

# The effects of whey protein on myostatin and cell cycle-related gene expression responses to a single heavy resistance exercise bout in trained older men

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**Abstract** Myostatin decreases muscle mass and this is accomplished, in part, by inhibiting muscle satellite cell proliferation and differentiation by regulating the expression of cell cycle-related proteins (e.g. p21 and cdk2) and myogenic regulatory factors (e.g. myogenin and MyoD). The purpose of this investigation was to determine whether protein ingestion before and after a resistance exercise (RE) bout affects myostatin and cell cycle-related gene expression. Strength-trained middle-aged to older men were divided into a protein group ( $61.4 \pm 4.3$  years,  $n = 9$ ) or a placebo group ( $62.1 \pm 4.2$  years,  $n = 9$ ). Muscle biopsies from the vastus lateralis muscle were taken at rest and 1 and 48 h after a  $5 \times 10$  repetition leg press RE bout. Protein (15 g whey) or non-caloric placebo was taken immediately before and after the RE bout. mRNA expression levels of myostatin and related genes (AcvrIIb, FLRG, p21, p27, cdk2, myogenin and MyoD) were determined by Taqman probe-based real-time RT-PCR and normalized to GAPDH mRNA. Myostatin mRNA decreased after a RE bout, but only in the placebo group ( $P \leq 0.05$ ). Conversely, myosta-

tin-binding protein FLRG and cell-cycle kinase cdk2 mRNA increased only in the protein group ( $P \leq 0.05$ ). p21 mRNA was increased at 1 h post-RE in placebo ( $P \leq 0.05$ ) and tended to be increased in the protein group ( $P = 0.08$ ). Myostatin, its binding protein and cell cycle-related gene expressions are affected by single RE bout and these responses are further modified by whey protein intake. Therefore, controlling nutrition intake is important when studying gene expression responses to exercise.

**Keywords** Cdk2 · FLRG · Muscle hypertrophy · Nutrition · p21 · Satellite cells · Strength training

## Introduction

Myostatin is a TGF- $\beta$  superfamily member (McPherron et al. 1997) and is a negative regulator of muscle mass in humans (Schuelke et al. 2004) and in other mammals (Bogdanovich et al. 2002; McPherron et al. 1997). Myostatin mediates its signals through different activin receptors, of which activin IIb may be the most important (Lee and McPherron 2001; Yang et al. 2007). Myostatin has many binding proteins such as follistatin and follistatin-related gene protein (FLRG) that inhibit its secretion, activation and receptor binding (Hill et al. 2002; Joulia-Ekaza and Cabello 2007). The current knowledge is that myostatin inhibits skeletal muscle mass growth by decreasing satellite cell proliferation and differentiation and myonuclear addition as well as attenuating muscle protein synthesis (Taylor et al. 2001; McCroskery et al. 2003; Welle et al. 2006; Yang et al. 2007). Muscle satellite cells are undifferentiated mononuclear myogenic cells that are important in skeletal muscle growth (e.g., Adams et al. 2002). After stimulation, satellite cells enter cell cycle, divide and add new myonuclei

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to the skeletal muscle fibers via fusion with existing muscle fibers (Allen et al. 1999). Satellite cell cycle is regulated, in part, by myogenic regulatory factors myogenin and MyoD, and by cyclin dependent kinases (cdk) such as cdk2, which in turn are inhibited by cdk inhibitors such as p21 and p27 (Malumbres et al. 2000; McCroskery et al. 2003; Rios et al. 2002). Myostatin decreases muscle satellite cell as well as myoblast proliferation and differentiation by regulating cell cycle proteins (e.g. p21 and cdk2) and myogenic regulatory factors (e.g. myogenin and MyoD; Joulia et al. 2003; Langley et al. 2002; McCroskery et al. 2003; Rios et al. 2002).

