Impact of resistance loading on myostatin expression and cell cycle regulation in young and older men and women

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Impact of resistance loading on myostatin expression and cell cycle regulation in young and older men and women. Am J Physiol Endocrinol Metab 288: E1110–E1119, 2005. First published January 11, 2005; doi:10.1152/ajpendo.00464.2004.—Myostatin inhibits myoblast proliferation and differentiation in developing muscle. Mounting evidence suggests that myostatin also plays a limiting role in growth/repair/regeneration of differentiated adult muscle by inhibiting satellite cell activation. We tested the hypothesis that myostatin mRNA expression would decrease after resistance loading (RL) with a blunted response in older (O) females (F) who have shown minimal hypertrophy [vs. males (M)] after long-term RL. As myostatin is thought to modulate cell cycle activity, we also studied the response of gene transcripts key to stimulation (cyclin B1 and D1) and inhibition (p21cip and p27kip) of the cell cycle, along with the muscle-specific load-sensitive mitogen mechano-growth factor (MGF).

Twenty young (Y; 20–35 yr, 10 YF, 10 YM) and 18 O (60–75 yr, 9 OF, 9 OM) consented to vastus lateralis biopsy before and 24 h after a bout of RL (3 sets × 8–12 repetitions to volitional fatigue of squat, leg press, knee extension). Gene expression levels were determined by relative RT-PCR with 18S as an internal standard and analyzed by age × gender × load repeated-measures ANOVA. A load effect was found for four transcripts (P < 0.005) including myostatin, cyclin D1, p27kip, and MGF as mRNA levels decreased for myostatin (−44%) and p27kip (−16%) and increased for cyclin D1 (34%) and MGF (49%). For myostatin, age × load and gender × load interactions (P < 0.05) were driven by a lack of change in OF, while marked declines were noted in YM (−56%), YF (−48%), and OM (−40%). Higher cyclin D1 levels in OF led to a main age effect (36%, O > Y) and an age × gender interaction (66%, OF > YF, 10%, OM > YM; P < 0.05). An age × gender × load interaction (P < 0.05) for cyclin D1 resulted from a 48% increase in OF. Post hoc testing within groups revealed a significant increase in MGF after RL in YM only (91%, P < 0.05). Higher levels of cyclin B1 in O (27%, O > Y) led to a main age effect (P < 0.05). An age × load interaction for cyclin B1 (P < 0.05) was driven by a 26% increase in Y with no change in O after RL. No age or gender differences, or load-mediated changes, were detected in levels of p21cip mRNA expression. These data clearly demonstrate that RL downregulates myostatin expression and alters genes key to cell cycle progression. However, failure to reduce myostatin expression may play a role in limiting RL-induced hypertrophy in OF.

Sarcopenia; mechano-growth factor; p27kip; aging; resistance exercise

Sarcopenia (From the Greek sарx for flesh and penia for loss) refers to the gradual loss of skeletal muscle mass and strength during the aging process. The resultant decline in functional capacity contributes to falls, fractures, and impaired mobility in older individuals (reviewed in Ref. 23). In differentiated adult skeletal muscle, chronic resistance loading (RL) is known to partially attenuate these sarcopenic phenomena by inducing increases in the size and strength of muscle. Although the mechanisms leading to RL-mediated hypertrophy are not fully understood, the process appears to require activation of normally quiescent myoblast precursor (i.e., satellite) cells (1). Satellite cell activity is modulated by a host of endocrine and autocrine/paracrine factors, some of which are responsive to mechanical load, including mechano-growth factor \[ MGF = IGF-1Ec \text{ (insulin-like growth factor-I (IGF-I) isofrom)} \]. We and others previously found a blunted myofiber hypertrophic response in older women compared with older men during long-term resistance training (reviewed in Ref. 16), suggesting that load-mediated satellite cell activity may differ by gender. Clearly, the muscles of young men acutely respond to mechanical load with upregulated expression of mRNAs thought to be important in the hypertrophic process (5, 14, 28, 41, 42). We suspect that a blunted response of stimulating factors and/or a failure to downregulate inhibitory factors may occur in older women.

First characterized in 1997 (25), myostatin, or growth and differentiation factor-8 (GDF-8), is a member of the transforming growth factor-β superfamily that is known to play an essential role in the regulation of skeletal muscle mass. Myostatin mutation leads to massive hypertrophy and/or hyperplasia in developing animals, as evidenced by knockout experiments in mice (25) and by the well-known “double-muscling” phenotype seen in myostatin-null cattle (26). Additionally, myostatin mutation has been recently identified in a young child with overt muscle hypertrophy (32). Myostatin impairs muscle growth by inhibiting myoblast proliferation (36, 37) as well as differentiation (21, 30) in developing muscle. Additionally, myostatin has been shown to inhibit satellite cell activation (24), which would presumably limit growth of terminally differentiated adult myofibers. Myostatin may therefore play a key role in the inhibition of hypertrophic processes (17). Although the mechanisms are not fully understood, it most likely acts by modulating key regulators of the cell cycle such as cyclins (e.g., cyclins B1 and D1) and cyclin-dependent kinase inhibitors (e.g., p21cip and p27kip). For example, myostatin inhibition of satellite cell activation appears to be specific to negative regulation of G1-S progression (24), which we suggest may point to a potential interaction between myostatin

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and p27kip. Levels of myostatin mRNA expression have been found to be reduced after long-term resistance training (31) and elevated by disuse (29), with the magnitude of elevation significantly related to the magnitude of disuse myofiber atrophy. These findings suggest myostatin expression is tightly coupled to mechanical load.

