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HIGHLIGHTED TOPIC | *Regulation of Protein Metabolism in Exercise and Recovery*

Resistance exercise with whey protein ingestion affects mTOR signaling pathway and myostatin in men

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Hulmi JJ, Tannerstedt J, Selänne H, Kainulainen H, Kovanen V, Mero AA. Resistance exercise with whey protein ingestion affects mTOR signaling pathway and myostatin in men. *J Appl Physiol* 106: 1720–1729, 2009. First published March 19, 2009; doi:10.1152/japplphysiol.00087.2009.—Signaling pathways sense local and systemic signals and regulate muscle hypertrophy. The effects of whey protein ingestion on acute and long-term signaling responses of resistance exercise are not well known. Previously untrained young men were randomized into protein ($n = 9$), placebo ($n = 9$), and control ($n = 11$) groups. Vastus lateralis (VL) muscle biopsies were taken before and 1 h and 48 h after a leg press of 5 × 10 repetitions [resistance exercise (RE)] and after 21 wk (2 times per week) of resistance training (RT). Protein (15 g of whey) or nonenergetic placebo was ingested before and after a single RE bout and each RE workout throughout the RT. The protein group increased its body mass and VL muscle thickness (measured by ultrasonography) already at *week 10.5* ($P < 0.05$). At *week 21*, the protein and placebo groups had similarly increased their myofiber size. No changes were observed in the nonexercised controls. However, the phosphorylation of $p70^{S6K}$ and ribosomal protein S6 (rpS6) were increased at 1 h post-RE measured by Western blotting, the former being the greatest with protein ingestion. Mammalian target of rapamycin (mTOR) phosphorylation was increased after the RE bout and RT only in the protein group, whereas the protein ingestion prevented the post-RE decrease in phosphorylated eukaryotic initiation factor 4E binding protein 1 (p-4E-BP1). Akt phosphorylation decreased after RT, whereas no change was observed in phosphorylated eukaryotic elongation factor 2. A post-RE decrease in muscle myostatin protein occurred only in the placebo group. The results indicate that resistance exercise rapidly increases mTOR signaling and may decrease myostatin protein expression in muscle and that whey protein increases and prolongs the mTOR signaling response.

hypertrophy; training; nutrition; S6K1; skeletal muscle

ADEQUATE MUSCLE MASS is crucial for human well-being. It is, therefore, important to identify the mechanisms that stimulate muscle hypertrophy or prevent atrophy. The most efficient way to increase the size of a skeletal muscle is by resistance training (RT) in combination with protein-containing nutrition. Muscle hypertrophy due to RT and protein nutrition seems largely to result from cumulative acute increases in muscle protein synthesis. One resistance exercise (RE) bout can within 1 h increase muscle protein synthesis (9), which can last up to 72 h after exercise (39). Protein ingestion before or after a bout of RE has been shown to significantly enhance this response (52) and be possibly more beneficial in terms of muscle hypertrophy than nutrient ingestion at other times of day (7, 11). It would thus be important to understand how protein ingestion affects pathways regulating intracellular hypertrophy of muscle in the context of a bout of RE and long-term RT.

Muscle protein synthesis and hypertrophy are stimulated by the mammalian target of rapamycin (mTOR) pathway protein kinase enzymes that are activated or inactivated by phosphorylation or dephosphorylation at different amino acid sites (13, 28). In this pathway, phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1) and $p70^{S6K}$/S6K1 (p70 ribosomal S6 kinase) by mTOR have been shown to be important in muscle protein synthesis and hypertrophy (2, 12, 28, 33, 35, 40, 43, 51). The importance of 4E-BP1 is due to the fact that its phosphorylation prevents the interaction and inhibition of 4E-BP1 with eIF4E and therefore increases translation of the protein synthesis (24, 42). On the other hand, $p70^{S6K}$ affects muscle hypertrophy at least through ribosomal protein S6 (rpS6) as well as possibly through some other proteins such as eukaryotic elongation factor 2 (eEF2) (44, 45).

Ingestion of protein with carbohydrate or only branched-chain amino acids in the context of a bout of RE has been shown to increase the phosphorylation of mTOR (3), $p70^{S6K}$ (26, 30), and rpS6 (26, 30) at 0–4 h post-RE in humans. The effect of an intact protein source alone, such as whey, on this pathway in humans, and especially in the longer term after a bout of RE, i.e., from 12 to 72 h or after months of resistance training, is unknown.

The mTOR pathway is opposed by myostatin signaling, which inhibits muscle growth (37, 46), partially possibly through inhibiting mTOR signaling (1). The only published studies so far on the myostatin response in humans to a bout of RE or long-term RT combined with protein ingestion are based on studies carried out recently in our laboratory (21, 22). These studies suggested that protein ingestion may acutely hinder the RE-induced decrease in myostatin mRNA expression in both young and old men; however, it remains unknown whether that would also lead to a change in a protein level of myostatin.

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The purpose of this randomized controlled and double-blinded trial was to examine acute and long-term/chronic responses to resistance training in terms of protein signaling known to be related to muscle hypertrophy. Specifically, our main focus was to investigate these pathways when a high-quality milk protein fraction, whey (15), is supplemented to a normal diet both immediately before and after a resistance exercise workout. We hypothesized that ingestion of whey proteins immediately before and after a resistance exercise bout would have fast acute but not long-lasting effects on the phosphorylation levels of the mTOR signaling pathway and on myostatin protein expression.

MATERIALS AND METHODS

Subjects

The subjects were randomly assigned to either a whey protein group ($n = 9$, age 24.7 ± 5.0 yr), placebo group ($n = 9$, 27.4 ± 3.1 yr), or control group ($n = 11$, 25.2 ± 2.7 yr). Anthropometric details of the subjects are presented in Table 1. The subjects were recruited for the study by advertising in newspapers and through e-mail lists. A subgroup from a previous study (22) was used in the present study.

