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ORIGINAL ARTICLE

Acute and long-term effects of resistance exercise with or without protein ingestion on muscle hypertrophy and gene expression

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Abstract The effects of timed ingestion of high-quality protein before and after resistance exercise are not well known. In this study, young men were randomized to protein ($n = 11$), placebo ($n = 10$) and control ($n = 10$) groups. Muscle cross-sectional area by MRI and muscle forces were analyzed before and after 21 weeks of either heavy resistance training (RT) or control period. Muscle biopsies were taken before, and 1 and 48 h after 5 × 10 repetition leg press exercise (RE) as well as 21 weeks after RT. Protein (15 g of whey both before and after exercise) or non-energetic placebo were provided to subjects in the context of both single RE bout (acute responses) as well as each RE workout twice a week throughout the 21-week-RT. Protein intake increased ($P \leq 0.05$) RT-induced muscle cross-sectional area enlargement and cell-cycle kinase cdk2 mRNA expression in the vastus lateralis muscle suggesting higher proliferating cell activation response with protein supplementation. Moreover, protein intake seemed to prevent 1 h post-RE decrease in myostatin and myogenin mRNA expression but did not affect activin receptor IIb, p21, FLRG, MAFbx or MyoD expression. In conclusion, protein intake close to resistance exercise workout may alter mRNA expression in a manner advantageous for muscle hypertrophy.

Keywords cdk2 · Myostatin · Activin receptor IIb · Skeletal muscle · Whey

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Introduction

One of the hallmarks of resistance training is an increase in muscle cross-sectional area and improved maximal force production, especially in previously untrained subjects (for a comprehensive review, see Wernbom et al. 2007). In addition to resistance training, protein ingestion may play an important role as a regulator of muscle mass and recovery from exercise. The timing of the nutrient intake seems to be also of importance. Nutrient intake before and/or immediately after a resistance exercise (RE) session may be more beneficial in terms of muscle protein anabolism than nutrient ingestion at other times such as in the morning and late evening at least 5 h before or after the workout (Cribb and Hayes 2006) or 2 h after the workout (Esmarck et al. 2001). Especially whey/milk protein supplementation may be advantageous for gaining muscle size (Andersen et al. 2005; Hartman et al. 2007) and improving muscle protein balance after a RE bout (Tipton et al. 2007; Wilkinson et al. 2007).

Fast recovery from RE-induced myofibrillar disruption (Gibala et al. 1995) is also important. Many molecular factors are important in the recovery process from exercise as well as in the regulation of muscle hypertrophy per se.

Of these, myostatin, a well-known negative regulator of muscle size (McPherron et al. 1997) and proteins downstream to myostatin such as myogenic regulatory factors and cell-cycle kinases as well as their inhibitors all have been shown to be crucial (Charge and Rudnicki 2004; Kuang et al. 2006; McCroskery et al. 2003; Rios et al. 2002; Wagner 2005). A single heavy RE bout provides a high loading stimulus to skeletal muscle, from which complete recovery takes usually at least 2–4 days, while also affecting myostatin, myogenic regulatory factors, and other cell-cycle related factors (Hulmi et al. 2007; Kim et al. 2007; Mascher et al. 2008). However, it is not known whether high-quality protein such as whey (Ha and Zemel 2003) intake close to a resistance exercise modifies exercise-induced gene expression responses both acutely and after some months of systematic training.

In the present study we investigated whether supplementation of high-quality whey protein has additive effects compared to normal dietary intakes only, when ingested in conjunction with RE. The rational for the timed addition of a high-quality protein such as whey, is the possibility that it could improve the muscle protein synthesis response to exercise without interfering with the response to normal food. Indeed, recent results in an acute design by Paddon-Jones et al. (2005) suggest that an essential amino acid and carbohydrate supplement does not acutely interfere with the normal muscle protein synthesis response to a mixed meal. Whey is considered to be a high-quality protein source containing large amounts of essential amino acids, important in the protein synthesis (Borsheim et al. 2002), and also fast acting compared to many other protein sources such as casein (Boirie et al. 1997). It is thus possible that addition of whey, when used chronically in conjunction with RE may be more anabolic for skeletal muscle than ingesting only normal mixed meals throughout the day. Therefore, the purpose of this investigation was to examine long-term adaptations from resistance training in terms of whole-muscle size, force production, and muscle hypertrophy related gene expression that may occur when high-quality protein is added to a "normal diet" both immediately before and after each resistance exercise session. To the best of our knowledge, this is also the first study combining both acute and long-term gene expression responses with nutrition and cross-sectional area and maximal force of trained muscles. We hypothesized that whey protein intake immediately before and after a resistance exercise bout has both acute and long-term effects on possible resistance exercise-induced myostatin and cell-cycle related gene expression responses and this timed protein ingestion also increases whole-muscle hypertrophy response from 21 weeks of resistance training.

Materials and methods

Subjects

The subjects were randomly assigned after control testing sessions to either the whey protein group ($n = 13$), placebo group ($n = 14$) or to control group ($n = 11$). There was no RT in the controls but they continued their habitual activity such as jogging, swimming or ball-games. The number of subjects who completed the study was 31. The average age in the three groups were as follows: protein: 25.2 ± 5.2 years ($n = 11$), placebo: 27.2 ± 3.0 years ($n = 10$) and control: 24.9 ± 2.7 years ($n = 10$) (Table 1).

All the subjects were examined by a physician and none of them had medical problems that would confound the results of this investigation. All subjects were also free from neuromuscular dysfunction and thus were cleared to perform heavy RT. None of the subjects had prior heavy RT experience. Prior to the investigation, each subject was informed about the experimental design and the associated risks and discomforts that may occur. Each then signed an informed consent document to participate in the study, which was approved by the local Ethics Committee of the University and was done in accordance with the Declaration of Helsinki.

