Postexercise Myostatin and Activin IIb mRNA Levels: Effects of Strength Training

JUHA J. HULMI[1], JUHA P. AHTIAINEN[1], TUOMAS KAASALAINEN[3], EIJA PÖLLÄNEN[2,4], KEIJO HÄKKINEN[1], MARKKU ALEN[2], HARRI SELÄNNE[5], VUOKKO KOVANEN[2,4], and ANTTI A. MERO[1]

[1]Department of Biology of Physical Activity and Neuromuscular Research Center, [2]Department of Health Sciences, [3]Department of Chemistry, and [4]Finnish Centre for Interdisciplinary Gerontology, University of Jyväskylä, Jyväskylä, FINLAND; and [5]LIKES Research Center, Jyväskylä, FINLAND

ABSTRACT

HULMI, J. J., J. P. AHTIAINEN, T. KAASALAINEN, E. PÖLLÄNEN, K. HÄKKINEN, M. ALEN, H. SELÄNNE, V. KOVANEN, and A. A. MERO. Postexercise Myostatin and Activin IIb mRNA Levels: Effects of Strength Training. *Med. Sci. Sports Exerc.*, Vol. 39, No. 2, pp. 289–297, 2007. **Purpose:** Muscle hypertrophy is likely to result from the cumulative effects of repeated bouts of resistance exercise (RE) on postexercise molecular responses. Therefore, we determined muscle growth- and regeneration-related mRNA expression in response to a single RE bout both before and after a strength-training (ST) period. By means of this novel longitudinal setting, we examined whether postexercise gene expression at the transcriptional level is different in the trained and untrained state. **Methods:** Eleven untrained healthy older men and 11 controls (age 62.3 ± 6.3 yr) volunteered as subjects. Muscle biopsies from the vastus lateralis muscle were taken at rest and 1 and 48 h after five sets of 10-repetition leg press RE both before and after 21 wk of supervised ST. **Results:** Myostatin and myogenin mRNA expression, determined by real-time RT-PCR, increased ($P < 0.05$) after ST. Conversely, the single RE bout decreased myostatin mRNA after ST, with the decrease showing a negative correlation ($r = -0.65$, $P < 0.05$) with the long-term increase in myostatin during ST. Furthermore, RE before ST increased myogenin mRNA ($P < 0.05$) and tended to increase after ST ($P = 0.08$). Myostatin receptor activin IIb mRNA levels were decreased at 1 h after RE in the pre-ST condition ($P = 0.05$) and also tended to decrease in the post-ST condition ($P = 0.07$). RE-induced downregulation in myostatin mRNA correlated with the ST-induced increase in total body muscle mass ($r = -0.82$, $P = 0.002$). **Conclusions:** A single bout of RE in older men can downregulate the expression of myostatin receptor activin IIb mRNA. ST influences the response of myostatin to RE, as short-term RE-induced downregulation of myostatin was observed only after ST. The results also indicate that RE-induced alterations in myostatin mRNA expression may have a role in ST-induced muscle hypertrophy. **Key Words:** GENE EXPRESSION, RESISTANCE EXERCISE, MUSCLE HYPERTROPHY, MYOGENIN, MYOD

Aging is related to a gradual loss of skeletal muscle mass, strength, and power, a condition called sarcopenia. Consequently, older people are predisposed to different disabilities, increased incidence of falls, and decreased capacity to perform daily tasks (8). Importantly, in older adults, repeated resistance exercise has well-established beneficial effects on body composition and physical fitness (10,11) and on the capacity to perform daily tasks of living (11). These training adaptations are likely to result from the cumulative effects of repeated bouts of resistance exercise (RE) on postexercise molecular responses (9).

Address for correspondence: Juha J. Hulmi, Department of Biology of Physical, Activity University of Jyväskylä, Rautpohjankatu 8, Viveca, 40700 Jyväskylä, Finland; E-mail: Juha.Hulmi@Sport.jyu.fi.
Submitted for publication April 2006.
Accepted for publication August 2006.

0195-9131/07/3902-0289/0
MEDICINE & SCIENCE IN SPORTS & EXERCISE®

DOI: 10.1249/01.mss.0000241650.15006.6e

One of the hypothetical pathways by which repeated RE-induced short-term molecular responses may eventually lead to muscle hypertrophy involves gene regulation via the myostatin pathway (13). Myostatin, a member of the TGF-β superfamily, is a negative regulator of muscle mass (20,26). Strength training (ST)-induced muscle hypertrophy involves satellite cell proliferation, differentiation, and fusion with existing myofibers, which is important for the maintenance of adequate nuclear/cytoplasmic ratio during muscle growth (1). Myostatin seems to work in adult muscle in part by inhibiting satellite cell proliferation and differentiation (20). Myostatin probably acts in this process by downregulating the family members of myogenic regulatory factors MyoD and myogenin (24) as well as cyclins and cyclin-dependent kinases (cdk) and by increasing the expressions of cdk inhibitor $p21^{cip}$ and, possibly, also $p27^{kip}$ (13,31), all these affecting satellite cell cycle regulation (24,31). Myostatin acts by binding to and signaling through the activin IIb receptor (AcvrIIb) (16) and is inhibited in part by the follistatin-related gene (FLRG) protein and myostatin propeptide (12).

Gene expression levels of the muscle growth- and regeneration-related factors myostatin, myogenin, MyoD, and $p27^{kip}$ have been shown to be responsive both to a

single bout of RE (7,13,22,23) and to long-term ST in humans (25,29,30). However, to our knowledge, no mRNA-level studies investigating the responses of myostatin receptor AcvrIIb and binding protein FLRG to a single exercise session or to long-term training have been published. Consequently, in the present study, we investigated the expressions of myostatin, AcvrIIb, FLRG, $P27^{kip}$, MyoD, and myogenin at transcript levels in response to a) a single heavy bout of RE (which is known to induce myofiber hypertrophy) and b) long-term ST. Most importantly, we were interested in the specific effects ST has on gene expression responses to a single bout of RE in older men. Previously, it has been shown in our laboratory that ST has an influence on the RE-induced short-term growth hormone response (10), showing that short-term physiological responses to RE are affected by previous training experiences. The same phenomenon has also been shown recently at the total muscle protein–synthesis level (14). Moreover, very recent studies comparing people with different training backgrounds in a *cross-sectional* setting show that short-term molecular responses to RE may also change depending on the previous type of training background (6,7). However, to the best of our knowledge, no *longitudinal* human studies are available in which this phenomenon has been investigated at the level of gene expression. In the present study, we addressed this issue by investigating short-term post-RE responses both before and after 21 wk of ST. We hypothesized that ST would enhance the short-term responses of specific muscle hypertrophy and regeneration factors to a single bout of RE at the mRNA level in older men.