Aging leads to decreases in muscle mass and strength (Greenlund and Nair 2003) and an old muscle recovers slower from exercise or injury than younger muscle (Brooks and Faulkner 1990), in which differences in satellite cell regulation may have a role (Dreyer et al. 2006). Heavy strength training is important for older subjects because it counteracts sarcopenic processes (e.g. Häkkinen et al. 1998). In addition to training, nutrition has probably an important role as a regulator in muscle mass and recovery. Single resistance exercise (RE) bout stimulates muscle protein synthesis but in the absence of nutritional intake the balance between protein synthesis and breakdown remains negative after RE (Tipton and Wolfe 2001). Recent studies have shown that nutrient intake before and/or immediately after a RE session is more beneficial in terms of muscle protein anabolism than nutrient ingestion at other times (Cribb and Hayes 2006; Esmarck et al. 2001). Especially, whey/milk protein supplementation has been shown to be advantageous for gaining muscle size (Andersen et al. 2005; Burke et al. 2001) and improving protein balance after a RE bout (Tipton et al. 2007). In addition to the effects of muscle protein synthesis per se, fast recovery from exercise-induced myofibrillar disruption (Gibala et al. 1995) is important, especially in the elderly. Myostatin and myogenic regulatory factors and therefore also satellite cell activation are probably important in the process of muscle recovery after acute micro- or macro-injury (Wagner et al. 2005). 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 and myogenic regulatory factor expression (e.g., Hulmi et al. 2007; Kim et al. 2005; Raue et al. 2006) and satellite cell proliferation (Dreyer et al. 2006). Protein ingestion may accelerate the recovery from a RE bout by possibly reducing the amount of muscle damage (Nosaka et al. 2006) and also possibly reducing protein degradation during the exercise (Tipton and Wolfe 2001). Protein intake may also have positive effects on muscle satellite cell regulation (Olsen et al. 2006). Interestingly, myostatin expression has been shown to be responsive to nutrition in some (e.g., Guernec et al. 2004; Jeanplong et al. 2003; Nakazato et al. 2006; Terova et al. 2006) but not all animal studies

(e.g., Chauvigne et al. 2003). Besides the effects of myostatin-binding supplements on serum myostatin levels (Willoughby et al. 2004), the effects of nutrition on human myostatin and cell cycle-related gene expression levels have not been reported in the literature. Therefore, the present study examined myostatin and cell cycle gene expression responses to a single resistance exercise bout with either 15 g of whey protein or placebo taken immediately before and after a RE bout in 57- to 72-year-old men.

## Methods

### Experimental design and subjects

The subjects trained with supervision heavy whole-body strength training for 5 months, two times per week. Details of the training procedure have been explained by Hulmi et al. (2007). Two weeks after the training, a subsample (age 57–72 years) was identified who were willing to take part in the present study. These subjects were matched according to age, body mass and maximal 1RM strength, and then randomly assigned to either a whey protein ( $n = 9$ ) or a placebo ( $n = 9$ ) group. Thus, previous training experience during the previous months was highly standardized among subjects. Six additional control subjects were used as controls who took part in the earlier study (Hulmi et al. 2007). The average age, weight, height and body weight adjusted dynamic one repetition maximum in the leg press in the three groups were as follows: for protein group  $61.4 \pm 4.3$  years,  $85.8 \pm 9.4$  kg,  $176.1 \pm 5.7$  cm and  $2.4 \pm 0.6$  kg/kg body weight, for placebo group  $62.1 \pm 4.2$  years,  $79.6 \pm 3.2$  kg,  $176.7 \pm 3.2$  cm,  $2.3 \pm 0.2$  kg/kg, and for controls  $66.5 \pm 7.1$  years,  $72.1 \pm 9.8$  kg,  $170.0 \pm 6.8$  cm,  $2.1 \pm 0.3$  kg/kg.

All the subjects went through medical examination and none of them had contraindications to perform heavy strength training. All subjects were physically rather active. The subjects were carefully informed about the design of the study with special information regarding possible risks and discomfort that might result. All the subjects signed the written informed consent 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.