The aims of the present study were to test the hypotheses that 1) acute RL using a regimen known to induce myofiber hypertrophy when performed 2–3 days/wk for several weeks (3 sets × 8–12 repetitions to volitional fatigue of squat, leg press, and knee extension) would suppress myostatin mRNA expression and elevate MGF mRNA expression in vastus lateralis samples and 2) the RL-mediated myostatin and/or MGF responses would be blunted in older women compared with both age-matched men and younger men and women. We studied additional transcripts involved in cell cycle progression (cyclins B1 and D1) and inhibition (p21cip and p27kip) to gain a better understanding of the influence RL exerts onto mitogenic processes. Expression levels of gene transcripts were determined by relative RT-PCR using 18S ribosomal RNA as an internal standard.

**METHODS.**

Subjects. Thirty-eight adults were recruited from the Birmingham, Alabama metropolitan area into two age groups. Age ranges were 60–75 yr for the older group (9 males, OM; 9 females, OF) and 20–35 yr for the younger group (10 males, YM; 10 females, YF). All subjects completed a detailed health history appraisal and all older subjects passed a comprehensive physical exam conducted by a geriatrician and a diagnostic stress test monitored by a cardiologist. Subjects were free of any musculoskeletal or other disorders that might have affected their ability to complete resistance training and testing for the study. Subjects were not obese (BMI <30) and none of the subjects had leg resistance training experience within the past 5 yr. Of the older postmenopausal women, four of nine women were on estrogen replacement therapy; however, this has been shown not to influence single myofiber function (40) nor resistance training adaptation (10). None of the subjects was being treated with exogenous testosterone or other pharmacological interventions known to influence muscle mass. The study was approved by the Institutional Review Boards of both the University of Alabama at Birmingham (UAB) and the Birmingham Veterans Affairs Medical Center. Written informed consent was obtained before participation in the research.

**RL stimulus.** We have described the RL stimulus in detail previously (4). Briefly, after a preexercise vastus lateralis muscle biopsy, subjects attended five successive visits to the laboratory on alternate days. The sequential sessions consisted of an introduction/familiarization to the 3 movements (squat, leg press, knee extension), a second familiarization session including practice one-repetition maximum (1RM) strength tests, 1RM assessment followed by 1 set × 8–12 repetitions of each exercise performed at 70% of 1RM, 2 sets × 8–12 repetitions to volitional fatigue, and, during the fifth session, 3 sets × 8–12 repetitions to volitional fatigue. All sets were separated by 90-s rest intervals. We designed this progressive protocol to prepare subjects for the full loading bout performed during the fifth exposure to the resistance exercises. As our objective was to study the effects of loading per se and not the effects of acute myofiber damage/inflammation, the progressive nature of this protocol was aimed at protecting subjects, at least in large part, from the acute inflammatory response that typically follows unaccustomed resistance exercise.

**Body composition.** Thigh lean mass (TLM), total body lean mass, and body fat percentage were determined by dual-energy X-ray absorptiometry (DEXA) using a Lunar Prodigy (model 8743; GE Lunar, Madison, WI). Analyses were conducted according to manufacturer’s instructions using enCORE 2002 software (version 6.10.029). DEXA scans were performed on all but one young male (thus n = 37 for DEXA analyses). For age and gender comparisons, 1RM strength results were adjusted for TLM to yield estimates of specific strength.

**Tissue collection.** Muscle samples were collected in the Pittman General Clinical Research Center at UAB. Muscle tissue was removed under local anesthetic (1% Lidocaine) from vastus lateralis muscle by percutaneous needle biopsy using a 5-mm Bergstrom biopsy needle under suction as previously described (9). To avoid any residual effects of the initial biopsy taken from the left leg, the postexercise biopsy was taken from the right leg 24 h after the first full bilateral loading bout (4 progressive bouts preceded this one). Samples were quickly blotted with gauze, dissected free of visible connective and adipose tissues, weighed, and snap-frozen in liquid nitrogen. All samples were stored at −80°C.

**Total RNA isolation.** Frozen muscle samples (average = 35 mg) were homogenized, and total RNA was extracted using the TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol, which is based on the method by Chomczynski and Sacchi (7). Extracted RNA was precipitated from the aqueous phase with isopropanol and, after two ethanol washes, dried and suspended in nuclease-free water (Promega, Madison, WI) at a ratio of 0.8 μg/mg muscle. RNA concentration was determined with a fluorometer (TD-700, Turner Designs, Sunnyvale, CA) using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol. RNA concentration of each sample was determined by linear regression using ribosomal RNA standards from the RiboGreen RNA Quantitation Kit and expressed as total RNA per milligram of muscle. RNA samples were stored at −80°C for the subsequent analyses of specific mRNAs by relative RT-PCR procedures.

