Skeletal Muscle Hypertrophy and Muscle Myostatin Reduction After Resistive Training in Stroke Survivors

Alice S. Ryan, PhD; Frederick M. Ivey, PhD; Steven Prior, PhD; Guoyan Li, MD; Charlene Hafer-Macko, MD

Background and Purpose—Stroke survivors experience disproportionate muscle atrophy and other detrimental tissue composition changes on the paretic side. The purpose was to determine whether myostatin levels are higher in paretic vs nonparetic muscle and the effects of resistive training (RT) on paretic and nonparetic mid-thigh muscle composition and myostatin mRNA expression in stroke survivors.

Methods—Fifteen stroke survivors (50–76 years) underwent bilateral multi-slice thigh CT scanning from the knee to the hip, bilateral vastus lateralis skeletal muscle tissue biopsies, a total body scan by dual-energy X-ray absorptiometry, and 1-repetition maximum strength test before and after a 12-week, (3 times/week) RT intervention.

Results—Total body fat mass and fat-free mass did not change. Bilateral leg press and leg extension 1-repetition maximum strength increased 31% to 56% with RT (P<0.001). Paretic and nonparetic muscle area of the mid-thigh increased 13% (P<0.01) and 9% (P<0.05), respectively, after RT. Muscle attenuation of the mid-thigh increased 15% and 8% (both P<0.01) in the paretic and nonparetic thigh, respectively, representing reduced intramuscular fat. Muscle volume increased 14% (P<0.001) in the paretic thigh and 16% (P<0.05) in the nonparetic thigh after RT. Myostatin mRNA expression levels were 40% higher in the paretic than nonparetic muscle (P=0.001) at baseline and decreased 49% in the paretic muscle (P<0.005) and 27% in the nonparetic muscle (P=0.06) after RT.

Conclusions—Progressive RT stimulates significant muscle hypertrophy and intramuscular fat reductions in disabled stroke survivors. The increased myostatin mRNA in the paretic thigh and reduction with RT imply an important regulatory role for myostatin after stroke. (Stroke. 2011;42:416-420.)

Key Words: exercise, myostatin, skeletal muscle, stroke

Sarcopenia, or the loss of fat-free mass with age, increases the risk for subsequent injury and disability.1 The progression and consequences of sarcopenia may be especially severe after a stroke because of the relative inactivity and reduced strength and fitness levels in stroke survivors. In an earlier study,2 we demonstrated that reduced muscle mass and greater severity of hemiparetic gait deficits were independent determinants of lower peak oxygen consumption in chronically disabled hemiparetic stroke survivors, illustrating a close relationship between sarcopenia and reduced fitness and frailty in stroke. Furthermore, the paretic thigh of stroke survivors had 20% lower muscle area and 25% higher intramuscular fat than the nonparetic thigh,3 demonstrating substantial atrophy and muscle composition change.

Although resistive training (RT) has proven effective for altering muscle mass in healthy elderly individuals,4–6 no evidence currently supports the use of RT for reversing muscle atrophy and increased intramuscular fat after stroke. It remains unclear whether disabled stroke survivors can perform RT at an intensity level sufficient to produce meaningful changes in tissue composition. Progressive RT after stroke results in significant increases in strength, power, and function.7,8 To our knowledge, there are no studies that have characterized body composition changes in hemiparetic stroke patients after RT.

It is also important to consider the molecular regulators of stroke-induced atrophy and adaptation with RT after stroke. Myostatin is a member of the transforming growth factor-β family of secreted growth factors and, thus, is a significant regulator of skeletal muscle development and size.9 Mutations in the myostatin gene (Mstn), which knockout myostatin expression, have led to dramatic increases in muscle mass in animals10,11 and were documented in a child.12 Both acute and chronic resistive exercise reduce basal myostatin levels in healthy individuals.13–16 providing evidence to support our hypothesis that RT may have the same effect in chronic stroke. Because of the muscle atrophy in the paretic thigh,3 we also hypothesized that myostatin levels would be elevated in paretic muscle in stroke survivors. Thus, the purpose of this study was to determine whether myostatin levels are...
higher in paretic vs nonparetic muscle and to determine the effects of a relatively intense 3-month RT program on paretic and nonparetic mid-thigh muscle composition and myostatin mRNA expression in stroke survivors.