### Maximal force testing and anthropometry

The force signal was recorded and analyzed with a Signal software version 2.15 (Cambridge Electronic Design, Cambridge, UK). Maximal isometric force of the bilateral leg extensor muscles was measured on an electromechanical dynamometer with a hip angle of  $110^\circ$  and a knee angle of

107° (Hulmi et al. 2007; Häkkinen et al. 1998). The intra-assay CV% for this device was with the present subjects 6.4% and Pearson’s correlation coefficient 0.94.

### Heavy resistance exercise (RE) protocol

The heavy RE bout was carried out in the bilateral leg press machine (David 210, David Fitness and Medical: Hulmi et al. 2007; Häkkinen et al. 1998). A 2-min warm-up with bicycle was conducted before the leg press protocol. The total set number in the leg press was five (5 × 10 repetition maximums). Recovery time between the sets was 2 min. The loads were adjusted during the course of the session so that each subject would be able to perform 10 repetitions for 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 before and after each set bilaterally with an electromechanical dynamometer with a knee angle of 107° (Hulmi et al. 2007; Häkkinen et al. 1998). The force signal was recorded and analyzed with a Signal software version 2.15 (Cambridge Electronic Design, Cambridge, UK). The intra-assay CV% for this device used for 1RM testing was with the present subjects 2.6% and Pearson’s correlation coefficient 0.95. Subjects were asked to rate their perceived exertion using Borg’s subjective scale (6–20) after the RE bout.

### Muscle biopsies

Muscle biopsies were obtained 0.5 h before and 1 as well as 48 h after the RE session (Fig. 1). Biopsies were taken from the VL muscle, midway between the patella and greater trochanter. The pre-RE biopsy and the 48-h post-RE biopsies were taken from the left leg. Avoiding any residual effects

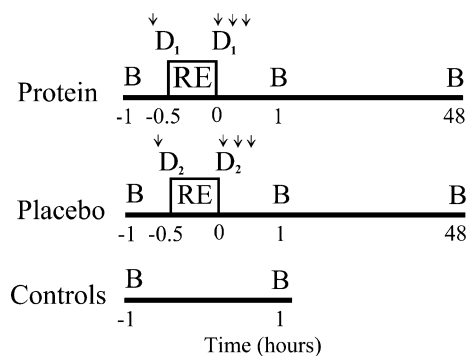
of the pre-biopsy, the 1-h post-RE biopsy was taken from the right leg and the 48-h biopsy was taken 2 cm above the previous biopsy location. Biopsy times were matched in the protein and placebo groups. Two biopsies, separated by 2 h of rest, were taken from six control subjects as has been explained earlier (Hulmi et al. 2007). The muscle sample was cleaned of any visible connective and adipose tissue, and blood. It was then quickly frozen in liquid nitrogen and stored at –80°C for future mRNA analysis.

### Nutritional protocol

The subjects fasted for 3 h before the first biopsy. Protein-rich foods (e.g., meat, dairy products, etc.) were not allowed during 5 h before the biopsy. Either a 250-ml whey isolate protein or an equivalent amount of placebo was ingested immediately before and after the bout of RE (Fig. 1). The drinks were provided to the subjects in a double-blind fashion. There were no calories in the placebo (exotic fruit, acesulfame-K, xanthane gum and betacarotene for flavor, viscosity and color). The protein was composed of 15 g of whey protein isolate (Protarmor 907 LSI, Armor Proteins, Brittany, France) with trinitriumsitrate, exotic fruit, acesulfame-K, xanthane gum and betacarotene for flavor and color. Whey is the most popular protein supplement used by people training at the gym and increases effectively net muscle protein balance when consumed before or after RE session at about similar doses used in this study (Tipton et al. 2007). All the supplements (Härmä Food) looked and tasted as identical as possible and had similar viscosity. Dietary intake of the subjects was registered by dietary diaries for 3 days before the first biopsy day and also during the biopsy day and the day thereafter and then analyzed using the Micro Nutrica nutrient-analysis software version 3.11 (The Social Insurance Institution of Finland).