All the subjects were examined by a physician, and none had medical problems that might confound the results of this investigation. None of the subjects had any regular RT experience, but they were moderately active. Their normal habitual activities included walking, jogging, swimming, or ball games, and they were urged to continue the normal activities and daily living exactly the same during the experimental period. The subjects had a typical Finnish diet containing a rather large amount of protein and moderate amounts of fat and carbohydrates. The subjects were urged to continue their normal diet throughout the project. Before the investigation, each subject was informed about the experimental design and possible associated risks and discomforts. Each then signed an informed consent to participate in the study, which was approved by the local Ethics Committee of the University of Jyväskylä and was conducted in accordance with the Declaration of Helsinki.

Design

This investigation examined acute and long-term responses of adding high-quality protein to a normal diet (with no other nutritional supplements). The experimental design involved both a single bout of RE and 0- to 48-h postexercise responses to it, and following that, 21 wk of RT consisting of 42 RE workouts (Fig. 1). A control group was included, and all the measurements were always carried out at the same time of day to exclude the effects of biopsy sampling or effects of time of year or daily variations (47, 57). All the measurements were preceded by at least 2 days of rest from physical activity.

Experimental RT

Whole body heavy RE workouts were carried out twice a week. A minimum of 2 days of rest was required between workouts. All training sessions were supervised by experienced trainers who ensured that proper techniques and progression were used in each exercise (32). The leg exercises included two exercises for the leg extensor muscles, the bilateral leg press and bilateral knee extension, and one exercise for the leg flexors, bilateral knee flexion. The RT program also included exercises for the other main muscle groups: chest and shoulders (bench press), upper back, trunk extensors and flexors, upper arms, ankle extensors, and hip abductors and adductors. RT was performed with progressive training loads of 40–85% of the subject's one-repetition maximum (1RM) in a periodized training program. For each exercise in a workout the number of sets increased (from 2–3 to 3–5) and the number of repetitions in each set decreased (from 15–20 to 5–6) during the 21-wk RT period. The loads were individually determined throughout the RT period. Recovery between

Table 1. *Anthropometry: height, body mass, fat percent, muscle fiber cross-sectional area, and muscle thickness of the protein, placebo, and control groups*

Variable/Group	Baseline	10.5 wk	21 wk	P Value$_{pre\ vs.\ 21}$	P Value$_{\Delta\%group21}$
Height, cm					
Protein	181.8±6.9				
Placebo	181.0±6.2				
Control	181.9±4.7				
Body mass, kg					
Protein	76.7±8.1	80.1±9.5*†	80.1±9.6*†	0.009	<0.001
Placebo	75.9±8.0	77.6±8.5	78.2±8.9*†	0.03	0.02
Control	74.5±7.8	75.6±8.4*	74.5±8.1	0.93	
Fat, %					
Protein	16.8±4.0	17.7±4.3	17.5±4.6	0.35	0.50
Placebo	17.3±3.9	17.2±4.5	17.1±3.9	>0.99	0.46
Control	16.6±3.5	17.5±4.5	16.9±4.6	0.64	
Fiber size: type I, μm^2					
Protein	4,650±178		6,582±511*†	0.009	0.03
Placebo	4,198±185		5,910±288*†	0.003	0.04
Control	4,940±411		5,099±391	0.21	
Fiber size: type II, μm^2					
Protein	5,021±402		7,599±576*†	0.002	0.01
Placebo	4,617±336		6,951±484*†	0.003	0.04
Control	5,501±407		5,629±380	0.72	
Muscle thickness, cm					
Protein	2.61±0.14	2.89±0.18*†	2.93±0.19*†	0.003	0.03
Placebo	2.47±0.22	2.72±0.32	2.74±0.25*	0.046	0.12
Control	2.68±0.15	2.65±0.17	2.78±0.13	0.10	

Values are means ± SD, except muscle size variables, which are means ± SE. P value$_{pre\ vs.21}$ designates Holm-Bonferroni corrected P values compared with baseline. P value$_{\Delta\%group21}$ designates the difference between the training group and the control group in percent change between the baseline and post-21 wk values. *Significant ($P < 0.05$) P value vs. pre. †Significant value compared with percentage change vs. the control group. See text for further description of groups and time points.

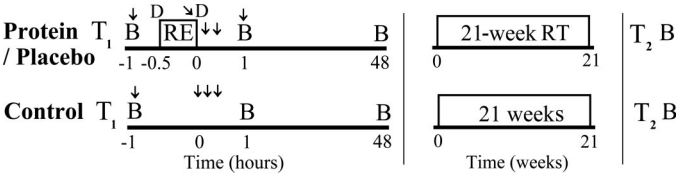

Fig. 1. Experimental design. B, vastus lateralis muscle biopsy; T_1, testing at baseline; T_2, testing at post 21 wk; ↓, venous blood sampling; RE, resistance exercise bout [5 × 10-repetition maximum (RM) leg press]; RT, heavy and progressive resistance training; D, protein (15 g of whey protein) or placebo (no energy) drink.

the sets was 2–3 min. No RT was done in the control group; instead they continued their habitual activity such as jogging, swimming, or ball games.

Nutritional Supplementation During RT

The subjects ingested immediately before and after each RE workout in the gym either 15 g of whey isolate protein with minimal lactose and fat (Protarmor 907 LSI, Armor Proteins, Brittany, France) dissolved in 250 ml of water or an equivalent volume of nonenergetic placebo. The drinks were provided for the subjects in a double-blind fashion. The essential amino acid composition of the protein drink (15 g) was as follows: histidine (0.2 g), isoleucine (1.0 g), leucine (1.7 g), lycine (1.4 g), methionine (0.4 g), phenylalanine (0.5 g), threonine (1.0 g), tryptophan (0.2 g), and valine (0.8 g). Both of the drinks contained equal amounts of exotic fruit (flavor), acesulfame-K (sweetener), and beta-carotene (color). The drinks were as identical as possible, differing mainly in the amount of the added viscosity substance (xanthane gum 3 g/l in the placebo and 1 g/l in the protein) and obviously in the protein content. Protein drink contained also trinatriumsitrate [to increase its pH to be equal with placebo (pH 7)]. The reason for the selection of a nonenergetic placebo drink instead of isocaloric carbohydrate drink was because carbohydrates per se can have also many effects on many of the studied variables (6).

