Myogenic protein expression before and after resistance loading in 26- and 64-yr-old men and women

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Submitted 22 December 2003; accepted in final form 21 May 2004

Bamman, Marcas M., Ronald C. Ragan, Jeong-su Kim, James M. Cross, Vernishia J. Hill, S. Craig Tuggle, and Richard M. Allman. Myogenic protein expression before and after resistance loading in 26- and 64-yr-old men and women. J Appl Physiol 97: 1329–1337, 2004. First published May 21, 2004; 10.1152/japplphysiol.01387.2003.—Based on the growing body of evidence implicating an important role for myogenic regulatory factors (MRFs) in the adaptive responses of skeletal muscle to mechanical load, we tested the hypothesis that protein concentrations of MRFs as well as cell cycle proteins (i.e., cyclins and cyclin-dependent kinase inhibitors) would be altered after heavy leg resistance exercise (RE). Because we and others, however, have shown a blunted adaptive response to long-term resistance training in older (O) women [females (F)] compared with men (M), we also tested the hypothesis that these myogenic responses to RE would be influenced by age and gender. Twenty-two younger (Y) adults (20–35 yr, 11 YF, 11 YM) and 20 O adults (60–75 yr, 9 OF, 11 OM) consented to vastus lateralis muscle biopsy before and 24 h after a bout of RE using a regimen known to induce myofiber hypertrophy when performed 2–3 days/wk for several weeks (3 sets of 80% one-repetition maximum for squat, leg press, and knee extension). Protein concentrations of MRFs (MyoD, myogenin, myf-6), cyclin D1, cyclin B1, α-actin, and the cyclin-dependent kinase inhibitor p27kip were determined by immunoblotting. Data were analyzed by using age × gender × load repeated-measures ANOVA. Myogenin expression was 44% higher (P < 0.05) in O compared with Y, and myf-6 tended to be higher in OF compared with YF (95%, P = 0.059). A significant gender × load interaction indicated that, in F, RE led to a reduction in p27kip (20%; P < 0.05), which was driven mainly by a 27% drop in OF. Levels of cyclin D1, cyclin B1, MyoD, myf-6, and α-actin were not influenced by age, gender, or loading. We report a novel finding in humans of markedly higher myogenin protein content in older sedentary muscle. The results do not, however, support the hypothesis that myogenic protein expression is altered 24 h after RE, irrespective of age or gender. Although the time point of postexercise muscle biopsy could be viewed as too early to capture maximal effects for most of these proteins, the significant decline in p27kip concentration found in OF suggests that mechanical load may provide one means of overcoming the inhibitory influence of p27kip.

Myogenin; MyoD; myf-6; p27kip; cyclin D1; cyclin B1; elderly; sarcopenia

AGE-RELATED SARCOPENIA LEADS to declines in muscular strength, power, and endurance that impair weight-bearing functional abilities and ultimately may cause dependence and disability.

As such, interventions, including systemic hormone treatment (e.g., testosterone, growth hormone, IGF-I) and resistance training, have received significant attention of late (reviewed in Ref. 14). Resistance training has been shown to induce substantial myofiber hypertrophy in aging muscle, but the efficacy of this type of training may be inversely proportional to the progression of muscle loss. For example, the magnitude of training-induced hypertrophy is minimal relative to strength gains in frail elderly (34), and, in gender comparisons, we and others have found a blunted hypertrophic response in sarcopenic older women compared with age-matched older men (7, 21, 38, 39). Additionally, animal models have provided evidence that the regenerative capacity of older muscles following contraction-induced injury is impaired (12). These findings suggest that the mechanotransduction mechanism(s) leading to load-induced myofiber growth and regeneration may be impaired with advancing sarcopenia.

In recent years, intensive investigation into the pathways underlying adaptive responses of skeletal muscle to changes in loading state has led to significant advances. It is largely accepted that satellite cell activation is integral to the growth and repair of myofibers subjected to increased mechanical load, as these myogenic precursor cells proliferate and differentiate to serve as donor nuclei to a growing postmitotic myofiber, thereby maintaining the myonuclear domain. It is therefore possible, if not probable, that, in aging muscle, the local stimuli for satellite cell activation are impaired and/or inhibitory factors are more prevalent, as the in vitro proliferative potential is hampered in satellite cells isolated from aged animals (36). Entrance of the normally quiescent (G0) satellite cell into the cell cycle is regulated by a host of locally produced growth factors, some of which are expressed in a load-sensitive manner. Two mitogenic factors that may be particularly important in this activation step are hepatocyte growth factor (9) and the load-sensitive isoform of IGF-I expressed in muscle (termed mechanogrowth factor) (1). There is evidence to indicate that load-induced mechanogrowth factor expression declines with age (19, 31). Progression of the cell cycle is regulated at multiple control points by a group of cyclin-dependent kinases (CDKs). Activation of these CDKs requires binding to a specific cyclin protein to form a cyclin-CDK complex, which then possesses catalytic activity. Whereas CDKs are constitutively expressed, cyclin levels vary across the stages of the cell cycle, and cyclin expression is influenced...
by external stimuli such as mechanical load. For example, muscle cyclin D1 mRNA increases in animal models of resistance exercise (RE) (17) or compensatory overload (2), which is consistent with the onset of and progression through G1 for a population of cells. In a model used to evaluate various rest intervals, Haddad and Adams (17) reported marked increases in cyclin D1 mRNA expression 16 h after two bouts of RE (separated by 48 h), with peak expression 24 h after the second bout. A trend for elevated expression was evident 12 h after a single bout. Recent findings in humans indicate that isometric contractions via electromyostimulation do not increase cyclin D1 mRNA expression (10). Cyclin B is important late in the cell cycle as it regulates progression through the G2-M boundary (mitosis) (3), making it an attractive target to study. Surprisingly, cyclin B has not received attention in studies of muscle myogenesis or satellite cell activation. Additionally, as yet there have been no animal or human reports of cyclin D1 mRNA increases within 0–24 h after mechanical load in rodents (2, 17) and in humans (10), and this finding has generally been viewed as an index of enhanced cellular differentiation. One inhibitor, p27kip, has been implicated 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-1, a potent mitogen (36). To our knowledge, the expression of p27kip and its response to mechanical load have not been studied in humans.