### Total RNA isolation, reverse transcription and cDNA synthesis

Homogenization of the muscle samples were done with FastPrep (Bio101 Systems, USA) tubes containing Lysing Matrix D (Q-Biogene, USA). Total RNA was extracted from ~50 mg of muscle tissue using the Trizol-reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. OD<sub>260</sub>/OD<sub>280</sub> ratio of approximately 2.0 and gel electrophoresis showed that our extraction yielded DNA-free and un-degraded RNA, respectively. Five microgram of total RNA was reverse transcribed to synthesize cDNA according to the manufacturer’s instructions in a total volume of 50 µl using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA).



**Fig. 1** Experimental design. Subjects ( $n = 24$ ) were 57- to 77-year-old men (RE bout groups 57–72 years, controls 58–77 years). RE heavy resistance exercise (5 × 10 repetition leg press exercise), B vastus lateralis muscle biopsy which was obtained before and 1 and 48 h after RE, arrow blood sample obtained pre, post 0, 15 and 30 min after RE, D<sub>1</sub> protein and D<sub>2</sub> placebo drink ingested before and after RE bout

## Real-time RT-PCR

The mRNA expression levels were quantified with a real-time reverse transcriptase-PCR (RT-PCR) assay, based on the 5' nuclease activity of the Taq polymerase and using an Abi Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The probes and primers used were pre-designed transcripts (so-called inventoried assays) 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 (P27), NM 001106 and Hs00609603\_m1 (AcvrIib), NM 005860 and Hs00610505\_m1 (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), and NM002046, Hs99999905\_m1 (GAPDH), X03205.1 and X03205.1 and Hs99999901\_s1 (18sRNA). Each sample was analyzed in triplicates. An identical set of PCR cycle parameters was 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 that has been validated by Applied Biosystems. GAPDH mRNA was used as an endogenous control because it had lower CV% (4.4 vs. 5.9%) and it was more stable across different timepoints and behaved more similarly in PROT versus PLAC compared to 18sRNA. GAPDH/18sRNA was also stable through pre to post-1 h and pre- to post-48 h samples ( $P > 0.25$ ). For each subject, all samples were run simultaneously to permit relative comparisons. The amplification efficiency-based method (Liu and Saint 2002) was used for quantification and normalization. Gene transcript results were calculated according to the Liu and Saint (2002) mathematical model that has been shown to be very valid in studying the initial amount of gene transcript (Liu and Saint 2002). SigmaPlot (version 9.0, Systat Software, Richmond, CA, USA) was used as a curve fitting software needed in the Liu and Saint method. The intra-assay CV%:s for the triplicate-samples in the PCR runs were as follows: GAPDH (4.4%), 18sRNA (5.9%), myostatin (9.5%), FLRG (13.8%), AcvrIib (7.7%), p21 (6.7%), p27 (7.9%), cdk2 (8.8%), myogenin (6.9%), and MyoD (7.3%).

## Serum insulin

The blood samples for the determination of serum insulin were drawn from the antecubital vein before RE, 0, 15 and 30 min after RE. Serum samples were kept frozen at  $-80^{\circ}\text{C}$  until assayed. Serum insulin concentrations were analyzed by an immunometric chemiluminescence method with Immulite<sup>®</sup> 1000 (DPC, Los Angeles, CA, USA). The sensitivity of the assay for insulin is 2 mIU/l and CV 3.4%.