The dietary intake was recorded in diaries for 3 days before the first biopsy day at the start of the study, on the biopsy day, and on the day thereafter (pre; 5 days overall), after 10.5 wk (mid; 4 days), and again before the 21st-week biopsy (post 21 wk; 3 days before, and on the biopsy day). The diaries were analyzed using the Micro Nutrica nutrient-analysis software (version 3.11, Social Insurance Institution of Finland). The subjects did not eat anything 1 h before and 0.5 h after the experimental exercise workouts during the RT period. Food restriction during these time periods was used to ascertain whether the supplementation of whey, considered a fast-acting and high-quality protein, has an additive effect where the normal meal ingestion is not forbidden ~2–3 h before and after each RE bout.

Heavy RE Protocol and Nutritional Supplementation Before and After a Bout of RE

A bilateral leg press machine (David 210, David Fitness and Medical) was used for the single heavy RE bout carried out before the experimental RT period. The RE bout protocol was same as in earlier studies (19, 21, 22). The total number of sets was five. Each set contained 10 repetition maximums. Recovery time between the sets was 2 min. The first set started with the 75% 1RM load based on the two earlier strength tests to measure baseline strength of the subjects (22). The loads were adjusted during the course of the RE bout due to fatigue so that each subject would be able to perform 10 repetitions at each set. If the load was too heavy, the subject was assisted slightly during the last repetitions of the set. Either 15 g of whey protein or the placebo was ingested immediately before and after the bout of RE.

Anthropometry

After an overnight fasting, body mass (kg) and fat percentage were measured. Body fat was measured using a skinfold caliper (biceps and triceps brachii, subscapularis, and iliac crest) (10). Vastus lateralis (VL) muscle thickness (at the middle of the VL muscle) was measured by ultrasonography in a standardized supine position (Aloka SSD-2000, Tokyo, Japan). The scanning head was coated with transmission gel to provide acoustic contact without depressing the dermal surface. The distance between the subcutaneous adipose tissue-muscle interface and intramuscular interface (i.e., aponeurosis) was defined as VL muscle thickness. The ultrasonography (US) measurement site was tattooed to ensure that the same site was used both before and after training. Intraclass correlation coefficient for body weight was $r = 0.996$, for fat percent was $r = 0.982$, and for the VL muscle thickness in US was $r = 0.914$.

Muscle Biopsies

Muscle biopsies were obtained 0.5 h before (pre) and 1 h (post 1 h) and 48 h (post 48 h) after the bout of RE, or resting in the control group, before the RT period (Fig. 1). The post-1 h biopsy time point represents fast responses of the RE bout and the 48-h time point the more delayed responses. The biopsy after RT (post 21 wk) was taken 4–5 days after the last exercise workout to minimize the effects of the last exercise workout on the post-RT biopsy. Biopsies were taken from the VL muscle with a 5-mm Bergström biopsy needle, midway between the patella and greater trochanter. The pre-RE and the 48-h as well as post-21 wk biopsies were taken from the right leg. To avoid any residual effects of the prebiopsy, the 1-h post-RE biopsy was taken from the left leg and the 48-h biopsy was taken 2 cm above the previous biopsy location. Before the baseline and the 21-wk biopsy, a 3-h fasting period was required. Of 11 control subjects, for 5 subjects only pre and post-21 wk biopsies were available.

The muscle sample was cleaned of any visible connective and adipose tissue as well as blood and frozen immediately in liquid nitrogen (−180°C) and stored at −80°C. The pre-21 wk and post-21 wk samples for immunohistochemical analysis were obtained with another needle, and they were immediately mounted on a cork, and frozen rapidly in isopentane cooled to −160°C in liquid nitrogen and thereafter stored at −80°C.

Tissue Processing

Muscle biopsy specimens were hand-homogenized in ice-cold buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% Triton X-100, 0.2% sodium deoxycholate, 30 μg/ml leupeptin, 30 μg/ml aprotinin, 60 μg/ml PMSF, and 1% phosphatase inhibitor cocktail (P 2850; Sigma, St. Louis, USA)] at a dilution of 15 μl/mg of wet weight muscle. Homogenates were rotated for 30 min at 4°C, centrifuged at 10,000 *g* for 10 min at 4°C to remove cell debris, and stored at −80°C. Total protein was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL).

Western Immunoblot Analyses

Aliquots of muscle lysate were solubilized in Laemmli sample buffer and heated at 95°C to denaturate proteins. For 4E-BP1, but not others, homogenates were first heated 10 min at 95°C, centrifuged at 7,000 *g* for 30 min at +4°C, and then continued with the Laemmli buffer and heating similarly as the other proteins (9).

Samples containing 30 μg of total protein were separated by SDS-PAGE for 60 to 90 min at 200 V using 4–20% gradient gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). All four samples from each subject were run on the same gel. Proteins were transferred to PVDF membranes at 300-mA constant current for 3 h on ice at 4°C. The uniformity of protein loading was checked by staining the membrane with Ponceau S. Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 1 h and then incubated overnight at 4°C with commercially available rabbit polyclonal primary phosphospecific antibodies. Antibodies rec-

ognized phosphorylated Akt on Ser^{473}, mTOR on Ser^{2448}, p70^{S6K} on Thr^{389}, rpS6 on Ser$^{235/236}$, 4E-BP1 on Thr$^{37/46}$, and eEF2 on Thr^{56} (Cell Signaling Technology, Beverly, MA) and COOH-terminal myostatin protein (Chemicon/Millipore AB 3239) (38). The rabbit polyclonal antibody used was raised against a peptide residing in the COOH terminus of myostatin corresponding to amino acids 349–364 of human myostatin and, therefore, being a similar antibody to those used previously (14, 50).

All the antibodies were diluted 1:2,000 (except eEF2 on Thr^{56}, which was 1:3,000) in TBS-T containing 2.5% nonfat dry milk. Membranes were then washed in TBS-T, incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Cell Signaling Technology) diluted 1:5,000 in TBS-T with 2.5% milk for 1 h followed by washing in TBS-T. Phosphorylated proteins were visualized by ECL according to the manufacturer's protocol (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology) and quantified using a ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories).