Design

This investigation examined long-term adaptations of adding high-quality protein to a "normal diet" (including no nutritional supplements) to increase its bioactivity. Because both acute and long-term molecular responses of resistance exercise without and especially with protein have not been carefully studied, several different muscle hypertrophy related gene transcript levels were examined both acutely after a single RE bout but also after long-term RT consisting of more than 40 RE workouts with either protein or placebo supplementation. The study design included a control group and all measurements were performed always at the same time to exclude the effects of biopsy sampling or effects of time of a year or daily variations (Sedliak et al. 2007; Vissing et al. 2005). The total duration of the present study was 23 weeks from which the first 2 weeks was a control period in which no experimental RT was carried out but the subjects maintained their normal recreational activities. All of the measurements (muscle force, muscle cross-sectional area, anthropometry and muscle biopsies) were preceded by at least 3 days of rest from physical activity. The experimental design is depicted in Fig. 1.

Table 1 Anthropometry. *P* $value_{pre}$ designates Holm–Bonferroni corrected *P* values compared to baseline

Variable	Group	2-week-control period		21-week-resistance training			*P* $value_p$	*P* $value_{\Delta group}$
		Control Mean ± SD	Baseline Mean ± SD	10.5 weeks Mean ± SD	21 weeks Mean ± SD	ΔChange Mean ± SD		
Height (cm)	PROT	182.2 ± 6.2						
	PLAC	181.0 ± 5.8						
	CONT	182.6 ± 4.8						
Body mass (kg)	PROT	76.1 ± 7.6	76.5 ± 7.3	79.5 ± 8.7	79.7 ± 8.7	3.2 ± 2.0	0.003*	0.001*
	PLAC	74.7 ± 8.5	74.8 ± 8.4	76.5 ± 8.7	77.3 ± 8.9	2.6 ± 2.1	0.008*	0.01*
	CONT	75.5 ± 8.1	75.7 ± 8.3	76.7 ± 8.6	75.9 ± 8.8	0.2 ± 1.5	0.65	
Fat (%)	PROT	17.0 ± 3.7	17.1 ± 3.8	17.5 ± 4.0	17.4 ± 4.2	0.3 ± 1.5	1	0.85
	PLAC	16.9 ± 4.4	16.6 ± 4.4	16.5 ± 4.7	16.6 ± 4.0	0.1 ± 0.9	1	0.55
	CONT	17.3 ± 3.8	16.7 ± 3.4	17.7 ± 4.3	17.1 ± 4.5	0.4 ± 1.5	0.86	

P $value_{\Delta group}$ designates the difference between the training groups and control group in the change between baseline and post 21-weeks values. ΔChange is the absolute difference from baseline to 21 weeks

* Significant ($P \leq 0.05$) *P* value

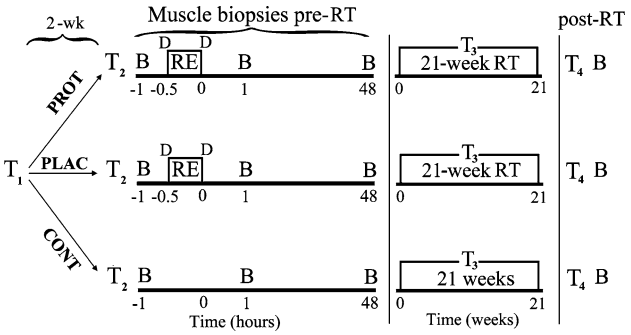

Fig. 1 Experimental design. *B* Vastus lateralis muscle biopsy, *T* testing, *RE* resistance exercise bout (5 × 10 RM leg press), *RT* heavy and progressive resistance training and *D* protein (15 g of whey protein) or placebo (no energy) drink. MRI was measured in T_2 and T_4 and muscle forces and anthropometry in $T_{1–4}$

Experimental resistance training

During the 21-week-RT period, total-body heavy RE workouts were carried out twice a week. A minimum of 2 days of rest was required between the two sessions each week. All training sessions were supervised by experienced trainers making sure that proper techniques and progression was used in each exercise (Kraemer et al. 2002). The training program was especially focused on knee extensors since the analysis of muscle cross-sectional area and muscle biopsies were obtained from the knee extensor muscle (i.e., vastus lateralis). The following exercises were used in each training session: two exercises for the leg extensor muscles, 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 of the body: chest and shoulders, upper back, trunk extensors and flexors, upper arms, ankle extensors, and hip abductors and adductors. Both leg press and knee extension exercises were thought to activate especially the VL muscle and it is the muscle in which the biopsy was taken. These exercises, previously utilized by our laboratory, produce somewhat larger hypertrophy responses in the VL and VM muscles compared to the other two quadriceps muscles during a comparable 21-week-RT program (Häkkinen et al. 2001).

The first two exercises in each workout were always the leg press and bench press. Recovery between the sets was 2–3 min. RT was performed with progressive training loads of 40–85% of the subject's 1 RM in a periodized training program. The number of sets of each exercise during RE workout increased (from 2–3 to 3–5) and the number of repetitions in each set decreased (15–20 to 5–6) during the 21-week-RT period. The loads were individually determined throughout the RT period.

Nutritional provision during resistance training

Either 15 g of whey isolate protein (Protarmor 907 LSI, Armor Proteins, Brittany, France, with minimal lactose and fat) dissolved in 250 ml of water or an equivalent amount of non-energetic placebo was ingested immediately before and after each bout of RE in the gym (Fig. 1). Whey is the most popular protein supplement by those resistance training and it effectively increases net muscle total protein synthesis and balance when consumed before or after RE bout at about similar doses used in this study (Tipton et al. 2007). The essential amino-acid composition of the protein drink (15 g) was as follows: histidine (0.2 g), isoleucine (1.0), leucine (1.7), lycine (1.4), methionine (0.4), phenylalanine (0.5), threonine (1.0), tryptophan (0.2) and valine (0.8). The drinks were provided for the subjects in a double-blind fashion. The drinks were made in our own

laboratory by the personnel who coded the drinks for the training supervisors. Drinks contained exotic fruit, trinatriumsitrate, acesulfame-K, xanthane gum and betacarotene for flavor, viscosity and color. The protein and placebo supplements looked and tasted as identical as possible.

