Stimulation of skeletal muscle myofibrillar protein synthesis, p70 S6 kinase phosphorylation, and ribosomal protein S6 phosphorylation by inhibition of myostatin in mature mice

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For muscle fiber enlargement to be functionally useful, the mass of myofibrils must increase along with the overall muscle size. Increased myofibrillar mass can occur only if the rate of myofibrillar synthesis exceeds the rate of degradation. In cultured myoblasts and myotubes, myostatin has an inhibitory effect on overall protein synthesis but does not affect the rate of proteolysis (24). In neonatal rats, infusion of follistatin, an inhibitor of myostatin, increases muscle protein synthesis (22). Mice with constitutive myostatin knockout have increased myofibrillar protein synthesis but normal myofibrillar half-life (25). There have been no studies of myofibrillar or total muscle protein synthesis after myostatin activity in mature animals has been reduced following normal muscle development. Because effects of postdevelopmental myostatin inhibition should not be inferred from studies of mice with constitutive myostatin knockout or addition of myostatin to cultured myotubes, we examined the effect of the anti-myostatin antibody on myofibrillar protein synthesis.

In principle, reducing myostatin activity could affect the rate of protein synthesis by several different mechanisms. One way would be to add new nuclei to the myofibers, thereby increasing the rate of RNA production per fiber. However, postnatal inhibition of myostatin activity causes fiber enlargement primarily by increasing the fiber volume per myonucleus rather than by increasing the number of nuclei per fiber (2, 26, 28). Another way to increase protein synthesis would be to increase the rate of transcription of genes encoding translation initiation or elongation factors or other components of the protein synthetic machinery. A microarray study indicated that mice with constitutive myostatin knockout have elevated expression of several genes encoding proteins involved in translation (21). The same approach was used in the present study to examine expression of these genes. Yet another potential mechanism for increasing the rate of protein synthesis is activation of the mammalian target of rapamycin (mTOR), a key integrator of nutrient and growth factor signals that determine cell size and protein metabolism (5, 18). Activated mTOR promotes phosphorylation of p70 S6 kinase (S6K) and translational initiation factor 4E-binding protein-1 (4E-BP1). Therefore, effects of the anti-myostatin antibody on phosphorylation of S6K, its target ribosomal protein S6 (rpS6), and 4E-BP1 were examined in the present study. Rapamycin was used to determine whether the effect of the anti-myostatin antibody on myofibrillar synthesis is mTOR dependent. Akt (also known as protein kinase B), which is in a signaling pathway that can activate mTOR, was

GENETIC MUTATIONS CAUSING LOSS of myostatin activity lead to marked hypermuscularity in several mammalian species, including humans (3, 12, 13, 19, 20). When myostatin activity is inhibited in mice several weeks or months after birth, there is a more modest muscle growth (2, 9, 23, 26–28). The postdevelopmental effects of myostatin are of great interest with respect to potential clinical applications of myostatin inhibitors. The early responses to myostatin inhibition are important in understanding the mechanism of hypertrophy and could be useful biomarkers for preclinical or initial clinical trials of the efficacy of potential anti-myostatin agents. Thus the present study was done to search for early molecular changes in adult skeletal muscle after inhibition of myostatin activity with an anti-myostatin antibody.

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examined because there is evidence that myostatin inhibits Akt phosphorylation (1, 11, 15).

MATERIALS AND METHODS

Male mice with a predominantly C57BL/6 background were used for this study. They were siblings of mice being generated for studies of Cre-mediated postdevelopmental knockout of a floxed myostatin exon (26). The mice used in the present study did not have a Cre transgene. Male homozygous for the floxed exon (Mstn\(^{\text{f/f}}\)) have normal myostatin expression if they do not have a Cre transgene (26). The study included 20 mice with the wild-type myostatin gene (Mstn\(^{\text{+/+}}\)), six Mstn\(^{\text{f/f}}\) mice, and six Mstn\(^{\text{+/+}}\) mice. Treatment groups were balanced with respect to the Rochester Mouse Research Committee and was compliant with all applicable federal and state regulations for humane use of animals in research. The mice were housed in microisolator cages with food and water available at all times.