Subjects and Methods
Subject Selection
Twenty-one individuals with a history of ischemic stroke (>6 months latency) enrolled. Six individuals did not complete the study because of either time constraints or medical issues unrelated to study participation. The 15 individuals (10 men, 5 women) who completed the study were between 50 and 76 years old and had body mass index between 23 and 39 kg/m^2. All stroke survivors had mild to moderate hemiparetic gait deficits and had completed conventional rehabilitation therapy. Evaluations included medical history, physical examination, fasting blood profile, and screening for dementia and depression to ensure adequate informed consent. Stroke participants were excluded if they had unstable angina, congestive heart failure (NYHA II), severe peripheral arterial disease, major poststroke depression, dementia, severe receptive aphasia, and orthopedic or chronic pain conditions. All tests were performed before and after the 3-month training intervention. All methods and procedures were approved by the Institutional Review Board of the University of Maryland and the Veterans Affairs Research and Development Committee. Each participant provided written informed consent.

Body Composition
Height and weight were measured. Fat mass, lean tissue mass, and percentage of body fat were determined by dual-energy X-ray absorptiometry. Thigh CT scans were performed every 4 cm starting at the patella and ending at the femoral head. Scans were analyzed using Medical Image Processing, Analysis, and Visualization (version 7.0, NIH). The cross-sectional area of each axial slice was multiplied by the distance between slices (4 cm) and summed across slices representing volume expressed in cm^3.

Exercise and Functional Tests
Exercise testing with open-circuit spirometry was conducted to measure peak oxygen consumption using a graded submaximal treadmill test. A standardized 6-minute walk test recorded the distance traveled, with stroke participants walking at their comfortable self-selected walking speed using their usual assistive devices. This walking test was chosen as a functional measure of gait deficit and to maximize the intensity of the training. Participants trained each leg separately to account for differences in paretic and nonparetic thighs. Scans were analyzed using Medical Image Processing, Analysis, and Visualization (version 7.0, NIH). The cross-sectional area of each axial slice was multiplied by the distance between slices (4 cm) and summed across slices representing volume expressed in cm^3.

Skeletal Muscle Biopsies and Analysis
Vastus lateralis biopsies of the paretic and nonparetic muscle were performed under local anesthesia in 9 subjects after a 12-hour fast for the measurement of gene expression for myostatin and insulin-like growth factor-1 (IGF-1). Muscle biopsies of the paretic and nonparetic muscle were also performed after the 3-month intervention. 24 to 36 hours after the last bout of RT in these 9 individuals. Muscle was immediately freeze-clamped and stored at −80°C. Approximately 50 to 80 mg of muscle was used for RNA isolation.

RNA Extraction and Reverse-Transcription for Real-Time Reverse-Transcription Polymerase Chain Reaction
Total RNA was extracted from skeletal muscle by the guanidinium isothiocyanate/phenol/chloroform method developed by Chomczynski and Sacchi. The RNA pellet was resuspended in RNAsafe resuspension solution (7010; Ambion) and RNA concentrations were measured in a spectrophotometer; 1 μg of total RNA for each sample was reverse-transcribed into first-strand cDNA using Transcriptor First Strand cDNA Synthesis Kit (04 896 866 001; Roche Applied Science) according to the detailed manufacturer’s protocol. The random primer was used as the primer and the RT reaction was performed at 10 minutes at 25°C and then 55°C for 30 minutes in a volume of 20 μL. The reaction was inactivated by incubating at 85°C for 10 minutes and stopped by placing the tube on ice. A reverse-transcription control (master mix without reverse-transcription enzyme) was performed.

Quantitative real-time polymerase chain reaction and data analysis for myostatin and IGF-1 were performed in a LightCycler 480 real-time polymerase chain reaction system with LightCycler 480 software (Roche Applied Science). LightCycler 480 Multiwell plate 384 (04 729 748 001; Roche Applied Science), LightCycler 480 Probes Master kit (04 887 301 001; Roche Applied Science), and Taqman gene expression primer/probe set (Applied Biosystems) were used. Each quantitative polymerase chain reaction was performed in a final volume of 10 μL, consisting of 2 μL 1:4 diluted template cDNA, 5 μL LightCycler 480 Probes Master, 0.5 μL Taqman gene expression primer and probe mix, and 2.5 μL nuclease-free water. Water instead of cDNA served as the no template control. According to the manufacturer’s instruction, the quantitative polymerase chain reaction protocol was adopted for all samples after incubation at 95°C for 10 minutes to activate the DNA polymerase, 45 cycles of 95°C for 10 seconds, and 60°C for 30 seconds each were performed to facilitate the polymerase chain reaction. For normalization, 36B4 served as an internal control. Data acquisition occurred in real-time during the annealing/elongation incubation at 60°C. All samples were amplified in triplicate from the same RNA preparation. Gene expression data were analyzed by Roche LightCycler 480 system software version 1.5 advanced relative quantification program. The average of 3 determinations for each sample and the normalized ratio of target-to-reference were used in statistical analyses.