Dietary intake of the subjects was registered with dietary diaries for 3 days before the first biopsy day at the start of the study, on the biopsy day, and the day thereafter (pre, 5 days overall), after 10.5 weeks (mid, 4 days) and again before the 21st week biopsy (post 21 weeks, 3 days before, and on the biopsy day). All of the diaries were analyzed using the Micro Nutrica nutrient-analysis software version 3.11 (The Social Insurance Institution of Finland). The subjects in either protein or placebo group did not eat anything 60 min before and 30 min after experimental exercise workouts during RT period. Food restriction during only these time periods was utilized to ascertain whether *addition* of a whey, considered fast acting and high-quality protein, has an *additive* effect even if the normal meal ingestion is not forbidden ~2–3 h before and after each RE bout.

Muscle cross-sectional area and anthropometry

The muscle cross-sectional area (CSA) of the right quadriceps femoris muscle was determined before and after the 21-week-period from both RT and control subjects using magnetic resonance imaging (MRI) (GE Signa Exite HD 1.5 T) at a local MRI center (Keski-Suomen Magneettikuvaus). During the measurement, the subjects' legs were kept parallel and strapped with a belt and a special cast designed to standardize the measurement as well as possible. Four axial-plane MRI scans were taken. The first image was taken 4 cm above the midway between the patella and greater trochanter ($image_1$) and thereafter the next three scans were taken at 2, 4 and 6 cm towards patella ($images_{2–4}$). All the MRI images were analyzed by the same experienced researcher with OsiriX (version 2.7.5) software.

After an overnight fasting, body mass (kg) and fat percentage were measured. Body fat was measured with skinfolds (biceps and triceps brachii, subscapular and iliac crest) (Durnin and Womersley 1974) by the same research assistant each time. Pearson Product correlations from control measurements spaced 2 weeks apart ($n = 8$ for MRI and $n = 38$ for skinfolds) showed high reproducibility for the measurement of quadriceps femoris CSA ($r > 0.96$) and for body fat percentage ($r = 0.97$).

Maximal force

Maximal isometric force of the bilateral leg extensor muscles was measured on an electromechanical dynamometer with knee angle of 107° and hip angle of 110° (Häkkinen et al. 2001; Hulmi et al. 2007). Unilateral isometric knee extension and flexion as well as bilateral bench press tests were performed with a David 200 system (David Fitness and Medical, Finland) (Häkkinen et al. 1998). The knee and elbow angles were 90°. A minimum of three trials were completed for each subject and the best performance trial was used in the subsequent statistical analysis. The force signal of the isometric measurements was recorded and analyzed with a Signal software version 2.15 (Cambridge Electronic Design Ltd., Cambridge, UK). A David 210 dynamometer (David Fitness and Medical, Finland) was used to measure maximal bilateral concentric force production for leg extensors (hip and knee extensors). Separate trials were performed for concentric 1 repetition maximum (RM) testing. After each repetition, the load was increased until the subject was unable to extend his legs to the full-extended ~180° knee angle position. The highest acceptable load was determined as the 1 RM. The subjects were carefully familiarized with the test procedures and had several warm-up contractions in all devices. Intra-class correlation coefficients with the present subjects ($n = 31$) from the control to baseline measures was 0.97 for bilateral isometric leg extension force, 0.98 for both 1 RM leg extension and isometric bench press and 0.90 for both knee extension and flexion.

Heavy RE protocol and nutritional supplementation when studying the acute effects of protein and RE bout

The heavy RE bout was carried out using the bilateral leg press machine (David 210) with similar protocols as described in earlier studies (Häkkinen et al. 2001; Hulmi et al. 2007, 2008) and the total number of sets for the leg press was five (each with 10 repetition maximums). Recovery time between sets was 2 min. The loads were adjusted during the course of the RE bout due to fatigue so that each subject would be able to perform ten repetitions at each set. If the load was too heavy, the subject was assisted slightly during the last repetitions of the set. Maximal isometric force was measured bilaterally before and after each set with an electromechanical dynamometer with a knee angle of 107° (Hulmi et al. 2007, 2008).

The details of the nutrition for this acute part of the study were exactly the same as in our earlier study with older men investigating acute gene expression responses to a single RE bout (Hulmi et al. 2008). The subjects fasted for 3 h before the first biopsy. Either a 250 ml of whey protein isolate or an equivalent amount of placebo was ingested immediately before and after the bout of RE (15 g of protein before and after the RE bout). The drinks were provided to the subjects in a double-blind fashion. Details of the drinks and dietary diaries were explained above in this article.

Muscle biopsies

Muscle biopsies were obtained 0.5 h before and 1 and 48 h after the RE session before RT as well as 4–5 days after the last RE workout from 21 weeks of RT (Fig. 1). The 1 h post-biopsy time point was selected to represent fast responses of RE bout and the 48 h post-time point the more delayed responses. We wanted to minimize the effects of the last exercise workout and the protein ingestion on the post-training biopsy. Therefore, the biopsy after RT was taken 4–5 days after the last exercise workout. Biopsies were taken from the VL muscle with a 5-mm Bergström biopsy needle, midway between the patella and greater trochanter. The pre-RE biopsy and the 48 h post-RE biopsies were taken from the right leg. Avoiding any residual effects of the pre-biopsy, 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. The 21-week-biopsy was taken from the same leg as the baseline biopsy (right). The muscle sample was cleaned of any visible connective and adipose tissue as well as blood. It was then immediately frozen in liquid nitrogen and stored at −80°C for future mRNA analysis.