The myogenic regulatory factor (MRF) family of basic helix-loop-helix transcription factors regulates the expression of muscle-specific genes and is involved early in differentiation or commitment of muscle precursor cells to the myogenic lineage (MyoD, myf-5), as well as in the terminal differentiation of these cells (myogenin, myf-6). Load-mediated increases in MRF expression have been shown in young adult humans (10, 20, 33) and in animals (2, 13, 17, 26). In animal models, however, these increases are blunted in aged muscle (27, 37). Increases in MRF mRNAs in humans generally peak within the first 6–24 h after loading and have been detected as early as 0 h (MyoD), 2 h (myf-6), and 6 h (myogenin) after loading (33). Hespel et al. (20) studied MRF proteins in a group of young adults before and after exercise training and found increases in myogenin and myf-6 after 10 wk of training, but the precise time interval between the final loading bout and muscle biopsy was not reported (the methods state “at least 48 h”). There have been no studies designed to test the effects of age and gender on basal or on load-mediated cell cycle activity and MRF protein expression.

Findings in both human and animal models indicate that older muscle may be under heightened stress in the basal state and may, therefore, be less responsive to the stress of acute mechanical load. Jozsi et al. (22) reported higher basal expression levels of genes associated with stress or damage [e.g., heat shock protein 27, natural killer cell enhancing factor, mitogen-activated protein (MAP) kinase kinase 3, thymosin β10] in older men vs. younger men and further demonstrated that RE-mediated gene expression was impaired in the older men. Additional findings at the protein level indicate heightened activity of the MAP kinase signaling pathway in resting muscles of older vs. younger men (i.e., greater phosphorylation state of several intermediates), but a blunted phosphorylation response to RE (41). In rodent models, higher gene expression levels of myogenin (28, 30), MyoD (28, 30), and myf-6 (28) have been noted in resting senescent muscle. We suggest that these age differences in resting muscle may be the result of an ongoing compensatory effort to maintain muscle mass and phenotype in the face of stress associated with gradual denervation and deterioration.

The three primary objectives of this study were to 1) test the hypothesis that protein expression of MRFs (myogenin, MyoD, myf-6) and cyclins (D1 and B1) would be elevated 24 h after a bout of heavy RE, suggesting early activation of load-induced myogenesis; 2) test the hypothesis that these “myogenic” responses to RE would be influenced by age and gender, with blunted responses in older subjects expected to be revealed primarily in older women; and 3) test the hypothesis that basal (pre-RE) expression of factors regulating terminal differentiation of satellite cells (myogenin, myf-6) and general cell cycle arrest (p27kip) would be higher in older adults, suggesting a blunted proliferative potential. As a secondary objective, we have provided a phenotypic characterization of sarcopenia in our subjects based on lean mass (LM), thigh muscle mass, serum anabolic hormone levels, and specific strength.

**METHODS**

**Subjects.** Forty-two adults were recruited from the Birmingham, Alabama metropolitan area into two age groups. Age ranges were 60–75 yr for the older group [11 men (OM); 9 women (females, OF)] and 20–35 yr for the younger group [11 men (YM); 11 women (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 (body mass index <30), and none of the subjects had leg resistance training experience within the past 5 yr. Of the postmenopausal OF, four of nine women were on estrogen replacement therapy; however, this has been shown not to influence single myofiber function (40) or resistance training adaptation (16). 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 and the Birmingham Veterans Affairs Medical Center. Written, informed consent was obtained before participation in the research.

**RE stimulus.** After a fasted morning blood draw and vastus lateralis muscle biopsy, subjects attended five successive visits to the laboratory on alternate days. The sessions were defined as follows: 1) an introduction and familiarization to the bilateral REs selected to overload the vastus lateralis (squat, leg press, knee extension); 2) a second familiarization session, including practice strength tests to familiarize the subjects with our one-repetition maximum (1-RM) test protocol; 3) 1-RM assessment followed by 1 set of 8–12 repetitions of each exercise performed at 70% of 1 RM (1 RM was defined by two failed attempts at a given load); 4) two sets of 8–12 repetitions at 75% of 1 RM; and 5) three sets of 8–12 repetitions at 80% of 1 RM. 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 REs. Each session was supervised by certified laboratory personnel, and each exercise session began with a 5-min warm-up on either a bicycle ergometer or treadmill at a low intensity.