METHODS

Subjects. Twenty-two men recruited from a larger study (Ahtiainen et al., unpublished data, 2006) were randomly assigned to either an ST group (N = 11) or a control group (N = 11). The subjects' characteristics (N = 20, not including two dropouts from the control group; mean ± SD) were age, 62.3 ± 6.3 yr; height, 174.4 ± 5.6 cm; and body mass, 78.2 ± 9.8 kg. All the subjects were screened by a medical doctor. Exclusion criteria included the following: 1) age under 50 or over 77, 2) those who had engaged in moderate to heavy resistance ST within the past 5 yr, 3) vegetarians, 4) those who had regularly ingested nutritional supplements or pharmacological substances that might affect the responses, 5) elite veteran athletes, 6) obesity (body mass index > 30), and 7) neuromuscular and cardiovascular diseases. The subjects were moderately active. In the last few years they had taken part in various low-intensity physical activities such as walking, bicycling, and swimming. The subjects were carefully informed about the design of the study with special information on the possible risks and discomfort that might result. Thereafter, the subjects signed a written informed consent to participate in the study, which had been approved by the ethics committee of the University of Jyväskylä, Finland. The study was conducted according to the Declaration of Helsinki.

Experimental design. The total duration of the study was 24 wk, of which the first 3 wk were a control period in which no experimental ST was carried out and during which the subjects continued their normal recreational activities. After the pre-ST test period, the subjects were randomly assigned to either the ST group or the control group. The controls had no ST but continued with their habitual levels of activity. The supervised ST period was 21 wk in duration.

Heavy RE protocol. The heavy RE bout before and after the ST period (Fig. 1) consisted of a bilateral leg press exercise on a machine (David 210, David Fitness and Medical). The RE bout started with a 2-min warm-up on a cycle ergometer. Thereafter, in the actual leg press RE, subjects started from the flexed-knee position (70°), extended the knees concentrically to full extension (180°), and then lowered the load eccentrically back to the starting position. The total number of sets was five (5 × 10-repetition maximum), with a 2-min recovery period between each set. The loads were adjusted during the course of the session so that each subject would be able to perform 10 repetitions per set. If the load was too heavy, the subject was provided assistance during the last repetitions of the set. In these assisted repetitions, the assistant tried to maintain the same velocity of movement and, thus, contraction time as in the first repetitions of the set. The same assistant was in attendance at every RE performed in the present study. Bilateral isometric maximal force was measured before and after each set with a David 200 electromechanical dynamometer at a hip angle of 110° and knee angle of 107°, in accordance with the previous studies in our laboratory (10,11). The RE was performed at the same time of day for each subject both before and after ST. Subjects were asked to rate their perceived exertion (RPE) using Borg's subjective scale (6–20) after the RE bout, both before and after ST.

Experimental ST. During the 21-wk ST period, RE sessions were carried out twice a week. A minimum of 2 d of rest was required between the two sessions each week. All training sessions were supervised by the authors or assistant researchers. The following exercises were used in each training session: two exercises for the leg extensor muscles, the bilateral leg press and bilateral knee extension; and one exercise for the leg flexors, the bilateral knee flexion. The ST program also included exercises for the other main muscle groups of the body: chest and shoulders, upper back, trunk extensors and flexors, upper arms, ankle extensors, and hip abductors and adductors. ST was performed with progressive training loads of 40–85% of each subject's 1RM. The number of sets of each RE performed increased (from 2–3 to 3–5) and the number of repetitions in each set decreased (15–20 to 5–6) during the 21-wk ST period. In the all exercises, the loads were individually determined during the training sessions throughout the ST period.

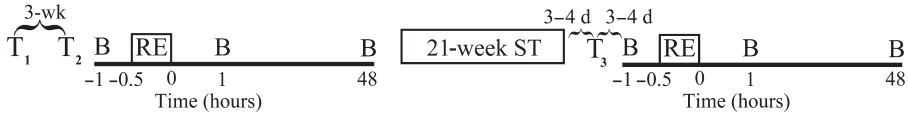

FIGURE 1—Experimental design. RE, heavy resistance exercise (five sets of 10-repetition leg press exercise); ST, heavy strength-training period; B, vastus lateralis muscle biopsy obtained before and 1 and 48 h after RE; T, strength and anthropometric tests (T_1, before control period; T_2, after control period; T_3, after 21 wk of ST).

Nutrition before and after RE protocol. The subjects had written instructions to eat similarly for 3 d before, during, and for 2 d after the RE day, both before and after ST. The subjects fasted for 3 h before the first biopsy, both before and after the ST program. Subjects were instructed to follow their normal diet during the ST period. Dietary intake was obtained from food diaries registered for 5 d preceding the 48-h biopsy and were analyzed using Micro Nutrica nutrient-analysis software, version 3.11 (The Social Insurance Institution of Finland).

Maximal force testing. The force signal was recorded and analyzed with Signal software, version 2.15 (Cambridge Electronic Design Ltd., Cambridge, UK). Maximal isometric force of the bilateral leg extensor muscles was measured on an electromechanical dynamometer with a hip angle of 110° and knee angle of 107°, in accordance with procedures used in previous studies (10,11). In the testing of the concentric one-repetition maximum (RM), separate trials were performed. After each repetition, the load was increased until the subject was unable to extend his legs to the fully extended (~180°) knee angle position. The highest acceptable load was determined as the 1RM.

Anthropometry. After an overnight fast, the body fat percentage and amount of total body muscle mass (kg) were measured by bioelectrical impedance using an Inbody720 machine with a multifrequency current (Seoul, Korea). Bioelectrical impedance was measured twice in each subject before ST, and interday reliability for these duplicates was tested with the interassay coefficient of variation (CV). The CV was 3.57% for fat and 0.15% for muscle mass (kg). The thicknesses of the vastus lateralis and intermedius muscles (CV: 12.54%) for the correlation analysis were measured with an ultrasound device in a standardized supine position (Aloka SSD-2000, Aloka Co, Tokyo, Japan). The ultrasound measurement site was tattooed to ensure that the same site was used both before and after training. All measurements were preceded by at least 2 d of rest from physical activity. The subjects were advised to drink normally but to abstain from anything with a dehydrating effect (e.g., sauna, physical work, or alcohol) during the day preceding the bioimpedance measurement. Subjects were permitted to drink one glass of water before the measurements conducted during the fasting state. The same researcher did all the bioimpedance and ultrasound measurements, increasing the repeatability of the procedure.