Analysis of muscle messenger RNA

Total RNA isolation, reverse transcription and cDNA synthesis. Homogenization of the muscle samples were done with FastPrep (Bio101 Systems, USA) tubes and total RNA was extracted using the Trizol-reagent (Invitrogen, Carlsbad, CA, USA). An OD_{260}/OD_{280} ratio of 1.8–2.0 and gel electrophoresis showed that our extraction yielded DNA-free and un-degraded RNA, respectively. A total of 3 μg of total RNA was reverse transcribed to synthesize cDNA according to the manufacturer's instructions using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA).

Real-time RT-PCR. The mRNA expression levels were quantified with a real-time reverse transcriptase-PCR (RT-PCR) assay using an Abi 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The probes and primers used were pre-designed transcripts 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 001106 and Hs00609603_m1 (activin receptor IIb), NM 005860 and Hs00610505_m1 (follistatin related gene protein: FLRG), NM 002478 and Hs00159528_m1 (MyoD), NM 002479 and Hs00231167_m1 (myogenin), NM_078467.1 and Hs00355782_m1 (p21), NM_052827.1 and Hs00608082_m1 (cdk2), NM002046, Hs99999905_m1 (GAPDH), NM_148177.1 and NM_058229.2 and Hs00369714_m1 (Muscle Atrophy F-Box: MAFbx/atrogin-1) (Hulmi et al. 2007, 2008; Mascher et al. 2008).

Each sample was analyzed in triplicates. PCR cycle parameters used were for all genes: 50°C for 2 min, +95°C for 10 min, 37–45 (depending on the mRNA analyzed) cycles at 95°C for 15 s, and 60°C for 1 min. GAPDH mRNA was used as an endogenous control because it was shown to be rather stable and better as a housekeeping gene than 18sRNA in our previous study (Hulmi et al. 2007, 2008). Moreover, in the present study, GAPDH mRNA and total RNA (μg/mg wet muscle) were stable across all data points in both protein and placebo groups ($P > 0.17$). Gene transcript results were calculated according to the Liu and Saint (2001) mathematical model (Liu and Saint 2002). SigmaPlot (version 9.0, Systat Software inc., Richmond, CA, USA) was used as a curve fitting software needed in the method. The intra-assay CV%s for the triplicate-samples in the PCR runs were as follows: GAPDH (5.8%), myostatin (9.3%), FLRG (14.3%), activin receptor IIb (7.9%), p21 (9.1%), cdk2 (8.9%), myogenin (7.6%), MyoD (10.6%), MAFbx (9.3%).

Statistical analyses

All data are expressed as mean ± SD, except where designated. The data were analyzed by a two-factor repeated measures General Linear Model (GLM) ANOVA. Any violations of the assumptions of sphericity were explored and, if needed, corrected with a Greenhouse-Geisser or Huynh-Feldt estimator. In muscle cross-sectional area measurements there were only two levels in the sample time factor and therefore dependent *t* tests were used for their analyses. Shapiro–Wilk test revealed that mRNA data were not normally distributed and therefore for the statistical tests, all the mRNA values were log-transformed. Holm–Bonferroni post hoc tests were performed to localize the effects. All the analyses were performed by means of SPSS 14.0. The level of significance was set at $P \leq 0.05$.

Results

Daily nutrient intake

There were no statistically significant differences in the total absolute or body weight adjusted energy consumption or any macronutrient (protein, carbohydrate or fat) intake between the protein and placebo conditions at weeks 0, 10.5 or 21 ($P > 0.23$) (Table 2). The subjects habitually consumed protein 1.5 ± 0.4 g/kg in the protein group and 1.4 ± 0.4 g/kg in the placebo group (assessed via an

Table 2 Daily dietary intake: at the beginning of the study, at week 10.5, and during the last week (week 21)

Variable	Week 0			Week 10.5			Week 21		
	Protein	Placebo	*P* value	Protein	Placebo	*P* value	Protein	Placebo	*P* value
E (1,000 kJ)	10.4 ± 1.6	9.6 ± 2.0	0.37	10.5 ± 2.1	9.7 ± 3.2	0.60	10.2 ± 0.3	11.6 ± 3.2	0.48
E (kJ/kg bw)	136.6 ± 20.3	131.2 ± 20.9	0.57	139.3 ± 34.3	125.4 ± 37.1	0.50	145.7 ± 11.9	154.8 ± 36.7	0.70
Prot (g/kg bw)	1.4 ± 0.3	1.3 ± 0.3	0.67	1.5 ± 0.4	1.5 ± 0.5	0.91	1.7 ± 0.4	1.5 ± 0.4	0.59
CHO (g/kg bw)	3.9 ± 0.6	3.7 ± 0.7	0.35	4.0 ± 0.8	3.5 ± 1.0	0.34	3.4 ± 0.7	4.4 ± 1.1	0.23
Fat (g/kg bw)	1.2 ± 0.3	1.2 ± 0.3	0.47	1.1 ± 0.4	1.1 ± 0.4	0.99	1.5 ± 0.3	1.3 ± 0.4	0.54

Dietary diaries were kept at week 0 (3 days before the biopsy), on the biopsy day, and the day thereafter. At week 21 the dietary diary was recorded during the 3 days before the biopsy and the biopsy day. Week 10.5 also included a 4-day diary. *E* energy, *g/kg bw* g per kg body mass, *Prot* protein, *CHO* carbohydrates. *P* value is statistical difference between the protein and placebo groups. There were no differences between weeks 0, 10.5 and 21 in the macronutrient consumption in either protein or placebo groups ($P > 0.05$)

average of all food diaries: week 0, week 10.5 and week 21) ($P = 0.71$).

Anthropometric measurements

Body mass increased significantly during RT in both protein and placebo groups ($P < 0.01$) while there was no change in the control group ($P = 0.65$) (Table 1). Body fat% did not change significantly in any group ($P > 0.86$).