**Body composition.** Thigh LM (TLM), total body LM, and body fat percentage were determined by dual-energy X-ray absorptiometry by using a Lunar Prodigy (model no. 8743, GE Lunar, Madison, WI). Analyses were conducted according to manufacturer’s instructions with the use of enCORE 2002 software (version 6.10.029). For age and gender comparisons, 1-RM strength results were adjusted for TLM to yield estimates of specific strength. The TLM-to-total body LM ratio was tested as an index of preferential lower limb atrophy in older adults. This ratio is defined as relative TLM.

**Tissue collection.** Fasted morning blood and muscle samples were collected in the Pittman General Clinical Research Center at the University of Alabama at Birmingham. Ten milliliters of blood were withdrawn from an antecubital vein, and serum was aliquoted and frozen at −80°C. Muscle tissue was isolated under local anesthesia (1% lidocaine) from *m. vastus lateralis* of the left leg by percutaneous needle biopsy by using a 5-mm Bergstrom biopsy needle under suction, as previously described (15). To avoid any residual effects (on muscle damage or myogenic protein expression) of the initial biopsy taken from the left leg, the postexercise biopsy was taken from the right leg 24 h after the bilateral loading bout. Samples were quickly frozen, dissected, and snap frozen in liquid nitrogen. All samples were stored at −80°C until analysis.

**Muscle protein immunoblotting.** Frozen muscle samples (range 28–38 mg) were powdered by using a liquid nitrogen-cooled mortar and pestle and homogenized in 3 μl/mg muscle of ice-cold lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 100 μM PMSF, and 0.5× protease inhibitor cocktail containing bestatin, leupeptin, and aprotonin (P2714, Sigma-Aldrich, St. Louis, MO)]. After two centrifugation steps (15,000 g for 20 min at 4°C), supernatant samples were assayed for total protein by using the bicinchoninic acid technique with BSA as a standard, as our laboratory has described previously (6). Twenty micrograms (for MyoD and myf-6), 30 μg (for actin, myogenin, and p27kip), or 40 μg (for cyclins D1 and B1) of total protein diluted in Laemmli buffer were separated by SDS-PAGE (120-V constant) in 4–20% gradient or 12% minigels (Bio-Rad MP3, Bio-Rad Laboratories, Hercules, CA). Proteins were transferred at constant current (1 mA per cm2 × 30 min) to PVDF membranes by using a semidy transfer cell (Trans-Blot SD, Bio-Rad Laboratories). Within subjects, pre- and postexercise samples were loaded in adjacent lanes. To control for age or gender bias, each gel contained pre-post paired samples for one subject per group (i.e., YM, YW, OM, OW) with the different subject groups loaded in random order on each gel. Equal loading across lanes and equal transfer were verified by staining all gels (after transfer) with Coomassie blue and staining randomly selected membranes with Ponczoe S.

Before immunoblotting of study samples, primary antibody specificities and target antigen migration patterns (i.e., molecular weights) were confirmed in control experiments by using recombinant proteins, lysate from cultured rat myoblasts, and human muscle lysates from surgical samples blotted adjacent to a biotinylated protein ladder. Immunoblotting was carried out by using mouse monoclonal antibodies against muscle-specific α-actin (1:1,000, NCL-MSA), cyclin D1 (1:1,000, sc-8396), and cyclin B1 (1:1,000, sc-245) and rabbit polyclonal antibodies against MyoD (1:1,000, sc-760), myogenin (1:1,000, sc-576), myf-6 (1:1,000, sc-301), and p27 (1:2,500, sc-528). In control experiments, multiple secondary antibodies were evaluated, and blocking buffers were optimized to minimize nonspecific binding of the secondary antibodies selected. Membranes treated with mouse monoclonal antibodies were blocked with 10% goat serum in PBST (PBS and 0.1% Tween-20), whereas primary and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary (1:50,000) antibodies were diluted in 1% goat serum in PBST. Membranes treated with rabbit polyclonal primary antibodies were blocked with 2% BSA and 2% milk in PBST, whereas primary and HRP-conjugated goat anti-rabbit secondary (1:50,000) antibodies were diluted in 0.5% BSA and 0.5% milk in PBST.

Membranes were blocked overnight at 4°C under gentle agitation followed by incubation in primary and secondary antibodies for 1 h each at room temperature, with a PBST rinse protocol (1 quick rinse, 1 × 15 min, 2 × 5 min) following each treatment. The sample lane on the membrane containing the biotinylated protein ladder was marked before transfer, cut away after transfer, and incubated in avidin-HRP (1:50,000) for 1 h at room temperature. All bands were visualized by chemiluminescence (Super West Dura kit, Pierce Biotechnologies, Rockford, IL) in a Bio-Rad ChemiDoc imaging system, and band densitometry was performed by using Bio-Rad Quantity One software. Parameters for image development in the ChemiDoc were consistent across all membranes when predefined saturation criteria were used for the charge-coupled device camera. The vertical strips containing biotinylated protein ladders were imaged separately as these develop rapidly (4–6 s). Both portions of a given membrane were imaged in the same vertical position inside the ChemiDoc system to accurately align target proteins with the molecular weight markers. This was accomplished by using a small rectangular tray secured inside the chamber. For each imaging session, serial imaging ceased at the first point of saturation on the developing image. This standardization, combined with equal distribution of the four age and gender groups across all membranes, enabled us to accurately test for age, gender, and loading effects.