**RT.** One microgram of RNA was reverse transcribed in a total volume of 20 μl using SuperScript II Reverse Transcriptase (Invitrogen, GibCO-BRL, Carlsbad, CA) with a mix of oligo(dt) (100 ng/reaction) and random primers (200 ng/reaction), according to the method described by Bickel et al. (5). After the RT reaction, mixtures were incubated at 45°C for 50 min, they were heated at 90°C for 5 min to denature the reaction and then stored at −80°C for subsequent PCR analyses.

**PCR.** A relative RT-PCR method was applied in the present study using 18S ribosomal RNA as an internal standard (Invitrogen) to determine relative expression levels of mRNAs for myostatin, MGF, cyclin B1, cyclin D1, p21cip, and p27kip. The primer sequences for the specific target mRNAs are shown in Table 1. Primers were designed using the Primer Select computer program (DNAStar, Madison, WI) and prepared by Invitrogen (GIBCO). In preliminary experiments, we confirmed that each target mRNA primer set was compatible with the alternate 18S primers.

For each PCR reaction, 18S (with a 324-bp product) was coamplified with each target cDNA (mRNA) to express each mRNA as a ratio of target mRNA/18S. The 18S primers were mixed with competitors to ensure that 18S and each target mRNA coamplified in the linear range. The ratio of this primer–competitor mixture was optimized in preliminary experiments and ranged from 1:25 to 1:120 depending on the abundance of the target mRNA. A representative linearity test for the validation of PCR cycle number is shown in Fig. 1. In this myostatin linearity test, 36 was chosen as the optimum number of cycles, as 18S and myostatin levels deviated from linearity past 38 and 40 cycles, respectively (not shown).

The conditions of both RT and PCR reactions were standardized by using the same premixed reagents for all 76 samples to be compared. Based on the number of PCR reactions, a PCR premix was prepared with 2 mM MgCl2, PCR buffer (GIBCO) 0.2 mM 2-deoxyribonucleotide 5′-triphosphate, 1 μM specific primer set, 0.5 μM 18S primer–competitor mix, and 0.75 unit of Biolase DNA Taq polymerase (GIBCO) in 25 μl of total volume. For each PCR, 1 μl of RT product...
Table 1. Sequences of the specific sets of primers used

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>PCR Primer Sequence 5′ → 3′</th>
<th>Product Size, bp</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myostatin</td>
<td>5′ sense: CTGACAGGAAGAAATGTTTCC ACCA</td>
<td>243</td>
<td>NM_005259</td>
</tr>
<tr>
<td></td>
<td>3′ sense: GATGAGAGGTGAGGAGTCGGTGTTT</td>
<td>282</td>
<td>U40870</td>
</tr>
<tr>
<td>MGF</td>
<td>5′ sense: CGTGGCAAAATGTTTCC ACCA</td>
<td>222</td>
<td>NM 031966</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>5′ sense: GCCTTGCGACCATCTCTTTGAC</td>
<td>269</td>
<td>NM 001758</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>5′ sense: GCCCCCGGACGGTTTCTACAT</td>
<td>233</td>
<td>L25610</td>
</tr>
<tr>
<td>p21&lt;sup&gt;WAF1&lt;/sup&gt;</td>
<td>5′ sense: GCAGCGGAACCTGACCAGTCA</td>
<td>232</td>
<td>NM 004064</td>
</tr>
<tr>
<td>p27&lt;sup&gt;kip1&lt;/sup&gt;</td>
<td>5′ sense: GCCGACGAGGCGGAGGAT</td>
<td>243</td>
<td>NM 005259</td>
</tr>
</tbody>
</table>

MGF, mechano-growth factor.

(cDNA) was added into 24 μl of premix and topped with 50 μl of mineral oil (Sigma, St. Louis, MO). PCR was carried out in a DNA Engine (PTC-200) Peltier Thermal Cycler (MJ Research, Waltham, MA) with an initial denaturing step of 3 min at 96°C, followed by specific cycles (31–38 cycles depending on the results of linearity tests for each target mRNA and 18S) of 1 min at 96°C, 45 s at specific annealing temperatures (52–58°C depending on primers), 45 s at 75°C, and a final step of 3 min at 72°C. Immediately following PCR, 25 μl of PCR product (22 μl of the reaction mixture diluted with 3 μl of loading buffer) were separated by electrophoresis (100-V constant) in a 2% agarose gel for 1.5 or 2 h [depending on the band separation between 18S (324 bp) and specific target genes due to the size of each mRNA product]. To eliminate age group, gender, or RL bias, each 20-well gel contained pre-post paired samples for subjects within each group (i.e., YM, YF, OM, OF) with the different subject groups loaded in random order on each gel. Gels were run with molecular mass markers (100 bp Hyper Ladder IV; Genesee Scientific, San Diego, CA) to confirm the expected size of each mRNA. Ethidium bromide (0.1 μg/ml) was premixed in the 2% agarose gel, and images were captured under UV in a Bio-Rad ChemiDoc imaging system (Hercules, CA). Band densitometry was performed using Bio-Rad Quantity One software. Parameters for image development were consistent across all gels using predefined saturation criteria for the CCD camera. For each gel, total exposure time was determined by the first point of saturation on the image. This standardization, combined with equal distribution of the four age/gender groups across all gels, enabled us to accurately test for age, gender, and RL effects.