Muscle CSA

The cross-sectional area (CSA) of the quadriceps femoris (QF) increased significantly after 21 weeks of RT in both protein and placebo groups ($P < 0.01$) but not in the control group ($P > 0.05$) (Fig. 2a). The change of the average QF CSA was higher in the protein group (9.9 ± 7.4%) compared to placebo (7.5 ± 4.8%) but the difference did not reach statistical significance ($P > 0.05$). CSA of the VL muscle increased in all four axial-plane images in both protein and placebo groups ($P < 0.001$) but not in the control group ($P > 0.25$) (Fig. 2b). The average increase in the VL muscle (VL_{1-4}) was significantly higher in the protein group (relative increase: 14.8 ± 6.8%) compared to the placebo (11.2 ± 5.6%) ($P < 0.05$). In VI, VM and RF there were no significant differences in the CSA change between the protein and placebo groups in any of the CSA images ($P > 0.05$).

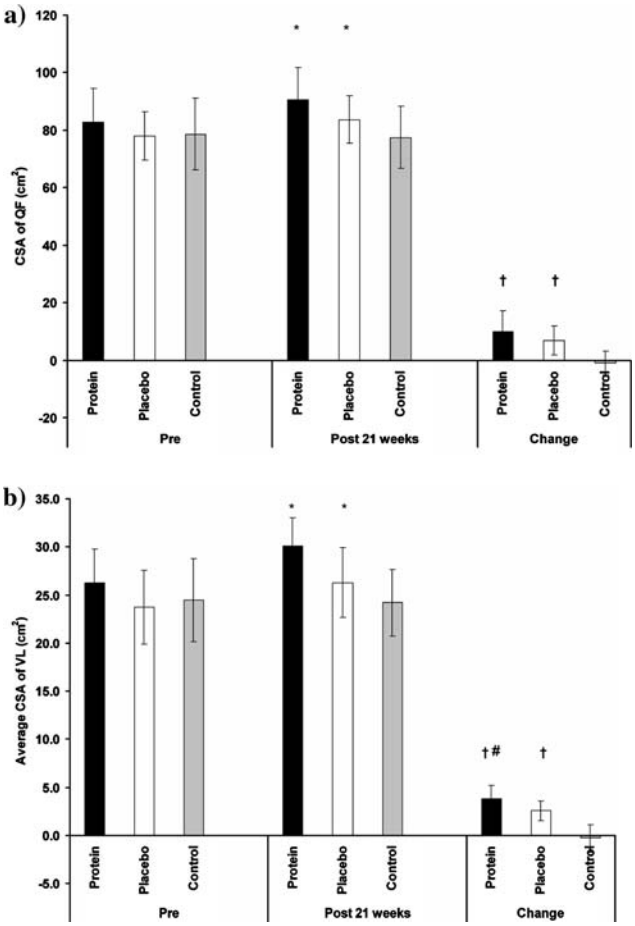

Fig. 2 The average CSA of **a** quadriceps femoris and **b** vastus lateralis from all four MRI images. Significantly ($P \leq 0.05$) different compared to the Pre (*asterisks*), to the Control (*daggers*) or to the Placebo (*hash*). All the values are mean ± SD

Muscle force

Maximal bilateral 1 RM leg extension, bilateral isometric bench press and unilateral isometric knee extension and flexion increased significantly and similarly during the 21-week-training period in both the protein and placebo groups ($P \leq 0.05$) (Table 3). However, compared to the control group (8.0 ± 9.5%, $P > 0.05$), isometric leg extension increased significantly only in the protein group (a relative increase of 24.3 ± 12.3%, difference between the groups $P = 0.02$), whereas the increase was not significant in the placebo group (19.3 ± 15.5%, $P = 0.23$).

Table 3 Muscle strength

Variable	Group	2-week-control period		21-week-resistance training			P value$_{pre}$	P value$_{\Delta group}$
		Control Mean ± SD	Baseline Mean ± SD	10.5 weeks Mean ± SD	21 weeks Mean ± SD	%Change Mean ± SD		
Dynamic (kg)	PROT	163.2 ± 31.8	168.6 ± 28.4	191.4 ± 33.0	200.9 ± 32.5	19.3 ± 4.7	<0.001*	<0.001*
1RM leg press	PLAC	161.0 ± 28.5	164.0 ± 29.9	184.5 ± 26.5	194.8 ± 26.3	19.8 ± 9.5	<0.001*	<0.001*
	CONT	165.5 ± 22.2	167.5 ± 24.4	171.5 ± 20.4	173.0 ± 21.6	3.7 ± 6.1	0.28	
Isometric	PROT	4017 ± 1533	3961 ± 1241	4485 ± 1548	4957 ± 1796	24.3 ± 12.2	0.002*	0.02*
Leg press (N)	PLAC	3647 ± 1333	3624 ± 1344	3823 ± 1350	4163 ± 1369	19.3 ± 15.5	0.007*	0.23
	CONT	3812 ± 925	3882 ± 978	3901 ± 815	4202 ± 1152	8.0 ± 9.5	0.06	
Isometric	PROT	853.3 ± 97.6	862.0 ± 104.5	981.1 ± 96.1	1029.1 ± 112.7	19.8 ± 8.6	<0.001*	<0.001*
Knee extension (N)	PLAC	813.5 ± 124.7	817.3 ± 96.6	956.1 ± 233.6	936.7 ± 117.5	14.9 ± 10.1	0.004*	0.05*
	CONT	841.0 ± 166.4	862.5 ± 194.1	860.0 ± 190.8	899.7 ± 209.6	4.4 ± 6.7	0.29	
Isometric knee flexion (N)	PROT	388.2 ± 54.2	399.5 ± 55.0	436.4 ± 56.9	461.9 ± 55.8	16.5 ± 12.7	0.004*	0.007*
	PLAC	422.3 ± 52.9	396.2 ± 66.2	441.4 ± 65.2	452.8 ± 73.9	15.3 ± 13.5	0.009*	0.01*
	CONT	394.3 ± 78.7	405.4 ± 95.9	397.6 ± 100.1	408.7 ± 95.6	1.3 ± 7.5	1.00	
Isometric bench press (N)	PROT	648.0 ± 141.5	655.0 ± 128.9	747.1 ± 154.9	803.9 ± 169.5	22.5 ± 8.3	<0.001*	<0.001*
	PLAC	628.6 ± 139.7	620.6 ± 149.1	716.0 ± 162.6	782.5 ± 174.0	25.2 ± 8.3	<0.001*	<0.001*
	CONT	613.2 ± 106.5	608.2 ± 118.3	633.5 ± 126.8	623.2 ± 130.1	2.4 ± 5.9	0.60	