**Serum hormone concentrations.** To characterize basal profiles of some key circulating anabolic and androgenic hormones, before any RE, we assayed serum for total IGF-I, IGF binding protein-3 (IGFBP-3), IGFBP-1, total testosterone, sex hormone binding globulin (SHBG), and androstenedione. Samples from subjects for a given hormone or binding protein were assayed in random order during a single run. IGF-I, IGFBP-3, IGFBP-1, and SHBG were assessed by immunoradiometric assays (Diagnostic Systems Laboratories, Webster, TX). The interassay coefficient of variation (CV), average intra-assay CV, and assay sensitivity for each hormone and binding protein measured by immunoradiometric assay were, respectively, as follows: IGF-I (4.04%, 3.20%, and 8.35 ng/ml), IGFBP-3 (3.29%, 4.66%, and 200 ng/ml), IGFBP-1 (8.77%, 10.49%, and 0.80 ng/ml), and SHBG (7.67%, 5.98%, and 6.50 nm). Total testosterone and androstenedione were determined by solid-phase radioimmunoassay (Diagnostic Systems Laboratories). For total testosterone, interassay CV was 6.07%, average intra-assay CV was 4.69%, and the average sensitivity (99% bound) was 0.45 ng/ml. For androstenedione, interassay CV was 7.71%, average intra-assay CV was 6.29%, and sensitivity was 0.10 ng/ml. Free testosterone levels were computed from total testosterone and SHBG by using an established formula (35).

**Data analysis.** Data are reported as means ± SE. Between-groups differences in preexercise descriptive variables were tested by using age × gender ANOVA. All variables measured before and after loading were analyzed by using age × gender × loading repeated-measures ANOVA. For each ANOVA model with a significant main or interaction effect, Tukey’s honestly significantly different tests were performed post hoc to localize the effect(s). To test whether age-related losses of muscle mass were related to circulating anabolic factors, zero-order correlations were tested between relative TLM and the serum factors that were determined to be continuous variables. A normal distribution was confirmed a priori for each variable tested in zero-order correlations.

For the various proteins assessed via immunoblotting before and after loading, zero-order correlations were tested among the change scores within subjects for these proteins. This secondary analysis was performed in an effort to potentially identify “responders” (i.e., subjects with larger change scores, suggesting a response to loading) and “nonresponders” (i.e., subjects who showed little to no change in
protein expression profiles after loading). Statistical significance was accepted at \( P \leq 0.05 \) for all tests.

**RESULTS**

Descriptive characteristics are shown in Table 1. In Figs. 1 and 2 and throughout the remaining text, the four age and gender groups are defined as YF, YM, OF, and OM. Within each age group, men and women were of similar age. Typical gender differences in height and weight were found, as main gender effects \(( P < 0.001)\) confirmed that men were taller and weighed more than women. A lower body fat percentage in YM compared with OM \(( P < 0.001)\) led to an age \(\times\) gender interaction for body fat \(( P < 0.05)\). Young subjects had more total body LM than their older counterparts \(( P < 0.05)\), with the effect most notable in men. TLM was greater in young subjects, but an age \(\times\) gender interaction revealed that this difference was primarily driven by men, as TLM was 24% higher in YM vs. OM \(( P < 0.001)\) and only 12% higher in YF vs. OF. 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 \(( P < 0.01)\) and YM \(( P < 0.001)\) compared with their older, within-gender counterparts. Because skeletal muscle comprises the bulk of the LM compartment in the limbs, these data support the concept that atrophy with aging occurs more rapidly in the weight-bearing lower limb musculature than in the remaining LM compartment.

We estimated specific strength by adjusting 1-RM strength for TLM (Table 1. Dual-energy X-ray absorptiometry-determined TLM is the sum of all muscles of the thigh, the bulk of which are knee and hip extensors and flexors. The TLM adjustment negated substantial age differences in absolute strength (data not shown) for both the squat and leg press (during which both knee and hip extensors are active), suggesting that strength differences by age in these two closed-chain movements were mainly the result of muscle atrophy rather than age differences in factors intrinsic to the muscle (i.e., “muscle quality”). For the knee extension movement, however, adjusted strength remained higher \(( P < 0.001)\) in younger adults (15% in YF vs. OF, 33% in YM vs. OM). Because only the knee extensors are agonists during this movement, these findings may point toward an age-related difference in muscle quality specific to the quadriceps. The main gender effect \(( P < 0.01)\) for adjusted squat strength (higher in women) may be the result of body mass distribution, in that men distribute a larger proportion of total body mass above the waist than women. This would introduce a gender bias in favor of women as only bar weight (and not upper body weight) was considered in the squat strength indexes.