## Statistical analysis

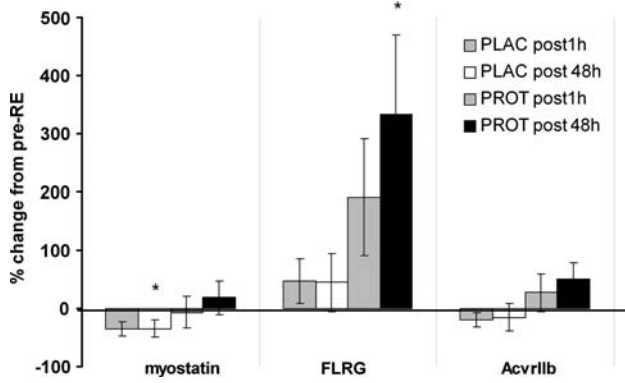
Standard statistical methods were used for the calculation of means, standard deviations (SD) and standard errors (SE). Correlation analysis was performed using the Pearson product moment method. The data from RE bout were analyzed by a two-factor repeated measures general linear model (GLM). There were three levels in sample time factor (pre-, post-1 h and post-48 h), so any violations of the assumptions of sphericity was explored and controlled according to the methods outlined in Atkinson (2001). Holm-Bonferroni post hoc tests were performed to localize the effects as suggested by Atkinson (2002). All the analyses of the strength training session were performed by means of SPSS 12.0. The level of significance was set at  $P \leq 0.05$ .

## Results

The total volume of the work in the RE bout (loads  $\times$  sets  $\times$  repetitions, mean  $\pm$  SD) was  $7,572 \pm 1,105$  kg in the placebo (PLAC) and  $8,094 \pm 1,378$  kg in protein (PROT, difference between the groups:  $P = 0.26$ ). There were no differences between Borg's subjective rate of perceived exertion following the RE bout in PLAC ( $17.0 \pm 0.6$ ) compared to PROT group ( $16.9 \pm 0.5$ ;  $P = 0.88$ ). Maximal isometric leg extension force decreased significantly ( $P < 0.01$ ) following the RE but there were no differences between the groups in this decrease of the force ( $P = 0.15$ ) after the RE bout (PLAC: from  $2,874 \pm 352$  to  $2,058 \pm 168$  N and PROT: from  $3,270 \pm 850$  to  $2,134 \pm 184$  N). There were no statistically significant differences in total absolute or body weight adjusted energy consumption or any macronutrient (protein, carbohydrate or fat) intake between protein and placebo conditions three days before a RE bout or in the nutrition during a RE day and the day thereafter ( $P > 0.23$ ). More specifically, average body weight adjusted protein intake (g/kg body mass) during three days before the RE bout was  $1.2 \pm 0.4$  and  $1.2 \pm 0.3$  in protein and placebo groups, respectively. Furthermore, average protein intake during the RE day and 1 day post-RE, not including 15 g of whey before and after the RE bout was  $0.9 \pm 0.3$  and  $1.0 \pm 0.3$  in protein and placebo groups, respectively.

## Muscle mRNA levels

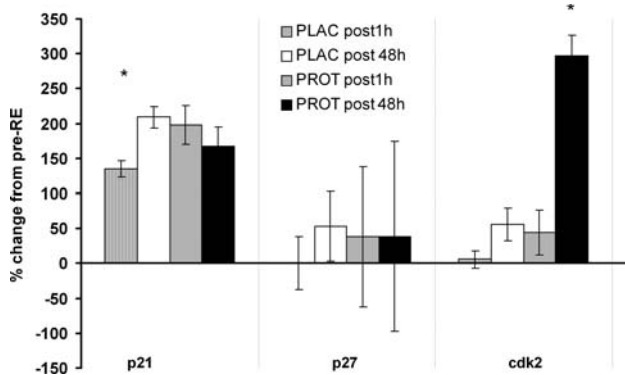
Heavy RE bout in the PLAC led to a decrease in myostatin mRNA ( $P = 0.03$ ), but not in PROT ( $P = 0.94$ ; Fig. 2). Post hoc analysis in PLAC revealed that the myostatin post-48 h levels were significantly lower compared to the corresponding pre-RE levels ( $P = 0.03$ ) and there was also a trend for a decreased myostatin mRNA at post-1 h ( $P = 0.06$ ).