RT Protocol
The training protocol was designed to provide a high volume, high-intensity training stimulus for maximal adaptation in skeletal muscle mass across a 3-month period. Subjects trained 3 times per week for 12 weeks, performing 2 sets of 20 unilateral repetitions on the leg press, leg extension, and leg curl machines (pneumatic resistance; Keiser) at every session. Generally, resistance was set at a level that would cause muscle failure somewhere between the repetitions 10 and 15. Resistance would then be gradually reduced to allow completion of the full 20-repetition set. Participants trained each leg separately to account for differences in strength and progression requirements between limbs. Resistance was gradually increased every 2 to 3 weeks to account for strength gains and to maximize the intensity of the training.

Statistical Analyses
Baseline myostatin levels between paretic and nonparetic thigh muscle were determined using paired Student t-tests. Changes in the paretic and nonparetic leg were assessed using repeated-measures ANOVA. All data were analyzed using SPSS 12.0. Data are presented as means±SEM. Probability P<0.05 is statistically significant.

Results
Physical Characteristics
Stroke survivors were mostly male (66%) but were racially mixed, with 47% white (n=7) and 53% black (n=8). Body weight, whole body fat mass, fat-free mass, and percentage...
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Intramuscular fat by dual-energy X-ray absorptiometry did not change significantly with RT. Muscle leg press improved 32% and 33% in nonparetic and paretic legs, respectively (P<0.001), with a group*time interaction (P<0.05). Leg extension increased 31% in nonparetic and 56% in paretic legs, respectively (P<0.001), with a greater increase in the paretic leg (P<0.001). Peak oxygen consumption, self-selected walking speed (mph) (2.11 vs 2.31) and nonparetic (33 vs 37) thighs did not change with RT.

Muscle Hypertrophy
Paretic muscle area of a single mid-thigh cross-section slice increased 13% after RT (P<0.01). Likewise, nonparetic mid-thigh muscle area increased 9% after RT (P<0.05). There were no significant changes in single-slice subcutaneous fat area and low-density lean tissue (Table 2). Muscle attenuation of the mid-thigh cross-section increased after RT in the paretic thigh by 15% (P<0.01) and nonparetic thigh by 8% (P<0.01), representing a decrease in intramuscular fat. Using multi-slice measurements of the thigh, we observed a 14% (P<0.001) increase in muscle volume in the paretic thigh and 16% (P<0.05) increase in the nonparetic thigh after RT (Figure 1). Subcutaneous fat volume of the paretic (1829±223 vs 1688±250 cm³) and nonparetic (1757±218 vs 1713±230 cm³) thighs as well as low-density lean tissue volume of the paretic (460±44 vs 463±53 cm³) and nonparetic (454±37 vs 433±45 cm³) thighs did not change with RT.

Skeletal Muscle Myostatin and IGF-1 Levels
Myostatin levels were 40% higher in the paretic than the nonparetic muscle (P=0.001; Figure 2). After RT, myostatin mRNA levels decreased 49% in the paretic muscle (P<0.005; Figure 2). In the nonparetic muscle, myostatin expression tended to decrease by 27% (P=0.06). The reduction in myostatin was greater in the paretic than nonparetic muscle (P<0.001) by repeated-measures ANOVA. IGF-1 mRNA was not different between paretic and nonparetic muscle before RT (3.57±0.48 vs 4.11±0.66 arbitrary units). IGF-1 levels did not change significantly before vs after RT in paretic (3.57±0.48 vs 3.37±0.47 arbitrary units) and nonparetic (4.11±0.66 vs 2.93±0.16 arbitrary units) muscle.