Table 2 summarizes age and gender differences in circulating anabolic hormones and their primary binding proteins. These serum factors were assessed to better characterize the phenotypes of the four age and gender groups. For this purpose, serum was collected under fasted, morning conditions in the basal state. Serum factor analysis at the 24-h postloading time point was deemed unnecessary, as we and others have shown that resistance loading does little to alter these hormones for an extended time period. Any changes noted are transient with restoration of baseline, typically within 15–120

**Table 1. Descriptive characteristics of each group**

<table>
<thead>
<tr>
<th></th>
<th>Young Women</th>
<th>Young Men</th>
<th>Older Women</th>
<th>Older Men</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Age, yr</td>
<td>26.2±1.1</td>
<td>26.5±1.2</td>
<td>63.6±1.0</td>
<td>64.5±1.4</td>
</tr>
<tr>
<td>Height,† cm</td>
<td>162.0±2.3</td>
<td>182.3±2.0</td>
<td>161.4±2.3</td>
<td>176.5±2.1</td>
</tr>
<tr>
<td>Weight,† kg</td>
<td>60.9±2.4</td>
<td>80.9±3.1</td>
<td>64.6±4.2</td>
<td>85.7±2.9</td>
</tr>
<tr>
<td>%Body fat,†‡</td>
<td>30.6±1.4</td>
<td>20.3±2.4</td>
<td>40.0±2.0</td>
<td>32.6±1.5</td>
</tr>
<tr>
<td>Total body LM,†‡ kg</td>
<td>36.9±1.5</td>
<td>61.4±1.3</td>
<td>36.5±1.7</td>
<td>55.0±1.8</td>
</tr>
<tr>
<td>Thigh LM,†‡ kg</td>
<td>8,814±444</td>
<td>15,504±414</td>
<td>7,873±416</td>
<td>12,514±476</td>
</tr>
<tr>
<td>Relative thigh LM,†‡</td>
<td>23.8±0.5</td>
<td>25.2±0.4</td>
<td>21.5±0.4</td>
<td>22.7±0.2</td>
</tr>
</tbody>
</table>

**Specific strength measurements [1 RM (kg)/thigh LM (kg)]**

<table>
<thead>
<tr>
<th></th>
<th>Young Women</th>
<th>Young Men</th>
<th>Older Women</th>
<th>Older Men</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knee extension*</td>
<td>4.17±0.24</td>
<td>4.15±0.14</td>
<td>3.62±0.12</td>
<td>3.11±0.20</td>
</tr>
<tr>
<td>Leg press</td>
<td>9.77±0.38</td>
<td>9.86±0.23</td>
<td>9.45±0.39</td>
<td>9.09±0.30</td>
</tr>
<tr>
<td>Squat†</td>
<td>6.76±0.31</td>
<td>5.86±0.31</td>
<td>6.41±0.20</td>
<td>5.62±0.31</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. LM, lean mass; 1 RM, one-repetition maximum strength. *Main age effect, \( P < 0.05 \). †Main gender effect, \( P < 0.05 \). ‡Age \(\times\) gender interaction, \( P < 0.05 \).

**Table 2. Profile of circulating anabolic hormones and their primary binding proteins**

<table>
<thead>
<tr>
<th></th>
<th>Young Women</th>
<th>Young Men</th>
<th>Older Women</th>
<th>Older Men</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>IGF-I,† ng/ml</td>
<td>326±33</td>
<td>385±43</td>
<td>170±20</td>
<td>170±21</td>
</tr>
<tr>
<td>IGFBP-3, ng/ml</td>
<td>4,480±187</td>
<td>4,461±299</td>
<td>4,532±191</td>
<td>4,076±134</td>
</tr>
<tr>
<td>IGFBP-1,† ng/ml</td>
<td>55.3±12.4</td>
<td>23.3±6.3</td>
<td>73.9±17.0</td>
<td>54.3±13.9</td>
</tr>
<tr>
<td>Total testosterone,†‡</td>
<td>22.5±4.0</td>
<td>259.7±49.1</td>
<td>32.3±11.5</td>
<td>460.0±28.6</td>
</tr>
<tr>
<td>SHBG,† µM</td>
<td>183±26</td>
<td>65±9</td>
<td>152±21</td>
<td>98±5</td>
</tr>
<tr>
<td>Free testosterone,†‡</td>
<td>4.7±0.8</td>
<td>316.6±28.3</td>
<td>6.2±2.9</td>
<td>156.6±10.4</td>
</tr>
<tr>
<td>Androstenedione, ng/ml</td>
<td>2.1±0.3</td>
<td>2.5±0.2</td>
<td>1.1±0.2</td>
<td>1.8±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. IGF-I, insulin-like growth factor I; IGFBP, IGF binding protein; SHBG, sex hormone binding globulin. *Main age effect, \( P < 0.05 \) (\( P = 0.06 \) for IGFBP-1). †Main gender effect, \( P < 0.05 \). ‡Age \(\times\) gender interaction, \( P < 0.05 \) (\( P = 0.069 \) for SHBG).
min (8, 18, 23). As expected, serum IGF-I was approximately twofold greater in younger adults \((P < 0.001)\), whereas its primary binding protein in circulation, IGFBP-3, did not differ by age or gender. Interestingly, IGFBP-1 levels were higher in women \((P = 0.05)\), and there was a strong trend toward higher IGFBP-1 in the older subjects \((P = 0.06)\). These combined data suggest that circulating free IGF-I levels were highest in YM and lowest in OF. An age \(\times\) gender interaction for total testosterone \((P < 0.05)\) was driven by 30% higher total testosterone in YM vs. OM \((P < 0.01)\), with no significant differences between YF and OF. For SHBG, only a gender difference was found, with higher levels noted in women \((P < 0.001)\). However, \(~50\%\) higher levels of SHBG in OM vs. YM led to a trend toward age \(\times\) gender interaction in SHBG \((P = 0.069)\). The lower levels of total testosterone and the trend toward higher levels of SHBG in OM vs. YM were perhaps best demonstrated in the computed free testosterone data. Age \((P < 0.001)\) and age \(\times\) gender \((P < 0.001)\) effects on free testosterone were primarily the result of a twofold higher level in YM vs. OM. No age or gender differences were noted for androstenedione.