P value$_{pre}$ designates Holm–Bonferroni corrected P values compared to baseline. P value$_{\Delta group}$ designates difference between the training groups and control group in the change between baseline and post 21 week values. ΔChange is the percentage difference from the baseline to 21 weeks

* Significant ($P \leq 0.05$) P value

Acute resistance exercise bout at the week 0

The total volume of the work in the RE bout (loads × sets × repetitions) was 6,722 ± 1,210 kg in the placebo and 6,753 ± 1,189 kg in protein group ($P = 0.96$) at week 0 of the study. Maximal isometric leg extension force was significantly decreased ($P < 0.01$) immediately following the RE session but there were no differences between the groups in this decrease after the RE bout ($P = 0.70$) (placebo: from 3,449 ± 1,118 N to 2,041 ± 369 N and protein: from 3,568 ± 1,184 N to 2,186 ± 431 N).

Muscle mRNA levels

There was no change in any measured mRNA values in the control group at any time point ($P > 0.05$) (Figs. 3, 4). A significant 31% decrease in myostatin mRNA was observed 1 h after the RE bout but only in the placebo condition ($P = 0.02$), not in the protein group ($P > 0.69$) (Fig. 3). The receptor of myostatin, activin receptor IIb mRNA, decreased in both protein and placebo groups after the RE bout being significant at 48 h after RE in both placebo and ($P = 0.04$) protein groups ($P = 0.01$).

A significant 340% increase in cdk2 mRNA was observed at 1 h after RE in the protein condition ($P = 0.01$) and there was also a trend for an increase both at 48 h post-RE (320%) and after 21 weeks of RT (120%) ($P = 0.08$) (Fig. 4). By contrast, there was a significant decrease after 21 weeks of RT in the placebo group ($P = 0.04$). Thus, the 21-week-responses in the cdk2 mRNA between the protein and placebo group were significantly different ($P = 0.04$).

p21 mRNA increased in both protein and placebo groups. The increase was significant in the placebo group both at post 1 h (679%, $P = 0.05$) and at post 48 h (976%, $P = 0.05$) after RE bout whereas significant increase was seen in the protein group at 1 h after RE (466%, $P = 0.003$). A significant decrease in myogenin mRNA was observed at 1 h after the RE bout (37%, $P = 0.005$) and after 21 weeks of RT (43%, $P = 0.02$) in the placebo group but not in the protein group ($P > 0.34$).

No significant change due to either protein, RE bout or 21 weeks of RT were observed in MAFbx ($P > 0.43$) (Fig. 3), MyoD ($P > 0.26$) and FLRG ($P > 0.10$) mRNA (MyoD and FLRG data not shown).

Discussion

The major findings of the present study investigating both acute and long-term effects in previously untrained young men were as follows: timed intake of 15 g of whey protein both immediately before and after each exercise session (1) further increased resistance training-induced vastus lateralis muscle hypertrophy measured by MRI without

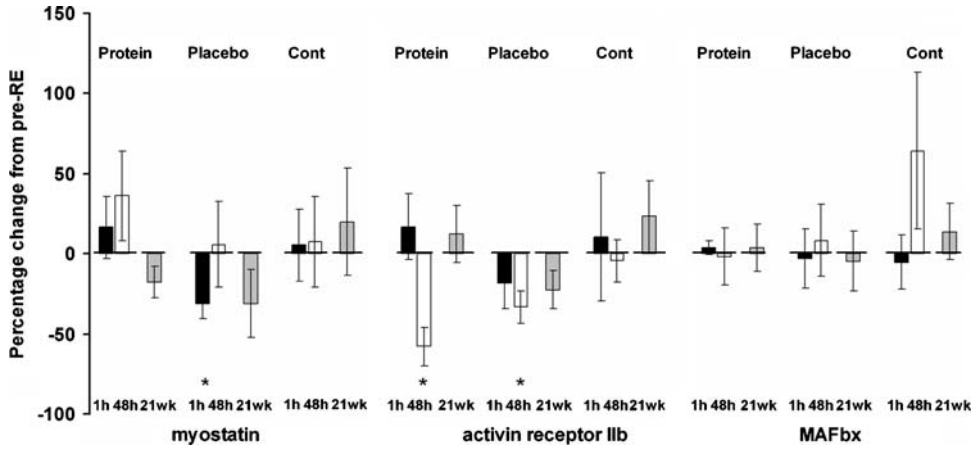

Fig. 3 Real-time RT-PCR results for myostatin, activin receptor IIb and MAFbx mRNA expressions before and after single RE bout (*black bar*: 1 h and *white bar*: 48 h) as well as after 21 weeks of RT (*gray bar*: 21 weeks) from vastus lateralis muscle in protein, placebo and control conditions. Results are normalized to GAPDH mRNA expression and changes are presented in relation to pre-RE levels. All the values are mean ± SE. *Asterisks* statistical ($P \leq 0.05$) difference compared to the pre-value in either protein or placebo condition and *hash* difference in the change between protein and placebo group. The results are shown as untransformed, whereas statistics were done with log-transformed values because the mRNA data were not normally distributed