Discussion
The present study is the first to our knowledge to show that RT results in lower extremity muscle hypertrophy and loss of

\[
\text{Myostatin} \quad \text{Pre} \quad \text{Post} \quad \text{Flux} \quad \text{Volume} \quad \text{Fat} \quad \text{Lean} \quad \text{Non-Paretic} \quad \text{Paretic} \quad \text{Total} \quad \text{Difference} \quad \text{Normalized}
\]

\[
\text{Figure 2. Skeletal muscle myostatin mRNA levels in the paretic and nonparetic thighs of stroke survivors pre and post resistive training (n=8).} \quad *P<0.01.
\]

Table 1. Characteristics of Stroke Survivors Before and After Resistive Training

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before</th>
<th>After</th>
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</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>65±2</td>
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<tr>
<td>Latency since stroke (yr)</td>
<td>8±2</td>
<td>…</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>27.7±1.2</td>
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<tr>
<td>Weight (kg)</td>
<td>83.1±3.9</td>
<td>83.6±4.3</td>
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<tr>
<td>Fat mass (kg)</td>
<td>29.6±2.6</td>
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<td>Fat-free mass (kg)</td>
<td>53.5±2.6</td>
<td>53.7±2.5</td>
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<td>Percent body fat (lb)</td>
<td>35.2±2.1</td>
<td>34.8±2.2</td>
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<td>VO₂ peak (mL · kg · min⁻¹)</td>
<td>20.3±1.7</td>
<td>20.9±1.7</td>
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<tr>
<td>Self-selected walking speed (mph)</td>
<td>2.11±0.27</td>
<td>2.18±0.31</td>
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<td>Paretic 1RM leg extension (lb)</td>
<td>53±8</td>
<td>82±11*</td>
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<tr>
<td>Nonparetic 1RM leg extension (lb)</td>
<td>105±8</td>
<td>138±8*</td>
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<tr>
<td>Paretic 1RM leg press (lb)</td>
<td>282±36</td>
<td>375±42*</td>
</tr>
<tr>
<td>Nonparetic 1RM leg press (lb)</td>
<td>422±33</td>
<td>555±33*</td>
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</table>

1RM indicates 1-repetition maximum; BMI, body mass index; VO₂ peak, peak oxygen consumption.

Values are means±SEM.

Note lb for strength levels are derived from pneumatic resistance equipment.

\[
1) 20.3±1.7 \quad 20.9±1.7
2) 1.58±0.16 \quad 1.54±0.19
3) 2.11±0.27 \quad 2.18±0.31
4) 53±8 \quad 82±11*
5) 105±8 \quad 138±8*
6) 282±36 \quad 375±42*
7) 422±33 \quad 555±33*
\]

Table 2. Mid-Thigh Muscle Composition Before and After Resistive Training in Stroke Survivors

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before</th>
<th>After</th>
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</thead>
<tbody>
<tr>
<td>Muscle area (cm²)</td>
<td>68.7±5.0</td>
<td>77.7±3.9†</td>
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<tr>
<td>Subcutaneous fat area (cm²)</td>
<td>78.8±10.4</td>
<td>77.3±10.9</td>
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<tr>
<td>Low-density lean tissue area (cm²)</td>
<td>25.0±2.6</td>
<td>24.0±2.9</td>
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<tr>
<td>Muscle attenuation (Hounsfield units)</td>
<td>29.8±1.5</td>
<td>34.2±1.9†</td>
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</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Paretic</th>
<th>Nonparetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle area (cm²)</td>
<td>68.7±5.0</td>
<td>88.1±6.8</td>
</tr>
<tr>
<td>Subcutaneous fat area (cm²)</td>
<td>78.8±10.4</td>
<td>73.8±10.3</td>
</tr>
<tr>
<td>Low-density lean tissue area (cm²)</td>
<td>25.0±2.6</td>
<td>23.5±2.4</td>
</tr>
<tr>
<td>Muscle attenuation (Hounsfield units)</td>
<td>29.8±1.5</td>
<td>36.4±1.5†</td>
</tr>
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</table>

Values are means±SEM.

*P<0.05 and †P<0.01 before vs after resistive training.
intramuscular fat in stroke survivors. We utilized multi-slice CT imaging to make muscle volume determinations, representing the most comprehensive approach for assessing regional skeletal muscle hypertrophy. In addition, we provide the first evidence to our knowledge that myostatin mRNA expression are higher in the paretic muscle than the nonparetic muscle, and that RT can reduce myostatin expression in stroke survivors. This suggests that myostatin is a key molecular regulator for paretic side muscle atrophy that can respond favorably to aggressive RT treatment interventions.