Correlational analyses suggest a role for some of these circulating factors in the constitutive maintenance of muscle mass with aging, as relative TLM was positively related to IGF-I \((r = 0.57, P < 0.001)\) and androstenedione \((r = 0.41, P = 0.01)\) and inversely related to IGFBP-1 \((r = -0.50, P = 0.001)\). Because total and free testosterone are obviously not continuous variables across gender, correlations between these and relative TLM were tested in men only. Relative TLM was significantly related to free testosterone in men \((r = 0.60, P = 0.005)\).

The results of protein immunoblotting studies are shown in Figs. 1 and 2. In general, the selected proteins of interest showed a remarkable degree of stability both within and between groups, as few differences were noted between age groups or genders, and only one significant change with loading was found. Figure 1 displays immunoblot results for the MRFs studied, as well as for muscle-specific \(\alpha\)-actin. No load-mediated changes in the levels of these proteins were detected at the 24-h time point. A significant aging effect was found for myogenin (Fig. 1A), which was 44\% higher in older adults \((P < 0.05)\), with no differences by gender. No significant effects were found for myf-6 (Fig. 1B) or MyoD (Fig. 1C). Levels of \(\alpha\)-actin (Fig. 1D) were likewise unaffected by age, gender, or load.

Of the MRFs studied, the concentrations of myogenin and MyoD were relatively stable within groups, whereas myf-6 showed a fair amount of variability within groups. This was particularly evident in older adults, as two older subjects showed no myf-6 expression (myf-6 was detected in all pre- and postloading samples in the 22 younger subjects). It is unlikely that myf-6 protein was present in these older adult samples at a concentration below the sensitivity of our detection system, as we have detected recombinant proteins diluted into the low-picogram range with the chemiluminescent detection system used (Super West Dura kit, Pierce Biotechnologies, Rockford, IL). In one OM, myf-6 was not detected at all before or after loading. Repeat blots confirmed this finding. Expression of the other MRFs was not impaired in this subject, as MyoD levels were above the means of the OM group both before \((+1.67 \text{ SD})\) and after \((+0.92 \text{ SD})\) loading, and myogenin content in this subject was close to the OM group mean.

\[\text{Fig. 1. Immunoblot results for myogenin (A), myf-6 (B), MyoD (C), and \(\alpha\)-actin (D).}\]

The blots above the histograms display an example of pre- and postloading protein levels for an individual subject within each group (young males (YM), young females (YF), older males (OM), and older females (OF)) run on the same gel. The order of samples on the blots corresponds with the order of bars in the histograms. Right lane on each blot is the corresponding biotinylated protein ladder run with each gel and imaged with the blot by using chemiluminescent detection. Values are means \(\pm\) SE. *Myogenin was significantly higher in older adults, \(P < 0.05\). Myf-6 tended to be higher in OF compared with YF, \(P = 0.059\). No effects of gender or loading were found.
both before (−0.00 SD) and after (−0.33 SD) loading. In one OF, myf-6 was undetectable before loading, whereas a fair amount of myf-6 was found after loading (−0.89 SD compared with OF group mean), suggesting de novo synthesis of myf-6 after loading in this subject. Myogenin and MyoD levels in this OF were within ±1 SD of the OF group means both before and after loading. With the exception of this single OF, myf-6 levels were generally high in OF relative to the other groups, as evidenced by the representative blot and the mean data in Fig. 1B.

Immunoblot results for two key cell cycle proteins and the CDK inhibitory protein p27kip are shown in Fig. 2. Cyclin D1 (Fig. 2A) expression tended to be higher in older adults (P = 0.069), but no statistically significant effects of age or gender were found for either cyclin D1 or cyclin B1 (Fig. 2B). Furthermore, cyclin expression was not altered 24 h after loading. For p27kip, a significant gender × load interaction was found for p27kip, which decreased in women after the loading bout, P < 0.05. No effects of age were found.

**DISCUSSION**

In this investigation, our first aim was to test whether a bout of heavy RE would lead to changes in muscle protein concentrations of MRFs and cell cycle proteins, and our second aim was to determine whether age or gender influenced these responses to loading. Subjects completed a RE protocol proven to induce myofiber hypertrophy when performed 2–3 days/wk for several weeks. It is important to reiterate that, by design (see METHODS), this bout of heavy RE was preceded by four prior exposures to the exercises, during which both workload and the number of contractions increased serially, leading up to the first “full” exercise bout. With this regimen, we met the aim of evaluating the acute responses to heavy loading while also facilitating any early summative effects that have been shown to occur (17).