Muscle biopsy. Muscle biopsies from the ST group (N = 11) were obtained 0.5 h before and 1 and 48 h after the RE session both before and after the 21-wk ST period. The timing of the biopsies is depicted in Figure 1. Biopsies were taken from the vastus lateralis muscle, midway between the patella and greater trochanter, in an area where the muscle is thickest and where no major nerves and blood vessels are located. The pre-RE biopsy and the 48-h post-RE biopsies were taken from the left leg. To avoid any residual effects of blood flow from the consecutive biopsy areas, the 1-h post-RE biopsy was taken from the right leg and the 48-h biopsy was taken 2 cm *above* the previous biopsy scar from the left leg. The post-ST biopsy was taken 1 wk after the last ST session to minimize the short-term effects of the last exercise bout. All biopsies were taken at the same time of the day for every subject on the pre-ST versus post-ST biopsy days. Concurrently with the ST-group biopsies, pre– and post–21 wk biopsies were taken from seven control subjects who did not train but who continued their previous habitual physical activity. To study the effects of obtaining the biopsy from the different legs (pre–1 h vs post–1 h biopsies in the ST group), two biopsies, separated by 2 h of rest, were taken from six subjects. All muscle samples were immediately frozen in liquid nitrogen and were then stored at −80° until analysis.

Total RNA isolation and cDNA synthesis. Muscle samples were homogenized with FastPrep (Bio101 Systems) tubes containing Lysing Matrix D (Q-Biogene). Total RNA was extracted from approximately 50 mg of muscle tissue with a monophasic solution of phenol and guanidine isocyanate using the Trizol-reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA concentration was determined by optical density (OD) at 260 nm. The muscle total RNA concentration was calculated on the basis of total RNA yield and the weight of the analyzed sample (3). Our procedure yielded nondegraded pure RNA determined by gel electrophoresis and free of proteins and DNA, as indicated by an OD_{260}/OD_{280} ratio of 1.8 to 2.0 in every sample. RNA amount and quality did not meet the qualification standards in one ST-group subject's post–1 h and 48-h samples during pre-ST condition or in the post–1 h sample during the post-ST condition. These three muscle samples were not included in the further mRNA analysis.

Five micrograms of total RNA were reverse transcribed to synthesize cDNA according to the manufacturer's instructions in a total volume of 50 μL using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). RNA were extracted and cDNA were synthesized by the same person to increase the reliability of the procedure.

Real-time PCR. mRNA expression levels were quantified with a real-time reverse-transcriptase PCR (RT-PCR) assay that was based on the 5 nuclease activity of the Taq polymerase, and using an Abi Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Every PCR run was made by the same person with triplicates to increase the reliability of the procedure. For each subject, all samples were run simultaneously to permit relative comparisons. The probes and primers used were predesigned (so-called inventoried assays) and validated by Applied Biosystems bioinformatics design pipelines. The gene-bank accession numbers and Applied Biosystems assay IDs, respectively, were NM 005259 and Hs00193363_m1 (myostatin), NM 004064 and Hs00153277_m1 (P27kip), NM 001106 and Hs00609603_m1 (AcvrIIb), NM 005860 and Hs00610505_m1 (FLRG), NM 002478 and Hs00159528_m1 (MyoD), NM 002479 and Hs00231167_m1 (myogenin), and NM002046 and Hs99999905_m1 (GAPDH). Applied Biosystems validated PCR cycle parameters were identically used for all genes: 50°C for 2 min + 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min; this cycle has been validated by Applied Biosystems. GAPDH mRNA was used as an endogenous control. The use of GAPDH mRNA was validated against other often used housekeeping gene 18sRNA. GAPDH/18sRNA was stable at all time points showing the eligibility of GAPDH as a housekeeping gene in this setting. GAPDH has been used previously in many RE studies and has been shown not to be affected by RE protocol (22). To determine whether the normalization to GAPDH levels affected the results of the present data, we also examined all the exercise mRNA data without normalization to GAPDH, as has been done recently in an RE study (7). We found, as expected, that the normalization did not significantly affect the changes in mRNA levels of any gene studied in the present study.

Gene transcript results were calculated according to the Liu and Saint (18) mathematical model that takes into account cycle-by-cycle PCR amplification efficiencies and that has been shown to be valid in studying the initial amount of gene transcript (18). SigmaPlot (version 9.0, Systat Software inc., Richmond, CA) was used as a curve-fitting software in the Liu and Saint method. Before analysis, the Liu and Saint method results were compared with the results obtained from the standard curve method, which produced similar results.

TABLE 1. Subject characteristics.

	ST Group (N = 11)		Control Group (N = 7)	
	0 wk	21 wk	0 wk	21 wk
Age (yr)	60.9 ± 5.0		63.9 ± 7.4	
Height (cm)	176.5 ± 2.9		171.8 ± 7.2	
Body weight (kg)	80.0 ± 1.6	79.9 ± 1.8	77.04 ± 14.2	76.8 ± 16.6
Muscle mass (kg)	36.1 ± 0.5	36.7 ± 0.5*	35.2 ± 5.4	35.1 ± 5.8
% fat	20.3 ± 1.4	19.2 ± 1.6*	18.6 ± 7.7	18.4 ± 7.6
Bilateral 1RM (kg)	157.2 ± 20.6	191.1 ± 27.3*	156.0 ± 12.8	161.5 ± 9.3
Bilateral isometric force (N)	2567 ± 563	3010 ± 494*	2721 ± 373	2819 ± 565

All values are mean ± SD. * Statistically significant difference ($P < 0.05$) between 0 and 21 wk.

TABLE 2. Real-time RT-PCR results for the control group.