**Statistical analysis.** Data are reported as means ± SE. Between-group differences in preexercise descriptive variables were tested using age × gender ANOVA. All variables measured before and after loading were analyzed using age × gender × load repeated-measures ANOVA. For each ANOVA model with a significant main or interaction effect, Tukey HSD tests were performed post hoc to localize the effect(s). Zero-order correlations were tested between levels of lean body mass (LM) or TLM and resting levels, as well as load-mediated changes, for each transcript studied. Correlations were also tested between load-mediated changes in mRNA levels for myostatin, MGF, cyclin B1, cyclin D1, p21<sup>WAF1</sup>, and p27<sup>kip1</sup> mRNA. For each correlation, we tested for the presence of outliers by residual analysis using a standard residual >2.5 SD. If one or more outliers were identified, these subjects were excluded and the correlation was retested. Results are summarized with the appropriate sample size for each correlation after removing outliers (if any). Statistical significance was accepted at P < 0.05 for all tests.

**RESULTS**

Descriptive characteristics of the subjects are shown in Table 2. The four age/gender groups are defined as YF (young females), YM (young males), OF (older females), and OM (older males) on all figures and throughout the remaining text. It is necessary to note that this study sample overlapped with our previous investigation of the effects of acute RL on protein expression (4); however, due to methodological limitations such as tissue availability, the present investigation includes several individuals not previously studied. Within each age group, males and females were of similar age. Typical gender differences in height and weight were found, as main gender effects (P < 0.0001) confirmed that men were taller and weighed more than women. A lower body fat percentage was found in young compared with older adults (P < 0.01). Young subjects had more total LM (P < 0.05) and TLM (P < 0.001) than their older counterparts, with the effect most notable in men. The age differences in both total body LM and TLM were not driven by height, as height was not different between young and older subjects within gender. Adjusting TLM for total body LM revealed significantly higher levels of relative TLM for both YF (P < 0.001) and YM (P < 0.01) compared...
with their older, within-gender counterparts. We previously interpreted age differences in this relative TLM index as an indication that atrophy with aging occurs more rapidly in the weight-bearing lower limb musculature than in the remaining LM compartment (4, 27). The similar estimates of specific strength (Table 2) among the groups indicate that all subjects exercised at the same relative loading intensity as a constant percentage of 1RM was used to set initial resistances. Lower knee extension-specific strength found in older subjects ($P < 0.05$) may, however, be indicative of age differences in quadriceps muscle quality.

The concentration of total RNA increased 13% after loading, from $0.24 \pm 0.01$ to $0.27 \pm 0.01 \mu g/mg$ muscle ($P < 0.05$). As the bulk of total muscle RNA is ribosomal, this could be considered as a marker of enhanced potential for protein synthesis (5). The results of specific transcript expression analyses are shown in Figs. 2–4. A main load effect ($P < 0.005$) was found for four transcripts including myostatin, MGF, cyclin D1, and $p27^{kip}$. We found a significant load-mediated decline in the levels of myostatin mRNA expression ($-44\%$, $P < 0.00001$) at the 24-h timepoint (Fig. 2A). In addition, age $\times$ load ($P < 0.01$) and gender $\times$ load ($P < 0.05$) interactions were found for myostatin mRNA, apparently driven by a lack of change in OF ($P = 0.69$). Levels of myostatin mRNA were markedly decreased in the other groups [OM ($-40\%$), YM ($-56\%$), and YF ($-48\%$), $P < 0.01$] after acute RL. By contrast, MGF mRNA expression increased markedly ($+49\%$, $P < 0.001$) after acute RL (Fig. 2B). Although no interactions were detected for MGF, the increase with load was clearly driven by YM ($91\%$) as post hoc tests revealed no other within-group increases.

Results of general cell cycle regulators are shown in Figs. 3 and 4. We assessed mRNA levels of cyclins key to initiation (cyclin D1) and progression through the G2-M boundary (cyclin B1). Results revealed a significant increase in cyclin D1 mRNA levels ($34\%$, $P < 0.001$) after RL (Fig. 3A). Higher cyclin D1 levels in OF led to a main age effect (36%, O $> Y$; $P < 0.01$) and an age $\times$ gender interaction (66%, OF $> YF$ vs. 10%, OM $> YM$; $P < 0.05$). An age $\times$ gender $\times$ load interaction ($P < 0.05$) for cyclin D1 resulted from a marked 48% increase in OF after RL. Higher levels of cyclin B1 mRNA expression (Fig. 3B) in older adults (27%, O $> Y$) resulted in a main age effect ($P < 0.05$). An age $\times$ load interaction for cyclin B1 ($P < 0.05$) resulted from a 26% increase in younger adults with no significant change in older adults after RL.

The results of two key cell cycle inhibitors are shown in Fig. 4. $p27^{kip}$ has been shown to arrest cultured satellite cells in G1 and has been implicated in the progression of sarcopenia (35). A main load effect revealed a modest but statistically significant reduction in $p27^{kip}$ mRNA ($-16\%$, $P < 0.01$) after RL (Fig. 4A). With this reduction, postloading levels of $p27^{kip}$ expression in OF reached approximately the basal level seen in the other three age-gender groups. No significant age, gender, or loading effects were detected for $p21^{kip}$ mRNA expression (Fig. 4B).