The membranes described above were incubated in Restore Western blot stripping buffer (Pierce Biotechnology) for 30 min and reprobed with appropriate antibodies for detection of the total expression levels of Akt and rpS6 (rabbit monoclonal) (Cell Signaling Technology) and p70^{S6K} (Santa Cruz Biotechnology) by immunoblot analysis as described above.

Blood analysis

The blood samples were drawn from the antecubital vein before and 0, 15, 30, and 60 min after the bout of RE using 21-gauge disposable needles. Blood was centrifuged at 3,500 rpm in 4°C for 10 min to separate serum and stored frozen at −80°C until assayed. Serum testosterone, sex hormone-binding globulin (SHBG), and insulin concentrations were analyzed by an immunometric chemiluminescence method with an Immulite 1000 (DPC, Los Angeles, CA). The sensitivity of the assay for testosterone and coefficient of variation (CV) are 0.5 nmol/l and 5.7%, for SHBG 0.2 nmol/l and 2.4%, and for insulin 2 mIU/l and 3.4%, respectively. Free testosterone was calculated from total testosterone and SHBG concentrations (56). The results are presented as uncorrected to plasma volume changes as there were no differences between the protein and placebo group in the decrease of the plasma volume during and after the bout of RE (data not shown).

Immunohistochemistry

Muscle fiber cross-sectional area. Serial 8-μm-thick transverse sections were cut on a cryomicrotome (Leica CM 3000) at −24°C. Fiber type was classified by staining using myofibrillar ATPase method according to the earlier study (31). Fiber sarcolemma was visualized with an antibody against dystrophin (DYS2, Novocastra Laboratories) using avidin-biotin peroxidase kit (Vectastain PK-4002, Vector Laboratories) with diaminobenzidine (Abbott Laboratories) as a chromogen. The measurements of fiber cross-sectional area (CSA) comprised an average of 125 ± 57 type I and 129 ± 61 type II muscle fibers. Stained cross sections were analyzed by Tema Image-Analysis System (Scan Beam) using a microscope (Olympus BX 50) and color video camera (Sanyo High Resolution CCD).

Immunohistochemical staining of rpS6 and mTOR. For immunohistochemical staining of rpS6 and mTOR, 8-μm longitudinal and cross sections before the RT period from resting state muscle of the present subjects were fixed 15 min with 4% PFA-PBS, permeabilized with 0.2% Triton-X for 10 min, and blocked 30 min with 3% BSA-PBS and thereafter incubated with primary antibodies overnight at +4°C. Double immunolabeling was performed using a rabbit monoclonal antibody against rpS6 or rabbit polyclonal antibodies against phospho-rpS6 on Ser$^{235/236}$ or phospho-mTOR on Ser^{2448} (Cell Signaling Technology; 1:40 in 1% BSA-PBS) with either mouse monoclonal antibody against human slow myosin heavy chain (MyHC I) [Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA: A4.951] diluted to 1:150 (18), or mouse monoclonal antibody against caveolin-3 (1:100) (BD Transduction Laboratories) to visualize muscle sarcolemma. Nuclei were stained by Hoechst 33258 (Sigma, St. Louis, MO). Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 or 546 and goat anti-mouse Alexa Fluor 546 or 488 (Molecular Probes, Eugene, OR). Negative controls were done by omitting the primary or secondary antibody. An Olympus BX-50F light microscope (Olympus Optical, Tokyo, Japan) with Olympus color CCD camera (Colorview III, Olympus Optical) and Analysis software (version 5.0, Soft-Imaging System, Munster, Germany) were used for the imaging and analysis. Two samples were also further analyzed using Olympus IX81 confocal microscope with imaging system and software (Olympus Fluoview 1.6a) (29).

Statistical Analyses

All data are expressed as means ± SD, except where designated. The data were analyzed by a repeated-measures general linear model ANOVA. Any violations of the assumptions of sphericity were explored and, if needed, corrected with a Greenhouse-Geisser or Huynh-Feldt estimator. The Shapiro-Wilk test revealed that Western blot data were not normally distributed, and therefore for the statistical tests, all those values were log-transformed. Holm-Bonferroni post hoc tests were performed to localize the effects. SPSS version 13.0 for Windows was used for statistical analyses (SPSS, Chicago, IL). The level of significance was set at $P < 0.05$.

RESULTS

Daily Nutrient Intake

Nutrient intake did not differ between the protein and placebo conditions at *weeks 0, 10.5*, or *21* or in the averaged values of those three time points (Table 2). The subjects habitually consumed 1.48 ± 0.35 g protein/kg body mass in the protein group and 1.41 ± 0.42 g/kg body mass in the placebo group.

Anthropometry

Body mass increased significantly in the training groups after 21 wk compared with the control group (Table 1). However, at the 10.5-wk time point, the protein group already showed an increase in body mass compared with the control group ($P = 0.01$), but the placebo group did not ($P = 0.56$). There was no change in the fat percent in any group. The protein group increased VL thickness after both 10.5 wk ($P < 0.05$) and 21 wk ($P < 0.01$) of RT, whereas the placebo group did so after 21 wk of RT ($P < 0.05$) but not after 10.5 wk ($P = 0.16$) (Table 1). As in the case of body mass, only the protein group increased its muscle thickness significantly after both

Table 2. *Dietary intake: averaged energy and macronutrient intakes in the protein and placebo groups throughout the 21-wk training period (week 0, week 10.5, and week 21)*

Variable	Protein Group	Placebo Group	*P* Value
E, ×1,000 kJ	10.5±1.5	10.2±3.0	0.73
E, kJ/kg body mass	140±23	135±34	0.57
Protein, g/kg body mass	1.5±0.3	1.4±0.4	0.57
CHO, g/kg body mass	3.9±0.7	3.8±1.0	0.63
Fat, g/kg body mass	1.2±0.3	1.2±0.4	0.91

Values are means ± SD. E, energy; CHO, carbohydrate. *P* value is statistical difference between the protein and placebo groups.

10.5 and 21 wk compared with the control group ($P < 0.05$), while the placebo group only approached a trend after 21 wk ($P = 0.12$).