(N = 7)	0 wk (Left Leg)	After 21 wk (Left Leg)	P Value†
MSTN	0.165 ± 0.053	0.147 ± 0.051	0.83
AcvrIIb	0.090 ± 0.018	0.104 ± 0.023	0.66
FLRG	0.002 ± 0.001	0.002 ± 0.001	0.83
P27kip	2.609 ± 0.396	2.711 ± 0.410	0.72
MyoD	0.540 ± 0.135	0.736 ± 0.160	0.20
Myogenin	1.397 ± 0.380	1.540 ± 1.871	0.65
(N = 6)	Before 2 h of Rest (Left Leg)	After 2 h of Rest (Right Leg)	P Value†
MSTN	0.106 ± 0.024	0.085 ± 0.032	0.72
AcvrIIb	0.089 ± 0.022	0.051 ± 0.010	0.22
FLRG	0.003 ± 0.001	0.008 ± 0.004	0.15
P27kip	3.102 ± 0.262	2.423 ± 0.319	0.18
MyoD	0.978 ± 0.149	1.171 ± 0.260	0.47
Myogenin	1.826 ± 0.201	1.486 ± 0.287	0.50

mRNA values for MSTN, activin IIb, FLRG, $P27^{kip}$, myogenin, and MyoD are calculated according to the mathematical model by Liu and Saint (18) and normalized to GAPDH mRNA (expressed as 10^3, means ± SE). Before and after the 21-wk study period, biopsies were taken from seven control subjects who did not train but continued their previous physical activity. In addition, two control biopsies from the different legs, separated by 2 h of rest, were obtained from six subjects. † P value of difference between pre and post conditions.

Statistical analysis. Standard statistical methods were used for the calculation of means, standard deviations (SD), standard errors (SE), and Pearson product–moment correlation coefficients, and the data from RE protocols were analyzed by a two-factor repeated-measures general linear model (GLM) by using SPSS 12.0. There were three levels in sample time factor (pre, post 1 h, and post 48 h), so any violations of the assumptions of sphericity were explored and controlled. For each GLM with a main or interaction effect, Holm–Bonferroni *post hoc* tests were performed to localize the effects as suggested by Atkinson (2). Statistical power in the significant mRNA findings ($P < 0.05$) exceeded 0.88, demonstrating an adequate sample size.

RESULTS

ST. There were no significant changes in total body weight attributable to ST, whereas a slight but consistent increase in total body muscle mass ($P = 0.01$) and a decrease in fat percentage ($P < 0.05$) were found (Table 1). The controls showed no change in any of these variables ($P > 0.05$). Furthermore, no significant changes took place in the maximal bilateral dynamic 1RM or isometric leg extension force, fat percentage, or muscle mass during the 3-wk nontraining control period preceding ST ($P > 0.05$), whereas maximal bilateral dynamic 1RM (22%, $P < 0.001$) and isometric leg extension force (17%, $P = 0.001$) increased significantly during ST but not in the control group ($P > 0.05$) (Table 1).

RE. The total volume of the RE bout (loads × sets × repetitions) was 6095 ± 926 kg (mean ± SD) before ST and 7578 ± 1046 kg after ST ($P < 0.001$). Maximal isometric leg extension force decreased significantly ($P < 0.001$) after RE in both pre-ST (27.1 ± 3%, from 2537 ± 425 to 1843 ± 95 N) and post-ST situations (28.5 ± 5%, from 2860 ± 334 to 2038 ± 159 N). The Borg subjective rate of perceived

exertion (scale of 6–20) after the RE bout in pre-ST (16.9 ± 0.4) was similar that after RE in the post-ST condition (16.8 ± 0.5). There were no statistically significant differences in total energy consumption or intake of any macronutrient between the pre- and post-ST conditions during the 5 d preceding the 48-h biopsy ($P > 0.20$). Total RNA concentration (micrograms of RNA extracted/milligrams of muscle wet weight) increased after the RE bout in the untrained condition (repeated-measures ANOVA main effect: $P = 0.01$) but no longer after ST ($P = 0.70$). *Post hoc* analysis localized the significantly increased total RNA concentration at 48 h after RE (18%, $P = 0.03$). The mRNA levels of the control subjects did not change during the 21-wk period or in the samples between the legs during the 2-h controlled resting period ($P > 0.15$). The results for the control subjects are presented in Table 2.

Myostatin and activin IIb mRNA. Myostatin mRNA decreased after the RE bout after 21 wk of ST (main effect: $P = 0.009$) but not before ST ($P = 0.49$) (Fig. 2A). After ST, myostatin levels at 48 h post-RE were significantly (48%) lower compared with the corresponding pre-RE levels ($P = 0.03$). A significant (57%) increase in basal (resting) myostatin mRNA levels was observed in response to ST ($P = 0.03$). The post–1 h mRNA levels of AcvrIIb were significantly decreased (42%, $P = 0.05$) compared with pre-RE levels in the pre-ST state, and there was also a trend in the post-ST state (29%, $P = 0.07$) (Fig. 2B). Figure 3 shows the relative change in AcvrIIb for every ST-group