Correlational analyses revealed some noteworthy associations. Using residual analysis to detect outliers as described in Methods led to the removal of no more than one outlier from any given correlation. For all subjects, the relative concentration of myostatin mRNA in resting VL muscles was positively related to LM ($r = 0.45$, $P < 0.01$, $n = 37$) and TLM ($r = 0.49$, $P < 0.01$, $n = 37$; Fig. 5A). Furthermore, the magnitude of reduction in myostatin mRNA correlated with LM ($r = -0.42$, $P < 0.05$, $n = 36$) and TLM ($r = -0.47$, $P < 0.01$, $n = 36$). Interestingly, these findings appeared to have been mainly driven by males. In men, resting myostatin levels were positively related to LM ($r = 0.68$, $P < 0.005$, $n = 18$) and TLM ($r = 0.63$, $P < 0.01$, $n = 18$; Fig. 5B), and there was a negative relationship between load-induced reduction in myostatin mRNA and muscle mass (TLM) ($r = -0.80$, $P < 0.01$, $n = 17$; Fig. 6A).

The findings of a positive association between resting myostatin mRNA levels and muscle mass suggest a novel paradox whereby individuals with an apparently advantageous potential for muscle growth and/or maintenance also express high levels of perhaps the single most potent “anti-growth” factor. This may, however, “prime” the muscle for growth, as load-induced reductions in myostatin mRNA were greatest in subjects with higher muscle mass. In support of this concept, we also found TLM in males was inversely related to the magnitude of load-induced change in $p27^{kip}$ mRNA expression ($r = -0.52$, $P < 0.05$, $n = 18$) and positively related to load-induced increases in cyclin D1 mRNA levels ($r = 0.47$, $P < 0.05$, $n = 18$). Furthermore, load-mediated changes in myostatin and $p27^{kip}$ mRNA levels were positively related in men ($r = 0.59$, $P < 0.01$, $n = 19$; Fig. 6B) but not in women ($r = 0.03$, $P > 0.05$, $n = 19$). This finding in males was primarily driven by

Table 2. Descriptive characteristics of each group

<table>
<thead>
<tr>
<th></th>
<th>Young Females (n = 10)</th>
<th>Young Males (n = 10)</th>
<th>Older Females (n = 9)</th>
<th>Older Males (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>27.9 ± 1.4</td>
<td>28.2 ± 1.2</td>
<td>64.3 ± 1.0</td>
<td>65.1 ± 1.7</td>
</tr>
<tr>
<td>Height, cm†</td>
<td>162.4 ± 2.4</td>
<td>178.6 ± 3.1</td>
<td>160.1 ± 2.1</td>
<td>177.0 ± 2.5</td>
</tr>
<tr>
<td>Weight, kg†</td>
<td>63.0 ± 3.2</td>
<td>79.5 ± 4.1</td>
<td>63.1 ± 3.2</td>
<td>83.2 ± 2.5</td>
</tr>
<tr>
<td>%Body fat†</td>
<td>34.2 ± 1.7</td>
<td>20.3 ± 3.2</td>
<td>38.8 ± 1.8</td>
<td>30.7 ± 1.9</td>
</tr>
<tr>
<td>Total body LM, kg*†</td>
<td>38.9 ± 0.9</td>
<td>59.5 ± 2.1</td>
<td>36.7 ± 1.8</td>
<td>55.1 ± 1.5</td>
</tr>
<tr>
<td>Relative thigh LM*†, %total body LM</td>
<td>24.0 ± 0.4</td>
<td>25.1 ± 0.4</td>
<td>21.7 ± 0.5</td>
<td>23.0 ± 0.3</td>
</tr>
</tbody>
</table>

Specific strength measurements, 1RM (kg)/thigh LM (kg)

- Knee extension*       | 3.85 ± 0.19            | 4.00 ± 0.17          | 3.59 ± 0.12           | 3.13 ± 0.22        |
- Leg press             | 9.46 ± 0.39            | 10.02 ± 0.46         | 9.25 ± 0.37           | 8.86 ± 0.26        |
- Squat                 | 6.08 ± 0.29            | 6.46 ± 0.47          | 6.27 ± 0.26           | 5.51 ± 0.25        |

Values are means ± SE. LM, lean mass; 1RM, one-repetition maximum strength. *Main age effect, $P < 0.05$. †Main gender effect, $P < 0.05$. 

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On the basis of these relationships, we speculate that not only are myostatin and p27kip related, the efficacy of load-mediated myogenesis may be determined by the degree to which both transcripts are synchronously downregulated. For all subjects, there was a positive correlation between load-mediated increases in MGF and cyclin D1 mRNA levels (r = 0.60, P < 0.01, n = 37). As cyclin D1 has been used extensively as a general marker of cell cycle activity, and MGF is a muscle-specific, load-sensitive mitogen, this relationship is suggestive of a load-induced increase in mitogenic activity within a population of myogenic cells. Partitioning by age or gender revealed significant relationships in young subjects (r = 0.80, P < 0.01, n = 19) and in males (r = 0.54, P < 0.05, n = 19).