The mice received two intraperitoneal (ip) injections of JA16 antibody \((n = 16)\) or vehicle (PBS; \(n = 16)\) at 4–5 mo of age (JA16 anti-myostatin antibody was generously provided by Wyeth Pharmaceuticals). To ensure effective inhibition of myostatin activity, they were injected with larger doses \((0.12 \, \text{mg/g body wt on day 1}, 0.09 \, \text{mg/g body wt on day 3})\) of the antibody than the dose reported to induce muscle hypertrophy \((0.06 \, \text{mg/g})\) when administered once/wk for several weeks \((27)\). Two days after the second antibody injection \((\text{day 5}, \text{between 0900 and 1030})\), the mice received an ip injection of L-\([\text{ring}-^3\text{H}]\)phenylalanine \((\text{D}5\text{Phe}, \text{purchased from Cambridge Isotope Laboratories; } 0.02 \, \text{ml/g body wt of 150 mM D5Phe in 75 mM NaCl})\). They were killed 30 min after the tracer injection by CO\(_2\)/H\(_2\)O inhalation and then cervical dislocation after cessation of breathing \((\sim 3 \, \text{min after start of CO}_2\) injection). The gastrocnemius muscles were removed as quickly as possible and immersed immediately in liquid nitrogen. Tissue was stored at \(-70^\circ \text{C}\).

Some of the mice \((n = 6)\) treated, 6 PBS treated also received ip injections of the mTOR inhibitor rapamycin \((\text{purchased from LC Laboratories; } 0.06 \, \text{mg/mouse daily from day 1 to day 4})\). The final injection was made \(\sim 16 \, \text{h before the D5Phe injection}\). The concentrated rapamycin solution in ethanol \((40 \, \text{mg/ml})\), stored at \(-15^\circ \text{C}\), was diluted to 0.1 mg/ml on the day of injection with 0.2% sodium carboxymethylcellulose/0.25% polysorbate 80 in water.

The rate of myofibrillar protein synthesis was determined by tracer incorporation, as described in detail previously (25). This involved determining the ratio of D5Phe to unlabeled phenylalanine for both the free amino acid pool and the myofibrillar protein preparation that was washed of all free amino acids before acid hydrolysis. t-Butyldimethylsilyl amino acid derivatives were separated by gas chromatography and analyzed with a quadrupole mass spectrometer to determine the ratio of D5Phe to unlabeled phenylalanine. The D5Phe enrichment of water-soluble proteins of several muscle samples also was determined as follows. Muscles were homogenized in water and centrifuged to pellet the myofibrils and other insoluble proteins. The supernatant was mixed with an equal volume of total protein from each sample were subjected to SDS-PAGE and transferred to nitrocellulose paper by semidry electrophoresis. Equal amounts of total protein from each sample were subjected to SDS-PAGE and transferred to nitrocellulose paper by semidry electrophoresis. Equal loading was confirmed by reversible staining of the blots with Membcode reagents (Pierce). The blots were blocked with 5% nonfat milk in Tris-buffered saline plus Tween-20 (TBST; pH 7.6 with 0.1% Tween-20), washed in TBST (5 min), incubated overnight at 4°C with primary antibody \((1,000:1 \text{ dilution of stock antibody in TBST with 5\% BSA; all antibodies purchased from Cell Signaling Technology})\, washed in TBST (4 × 5 min), incubated for 1 h at room temperature with secondary antibody \((\text{T}2,000:1 \text{ dilution of horse-radar peroxidase (HRP)-tagged goat anti-rabbit IgG in TBST with 5\% nonfat milk})\, and then washed in TBST (4 × 5 min) and water (10 min).

Blots were covered with HRP-dependent chemiluminescence reagents \((\text{SuperSignal West Dura; Pierce})\) and photographed in a dark box with a cooled charge-coupled device camera \((\text{Quantum Scientific Imaging, Model 504s})\) with a Fujinon HF9HA-1B lens. Pixel intensities were adjusted for bias, dark current, and position relative to lens. Background light intensity was subtracted from the sum of pixel intensities for each band. Values were converted to a percentage of the mean value for the three to four control samples \((\text{i.e., muscles from PBS-treated mice})\) on each blot.

RNA was extracted by homogenizing muscles in Trizol \((\text{Invitrogen})\) followed by precipitation and washing as recommended by manufacture, provided with the Trizol reagent. The RNA pellet was dissolved in RNase-free water. The total RNA concentration was determined by absorbance of 260-nm light \((\text{Nanodrop 1000 spectrophotometer})\). RNA integrity was confirmed by detection of strong 18S and 2S RNA signals with an Agilent Bioanalyzer.