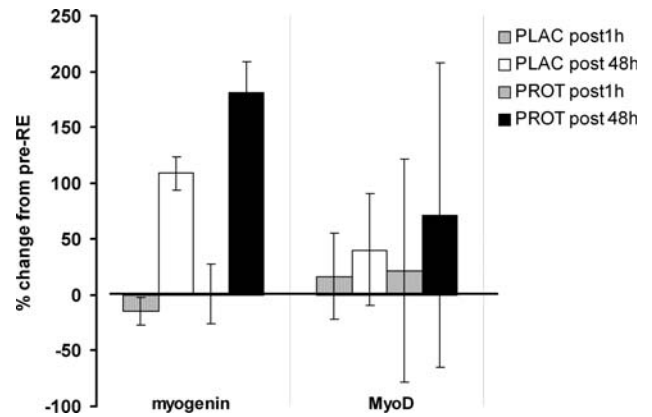
**Fig. 2** Real-time RT-PCR results for myostatin, FLRG and Acvr1Ib mRNA expressions before and after RE bout from vastus lateralis muscle under protein (PROT) and placebo (PLAC) conditions. Results are normalized to GAPDH mRNA expression and changes are presented in relation to pre-RE levels. Values are calculated according to Liu and Saint (2002) mathematical model. All the values are means ± SE. Asterisk indicates the Holm–Bonferroni corrected statistical  $P < 0.05$  difference compared to the pre-value in either protein or placebo condition

Conversely, FLRG mRNA increased only in PROT condition ( $P = 0.03$ ) while there was no change in PLAC ( $P = 1.00$ ; Fig. 2). Moreover, cell-cycle regulator cdk2 mRNA increased significantly only in PROT ( $P = 0.009$ ) while there was only a trend in PLAC ( $P = 0.075$ ; Fig. 3). The increase in both FLRG and cdk2 mRNA in PROT was localized at 48 h post-RE ( $P = 0.02$ ). Cdk inhibitor p21 mRNA was significantly higher compared to the pre-level at 1 h post-RE ( $P = 0.03$ ) in PLAC and there was a trend in PROT ( $P = 0.08$ , Fig. 3).

There was no RE loading effect for Acvr1Ib, p27, myogenin, MyoD, in either PROT or PLAC ( $P > 0.14$ ; Figs. 2, 3, 4); 2 h of resting did not change mRNA expression of myostatin, Acvr1Ib, FLRG, p27, myogenin, MyoD (Hulmi et al. 2007) or p21 ( $P = 0.38$ ) and cdk2 ( $P = 0.82$ , data not shown). To see the average RE loading effect from all ( $n = 18$ ) subjects with protein nutrition or not, the data were



**Fig. 3** Real-time RT-PCR results for p21, p27 and cdk2 mRNA expressions before and after RE bout. See further explanations in text for Fig. 2



**Fig. 4** Real-time RT-PCR results for myogenin and MyoD mRNA expressions before and after RE bout. See further explanations in text for Fig. 2

also pooled. The only significant results that were observed were for myogenin, showing a significant RE-loading effect ( $P = 0.05$ ). In addition, pooled data for p21 and myoD showed a trend for RE loading effect ( $P = 0.090$  and  $P = 0.096$ , respectively). Post hoc analysis localized the p21 response to especially post-1 h ( $P = 0.01$ ) but also with a trend to post-48 h ( $P = 0.07$ ).

#### Correlations between the studied mRNA responses

For correlation analysis, the data were pooled ( $n = 18$ ) and the correlations were analyzed for the pre- to post-1 h and for pre to post-48 h changes. Many significant positive correlations were found ( $P \leq 0.05$ ). For instance, cdk2 change at post-1 h ( $r = 0.67$ ,  $P = 0.002$ ) and at post-48 h ( $r = 0.75$ ,  $P < 0.001$ ) correlated with the corresponding changes in Acvr1Ib. The post-48 h change in myostatin correlated with the corresponding change in its receptor Acvr1Ib ( $r = 0.48$ ,  $P = 0.04$ ) and also with its binding protein FLRG (with FLRG at 1 h:  $r = 0.78$ ,  $P < 0.001$  and at 48 h:  $r = 0.53$ ,  $P = 0.02$ ). Moreover, the post-1 h change in myostatin correlated with the change in p27 (at 1 h:  $r = 0.821$ ,  $P < 0.001$  and at 48 h:  $r = 0.573$ ,  $P = 0.013$ ). Myogenin post-1 h change correlated significantly with corresponding change in MyoD ( $r = 0.49$ ,  $P = 0.04$ ), p21 ( $r = 0.46$ ,  $P = 0.05$ ), cdk2 ( $r = 0.76$ ,  $P < 0.001$ ), while myogenin post-48 h change correlated with the corresponding change in cdk2 ( $r = 0.79$ ,  $P < 0.001$ ) and Acvr1Ib ( $r = 0.55$ ,  $P = 0.02$ ). Interestingly, all the significant ( $P \leq 0.05$ ) and almost significant ( $P < 0.20$ ) correlations between the studied mRNA values at both post-1 h and at post-48 h were positive.