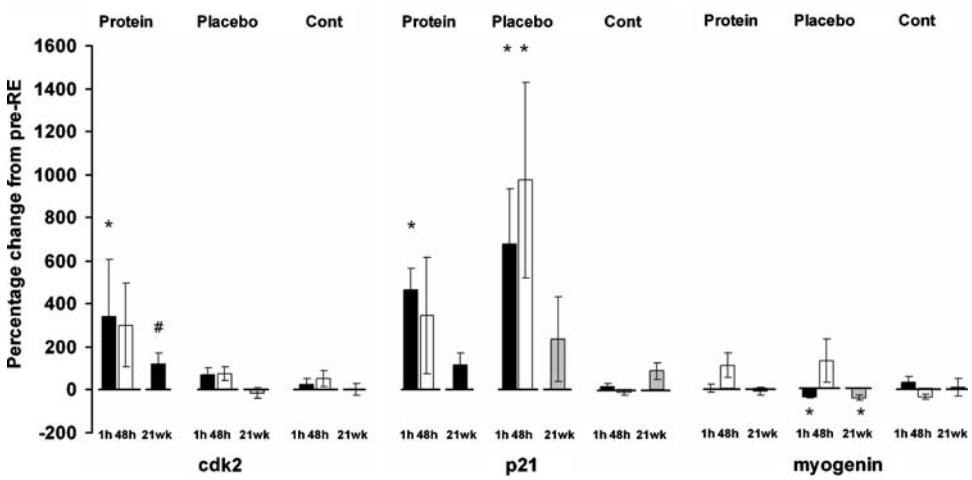

Fig. 4 Real-time RT-PCR results for cdk2, p21 and myogenin mRNA expressions before and after a single RE bout as well as after 21 weeks of RT. See further explanations in text for previous Fig. 3

significantly increasing cross-sectional areas of other quadriceps femoris muscles and also (2) increased cell-cycle related kinase cdk2 mRNA expression. Moreover, protein intake (3) seemed to prevent post-exercise decrease in myostatin and myogenin mRNA expression. The inclusion of the control group in the study assured that the results were not due to repeated biopsy effect, diurnal effect, or time of the year (Sedliak et al. 2007; Vissing et al. 2005).

Protein ingestion has been shown also previously to increase muscle myofiber CSA (Andersen et al. 2005; Cribb et al. 2007; Hartman et al. 2007) as well as lean or fat-free body mass (Burke et al. 2001; Candow et al. 2006a; Cribb et al. 2007; Hartman et al. 2007; Kerksick et al. 2006) during RT. This study was the first one to investigate the effects of timed protein nutrition close to a resistance exercise bout on training-induced whole-muscle hypertrophy by magnetic resonance imaging (MRI). MRI is considered a "gold standard" for cross-sectional area measurements of muscle size due to the high quality of the images and high reproducibility (Reeves et al. 2004). The current results demonstrate that subjects ingesting 15 g of whey both immediately before and after each RE workout, two times a week for 21 weeks, had larger quadriceps femoris (QF) muscle hypertrophy (~10%) than the placebo group (~7.5%) (ns). Of the individual QF muscles, protein ingestion significantly increased resistance training-induced muscle hypertrophy in vastus lateralis, one of the largest muscles in the body and largest of the four QF muscles (Häkkinen et al. 2001). The MRI results of the present study, therefore, further suggests the importance of high-quality protein consumption soon before and after each heavy RE workout (Andersen et al. 2005; Cribb and Hayes 2006). However, there were no statistically significant effects of

protein intake for other QF muscles although the most distal vastus medialis MRI-image showed signs for greater hypertrophy response in the protein group (16.9–19.9 cm^2 compared to 16.2–17.5 cm^2 for the placebo, $P = 0.059$, data not shown). The reason for observing the significant difference only in the VL muscle may be due to the fact that our exercise selection was designed to specifically load the VL muscle, the muscle from which muscle biopsies were taken. Indeed, compared to the VI and RF, the VL (and with a smaller extent also VM) exhibited the greatest hypertrophy, both absolutely and relatively, during the 21 weeks of RT (data not shown) supporting earlier results from our laboratory (Häkkinen et al. 2001).

Of the muscle strength variables, protein intake had a positive effect only in isometric leg force production in the leg press (the increase was significant compared to the controls only in the protein group). The finding that protein did not have consistent effect on maximal muscle force is in agreement with some (Andersen et al. 2005; Burke et al. 2001; Candow et al. 2006b; Kerksick et al. 2006), but not all previous studies (Candow et al. 2006a; Cribb et al. 2007). Accordingly, the effect of protein intake on improved muscle force in previously untrained subjects was only minor. This may be due to neural mechanisms, which may explain most of the force production enhancement during the first weeks of RT (Häkkinen et al. 2001). The effects of protein could, therefore, become significant and more consistent in terms of both muscle hypertrophy and muscle force production after much longer term training (e.g., 1–2 years), or possibly even faster with inclusion of already well-trained subjects or an amount larger than 2 × 15 g of protein per RE workout. These possibilities need further investigation.

In addition to muscle phenotype, many different gene transcript levels from the muscle were examined both acutely after the single RE bout and also after RT for 21 weeks. We found that cyclin-dependent kinase 2 (cdk2) mRNA levels increased significantly after the RE bout only in the protein group, the same phenomenon that has been shown earlier with older men in response to a similar RE bout and protein protocols (Hulmi et al. 2008). Interestingly, this increase in cdk2 mRNA remained elevated after 21 weeks of RT, but again only in the protein group. Therefore, it seems evident that protein ingestion close to the RE bout increases cdk2 mRNA expression both in young and old men.