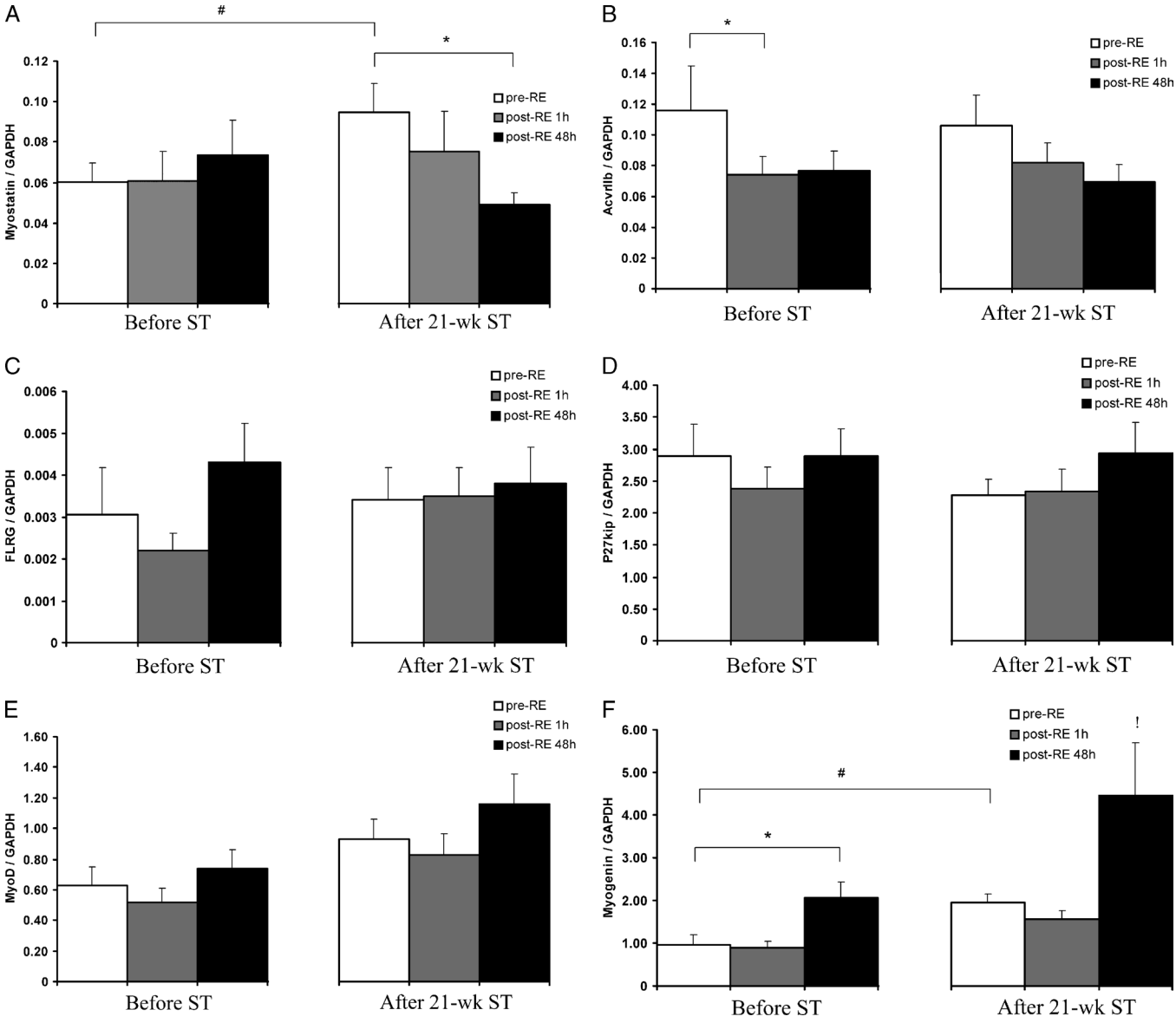

FIGURE 2—Real-time RT-PCR results for resistance exercise–induced A) myostatin, B) activin IIb, C) FLRG, D) $P27^{kip}$, E) MyoD, and F) myogenin mRNA expressions from vastus lateralis muscle. Results are shown both before and after 21 wk of ST ($N = 11$) and are expressed as normalized to GAPDH mRNA expression. Muscle biopsies were obtained before resistance exercise (pre-RE, *white bars*) and 1 h (post-RE 1 h, *gray bars*) and 48 h (post-RE 48 h, *black bars*) after exercise. Values are means ± SE ($N = 11$), calculated according to Liu and Saint's (18) mathematical model (expressed as 10^3). * Significantly different from corresponding pre value in RE; # difference between pre- and post-ST resting levels ($P < 0.05$); ! trend for myogenin mRNA increase also after ST ($P = 0.08$).

subject from whom high-quality RNA samples were available in all pre- and post-RE samples. Only one subject had a consistent increase in AcvrIIb response to RE, whereas all other subjects responded with a decrease at post–1 h or at post–48 h both before and after ST.

FLRG, P27^{kip}, MyoD, and myogenin mRNA. No significant changes attributable to ST or RE in either the pre- or post-ST state were observed in FLRG ($P > 0.27$) and P27^{kip} mRNA ($P > 0.40$) (Fig. 2C and 2D). However, MyoD levels tended to be increased ($P = 0.09$) at 48 h after RE in the pre-ST state (Fig. 2E). Moreover, an average (post 1 and 48 h) MyoD expression after RE was significantly higher in the post-ST condition compared with pre-ST (61%, $P = 0.04$). Myogenin mRNA increased in response to RE ($P = 0.01$) before ST, and there was a trend after ST ($P = 0.08$) (Fig. 2F). The *post hoc* analysis showed significantly increased myogenin mRNA levels at 48 h after RE in the pre-ST state (109%, $P = 0.05$) but not in the post-ST situation (127%, $P = 0.11$). A significant 103% increase in basal myogenin mRNA level was observed in response to ST ($P < 0.001$).

Specific correlations. In the pre-ST situation, the RE-induced 48-h change from pre-RE in myostatin mRNA levels correlated inversely with the increase in total body muscle mass during ST ($r = -0.82$, $P = 0.002$) (Fig. 4A). Supporting the validity of this result, the same myostatin decrease tended to correlate inversely with the percentage change in the thickness of the combined vastus lateralis and intermedius muscles ($r = -0.52$, $P = 0.12$). In the post-ST situation, the magnitude of the RE-induced 48-h reduction in myostatin mRNA correlated negatively with its levels before RE ($r = -0.94$, $P < 0.001$) (Fig. 4B) and with the ST-induced change in the basal levels of myostatin ($r = -0.65$, $P = 0.03$). The change in myostatin during ST correlated inversely with the change in MyoD mRNA during ST ($r = -0.60$, $P = 0.05$).

DISCUSSION

Main findings. In the present study, in a longitudinal setting, we present the novel finding that long-term supervised ST influences the short-term myostatin mRNA response to a single RE session. More specifically, downregulation of myostatin mRNA was observed only in the trained state. The short-term postexercise regulation of myostatin at the transcriptional level may be important because a high correlation was found between short-term downregulation of myostatin mRNA and muscle hypertrophy during ST. In addition to myostatin, we demonstrate the new finding in untrained older men that activin IIb (AcvrIIb), a receptor of myostatin, was significantly downregulated in mRNA level after a single RE.

Myostatin, activin IIb, FLRG, and P27^{kip}. Our results that myostatin mRNA increased after ST concomitant with gains in muscle strength and mass are in agreement with Willoughby (29), who reported increased myostatin mRNA levels after 6 and 12 wk of ST. Interestingly, in that study, the same response was seen also at the immunoreactive protein level in both muscle and in blood, suggesting that the myostatin mRNA response follows the same pattern as protein levels. However, there is uncertainty in this and other reported studies regarding the specificity of the antibodies used to measure myostatin levels (15). Therefore, because there seems to be no valid method available in humans at the moment, the myostatin protein levels were not measured in the present study. The study by Roth et al. (25) is, thus far, the only published study to show decreased muscle myostatin mRNA levels after ST in humans. This may be because the post-ST biopsies were obtained as soon as 48–72 h after the last RE bout in that study. This time period may reflect the transient downregulating effects of the last RE bout on myostatin mRNA levels. In the present data, in 10 of 11 subjects, the RE-induced decrease was seen 48 h after RE in the trained muscle condition. In our study, muscle biopsies were obtained 1 wk after the last supervised ST session, thus minimizing the short-term effects from this last bout. Only light recreational activity was allowed during this 1-wk period, and diet during the last few days was controlled. Therefore, we assume that the long-term adaptation of myostatin mRNA observed was attributable to the 21 wk of ST. However, it is also possible that different training programs (e.g., the 9 wk of unilateral knee extension used by Roth et al. (25) compared with 21 wk of whole-body training in the present study) have opposite effects on myostatin mRNA expression in response to ST.