YM (r = 0.69, P < 0.05, n = 10). On the basis of these relationships, we speculate that not only are myostatin and p27kip related, the efficacy of load-mediated myogenesis may be determined by the degree to which both transcripts are synchronously downregulated.

For all subjects, there was a positive correlation between load-mediated increases in MGF and cyclin D1 mRNA levels (r = 0.60, P < 0.01, n = 37). As cyclin D1 has been used extensively as a general marker of cell cycle activity, and MGF is a muscle-specific, load-sensitive mitogen, this relationship is suggestive of a load-induced increase in mitogenic activity within a population of myogenic cells. Partitioning by age or gender revealed significant relationships in young subjects (r = 0.80, P < 0.01, n = 19) and in males (r = 0.54, P < 0.05, n = 19).
DISCUSSION

To our knowledge, this study is the first to demonstrate the effects of age, gender, and acute RL on the mRNA levels of myostatin and a host of other key modulators of cell cycle progression and withdrawal. The results of the present study clearly demonstrate that acute RL markedly downregulates myostatin mRNA expression with a concomitant increase in MGF mRNA. Others have reported decreased myostatin protein levels in response to loading in rodents (19). RL also increased levels of gene expression for cyclin D1 (all subjects) and cyclin B1 (young subjects only). Although there were no changes in p21^{kip} expression levels, load-mediated downregulation of p27^{kip} gene expression was observed. These combined data suggest that, by some as yet unknown mechanism, mechanical load facilitates cell cycle progression by attenuating inhibitory factors and elevating stimulatory factors. Furthermore, the positive relationships between magnitudes of change in general and muscle-specific cell cycle promoters and inhibitors suggest that at least some enhanced cell cycle activity occurred in mitotically active myogenic cells such as satellite muscle cells. Based on the assumption that satellite cell activation is a requisite step in the hypertrophy process, these alterations may provide some valuable insight regarding load-mediated mechanisms of myofiber hypertrophy.

The capacity of young men for resistance training-induced myofiber hypertrophy is well known. Additionally, much of the recent work demonstrating acute load-mediated increases in proteins and/or mRNAs thought to be important for long-term growth/regeneration has been conducted in young men (14, 28, 41, 42). Our unique model enabled us to assess potential...
influences of age and/or gender on key transcriptional activities following loading. The overall trend of these data is that young men responded most positively to the loading program, demonstrating robust and consistent changes in factors thought to promote (MGF, cyclin D1, cyclin B1) and inhibit (myostatin, p27kip mRNA expression. The correlation suggests that subjects with higher muscle mass were more responsive to acute mechanical load (i.e., greater reduction in myostatin mRNA). B: positive relationship between load-mediated changes (chg) in mRNA levels of p27\(^{kip}\) and myostatin found in men only, indicating that greater declines in myostatin mRNA levels were associated with declines in p27\(^{kip}\) mRNA expression.

![Graph A](image1.png)

Fig. 6. A: inverse relationship in men between untrained, resting thigh muscle mass and the magnitude of load-mediated reduction in myostatin mRNA expression. The correlation suggests that subjects with higher muscle mass were more responsive to acute mechanical load (i.e., greater reduction in myostatin mRNA). B: positive relationship between load-mediated changes (chg) in mRNA levels of p27\(^{kip}\) and myostatin found in men only, indicating that greater declines in myostatin mRNA levels were associated with declines in p27\(^{kip}\) mRNA expression.

Although these data provide important insight regarding age and gender influences on load-mediated gene expression, two limitations are notable. First, our postloading analysis was limited to one timepoint (24 h). Additional postloading biopsies both earlier and later than 24 h may have revealed additional age and/or gender differences. With serial biopsies ranging from 0 to 48 h postloading, Psilander et al. (28) clearly demonstrated (in young men) that temporal postloading responses vary among transcripts. Second, our analyses were limited to mRNAs. Although our work is certainly not unique in this regard (5, 28), coupled analysis of both mRNA and its protein product is certainly a strength. However, we previously showed using the same model that 24 h after loading is probably too early to detect a host of changes at the protein level (4). A protocol with multiple postloading biopsies would have addressed both of these limitations but was simply not feasible.

**Myostatin and general cell cycle inhibitors.** We found no significant age differences in resting levels of myostatin mRNA, a finding that has been shown previously in men (39). One important finding was that, in response to the same relative loading stimulus, older women had an impaired ability to downregulate myostatin expression. Older men, however, showed a load-induced reduction in myostatin similar in magnitude to both younger men and women. We recently reported that older females showed a blunted myofiber hypertrophic response along with a blunted strength gain after 26 wk of progressive resistance training compared with older males (3). If myostatin downregulation is key to the promotion of hypertrophic processes, an attenuated acute response to loading in older women may contribute to an impaired long-term growth adaptation. Interestingly, older women have also been shown to be more susceptible to detraining following resistance training compared with young men, young women, and older men (18). A study of polymorphic variation in a cohort of older women showed that myostatin polymorphic variants were related to differences in strength levels in older women (34). This group further demonstrated that women with a less common myostatin allele exhibited a 68% larger increase in muscle volume in response to strength training (17), suggesting this allele resulted in either lower amounts of myostatin protein or an alternate protein product with reduced efficacy as a negative regulator.