Acute RE Bout at Week 0 and Training Volumes

The total volume of the work performed in the RE bout (loads × sets × repetitions) was similar in the placebo (88.7 ± 15.4 kg/kg body weight) and protein group (91.5 ± 15.7 kg/kg body wt) at *week 0*. Average training volumes (loads × sets × repetitions) for the leg extensor muscles (leg press and knee extension) were calculated for *weeks 1–7, 8–14*, and *15–21*. No significant difference was found between the protein and placebo groups (data not shown).

Western Blotting Results

ANOVA revealed a time effect in both the protein and placebo groups for the phosphorylation of $p70^{S6K}$ on Thr^{389}, rpS6 on $Ser^{235/236}$ and dephosphorylation of Akt on Ser^{473} ($P < 0.05$) (Fig. 2). In the protein group only, a significant time effect was also seen for the phosphorylation of mTOR at Ser^{2448} and in the placebo group for the dephosphorylation of 4E-BP1 on $Thr^{37/46}$ (Fig. 3*A*). In the control group, no time effect was seen in any of the studied proteins. The post hoc test revealed that the phosphorylation of $p70^{S6K}$ and rpS6 was increased in the protein and placebo groups 1 h after the RE bout. The change in the phosphorylation of $p70^{S6K}$ was significantly greater with the protein ingestion compared with the placebo group ($P < 0.001$). The phosphorylation of mTOR was increased only in the protein group, the increase persisting at all time points (post 1 h, post 48 h, and post 21 wk) ($P < 0.05$). There was a strong decreasing trend in the phosphorylation of 4E-BP1 in the placebo group at post 1 h ($P = 0.06$). The decrease was significant compared with the controls ($P < 0.05$). Of the individual changes of phosphorylated 4E-BP1 from pre to post 1 h, seven of nine subjects in the placebo group showed a decrease (average 43%) and two of nine subjects showed an increase (8%), whereas six of nine in the protein group showed an increase (112%) and three of nine

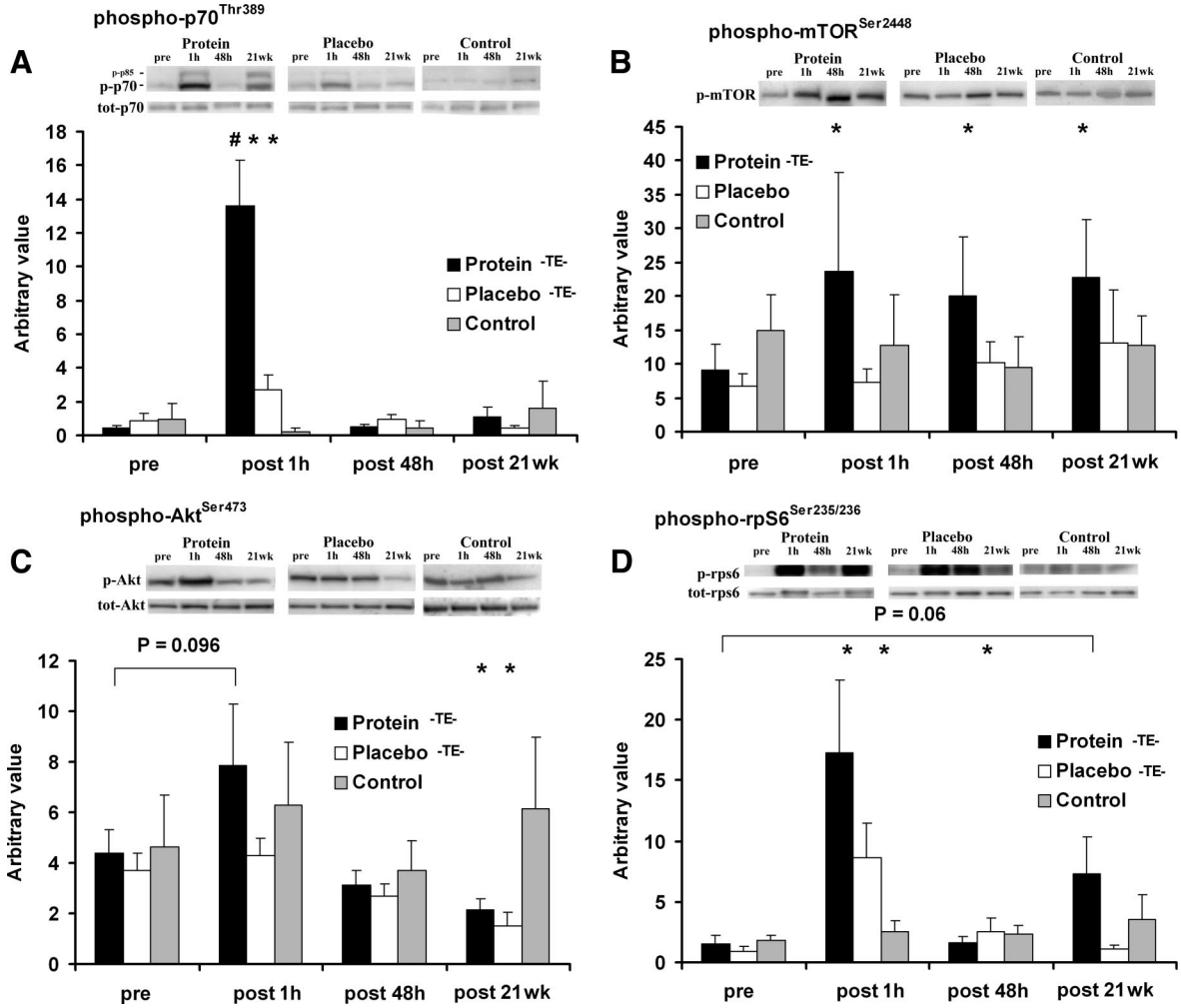

Fig. 2. Phosphorylation of $p70^{S6K}$ on Thr^{389} (p-p70; *A*), mammalian target of rapamycin (mTOR) on Ser^{2448} (p-mTOR; *B*), Akt on Ser^{473} (p-Akt; *C*), and ribosomal protein S6 (rpS6) on $Ser^{235/236}$ (p-rps6; *D*). Immunoblot of 1 individual is shown on top of *A–D*, as well as total (tot) forms of $p70^{S6K}$, Akt, and rpS6. $p70^{S6K}$ blot shows also that phosphorylation of other isoform of S6K1, $p85^{S6K}$ on Thr^{412} (p-p85), followed the same trend as $p70^{S6K}$. Values are arbitrary units (means ± SE). *$P < 0.05$ vs. pre. #$P < 0.05$, difference between protein and placebo. -TE-, $P < 0.05$ time effect of ANOVA. $n = 9$ for protein and placebo groups and $n = 6$ for control group. Five control subjects served only as pre and post-21 wk subjects and thus biopsies were obtained from 6/11 control subjects from all the 4 time points. See text for description of time points (pre, post 1 h, post 48 h, post 21 wk).