For microarray analysis, RNA was converted to biotin-labeled, fragmented cDNA with kits from NuGEN. The cDNA was hybridized overnight with Affymetrix Mouse Genome 430 2.0 arrays. The arrays were washed, stained with phycoerythrin-streptavidin, and scanned with standard protocols recommended by Affymetrix. Data were normalized to total mRNA mass, and expression levels were computed with Gene Chip Operating Software (Affymetrix). All microarray data, including the Affymetrix CEL files, are available from the Gene Expression Omnibus web site under accession no. GSE13707 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13707).
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For quantitative RT-PCR, 2 µg of RNA was reverse transcribed with the high-capacity cDNA reverse transcription kit (Applied Biosystems). Triplicate PCR reactions per cDNA were monitored with an ABI 7900HT thermal cycler/fluorescence monitor using the standard conditions recommended for Taqman PCR. Data were analyzed with the SDS 2.2 software (Applied Biosystems). Primer and probe sequences for Myh4 cDNA were published previously (26). Primers and probes for 18S rRNA cDNA (product no. 4308329) and Acta1 cDNA (Mm00808218_g1) were purchased from Applied Biosystems.

Levels of statistical significance for differences between treatment groups were determined by analysis of variance and t-tests.

RESULTS

Administration of the anti-myostatin antibody increased the mean rate of myofibrillar synthesis by 18.7% in mice that did not receive rapamycin (P = 0.003; Fig. 1). Synthesis of soluble proteins (not shown in Fig. 1) was 72% more rapid than myofibrillar synthesis regardless of treatment, so it was increased by JA16 administration to the same extent as myofibrillar synthesis (18.8%, P = 0.003). Rapamycin did not cause any reduction in mean myofibrillar synthesis rate in either saline-treated or JA16-treated mice (Fig. 1). Thus the stimulatory effect on myofibrillar synthesis of the anti-myostatin treatment was not significantly different in rapamycin-treated mice (14.8%, P = 0.03) than it was in the mice that did not receive rapamycin. Analysis of variance indicated that JA16 treatment was a highly significant source of variance, whereas rapamycin treatment and the JA16 × rapamycin interaction did not contribute significantly to variance (P values shown in Fig. 1).

Blocking myostatin activity did not significantly affect muscle concentrations of S6K, rpS6, 4E-BP1, or Akt (P > 0.10; Fig. 2). The JA16 antibody increased the level of phosphorylation of phospho-Thr389-S6K (1.7-fold, P = 0.02) and phospho-Ser235/236-rpS6 (2.3-fold, P = 0.003) but did not affect phosphorylation of 4E-BP1 (phospho-Thr37/46, phospho-Ser42, and phospho-Thr426) or Akt (phospho-Thr308 and phospho-Ser473) (Fig. 2). The proportions of S6K and rpS6 in the phosphorylated form were 1.9-fold greater in JA16-treated mice (P < 0.05). In mice that received rapamycin, there was no detectable phospho-S6K or phospho-rpS6 in skeletal muscle, and there was less phosphorylation of 4E-BP1 at Thr46 (−35% of normal) and Ser64 (−60% of normal) (Fig. 3).

Although levels of total RNA, ribosomal RNA, Acta1 mRNA (which encodes skeletal muscle α-actin), and Myh4 mRNA (which encodes the most abundant myosin heavy chain in mouse gastrocnemius, type 2b) were not consistently affected by JA16 treatment (P > 0.2), the mean levels per milligram tissue increased to approximately the same extent as the mean increase in myofibrillar synthesis rate (Table 1). Microarrays did not reveal any significant differences between saline-treated and JA16-treated mice in the expression of genes encoding other myofibrillar proteins (various isoforms of myosin heavy and light chains, troponins, tropomyosins, titin, and titin cap) relative to expression of either Acta1 or Myh4.

There were five genes encoding ribosomal proteins that were expressed at higher levels (normalized to total mRNA mass) in JA16-treated mice at nominal P < 0.05, but only three were increased >7% (Rpsa, +46%; Rpl22l1, +19%; Rps27l1, +16%). Rpsa is expressed at a very low level relative to other ribosomal proteins. The protein it encodes is also known as the 67-kDa laminin receptor, and it is unlikely to have a significant role in global protein synthesis. Because there were more than 200 probe sets for ribosomal protein genes, these effects might be “false positives” and must be considered tentative without confirmation in a prospective study. Except for a slight (7%) increase in expression of Elf5α, genes encoding translation initiation factors, translation elongation factors, and tRNA synthetases were not expressed at higher levels in JA16-treated mice at P < 0.05.