Reductions in myostatin mRNA have been shown previously following 9 wk of resistance training (31). On the basis of our finding of markedly reduced myostatin mRNA levels following acute RL, we suggest the bulk of the reduction found after 9 wk of training may have occurred very early in the training program (e.g., first 1–3 exercise bouts). In contrast to our findings and to those of Roth et al. (31), Willoughby (41) recently reported increased levels of myostatin protein and mRNA after both 6 and 12 wk of resistance training. One major difference in protocol design, however, was the collection of 6- and 12-wk biopsies only 15 min after an exercise bout (compared to 24 h in the current study).

As increased mechanical load has been shown to reduce myostatin, one might expect increased myostatin levels during states of reduced loading. Findings in both humans (29) and rodents (20) support this concept. Significantly elevated myostatin mRNA levels were observed in the vastus lateralis muscle of 12 patients with chronic disuse atrophy of type II myofibers (29). Furthermore, muscle atrophy during spaceflight has been shown to be associated with increased myostatin mRNA levels (20).

Striking from the present study was the finding that thigh muscle mass in healthy sedentary adults, irrespective of age or gender, was positively related to resting levels of muscle myostatin mRNA. At odds with this finding are reports that
serum levels of a “myostatin-immunoreactive protein” are higher in older adults and inversely related to muscle mass (44) and lean mass in frail elderly women (33) and to lean mass in HIV-positive men suffering from cachexia (11). However, the sequence of the 26-kDa “immunoreactive protein” has not been confirmed, and whether this protein is in fact myostatin in any form has been questioned (45). Our findings of a positive association between resting myostatin mRNA levels and muscle mass were revealed in a fairly large subject cohort. Although further study is certainly needed (including protein-level analyses), we consider this result combined with the finding that load-induced reductions in myostatin mRNA were greatest in subjects with higher muscle mass as a possible indicator of hypertrophic potential. It is also possible that the positive relationship seen between resting myostatin mRNA and thigh muscle mass was, in part, driven by myofiber type distribution. Previous studies in rodents have indicated preferential expression of myostatin in type II plantaris muscle (38) and a positive relationship between myostatin mRNA expression and MHCIIb abundance (6). Whether myostatin is more abundant in type II muscle among humans is unknown. If so, it is certainly possible that subjects with greater thigh muscle mass in our study also possessed a greater type II myofiber area distribution. In an ongoing study of over 40 young and older participants similar in age to the current subjects, we have found that the type II atrophy associated with sarcopenia is most notable in type IIX myofibers (M. Bamman, J.S. Kim, and D. Kasek, unpublished observations).

The cell cycle is regulated by a number of cyclin-dependent kinase inhibitors including the INK4 family and the CIP/KIP family (p21cip, p27kip, p57kip). p27kip is typically considered an inhibitor of cell cycle initiation (G1) (22), while p21kip inhibits the cell cycle at multiple control points, including progression through the G2-M boundary. In the present study, a load-induced reduction was found for p27kip mRNA levels as indicated by an overall main effect of loading. The mean data in Fig. 4A show that all groups except OM contributed to this effect. We reported a similar load-mediated reduction in p27kip protein concentration in women using the same acute loading model (4). Spangenberg et al. (35) previously suggested that p27kip may be a molecular mediator in the progression of age-related sarcopenia based on the findings that p27kip expression is higher in older satellite cells, and p27kip overexpression arrests cultured satellite cells in G1 despite the overexpression of IGF-I, a potent mitogen.

Lin et al. (22) previously demonstrated the inhibitory influence of p27kip on myogenic cells and its association with myostatin in developing muscle, as p27kip knockout mice showed markedly increased gastrocnemius muscle mass and a reduction in myostatin mRNA levels. This finding suggests that p27kip deficiency-induced muscle hypertrophy may be partially mediated by decreased myostatin. We report herein that not only were p27kip and myostatin mRNA levels reduced by mechanical load, the magnitudes by which these transcripts declined were positively related in men and especially in young men. If, in fact, p27kip and myostatin work in concert, our findings may provide some basis for a greater load-induced hypertrophic potential in young men vs. older women, as no coordinated decline in the two transcripts was seen in older women nor in all women combined.

The importance of p27kip in the inhibition of cell growth has been demonstrated by Coats et al. (8), suggesting that the activity of cdk2, which is involved in progression throughout the entire cell cycle from G1 to M, is mainly inhibited by p27kip and to a lesser degree by p21cip. Levels of p21cip mRNA did not change in our model. By contrast, p21cip mRNA levels have been shown to increase markedly in response to mechanical load in rodents (2, 12) and in humans (5). These investigators considered an increase in p21cip as indicative of enhanced differentiation. In the work by Bickel et al. (5), muscle biopsies were collected 24 h after the second of two bouts of electrically evoked isometric contractions. The timing between stimulus and biopsy was identical to our model but the contraction paradigm differed substantially. We do not know whether this difference is responsible for the markedly different results in p21cip expression between the two studies; however, it is noteworthy that results of both total RNA concentration and cyclin D1 mRNA levels also differed, as total RNA concentration (µg RNA/mg muscle) increased 13% and cyclin D1 mRNA increased 34% after our dynamic RL regimen but did not change significantly in the isometric contraction model of Bickel et al.