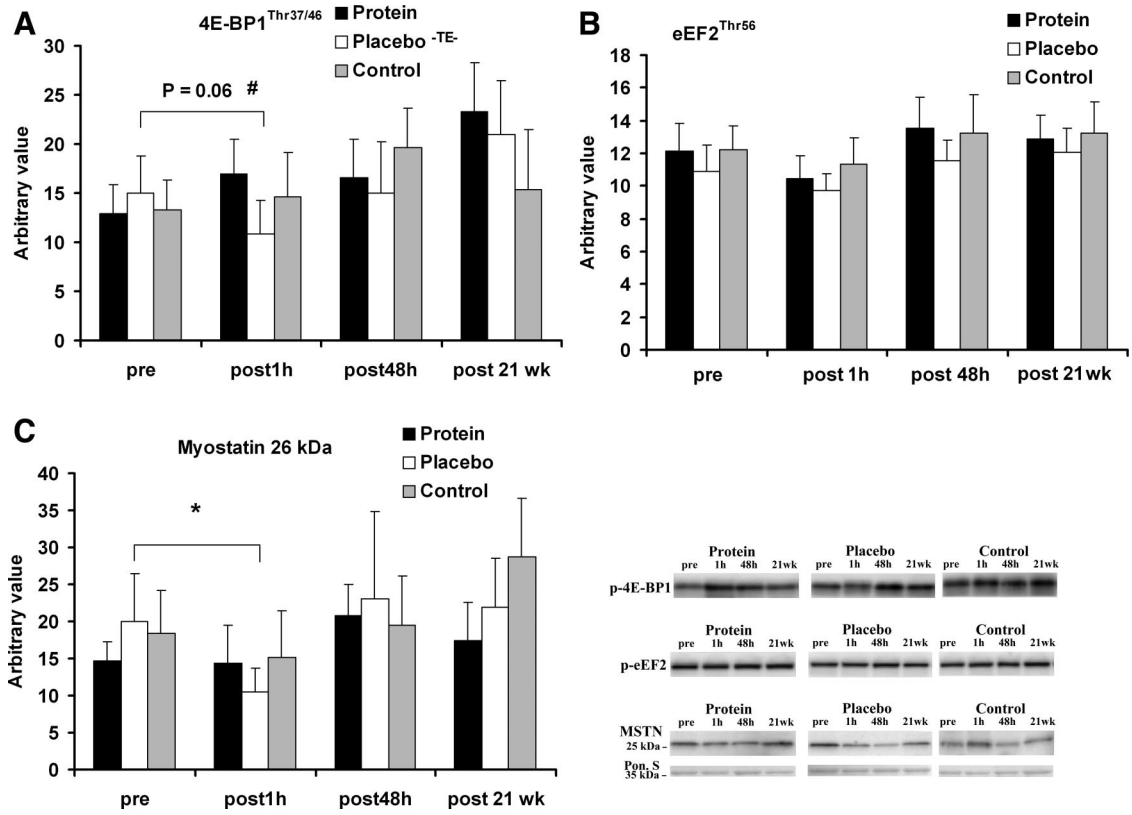

Fig. 3. Phosphorylation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1) on Thr$^{37/46}$ (p-4E-BP1; *A*), eukaryotic elongation factor 2 (eEF2) on Thr^{56} (p-eEF2; *B*), and myostatin 26 kDa (MSTN) COOH-terminal protein (*C*). Ponceau S (Pon S) staining shows equal protein loading. Immunoblot of 1 individual is shown at *right*. See text for further explanation. There was a between-group difference between the protein and placebo group and also between the placebo and control group in the change from pre to post 1 h (*$P < 0.05$).

a decrease (32%) (between-group difference in the change: $P = 0.03$). The phosphorylation of Akt decreased after 21 wk of RT in both training groups ($P < 0.05$). There was, however, no change in the phospho-eEF2 (p-eEF2) or total protein expression of p70^{S6K}, Akt, and rpS6. The myostatin COOH-terminal protein was decreased at post 1 h in the placebo group ($P = 0.02$) but not in the protein or control groups (Fig. 3*C*).

There were no significant correlations between the RE-induced change in the protein kinases or in the myostatin protein with corresponding changes in VL fiber size or muscle thickness (by ultrasonography) or VL CSA [by MRI previously (22)].

Immunohistochemistry

The CSA of type I and II fiber types increased significantly and similarly after 21 wk of RT in both the protein and placebo groups ($P < 0.01$) and also significantly ($P < 0.05$) compared with the control group (Table 1).

Both phosphorylated mTOR at Ser^{2448} and rpS6 at Ser$^{235/236}$ as well as total rpS6 were primarily localized close to the nuclei and sarcolemma, outside the area where contractile proteins are located (Fig. 4). The signal for these proteins emanated in large part from inside the muscle fibers but also to some extent from outside the sarcolemma. No clear cell-type difference was seen.

Serum Testosterone and Insulin

Compared with the control group, serum total testosterone concentration elevated significantly during the bout of RE only in the placebo group ($P = 0.04$). No differences between the groups were observed in free testosterone or in serum insulin (not shown).

DISCUSSION

The main findings of the present study were that ingestion of whey proteins before and after a bout of RE rapidly increased the phosphorylation of p70^{S6K} and also prevented the decrease in the phosphorylation of 4E-BP1. Moreover, the RE bout acutely decreased the active form of myostatin protein, but only when protein was not supplemented. The phosphorylation of mTOR remained increased after the RE bout from post 1 h to post 48 h and also after 21 wk of RT when the protein was ingested. However, RT itself decreased Akt phosphorylation. The control group results ensured that the results were not due to repeated biopsy, diurnal rhythm, or time of year (47, 57).

