes, myostatin and Smad3, and of the ubiquitin ligases, Atrogin-1 and MuRF-1, was also investigated in the EDL muscle (Fig. 5). There was no effect of disuse on myostatin and Smad3 mRNA expression, but PF-354 treatment induced a main effect for reduced myostatin ($P < 0.01$, Fig. 5A) and Smad3 mRNA expression compared with saline treatment ($P < 0.05$, Fig. 5B). Disuse induced a main effect of increased Atrogin-1 ($P < 0.05$, Fig. 5C) and MuRF-1 mRNA expression ($P < 0.05$, Fig. 5D), and treatment with PF-354 reduced Atrogin-1 (~68%, $P < 0.05$) and MuRF-1 mRNA expression (~78%, $P < 0.05$).

**DISCUSSION**

Counteracting the atrophy of skeletal muscle associated with disuse has significant implications for minimizing the wasting and weakness with plaster casting, joint immobilization, and other forms of limb unloading. For example, plaster casting is performed routinely to promote healing of traumatized limbs, and interventions that can attenuate wasting during the casting period have important clinical implications, especially for orthopedics, sports medicine, and plastic and reconstructive surgery. The most important finding of this study was that PF-354 antibody-directed myostatin inhibition attenuated the atrophy and loss of functional capacity of muscles from mice subjected to unilateral hindlimb casting. These effects were more significant during 14 days, but not 21 days of hindlimb casting, indicating that such an intervention may have greatest efficacy during the initial period of rapid loss of muscle mass and strength.

Whether deletion of myostatin has efficacy for attenuating disuse muscle atrophy has been equivocal, with one study reporting that Mstn$^{-/-}$ mice exhibited muscle atrophy similar to that in wild-type mice during hindlimb suspension (17), but another reporting a greater loss of muscle mass in Mstn$^{-/-}$ mice (24). We found that antibody-directed myostatin inhibition attenuated disuse atrophy compared with saline-treated mice and importantly, that myostatin inhibition attenuated the loss of muscle function with limb casting. Previous studies have shown that in young men undergoing unilateral lower-limb suspension, the loss of muscle strength was greatest during the first 2 wk, and then slowed during the third week of disuse atrophy (9). We found similar losses of muscle mass and functional capacity after 14 and 21 days of unilateral limb casting, indicating that most of the muscle wasting and weakness occurred during the first 14 days. We examined whether the protective effect of PF-354 antibody-directed myostatin inhibition for the disuse atrophy with unilateral hindlimb casting in mice was temporal and found that the preservations of muscle mass and functional capacity with PF-354 antibody-directed myostatin inhibition were more significant during 14 days than after 21 days of disuse. PF-354 treatment partially preserved the mass of the TA, EDL, and quadriceps muscles after 14 days but not after 21 days of plaster casting. Functional properties of soleus muscles were preserved after 14 and 21 days of treatment, but in the EDL muscles, function was...
preserved only after 14 days of disuse. The temporal effects of PF-354 in muscles from casted mice are similar to our findings in adult (12 wk old) dystrophic mdx mice, in which 5 wk but not 8 wk of PF-354 treatment enhanced muscle mass (27). PF-354 antibody-directed myostatin inhibition was therefore most beneficial for attenuating the loss of muscle mass and functional capacity during the initial stages of disuse, when the loss of muscle strength was most rapid. Our findings support the contention that interventions to limit muscle deterioration should commence within the first 14 days of disuse (9).