#### Serum insulin

There was a tendency towards an increase in serum insulin in response to RE bout both in PROT ( $53.0 \pm 24.5\%$ ,

$P = 0.08$ ) and in PLAC ( $22.6 \pm 10.2\%$ ,  $P = 0.10$ ; data not shown). The response between PROT and PLAC was similar ( $P = 0.33$ ).

## Discussion

This is the first study in humans to demonstrate that protein ingestion affects myostatin and cell cycle-related gene expression. More specifically, the present study showed that decrease in myostatin mRNA after a resistance exercise bout was prevented with whey protein ingestion in 57- to 72-year-old trained men. Moreover, protein intake increased the binding protein of myostatin, FLRG, and cyclin-dependent kinase 2 (cdk2) mRNA expressions after a RE bout.

In agreement with earlier studies, myostatin mRNA was found to decrease following a single RE bout when no extra-nutrition supplementation was provided (e.g., Hulmi et al. 2007; Kim et al. 2005; Raue et al. 2006). Furthermore, we found out that protein ingestion before and after a RE bout hinders decrease in myostatin mRNA in older or middle-aged men. Previously, a 48-h fasting decreased mRNA levels of myostatin while a 48-h re-feeding increased its levels back to normal level in chicken (Guernec et al. 2004). The effect of nutrition on myostatin may be mediated via protein nutrition because in rats, both mRNA and active form of myostatin protein was upregulated in the groups with increased amount of protein in the diet (Nakazato et al. 2006). Therefore, it can be speculated that the present decrease in myostatin observed in placebo condition after a RE session or in fasting/low protein in the previous studies (Guernec et al. 2004; Nakazato et al. 2006) could be a protective mechanism against muscle wasting (Guernec et al. 2004). This possible protective mechanism may not be needed when protein is ingested because protein such as whey increases net muscle protein balance (Tipton et al. 2007). However, the results from different animal species, ages and study settings on the effects of different nutritional intakes for the expression of myostatin gene family proteins are contradictory (e.g., Chauvigne et al. 2003; Guernec et al. 2004; Jeanplong et al. 2003; Nakazato et al. 2006; Terova et al. 2006). Therefore, more studies are needed to elucidate the complex effects of nutrition on myostatin expression.

Interestingly, some individual amino acids such as glutamine or glutamic acid, highly included in whey protein (combined 22% of all amino acids in our whey), may mediate the effects of protein on myostatin expression. Salehian et al. (2006) showed recently that feeding rats with glutamine or adding glutamine to growth medium of C2C12 myoblast cells increased myostatin protein levels, however only non-significantly. The results of that study are complicated to interpret since the glutamine intake also prevented

the glucocorticoid-induced increase in myostatin expression. Thus, the effect of single amino acids, or furthermore, the upstream signaling mechanisms for protein nutrition in affecting myostatin expression are currently unknown.