Whey protein rapidly increases mTOR signaling. In the present study, whey protein ingestion rapidly increased the phosphorylation of p70^{S6K} on Thr^{389} at 1 h post-RE, showing the activation of the TORC1 complex, including mTOR and its regulatory proteins (25, 54, 58). Probably also the activation of p70^{S6K} was increased especially in the protein group because

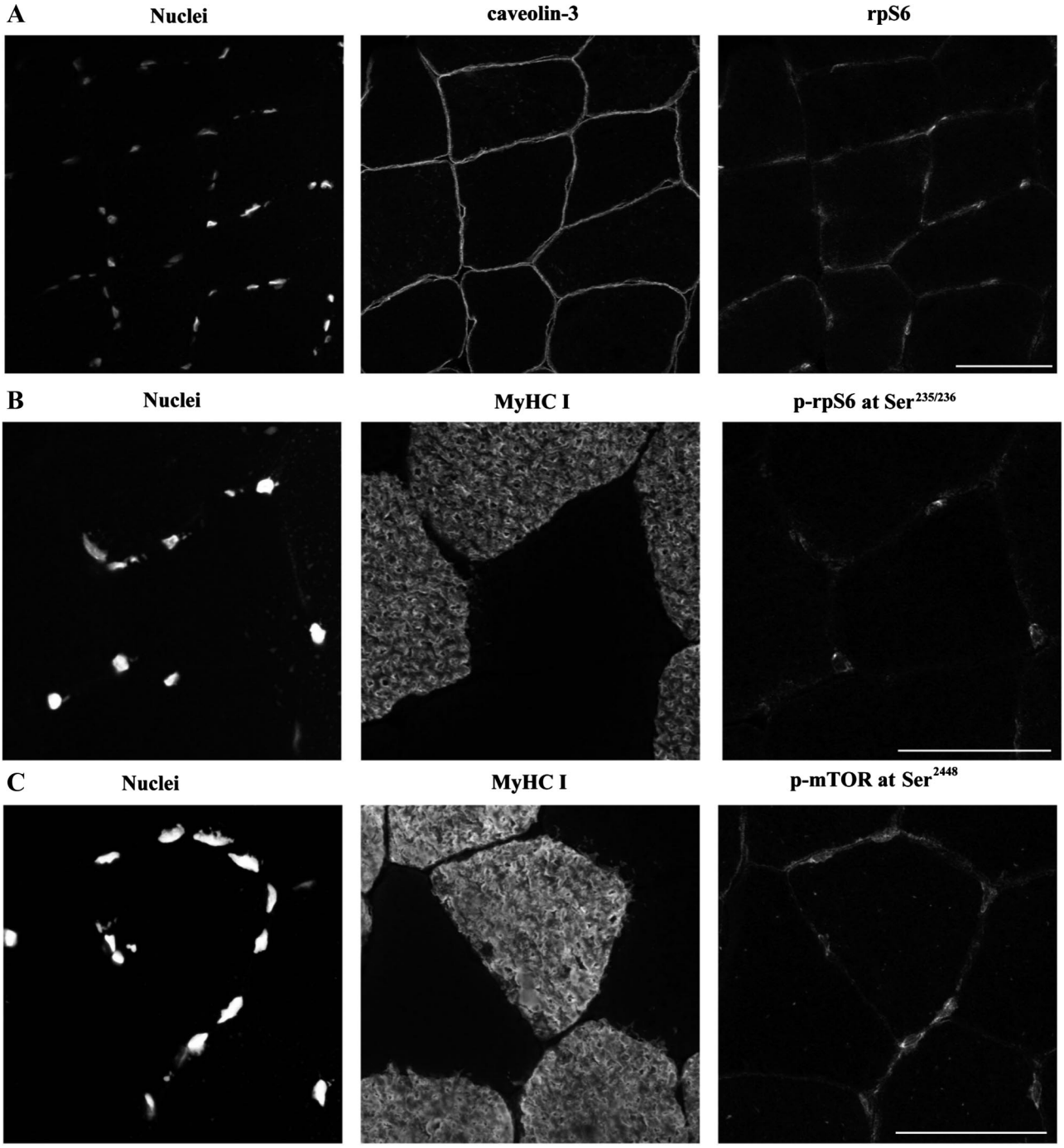

Fig. 4. Confocal microscopy images of localization of total (*A*) and phosphorylated rpS6 (*B*) and phopshorylated mTOR (*C*) in muscle cross sections. Nuclei were stained with Hoechst 33258, sarcolemma with antibody against caveolin-3, and myofibrillar area with an antibody against myosin heavy chain I (MyHC I). All images are representative of 2 subjects visualized with confocal microscope and of a total of 5 subjects with epifluorescence microscope. Phosphorylated mTOR at Ser^{2448}, rpS6 at $Ser^{235/236}$, and total rpS6 were primarily localized close to the nuclei and sarcolemma (caveolin-3), outside the area where contractile proteins are located (MyHC). The images were taken with the settings in which the secondary antibody (not shown) only gave minimal signal. Scale bars are 50 μm.

phosphorylation from this site is the chief event in the activation of $p70^{S6K}$ (44) and since there was also a tendency for larger phosphorylation of one of its downstream target rpS6 (protein vs. placebo, $P = 0.11$) and the increase in mTOR phosphorylation at Ser^{2448} was observed only in the protein group. This site of mTOR has been shown to be phosphorylated by $p70^{S6K}$ (5). The present results agree with those of a recent study showing that protein intake together with carbohydrate before, during, and 1 h after a RE bout increased phosphorylation of $p70^{S6K}$ at post 0–4 h compared with carbohydrate only (30). Interestingly, the phosphorylation of the second isoform of S6K1, $p85^{S6K}$, clearly followed the same pattern in the present study as that of $p70^{S6K}$ (see Fig. 2, protein blot image).