DISCUSSION

The present study indicates that myostatin exerts a tonic inhibitory influence on the rate of myofibrillar protein synthesis even after muscles are fully developed. Increased protein synthesis is probably the major mechanism for the muscle fiber hypertrophy, because previous studies suggested that myostatin does not influence muscle proteolysis (24, 25). The magnitude of the stimulatory effect of the anti-myostatin antibody on the fractional rate of myofibrillar synthesis was similar to the effect observed in young mice with constitutive myostatin knockout (25). However, the effect on the synthesis rate per muscle, which is what determines the myofibrillar mass when protein synthesis and breakdown are in equilibrium, was quantitatively much greater with constitutive knockout. The greater effect of constitutive knockout on protein synthesis per whole muscle reflects an increase in the number of muscle fibers during development in myostatin-deficient mice.

With the method used in the present study, myofibrillar synthesis rate depends not only on the rate of tracer incorporation into the individual proteins of the myofibrils but also on their assembly into structures that render them insoluble in the buffer used to wash away nonmyofibrillar proteins. Thus it is possible theoretically that the increase in D3Phe incorporation into myofibrils in JA16-treated mice reflects more rapid assembly of insoluble myofibrillar protein complexes rather than, or in addition to, more rapid production of the individual myofibrillar proteins. However, analysis of proteins soluble in water...
The anti-myostatin antibody increased phosphorylation of S6K and one of its targets, rpS6. This is consistent with reports that S6K phosphorylation is reduced in rat muscle with elevated myostatin expression (1) and is increased in neonatal rats treated with follistatin, which binds to myostatin and inhibits its activity (22). The anti-myostatin antibody did not stimulate phosphorylation of 4E-BP1, which often increases in concert with S6K phosphorylation because both are phosphorylated by mTOR. However, an increase in S6K phosphorylation without a similar effect on 4E-BP1 is not unprecedented (4, 7). Moreover, when myostatin is overexpressed in rat muscle, the modest depression of 4E-BP1 phosphorylation is approximately threefold less than the depression of S6 phosphorylation (1). S6K phosphorylation could have increased in JA16-treated mice without an increase in mTOR activity. Another potential explanation is that myostatin inhibits the activity of a phosphatase that normally offsets some of the S6K phosphorylation caused by other growth factors and nutrients signaling through mTOR.

Although rapamycin eliminated phosphorylation of S6K and rpS6 and attenuated phosphorylation of 4E-BP1, it did not block the stimulation of myofibrillar synthesis induced by anti-myostatin treatment. Thus mTOR activation does not appear to be necessary to overcome the tonic inhibitory effect of myostatin on myofibrillar synthesis. We recognize that the null hypothesis cannot be proven and that sampling and technical variance could have caused us to underestimate the effect of rapamycin on protein synthesis. On the basis of statistical confidence intervals, there is <5% probability that a repeat experiment would demonstrate complete inhibition of the effect of the JA16 antibody on myofibrillar synthesis and ~80%...

Table 1. RNA analyses

<table>
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<th>mRNA</th>
<th>Saline</th>
<th>JA16</th>
<th>%Δ</th>
<th>P</th>
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<td>Total RNA, µg/mg tissue</td>
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<td>18S rRNA/mg tissue, arbitrary units</td>
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<td>28S rRNA/18S rRNA</td>
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<td>+7</td>
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<td>Acta1 mRNAs/µg total RNA, arbitrary units</td>
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<td>1.20±0.23</td>
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<td>0.51</td>
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</table>

Values are means ± SE. Items expressed in arbitrary units were measured by real-time PCR with cDNA templates generated by reverse transcription of total RNA. Mean value in saline-treated mice defined as 1 unit. Total RNA was determined by UV absorbance. 28S/18S rRNA ratios were determined with Agilent Bioanalyzer.