Since muscles comprised predominantly of type I fibers exhibit larger reductions in strength with disuse atrophy than muscles comprised predominantly of type II fibers (33), and because myostatin expression is higher in type II than in type I fibers (7), we investigated whether antibody-directed myostatin inhibition during hindlimb casting could attenuate the loss of function and degeneration within the soleus (predominantly type I fibers) and EDL muscles (predominantly type II fibers). Similar functional and pathological improvements were evident in both muscles, with PF-354 treatment increasing submaximal force production after 14 days of disuse in both the soleus and EDL muscles, and after 21 days of disuse in soleus muscles. Median fiber CSA was partially preserved with PF-354 in both muscles, and the mass of the EDL, but not the soleus, was partially preserved with PF-354 treatment after 14 days of disuse. These findings contrast with those from another study in healthy mice where myostatin inhibition induced via intravenous administration of a myostatin propeptide viral vector (AAV8ProMyo) increased median fiber CSA of the soleus and EDL muscles to a similar extent (~35%), but only improved the functional capacity of the soleus muscle (10). Since PF-354 attenuated the atrophy and loss of functional capacity of both slow- and fast-twitch muscle fibers during hindlimb casting, antibody-directed myostatin inhibition may be a more efficacious approach for enhancing overall muscle function. Surprisingly, PF-354 treatment reduced median fiber CSA of the soleus and EDL muscles in uncasted mice. This finding contrasts with the higher mass and CSA of the EDL muscle and the unchanged mass and CSA of the soleus muscles from uncasted mice treated with PF-354. Although PF-354 treatment induced significant functional and pathological improvements in muscles of casted mice, there were only relatively minor improvements in the muscles of uncasted mice, which differs from the large 60% improvements in muscle mass in healthy C57BL/6 mice treated for 2 wk with a soluble form of the activin type IIB receptor (22). The greater potency of ACVR2B-Fc is not surprising given that it inhibits the signaling of multiple TGF-β ligands in addition to myostatin (22), whereas PF-354 only inhibits myostatin signaling. It would therefore be of interest to examine whether ACVR2B-Fc or another inhibitor of the activin type IIB receptor can elicit greater preservation of mass and function during casting than PF-354.

Myostatin signals through the intracellular Smad signaling pathway and has been shown to activate the E3 ubiquitin ligases, atrogin-1 (MAFbx) and MuRF-1 (23). The expression of these ubiquitin ligases is elevated in numerous conditions of muscle wasting, including hindlimb casting (19), and known targets of atrogin-1 include MyoD, a muscle regulatory factor important in muscle regeneration, and eIF-3f, a translation initiation factor, whereas MuRF-1 has been shown to directly target the myosin heavy chain for ubiquitination (12). To investigate the mechanisms underlying these responses, we examined the effect of PF-354 and disuse on the expression of these genes, including Smad3 and myostatin. Disuse increased atrogin-1 and MuRF-1 mRNA expression, but surprisingly, the apparent increase in myostatin and Smad3 mRNA expression with disuse did not reach statistical significance, which may reflect the variability associated with mRNA analyses (28). PF-354 treatment reduced the mRNA expression of myostatin, Smad3, atrogin-1, and MuRF-1, consistent with previous reports in aged mice exhibiting sarcopenia (21, 26). The reduction in myostatin mRNA expression with PF-354 may indicate the existence of a positive feedback mechanism caused by the reduced myostatin signaling, which is supported by the decreased Smad3 mRNA expression. Indeed, PF-354 treatment has been shown previously to reduce Smad3 phosphorylation in muscles from aged mice (21). PF-354 therefore attenuated the disuse-induced increases in mRNA expression of the ubiquitin ligases, atrogin-1 and MuRF-1, providing the first mechanistic insight into the therapeutic potential of this treatment for casting-induced muscle atrophy.

In conclusion, antibody-directed myostatin inhibition attenuated the atrophy and loss of functional capacity in muscles from mice subjected to unilateral hindlimb casting, and the effects were more significant during the first 14 days of disuse, when the reductions in muscle size and strength were most rapid. Antibody-directed myostatin inhibition combined with physical rehabilitation programs could be initiated within the first 2 wk of disuse to minimize loss of muscle mass and preserve muscle function, effects that could ultimately enhance quality of life and reduce mortality of the most severely affected patients. It remains to be determined whether the hypertrophic effects of myostatin inhibition persist when treatment is initiated after reloading and remobilization.

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