We evaluated three transcription factors known to be important in myogenic processes (myogenin, myf-6, MyoD) and found no significant changes in any of these proteins 24 h after loading. These novel data document for the first time age- and gender-specific MRF protein levels in humans at rest and 24 h after mechanical loading. Acute load-induced changes in MRF mRNAs (10, 33) and proteins (42) have been evaluated sparingly in humans, and no age or gender effects have been previously tested. Although we certainly recognize that mRNA levels and protein levels do not follow a direct 1:1 relationship, studies at the protein level are severely lacking; thus some discussion of studies at the mRNA level is obligatory. One time course study in young men reported increases in MRF mRNAs detected as early as 0 h (MyoD), 2 h (myf-6), and 6 h (myogenin) after a single bout of RE (33). Additionally, Bickel et al. (10) reported a nearly threefold increase in myogenin mRNA 24 h following two bouts of electromyostimulation. In rats, Peters et al. (32) reported marked increases in MyoD and myogenin mRNAs only 3 h after a single bout of eccentric loading, and others have found increased myogenin mRNA within 16 h of two successive RE bouts (17). From these studies, it is apparent that MRFs are rapidly transcribed fol-
lowing a loading bout. Whether the gene products led to increased protein translation cannot be determined from these studies.

At first glance, we suspected that 24 h may have been too early to detect any groupwide changes in MRF protein expression. The work of Willoughby and Nelson (42) is, to our knowledge, the only other evaluation of acute protein-level MRF responses to a single loading bout in humans. Using a RE bout essentially identical to ours, they report remarkably rapid increases in both myogenin and MyoD protein (via ELISA) only 0.5 and 6 h after the loading stimulus in a group of seven young men. Concurrent increases in myogenin and MyoD mRNAs were also noted. Based on the rapid rates of rise, it is unlikely that the increases in MRF protein resulted from increased translation. However, considering these data and the recent evidence supporting summation of mRNA responses over repeat exercise bouts (17), we were surprised to not find any increases in MRF protein in our model, particularly in young men.

We hypothesized that load-induced increases in MRF protein expression would be attenuated in older subjects. This expectation was based on numerous lines of evidence in animal models, although most of this prior work focused on MRF mRNAs rather than protein levels. Expression of MyoD protein and mRNA has been shown to be blunted in old rats vs. younger rats after a single bout of RE (37). Furthermore, the increases in MRF mRNA expression consequent to stretch overload, a proven hypertrophy stimulus in the quail model, are attenuated in fast-twitch muscles of older quails (27). In a study of muscle regeneration (following bupivacaine injection), Marsh et al. (28) found that the increases in myogenin mRNA early in the regeneration period were significantly less in old rats compared with younger animals. Also noteworthy was a prolonged elevated expression of both myogenin and MyoD transcripts in the old rats, perhaps suggesting a slower myogenic process during recovery. These data combined support our hypothesis that load-induced MRF protein expression would be blunted in older adults. However, we were unable to support this hypothesis as no significant changes in MRF protein levels were found in either age group after the loading stimulus. Clearly a limitation of this study is the lack of transcript level analyses. As a result, we do not know whether mRNA levels had increased as expected.

As markers of mitotic activity, we immunobotted for cyclin D1, which is integral to cell cycle initiation, and cyclin B1 as an index of late cell cycle activity (i.e., progression through the G2-M boundary). Previous findings of enhanced cyclin D1 mRNA expression in skeletal muscle following loading have been interpreted as indicative of increased proliferative activity in a population of cells within the muscle, which could be satellite cells or nonmuscle cells (e.g., cells to support adaptive responses in other tissues such as connective tissue) (17). We found a significant correlation between the basal concentrations of these two cyclins ($r = 0.44$, $P = 0.005$), indicating similar levels of cyclin expression within subjects. The likelihood of detecting a load-induced change in cell cycle activity was thus probably enhanced by analyzing both of these proteins. While cyclin D1 mRNA increases substantially in rats after two bouts of resistance loading (17) and after 24 h of compensatory overload (2), a recent report in humans described a trend but no significant increase in the cyclin D1 transcript following electromotystimulation (10). We found no changes in cyclin D1 or B1 protein after a resistance loading bout. Because Haddad and Adams (17) found a minimal increase in cyclin D1 mRNA after a single bout but a more than twofold increase after two successive bouts, we hypothesized that our model of progressive loading during four successive sessions leading up to a full overload stimulus would induce an increase in cyclin protein expression. Although the time point of muscle biopsy (24 h after loading) may not have been ideal, it is also possible, if not probable, that voluntary RE in humans (even when prescribed at a relatively high intensity) does not impose a loading stimulus nearly as intense as various overload models in rats (2, 17).

Of the proteins studied, only the CDK inhibitory protein p27kip showed a dynamic change with loading, as p27kip concentrations declined in women but not in men. This decline was driven primarily by a rather large drop in older women. Spangenburg et al. (36) have 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. The inhibitory influence of p27kip on myogenic cells has also been demonstrated in vivo in developing muscle, as knockout of the p27kip gene leads to markedly enhanced gastrocnemius muscle mass development (25). A load-induced reduction in p27kip in our older women suggests that mechanical load may provide one means of overcoming the inhibitory influence of p27kip.