![Fig. 2. Mean ± SE relative amounts of total and phosphorylated (p) p70 S6 kinase (S6K), ribosomal protein S6 (rpS6), 4E-binding protein-1 (4E-BP1), and Akt in 10 saline (Sal)-treated (gray bars) and 10 JA16-treated mice (black bars), as determined by immunoblotting. Representative bands are shown for proteins (S6K, rpS6) with increased phosphorylation (P < 0.05) in JA16-treated mice (P values for all proteins shown on right side of figure). The phosphorylated amino acids detected by the various anti-phospho 4E-BP1 and anti-phospho-Akt antibodies are shown next to the bars. The anti-phospho-S6K antibody detected phosphorylation at Thr (T)389. The anti-phospho-rpS6 antibody detected phosphorylation at Ser (S)235/236.](image1)

![Fig. 3. Efficacy of rapamycin treatment was demonstrated by elimination of S6K and rpS6 phosphorylation (representative blots are shown; no p-S6K or p-rpS6 was detected in any of the 12 rapamycin-treated mice). Phosphorylation of 4E-BP1 was attenuated but not eliminated by rapamycin.](image2)
probability that a repeat experiment would demonstrate <50% inhibition of the JA16 effect.

The failure of rapamycin to influence baseline protein synthesis (i.e., synthesis in mice that did not receive the anti-myostatin antibody) is consistent with previous reports that rapamycin does not affect the baseline rate of total protein synthesis in muscle (8) or liver (16). Thus mTOR-mediated phosphorylation of S6K is not essential to sustain the normal maintenance level of either global or myofibrillar protein synthesis. This conclusion is reinforced by the fact that myotubes derived from S6K-null mice have normal fractional rates of protein synthesis and global translation initiation (14). The only S6K target examined in the present study was rpS6. This target was chosen because its phosphorylation status is a marker of S6K activity, not because of compelling evidence that it promotes myofibrillar or global protein synthesis in muscle. The role of rpS6 in regulating protein synthesis is uncertain. Although it was originally postulated that phosphorylation of rpS6 promoted translation of mRNAs with 5' terminal oligopyrimidine tracts, including mRNAs encoding ribosomal proteins, this notion has been challenged by more recent studies (17). S6K has several other substrates in addition to rpS6, including upstream binding factor, which promotes rRNA transcription (6), and two proteins directly involved in translation, eEF2 kinase (which inhibits elongation by phosphorylating eEF2; eEF2 kinase is inactivated by p-S6K) and eIF4B (which enhances the processivity of eIF4A and could accelerate translation of mRNAs with long, structured 5' ends) (17). Activation of upstream binding factor and inhibition of eEF2 kinase should have global effects on protein synthesis, including myofibrillar synthesis. If the increase in S6K phosphorylation did activate upstream binding factor, the effect was insufficient to consistently increase rRNA levels (although a trend in that direction was observed). We did not examine these other S6K targets because elimination of S6K phosphorylation with rapamycin did not affect protein synthesis.

Myostatin has influenced the phosphorylation of Akt, an activator of mTOR, in some experiments. In cultured myotubes and cardiomyocytes and in rat muscle in vivo, myostatin has an inhibitory effect on Akt phosphorylation (1, 15). In contrast, myostatin stimulates Akt phosphorylation in cultured skeletal muscle fibroblasts (10). In the present study, we observed no significant effect of inhibiting myostatin on total levels or phosphorylation of Akt. Thus it does not appear that Akt activation is required for the initial increase in myofibrillar protein synthesis.

Although there was not a statistically significant difference in RNA abundance per milligram muscle tissue in JA16-treated and saline-treated mice, there was a trend for higher total RNA, rRNA, and myofibrillar mRNA levels in JA16-treated mice. Thus the rate of protein synthesis normalized to RNA levels was not significantly affected by inhibiting myostatin, and we cannot conclude that translational efficiency was increased.

It has been reported that several genes encoding translation initiation and elongation factors are expressed at higher levels in muscle of young mice with constitutive myostatin deficiency (21). The early response to myostatin inhibition in mature mice did not include altered expression of these same genes, except for a slight increase in expression of Eif5a (7% increase in expression in JA16-treated mice, 26% increase in mice with constitutive myostatin knockout). Mice with constitutive myostatin knockout also have increased expression of several ribosomal protein genes. The only one that also was expressed at higher levels in the present study was Rps27l (16% increase with JA16 treatment, 20% increase with myostatin knockout). Overall, the microarray data indicate that the increase in muscle protein synthesis induced by the anti-myostatin antibody cannot be explained by a concerted upregulation of genes encoding the translational machinery of the muscle fibers.

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