A decrease (48%) was observed in myostatin mRNA levels at 48 h after RE, but only after 21 wk of ST. This is in agreement with Kim et al. (13), who reported decreased (40%) myostatin mRNA levels 24 h after RE in older men. However, unlike the study of Kim et al., where only untrained subjects were used, in the present study, the same response was not seen in the untrained state. The major differences between our study and that by Kim et al. (13) were in the postexercise biopsy time (48 vs 24 h), the RE protocol (five sets of 10RM leg presses vs three sets of 8- to 12RM squats, leg presses, and knee extensions), longitudinal versus cross-sectional study design, and mRNA quantification method (real-time PCR vs end-point PCR), respectively. The major reason for the differences seen in these studies in untrained subjects may be the biopsy time; Raue et al. (23) and Coffey et al. (7) have shown decreased myostatin mRNA levels 3 or 4 h after a single low-volume bout of RE in untrained women and men, respectively. However, it is also possible that the five sets of 10RM used in this study are not a high enough volume to induce downregulated myostatin expression after RE in most untrained older men. The present study suggests that myostatin gene expression is not yet altered at 1 h after RE. The previous results in humans (7,13,23) suggest that RE can downregulate myostatin gene expression in humans starting at about 3–24 h after RE. The present data show that myostatin mRNA downregulation in

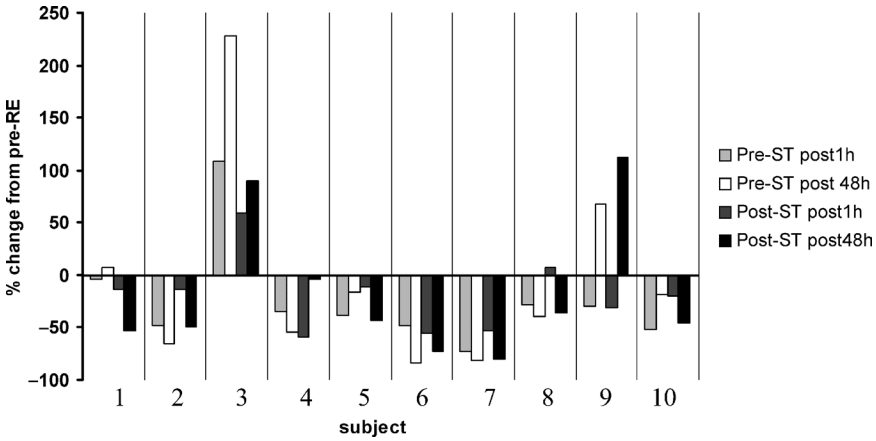

FIGURE 3—Relative individual changes induced by resistance exercise (RE) in activin IIb mRNA levels before strength training (pre-ST) and after strength training (post-ST) for all subjects from whom samples were available for analysis. Figure shows the RE-induced downregulation of activin IIb in all but subject number 3.

the trained condition lasts at least 48 h, suggesting that myostatin-related pathways may contribute to regeneration/recovery processes occurring after intensive RE. Indeed, the role of myostatin in regeneration processes attributable to short-term injury has been shown in rats (28). Unfortunately, we did not measure the protein levels of myostatin because of the lack of proper methods for analyzing bioactive myostatin protein in human samples. Consequently, no final statements can be made about the function of myostatin protein. Instead, we have shown that training affects myostatin gene expression in response to a single bout of RE.

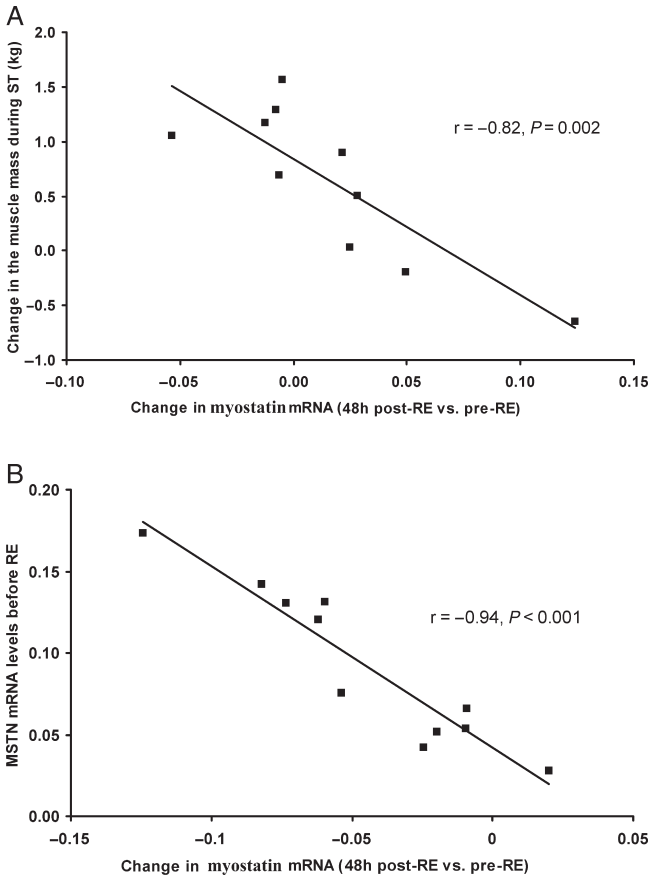

FIGURE 4—A) Correlations between untrained muscle resistance exercise (RE)–induced 48-h change in myostatin mRNA levels and after muscle mass change during ST measured by bioimpedance, and B) correlations between trained muscle resting levels of myostatin mRNA immediately before RE and after RE-mediated 48-h change in myostatin mRNA.