Sarcopenia is characterized by a gradual loss of myofibers and preferential type II myofiber atrophy. Reduced myofiber number may result from an impaired ability to repair damaged myofibers (i.e., necrosis) and/or apoptosis, and these two processes may occur simultaneously in aging muscle. Because the recovery time course following contraction-induced damage is prolonged in aging muscle (12), it is certainly possible that a reduced capacity for satellite cell activation with age (11) could increase susceptibility to irreparable myofiber damage and consequent necrosis. Based on this concept, our third aim was to test the hypothesis that basal (pre-RE) expression of factors regulating terminal differentiation of satellite cells (myogenin, myf-6) and general cell cycle arrest (p27kip) would be higher in older adults, suggesting a blunted proliferative potential. We found substantially higher levels of myogenin in the muscles of older adults (44%). This novel finding is supported at the mRNA level by studies in rats (4, 28) and mice (30). Whereas Alway et al. (4) found markedly elevated MRF mRNAs in aged rat muscles, protein levels were equal to or lower than those found in young animals. The dichotomy appeared to result from elevated Id repressor levels in the older muscles (4). Although purely speculative, the protein level differences between our myogenin data and those of Alway et al. could potentially be related to relative age differences. The 37-mo old rats studied by Alway et al. were relatively older and more sarcopenic (~50% lower muscle wet weight/body wt) than our 64-yr-old human subjects. It is possible that posttranscriptional inhibition of the MRFs occurs later in the aging time course than MRF upregulation.

Additional findings in humans support the idea that older muscle may be under heightened stress in the basal state but may be less responsive to the stress of acute mechanical load.

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Higher basal expression levels of genes associated with stress or damage (22) and heightened activity of the MAP kinase signaling pathway (i.e., greater phosphorylation state of several intermediates) (41) have been documented in resting muscles of older vs. younger men. These studies further demonstrated that RE-mediated responses were impaired in the older men. We suggest that these age differences in resting muscle may be consequent to a failing compensatory effort to maintain muscle mass and phenotype in the face of gradual denervation and deterioration.

In this study, we found higher basal expression of only myogenin in older muscle, whereas mRNA studies using rodent models have shown heightened levels of myogenin (28, 30), MyoD (28, 30), and myf-6 (28) mRNAs in the older animals. The mean age of the subjects in our older group was ~64 yr. This may have been too early in the aging time course to detect substantially elevated levels of multiple MRFs, which might be expected in frail elderly or in senescent muscle. In light of this, we consider the substantially elevated levels of myogenin in both OM and OF quite remarkable. Myogenin may be preferentially expressed in type I myofibers (29). In preliminary experiments using rodent muscles with extreme fiber-type differences, we noted remarkably higher concentrations of myogenin in soleus compared with plantaris in young adult rats (unpublished observation). One might argue that the elevated myogenin in the older muscles of the present study was, in part, a reflection of the well-known type II atrophy found in sarcopenic muscles, leading to a shift in the overall phenotype toward type I muscle. The phenomenon of denervation-reinnervation in aging muscle, which leads to myofiber-type grouping, could also increase the percentage of myofibers containing type I myosin (5, 24). To examine this possibility, we assessed (via immunoblot) in a subset of these subjects (10 YM and 8 OM) the relative quantities of protein isoforms differentially expressed in type I and type II myofibers. Skeletal muscle expresses isoforms of sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA), which are predominately, if not exclusively, found in type I (SERCA2a) and type II (SERCA1) myofibers. We found no significant age differences in the relative amounts of these isoforms (data not shown). We therefore suggest that the heightened myogenin protein levels found in older muscle are not driven by myofiber-type differences and are more likely indicative of a compensatory effort during an ongoing remodeling process in aging muscle. Although no significant group differences were noted in myf-6 levels, myf-6 expression was generally high in OF compared with the other groups. Excluding one OF with no detectable basal expression revealed 95% more myf-6 in OF compared with YF (P = 0.059). No such trend was found in men, suggesting a gender-dependent increase in myf-6 expression in aged muscle.

In summary, this study was designed to compare protein expression levels of myogenic markers in YM and YF vs. sarcopenic older adults before and after a resistance-loading stimulus. To our knowledge, this is the first test of potential age and gender differences in myogenic markers and the first study to evaluate the influence of mechanical loading on acute protein-level responses in YM, OM, YF, and OF. We characterized the sarcopenic phenotype in these older adults by confirming deficits in thigh muscle mass relative to whole body LM, specific strength, and serum anabolic hormone status. In the vastus lateralis, OM and OF expressed more myogenin than the younger subjects, and OF tended to express more myf-6 and p27Kip1 than the other groups. On the basis of these findings, we suggest that older muscles, and particularly those of OF, may have a blunted capacity for cellular proliferation. The loading bout did little to alter expression of the proteins studied. Although the time point of postexercise muscle biopsy (24 h) may not have been optimal to capture maximal effects at the protein level, a significant decline in p27Kip1 concentration was found in OF after the exercise bout. This finding suggests that mechanical load may provide one means of overcoming the inhibitory influence of p27Kip1. Future studies of transcript-level analyses and additional time points are needed to further explore the potential interactions of age and gender on load-mediated myogenic activity.

ACKNOWLEDGMENTS

We thank the subjects for their tireless effort and dedication. We thank S. Hall for assistance with data collection and analysis.

GRANTS

Grant support for this work was provided by National Institute on Aging Grant RO1 AG-17896 (M. M. Bamman) and General Clinical Research Center Grant M01 RR-00032.

REFERENCES


MYOGENIC PROTEIN EXPRESSION AFTER LOADING