The most important component responsible for the increased phosphorylation of $p70^{S6K}$ with the whey protein ingestion is probably its large content of branched-chain amino acids that can elicit a similar $p70^{S6K}$ response in the context of a bout of RE as observed in the present study (3, 26). S6K1/$p70^{S6K}$ has been shown in animal and cell models to be especially important in muscle hypertrophy (2, 40, 48). Moreover, in humans an acute increase in the phosphorylation of $p70^{S6K}$ after a bout of RE has also correlated with a long-term loading-induced increase in fiber size as well as fat-free mass in trained humans (51), and with a RE-induced myofibrillar protein synthesis (33).

Whey protein intake alone prevented a RE-induced decrease in the phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1). This supports a recent finding in humans with a slightly different time scale and nutrients (30). An RE bout per se has also previously decreased the phosphorylation of 4E-BP1 shortly after exercise when protein or amino acids are not supplemented (8, 9, 30, 36). Prevention of the dephosphorylation of 4E-BP1 after a bout of RE by ingestion of whey proteins probably prevents association of the 4E-BP1 with eIF4E (43). This allows a larger increase in protein synthesis (12, 28, 35, 43), a phenomenon previously observed after the ingestion of whey proteins (52, 53). It can be speculated that whey protein alone can affect mTOR signaling TORC1 dependently and independently as the phosphorylation of 4E-BP1 from this site ($Thr^{37/46}$) may occur independently of TORC1 unlike the phosphorylation of $p70^{S6K}$ (25, 54, 58). Our results suggest that these effects occurred independently of blood insulin or the phosphorylation of Akt at Ser^{473}.

The phosphorylated mTOR is localized mainly close to the sarcolemmal membrane as has been shown in rodents (17, 41) while rpS6 and phospho-rpS6 (p-rpS6) were mostly located very close to the nuclei as has been found earlier with p-rpS6 (30). Magnification showed that rpS6 usually surrounded the nuclei, which is theoretically optimal for efficient protein synthesis.

Protein and training affects the phosphorylation of mTOR and Akt, respectively. In contrast to the rapid changes, the RE bout itself did not seem to have a consistent effect on the phosphorylation of the mTOR pathway proteins at 48 h post-RE or after a longer term RT, supporting recent human studies investigating time points 48 h (36) and 24 h post-RE (8, 36), and ~4 days after a RT period (34, 59). The only long-lasting effect of protein ingestion was the increased phosphorylation of mTOR, which remained increased in the protein group from 1 to 48 h after the RE bout and also after 21 wk of RT. Surprisingly, the phosphorylation of Akt decreased ~0.5-fold in both training groups after 21 wk of RT. In contrast, in previous studies 8–10 wk of RT increased the phopshorylation of Akt at Ser^{473} (34, 59). This different response may, owing to possible complexity of the temporal pattern of the Akt phosphorylation state, depend on the timing of the biopsies or additionally on the length or type of the training period or possibly the nutritional state.

The time point 4–5 days post-RE when the biopsy was taken represents roughly the time point when the next RE workout would have taken place. Therefore, the pre and post-21 wk biopsies are comparable. The observed more "chronic" Akt and mTOR responses to the RT with or without protein ingestion were probably due to *1*) a very long-lasting 4- to 5–day effect from the last RE workout, and/or *2*) a more permanent RT-induced change in the basal state of phosphorylation of these proteins. These changes may affect the level of the protein synthesis of the resting muscle (59) and/or the amount of the signal needed to activate the cascade through these pathways. It seems obvious that the change in the state of phosphorylation of at least some of the mTOR pathway protein kinases can be rather chronic and less transient due to, e.g., aging or exercise (16, 34, 59) and, at least partially, also due to protein ingestion.

Muscle Hypertrophy after RT, and Myostatin

The earlier MRI results obtained from the present study design showed a larger increase in VL muscle hypertrophy with whey protein ingestion (22). The present study also showed a somewhat faster increase in VL muscle thickness and body mass with protein ingestion. However, a larger protein-induced increase after the full 21-wk RT in fiber size was not observed. Recently, in older men, there were no positive effects of 10 g of casein protein ingested also immediately before and after a RE workout (55). It is possible that larger effects on muscle size would have been seen in the present study using subjects with a higher level of RT background or whose habitual ingestion of protein is smaller than ~1.4–1.5 g/body weight (49). Therefore, while the positive effects of the protein or amino acid ingestion on muscle hypertrophy signaling can often be clear when studied acutely after each exercise, especially when the study was performed in a fasting state, the long-term positive effects may not be as robust with normal daily high protein consumption.

The present study is the first in humans showing that myostatin peptide concentration, thought to be the active form of myostatin, can follow the decreased mRNA transcript of myostatin after a RE bout. Interestingly, protein ingestion seemed to prevent the decrease in myostatin after the RE bout. This may have hindered larger hypertrophy in the protein group, which could have been predicted from the mTOR pathway results because myostatin inhibits muscle growth (14, 37, 46). The hindering effect of protein ingestion for down-regulating myostatin expression after the bout of RE supports our earlier mRNA-level findings among younger (22) and older men (21), suggesting that the change in myostatin was transcriptionally regulated. Indeed, with the present subjects the myostatin mRNA and protein level changes from pre to post 1 h also correlated positively ($r = 0.66$, $P = 0.007$). It is assumed that the detected ~26-kDa myostatin is a glycosylated tightly bound dimer of a 110-amino acid COOH-terminal peptide of myostatin and/or that the monomer of myostatin is strongly bound by some other protein (14, 50). Recently, a myostatin propeptide of size 28 kDa and myostatin protein complexes of size 50 kDa were not changed 24 h after a bout of RE in humans (27).

Whey protein also seemed to prevent the elevation in serum total testosterone seen in the placebo group after the bout of RE, thereby supporting the previous results of protein ingestion (4, 20, 23). The testosterone response may be due to a decrease in the synthesis/secretion of testosterone and/or an increase in metabolic clearance. As was the case with myostatin protein concentration, the effect of protein ingestion on testosterone

was, however, small. Thus the physiological significance of these responses warrants future studies.

In conclusion, resistance exercise rapidly increases mTOR signaling, and whey protein increases and prolongs the mTOR signaling response to exercise and training. Active form of myostatin peptide rapidly decreases after a RE bout when protein nutrition is not supplemented.

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