The opposing myostatin responses observed after the long-term ST and the single RE bout might indicate that these short- and long-term responses are interrelated. To investigate this, we calculated the correlation coefficients between these two responses. The results show that the greater the increase in myostatin mRNA during ST, the higher the decrease after the single RE. Importantly, the pre-RE levels of myostatin accounted for as much as 89% of the variance in its decrease after RE in the trained condition. Therefore, it is probable that the adaptive state of myostatin expression attributable to ST has a strong effect on myostatin gene expression in response to a short-term bout of RE, at least in older men. We were also interested to see whether the RE-induced myostatin response might have something to do with the subsequent muscle-mass adaptations during the 21-wk ST period. Indeed, the 48-h change in myostatin expression after RE was inversely related to the gain in muscle mass during ST. This inverse relationship is in accordance with the theoretical negative role of myostatin in regulating muscle mass (21,26,31). In agreement with our results, Kim et al. (13), in a cross-sectional study, recently reported a significant correlation between single RE-induced down-regulation of myostatin mRNA expression and the total lean body mass and thigh lean mass. Supporting the importance of mRNA transcript levels of myostatin in muscle growth, recent findings with short hairpin interfering RNA (shRNA) technology have shown that even low reduction in myostatin mRNA leads to an increase in the satellite cell number and muscle size in mice (19).

A slight 1-h and/or 48-h postexercise decrease (~30–40%, with moderate effect sizes 0.46–0.63) in AcvrIIb mRNA attributable to RE was observed in the present study, both before and after ST in all but one subject

(subject 3 in Fig. 3). Stevenson et al. (27) demonstrated that AcvrIIb receptor expression was increased after 1, 4, 7, and 14 d of hindlimb unloading in rats. Thus, muscle loading and unloading seem to have opposite effects on AcvrIIb expression. The untrained muscle AcvrIIb responses to a single RE bout after 1 h and 48 h were highly correlated with the corresponding AcvrIIb responses of the trained muscle ($r > 0.70$, $P < 0.02$). Therefore, the AcvrIIb response to RE seems to be highly consistent, indicating that training state may not have an effect on the response; however, it is possible that this response would be different in, for instance, younger men. To our knowledge, our results are the first reported findings on the behavior of myostatin receptor and binding protein (FLRG) mRNA levels after a single bout of exercise. Myostatin has a high binding affinity for AcvrIIb (16), and the disruption of myostatin signaling by a truncated form of AcvrIIb leads to an increase in muscle mass (16). A rate-limiting step for protein synthesis in response to exercise is the quantity of mRNA abundance (4). Thus, the combination of the downregulation of myostatin and AcvrIIb mRNA observed after the repeated RE protocols is theoretically advantageous for muscle growth processes.

Knockout of the $p27^{kip}$ gene in mice increases skeletal muscle mass (17) and may act in concert with myostatin (13,17). Therefore, the levels of $p27^{kip}$ mRNA were also studied in the present study. The gene expression levels of the cyclin-dependent kinase inhibitor, $p27^{kip}$, were not responsive to single RE or long-term ST in the present study. However, in contrast to older men, Kim et al. (13) showed a modest reduction after an RE protocol in younger males and females and in older females. It is obvious that the complex role of the single RE or ST in $p27^{kip}$ and other cell cycle regulators needs further study.

Myogenin and MyoD. Myogenin mRNA was found to increase after both the single RE and the long-term ST in the present study in older men. Furthermore, mean post-RE MyoD expression was significantly higher in the post-ST condition compared with the pre-ST condition. Myogenin and MyoD are known to modulate repair/regeneration responses after overload, and, in parallel with our study, the expression of myogenin mRNA has been shown to increase in the short-term after a bout of RE (22), after electrically stimulated contractions (3,9), and after ST (30).

The mechanism suggested for the inhibition of myoblast differentiation by myostatin is downregulation of MyoD and myogenin gene expression via the myostatin-induced pathway (24). Indeed, in the present study, myostatin responded inversely to RE and ST compared with the response of myogenin, and the increase in myostatin during ST correlated inversely with the corresponding change in MyoD. Theoretically, the observed post-RE upregulation of myogenin expression and the concurrent downregulation of myostatin and AcvrIIb expression could facilitate regeneration and/or the repair of the damaged myofibers attributable to RE (28). There was an increase in the resting levels of myogenin and in both MyoD and myostatin mRNA response to RE after 21 wk of ST in older men. A methodological limitation of the present study is that we did not measure protein levels. However, the observed changes in mRNA show that regulation occurs at the transcriptional level, allowing altered synthesis rates of these proteins because that quantity of an abundance of mRNA is an important factor determining protein synthesis in response to exercise (4). This suggests that post-RE muscle-regeneration capacity may be increased after ST in older men. This may be an important finding, especially in older people, because an old muscle is more susceptible to contraction-induced injury and regenerates more slowly than a younger muscle (5).

Muscle RNA. The concentration of total muscle RNA increased at 48 h after RE, but only in the untrained condition. Bickel et al. (3) found that RE increased total muscle RNA only in spinal cord–injured subjects, not in their able-bodied counterparts. Our results and those of Bickel et al. (3) suggest that previous training decreases total RNA responses to RE. The majority of cellular RNA consists of ribosomal RNA. Therefore, the concentration of RNA in muscle can be regarded as an indication of the translational capacity of the tissue. It is suggested that the capacity of total protein synthesis was increased in untrained but not in trained muscle after a single bout of RE. It is possible that there is no need to further increase the amount of ribosomal RNA in the trained state because the efficiency of myofibrillar protein synthesis in proportion to total protein synthesis may be increased after ST (14). The limitation of our study is that the control group did not perform the short-term RE bout before and after the 21-wk period. Therefore, it is possible that these short-term post-RE changes seen in the untrained and trained condition may have also existed after this single bout of RE. However, this is not probable, because all the subjects were familiarized with the protocol and had two ST sessions in the same leg press machine before the actual RE bout.

In conclusion, the present study showed specifically that a single heavy RE session can downregulate both myostatin and its receptor activin IIb mRNA and can upregulate myogenin mRNA expression in older men. However, the RE-induced gene expression of myostatin depends on the training state of the muscle, at least in older men. Interestingly, the change in myostatin mRNA attributable to long-term ST was inversely related to the single RE-induced myostatin mRNA response. The short-term myostatin mRNA–expression response to RE may be important because myostatin downregulation was related to ST-induced muscle growth.

The authors thank Tuovi Nykänen, Risto Puurtinen, Aila Ollikainen, and Erkki Helkala for their help in data collection and analysis. We also thank the very dedicated group of subjects and research assistants who made this project possible. The Finnish Ministry of Education and the Finnish Cultural Foundation supported this research.