Cyclin-dependent kinases are probably the most important regulators of cell proliferation (for review, see Malumbres et al. 2000). Cdk2 is especially important in the G1/S progression of the cell cycle (Berthet et al. 2003). Whereas cdk2 is a protein of which expression in both mRNA and protein levels as well as activity are increased in proliferating myoblasts (Hlaing et al. 2002; McCroskery et al. 2003; Ohkubo et al. 1994), it is possible that protein ingestion before and after a RE workout may increase satellite cell proliferation. This may lead to increased satellite cell count which is important in muscle recovery from micro-damage (Charge and Rudnicki 2004) and possibly also to myonuclear addition, a phenomenon important in muscle growth (Adams et al. 2002). Indeed, a recent study by Olsen et al. (2006) suggests that protein ingestion may have positive effects on the muscle satellite cell number during RT in humans. Moreover, Halevy et al. (2003) found that feeding increased DNA synthesis and satellite cell number in the culture of turkey breast muscle satellite cells when compared to a food-deprived state. Additional proof that protein possibly affects gene expression in human satellite cells in vivo comes from the present finding that protein ingestion seemed to prevent a small but rapid decrease in myogenin mRNA after the single RE bout and also a decrease after 21 weeks of RT, both observed only in the placebo group. This may be explained by an in vitro finding showing that feeding increases myogenin levels in satellite cell culture when compared to a food-deprived state (Halevy et al. 2003). Myogenin is a transcription factor expressed in myogenic cells, and like cdk2, is also downstream to myostatin (Rios et al. 2002). Myogenin is an important regulator for muscle satellite cell differentiation (Charge and Rudnicki 2004; Rios et al. 2002).

It is also possible that at least part of the observed higher cdk2 mRNA response with protein intake comes from proliferating cells other than satellite cells (or other muscle myogenic cells), such as fibroblasts and endothelial cells. Previously, one week of low protein intake *decreased* transcript levels in muscle positively relating to cell proliferation and *increased* transcript levels that negatively regulate cell proliferation (Thalacker-Mercer et al. 2007). The possibly larger cell proliferating capacity response could enhance muscle recovery after exercise workouts. The upstream mechanisms for the increased cdk2 gene expression response with whey protein are, however, unknown. It is possible that either (1) whey proteins in general or particular amino acids, (2) some bioactive peptides or other related functional components in it (Ha and Zemel 2003) or (3) energy in itself could affect cell-cycle regulators in skeletal muscle when the muscle metabolism is most active (i.e., during and after a RE bout when protein was provided). Whey proteins have a large amount of leucine, which was recently shown to activate myogenic satellite cells in pigs through the mTOR pathway (Han et al. 2008). Interestingly, cdk2 knockout mice are slightly smaller than wild-type mice (Berthet et al. 2003) and this difference could be related to possible positive effects of cdk2, a protein downstream of myostatin (McCroskery et al. 2003), on muscle mass.

In addition to cdk2, we also found effects of protein ingestion on myostatin mRNA itself. More specifically, the decrease in myostatin mRNA, occurring 1 h after the RE bout in the placebo group, was prevented with whey protein ingestion. We have observed that in older men, protein ingestion prevented the delayed post 48 h decrease in myostatin mRNA (Hulmi et al. 2008). Therefore, protein intake seems to affect muscle myostatin gene expression in healthy men, but possibly with a different time-scale in the young versus old. However, the results from various animal species and study settings on the effects of different nutritional protocols for the expression of myostatin are contradictory (Guernec et al. 2004; Jeanplong et al. 2003; Nakazato et al. 2006) and, therefore, more research is warranted. The *positive* effect of timed protein intake on vastus lateralis muscle growth was observed in the present study. Therefore, it is possible that the acute myostatin mRNA decrease in vastus lateralis muscle and protein ingestion effect on preventing this decrease may not have an especially important effect on muscle hypertrophy. This agrees with a recent study utilizing cluster analysis, which showed that subjects who had largest increase in muscle fiber CSA during a RT period did not have different post-RE myostatin mRNA response compared to individuals who experienced low to no increases in muscular hypertrophy (Kim et al. 2007).

We did not observe any effect of protein ingestion on myostatin binding protein FLRG mRNA response to the RE bout in contradiction with our earlier results with older men (Hulmi et al. 2008). Protein intake did not have a significant effect on the RE-induced response of cdk inhibitor p21 and activin receptor IIb mRNA, which supports our earlier results with older men (Hulmi et al. 2008). The significant down-regulation of activin receptor IIb 48 h after the RE bout confirms our previous findings with both untrained and trained older men (Hulmi et al. 2007). This RE-induced response is interesting since myostatin mediates its signals mainly through activin receptor IIb (Lee and McPherron 2001). Therefore, the decrease in activin receptor IIb mRNA gene expression after a RE bout may lead to lower myostatin signalling in muscle fibres, a response being theoretically advantageous for muscle growth. This possibility, however, needs further investigation. Since protein ingestion seems to have at least a minor acute effect on decreasing endogenous muscle protein degradation (Nagasawa et al. 1998; Tipton and Wolfe 2001), we were also interested in studying whether protein intake could affect transcription of enzymes regulating proteolysis. The results suggest that protein ingestion close to the RE bout does not have an effect on ubiquitin-ligase MAFbx mRNA expression (also called atrogin-1), a factor important in muscle proteolysis and atrophy (Bodine et al. 2001). This suggests that if protein affects RE-induced proteolysis it is not through transcriptional regulation of MAFbx.

In conclusion, high-quality whey protein intake before and after resistance exercise appears to further augment resistance training-induced muscle hypertrophy in previously untrained subjects. It also increased cyclin-dependent kinase 2 (cdk2) gene expression and may prevent an exercise-induced decrease in myostatin and myogenin mRNA. The increase in cdk2 gene expression suggests a higher proliferating cell activation response with protein supplementation that can be advantageous for muscle hypertrophy.

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