Numerous studies indicate that progressive RT is well-tolerated and increases muscle strength in stroke survivors. Our results confirm both the nature and magnitude of these RT-induced strength gains after stroke. Interestingly, we had similar relative improvements in leg press strength between the paretic and nonparetic legs, but the leg extension gains were almost 2-fold higher on the paretic side. This may have been attributed to the greater difference in strength between the 2 legs at baseline for the leg extension strength test. Alternatively, it may be indicative that factors unrelated to muscle mass (ie, muscle quality) play a greater relative role in the strength adaptations related to open-chain kinetic movements on the paretic side. Although we did not measure muscle power, other stroke studies have shown increases in lower body power after RT. According to a recent meta-analysis of 13 randomized, controlled trials, RT can also improve upper limb strength and function.

Our functional outcome results are consistent with randomized and nonrandomized trials that fail to show improvements in walking distance or gait velocity with RT. Specifically, we did not see any changes in fastest or usual pace walking speed with RT. In contrast, circuit weight training that includes exercises specifically designed to improve gait and balance resulted in a greater distance walked in stroke subjects compared to control. Yang et al also reported increased gait speed, stride length, and 6-minute walk distance after RT in stroke survivors compared to controls. Similarly, when progressive RT is combined with aerobic training, there are improvements in gait performance (6-minute walking speed). Last, there is some evidence that progressive functional strength training in the subacute stroke recovery period involving weight-bearing exercises may improve walking ability (speed) and muscle strength of knee flexors. Collectively, these studies suggest that RT alone may not change function but that hybrid interventions are effective and may work best to maximize the functional impact of RT after stroke.

The magnitude of hypertrophy with RT in stroke survivors is consistent with muscle hypertrophy after RT in healthy older individuals. Longer-duration RT studies are needed to answer whether RT can further diminish the differences in paretic and nonparetic muscle area. We did not see significant changes in whole-body fat-free mass by dual-energy X-ray absorptiometry after RT, although others have reported increased fat-free mass with RT in healthy elderly individuals. This is likely because our RT protocol was limited to training the lower extremities, making whole-body dual-energy X-ray absorptiometry not sufficiently sensitive enough to register the regional compositional change taking place in the thighs. Using multi-slice CT measurements of the thigh, we found significant comparable muscle hypertrophy across the thigh in the paretic and nonparetic thighs, demonstrating that the unilateral training was an effective stimulus for both legs. The atrophy in the paretic limb at baseline with subsequent hypertrophy of the muscle after the RT is encouraging and suggests that this type of exercise may be especially important in stroke survivors. Moreover, we observed an increase in muscle attenuation after RT in both paretic and nonparetic thighs, indicating a loss of adipose tissue interspersed around muscle. Muscle attenuation is associated with greater specific force production and muscular strength in elderly individuals of the Health ABC study. In addition, there is augmented fat infiltration within muscle (reduced muscle attenuation) in obesity. RT has been shown to increase the attenuation of muscle and strength in elderly women, but this is the first account to our knowledge of larger and leaner muscle in stroke survivors.

Our results suggest that the myostatin cascade is a signaling pathway involved in poststroke muscle atrophy. Myostatin is a negative key regulator of muscle mass as indicated in case studies of humans and animals. Myostatin knockout mice have increased muscle mass accounted for by increased muscle fiber size and number. The hypertrophic effect of myostatin inhibition may be partly attributable to increased activity of satellite cells. Our findings in the stroke model of atrophy in the paretic thigh compared to the nonparetic thigh coincide with the significantly higher myostatin expression in the paretic limb than the nonparetic limb and provide indirect evidence that myostatin contributes to human muscle atrophy. Our RT intervention resulted in a significant decrease in myostatin mRNA levels in the paretic limb and approached significance in the nonparetic thigh. These results corroborate investigations in healthy adults in whom myostatin mRNA expression has been reported to decrease after resistance training. We did not see changes in IGF-1 expression, indicating that this is a less important regulator in the context of RT-related hypertrophy after stroke, although other studies have shown it is important in healthy adults. Future studies could examine additional growth factors and satellite cell proliferation in paretic and nonparetic muscle in regulating muscle growth with RT in chronic stroke.

Conclusion

We are the first to our knowledge to report significant hypertrophy and improved skeletal muscle composition of the thigh with a 3-month RT program in older stroke survivors. Moreover, there is higher myostatin mRNA expression in the paretic skeletal muscle before RT that decreases with the intervention. Future investigations are necessary to elucidate the role of additional downstream factors involved in stroke-related muscle atrophy and to identify targeted exercise rehabilitation strategies that best-mitigate these muscle composition and gene expression effects.

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**Disclosures**

None.

**References**

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