REFERENCES

1. ALLEN, D. L., S. R. MONKE, R. J. TALMADGE, R. R. ROY, and V. R. EDGERTON. Plasticity of myonuclear number in hypertrophied and atrophied mammalian skeletal muscle fibers. *J. Appl. Physiol.* 78: 1969–1976, 1995.
2. ATKINSON, G. Analysis of repeated measurements in physical therapy research: multiple comparisons amongst level means and multifactorial designs. *Phys. Ther. Sport* 3:191–203, 2002.
3. BICKEL, C. S., J. M. SLADE, F. HADDAD, G. R. ADAMS, and G. A. DUDLEY. Acute molecular responses of skeletal muscle to resistance exercise in able-bodied and spinal cord-injured subjects. *J. Appl. Physiol.* 94:2255–2262, 2003.
4. BOOTH, F. W., and K. M. BALDWIN. Muscle plasticity: energy demand and supply processes. In: *Handbook of Physiology. Section 12: Exercise: Regulation and Integration of Multiple systems*, L. B. Rowell and J. T. Shepherd (Eds.). New York: Oxford University Press, pp. 1075–1123, 1996.
5. BROOKS, S. V., and J. A. FAULKNER. Contraction-induced injury: recovery of skeletal muscles in young and old mice. *Am. J. Physiol.* 258:C436–C442, 1990.
6. COFFEY, V. G., Z. ZHONG, A. SHIELD, et al. Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *FASEB J.* 20:190–192, 2006.
7. COFFEY, V. G., A. SHIELD, B. J. CANNY, K. A. CAREY, D. CAMERON-SMITH, and J. A. HAWLEY. Interaction of contractile activity and training history on mRNA abundance in skeletal muscle from trained athletes. *Am. J. Physiol. Endocrinol. Metab.* 290:E849–E855, 2006.
8. GREENLUND, L. J. S., and K. S. NAIR. Sarcopenia - consequences, mechanisms, and potential therapies. *Mech. Ageing Dev.* 124: 287–299, 2003.
9. HADDAD, F., and G. R. ADAMS. Selected contribution: acute cellular and molecular responses to resistance exercise. *J. Appl. Physiol.* 93:394–403, 2002.
10. HÄKKINEN, K., A. PAKARINEN, W. J. KRAEMER, A. HÄKKINEN, H. VALKEINEN, and M. ALEN. Selective muscle hypertrophy, changes in EMG and force, and serum hormones during strength training in older women. *J. Appl. Physiol.* 91:569–580, 2001.
11. HÄKKINEN, K., M. ALEN, M. KALLINEN, R. U. NEWTON, and W. J. KRAEMER. Neuromuscular adaptation during prolonged strength training, detraining and re-strength-training in middle-aged and elderly people. *Eur. J. Appl. Physiol.* 83:51–62, 2000.
12. HILL, J. J., M. V. DAVIES, A. A. PEARSON, et al. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *J. Biol. Chem.* 277: 40735–40741, 2002.
13. KIM, J. S., J. M. CROSS, and M. M. BAMMAN. 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.
14. KIM, P. L., R. S. STARON, and S. M. PHILLIPS. Fasted-state skeletal muscle protein synthesis after resistance exercise is altered with training. *J. Physiol.* 568:283–290, 2005.
15. LEE, S. J. Regulation of muscle mass by myostatin. *Annu. Rev. Cell Dev. Biol.* 20:61–86, 2004.
16. LEE, S. J., and A. C. MCPHERRON. Regulation of myostatin activity and muscle growth. *Proc. Natl. Acad. Sci. U. S. A.* 98:93069311, 2001.
17. LIN, J., M. A. DELLA-FERA, C. LI, et al. P27 knockout mice: reduced myostatin in muscle and altered adipogenesis. *Biochem. Biophys. Res. Commun.* 300:938–942, 2003.
18. LIU, W., and D. A. SAINT. Validation of a quantitative method for real time PCR kinetics. *Biochem. Biophys. Res. Commun.* 294: 347–353, 2002.
19. MAGEE, T. R., J. N. ARTAZA, M. G. FERRINI, et al. Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. *J. Gene. Med.* 8:1171–1181, 2006.
20. MCCROSKERY, S., M. THOMAS, L. MAXWELL, M. SHARMA, and R. KAMBADUR. Myostatin negatively regulates satellite cell activation and self-renewal. *J. Cell. Biol.* 162:1135–1147, 2003.
21. MCPHERRON, A. C., A. M. LAWLER, and S. J. LEE. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 387:83–90, 1997.
22. PSILANDER, N., R. DAMSGAARD, and H. PILEGAARD. Resistance exercise alters MRF and IGF-I mRNA content in human skeletal muscle. *J. Appl. Physiol.* 95:1038–1044, 2003.
23. RAUE, U., D. SLIVKA, B. JEMIOLO, C. HOLLON, and S. W. TRAPPE. Myogenic gene expression at rest and following a bout of resistance exercise in young (18–30 y) and old (80–89 y) women. *J. Appl. Physiol.* 101:53–59, 2006.
24. RIOS, R., I. CARNEIRO, V. M. ARCE, and J. DEVESA. Myostatin is an inhibitor of myogenic differentiation. *Am. J. Physiol. Cell Physiol.* 282:C993–C999, 2002.
25. ROTH, S. M., G. F. MARTEL, R. E. FERRELL, E. J. METTER, B. F. HURLEY, and M. A. ROGERS. Myostatin gene expression is reduced in humans with heavy-resistance strength training: a brief communication. *Exp. Biol. and Med.* 228:706–709, 2003.
26. SCHUELKE, M., K. R. WAGNER, L. E. STOLZ, et al. Myostatin mutation associated with gross muscle hypertrophy in a child. *N. Engl. J. Med.* 350:2682–2688, 2004.
27. STEVENSON, E. J., P. G. GIRESI, A. KONCAREVIC, and S. C. KANDARIAN. Global analysis of gene expression patterns during disuse atrophy in rat skeletal muscle. *J. Physiol.* 551:33–48, 2003.
28. WAGNER, K. R., X. LIU, X. CHANG, and R. E. ALLEN. Muscle regeneration in the prolonged absence of myostatin. *Proc. Natl. Acad. Sci. U. S. A.* 102:2519–2524, 2005.
29. WILLOUGHBY, D. S. Effects of heavy resistance training on myostatin mRNA and protein expression. *Med. Sci. Sports. Exerc.* 36:574–582, 2004.
30. WILLOUGHBY, D. S., and J. M. ROSENE. Effects of oral creatine and resistance training on myogenic regulatory factor expression. *Med. Sci. Sports. Exerc.* 35:923–929, 2003.
31. ZIMMERS, T. A., M. W. DAVIES, L. G. KONIARIS, et al. Induction of cachexia in mice by systemically administered myostatin. *Science* 296: 1486–1488, 2002.