The cdk inhibitors (p21cip, p27kip) we studied are often viewed as general markers of differentiation but, in addition to promoting withdrawal of actively proliferating cells, they specifically inhibit cell cycle initiation and/or progression. In a quiescent cell type such as satellite cells, our impression is that these cdk inhibitors may be responsible for inhibiting entry into the cell cycle (arresting in G1). Thus in the early response phase following loading, we speculate a suppression of cdk inhibitors might be advantageous by facilitating entrance into the cell cycle. Beyond an initial phase of proliferative activity (e.g., the first 24 h), an increase in cdk inhibitors may be advantageous to promote withdrawal (i.e., differentiation) of some cells as proliferation continues. The expression of p21kip mRNA has been shown to increase markedly within 12 h after the onset of compensatory overload (via synergist ablation) in rats (2). The compensatory overload model induces a much greater loading stimulus than our resistance exercise regimen, thus making it difficult to compare the two time courses.

**MGF and general cell cycle promoters.** In differentiated adult skeletal myofibers, RL-mediated myofiber hypertrophy involves increased muscle protein synthesis and activation of satellite cells. MGF is a load-sensitive autocrine/paracrine growth factor that likely works in concert with IGF-I to promote both of these processes (13, 15, 43). We found a marked increase in MGF mRNA expression (+49%) after acute RL; however, post hoc tests revealed a within-groups increase in young men only (91%). Others have also reported induction of MGF mRNA after acute RL in young men (5, 14, 28). Hameed et al. (14) found this acute response in young men only, as no change in MGF mRNA was noted in elderly men. In a separate study, however, this group found a substantial rise in MGF mRNA after 5 wk of resistance training in elderly men (13). The young men in the present study not only experienced the largest elevation in MGF but also the greatest reduction in myostatin mRNA, suggesting the muscles of young men were most responsive to the loading stimulus.

As markers of general proliferative activity, we examined cyclin D1 as integral to cell cycle initiation and cyclin B1 as an index of late cell cycle activity (i.e., progression through the
G2-M boundary). Our data indicate that acute RL induced an overall increase in levels of cyclin D1 gene expression while only young subjects increased cyclin B1 mRNA levels. Others found increases in cyclin D1 mRNA levels after acute loading in rodents (12). Certainly these changes in cyclin expression cannot be uniquely attributable to satellite cell activation, as these general cell cycle markers may also point toward upregulation of mitotic activity in non-muscle cells (e.g., fibroblasts) that would seem necessary for growth/repair/regeneration of supporting tissues within the muscle. However, by assessing both cyclins D1 and B1, our data may provide some important insight into the overall growth/regenerative capacity of young and older men and women in response to acute loading. Based solely on cycle D1 mRNA levels (Fig. 3A), one might hypothesize that older women would have the greatest mitotic potential; however, by also studying late cell cycle activity (cyclin B1; Fig. 3B), younger men emerge as the group with the greatest combined response. In support of this, we noted positive relationships between increases in cyclin D1 and MGF only in young subjects and in men.

If we accept these molecular markers as representative of some key processes in the regulation of muscle mass, our combined findings may provide some molecular basis for disparate hypertrophic adaptations to long-term resistance training among gender- and age-specific populations. Based on the aforementioned assumption, we have drawn the following conclusions from these data. First, in resting and otherwise healthy muscle not induced to hypertrophy and not exposed to acutely atrophic conditions, the concentration of myostatin mRNA was positively related to muscle mass. This novel “paradox” may lead to a greater potential to reduce myostatin (among those with higher muscle mass) when a hypertrophic stimulus is imposed, as the magnitude of load-mediated reduction in myostatin was inversely related to muscle mass and this relationship was strengthened when only men were included. Second, as additional evidence for a link between resting muscle mass and mitogenic potential, load-mediated increases in cyclin D1 and decreases in p27kip mRNAs were related to TLM in men only. Third, concurrent reduction in myostatin and p27kip, as seen in young men, may be optimal as a permissive signal for the induction of myogenesis. Fourth, the greatest load-driven increase in MGF mRNA was found in young men, and the magnitudes of increase in MGF and cyclin D1 mRNAs were correlated in all subjects, suggesting coordinated mitogenic signaling between the two.

Overall, our findings suggest that high- to moderate-intensity RL serves as a natural inhibitor of myostatin gene transcription. Myostatin may act in concert with p27kip and reductions in these cell cycle inhibitors are coincident with enhancements in cell cycle progression (cyclins D1 and B1) and myogenic potential (MGF). Although these load-mediated myofiber hypertrophic signals appear to be optimal in young males, older women show impaired capacity to reduce myostatin and enhance MGF gene expression levels in response to the same relative loading stimulus. These acute molecular responses may underlie age and gender differences in the capacity for load-mediated hypertrophy. Thus an important next step would be the evaluation of these responses during long-term resistance training to determine whether indeed these acute events are predictive of satellite cell activation and myofiber hypertrophy.

REFERENCES


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