Many binding proteins for myostatin have been found but their specific role in regulating myostatin activation and receptor binding is not clear (Hill et al. 2002; Joulia-Ekaza and Cabello 2007). One of the binding proteins and inhibitors of myostatin is FLRG. FLRG has been shown to inhibit myostatin activity in a concentration-dependent manner in vitro (Hill et al. 2002) and it has been shown to circulate in human blood (Hill et al. 2002) and expressed in human skeletal muscle (Hulmi et al. 2007). Lee (2007) showed recently that transgenic expression of FLRG in mice increased muscle mass in a dose-dependent manner. Those results also suggested that FLRG appears to act by blocking not only myostatin but also additional ligands (probably other TGF- $\beta$  family members) that affect muscle growth. In the present study, FLRG mRNA responded with an increase to a RE bout only in the protein group. Therefore, it seems that protein ingestion may increase FLRG gene expression. This response may, if seen also at the protein level, decrease myostatin activity (Hill et al. 2002) and therefore could compensate for the possibly increased protein levels of myostatin in the protein group compared to the placebo group. The higher increase in cdk2 mRNA and similar responses in p21, myogenin and myoD mRNA after a RE session in protein compared to placebo condition indicate that protein ingestion possibly did not increase myostatin signaling because these proteins are downstream to myostatin signaling (McCroskery et al. 2003). In fact, myostatin increases p21 and decreases cdk2, myoD and myogenin expression (Joulia et al. 2003; McCroskery et al. 2003; Rios et al. 2002). Unfortunately, we did not have enough high quality muscle samples from the subjects for protein level measurements. Thus the effect of a RE bout with or without protein ingestion on myostatin and its signaling proteins (Yang et al. 2007) should be further studied in the future.

Muscle satellite cells are myogenic progenitor cells that are shown to be important in skeletal muscle growth, at least after some extent of increase in muscle fiber size (e.g., Adams et al. 2002). When stimulated, normally quiescent satellite cells enter cell cycle (McCroskery et al. 2003) and eventually divide and add new myonuclei to the skeletal muscle fibers via fusion with existing muscle fibers (Allen et al. 1999). The satellite cell cycle is regulated, in part, by cyclin-dependent kinases (cdk) such as cdk2, which in turn are inhibited by cdk inhibitors such as p21 and p27 (for a review, see Malumbres et al. 2000). The cdk inhibitor p21 inhibits progression of the cell cycle and aids in the initiation of the differentiation (Malumbres et al. 2000). In the present study, the levels of p21 mRNA increased after RE bout. The same result has been seen previously in humans

24 h after two bouts of electromyostimulation (Bickel et al. 2003), and during compensatory hypertrophy in rats (Adams et al. 1999) and strength training in humans (Kadi et al. 2004). The increase in the p21 mRNA may reflect satellite cells that are stimulated after a RE bout. However, other cell types such as fibroblasts or infiltrating immune cells may also contribute to this change. Positive correlation between p21 and myogenin mRNA responses suggests that p21 mRNA response to RE could be from myogenic cells, e.g. satellite cells. Because p21 is also anti-apoptotic (Wang and Walsh 1996), it could be speculated that a RE bout provides a stimulus to inhibit muscle satellite or other muscle stem cells' apoptosis by up-regulating anti-apoptotic p21 expression fast after a RE bout. Increased transcript level of myogenin at 48 h after a RE bout (pooled data:  $P = 0.07$ ) suggests that muscle satellite cell differentiation is ongoing (Rios et al. 2002), but increased cdk2 mRNA may indicate that part of these cells are still proliferating (McCroskery et al. 2003), at least in the protein group.

Interestingly, cdk2 mRNA response to a RE session was higher in the protein group compared to placebo. Since cdk2 is a protein expressed when satellite cells are activated for cell cycle (Malumbres et al. 2000; McCroskery et al. 2003), it can be suggested that protein ingestion before and after a RE may increase satellite cell activation and therefore their proliferation. Indeed, Olsen et al. (2006) showed that protein ingestion may have a positive effect on muscle satellite cell number during long-term strength training. Also supporting our suggestion, Thalacker-Mercer et al. (2007) recently found that 1 week of low protein intake *decreased* transcript levels positively related to cell proliferation. Moreover, at the same time, low-protein intake *increased* transcript levels that negatively regulate cell proliferation. The possible effect of protein ingestion on proliferating satellite cells could be especially important in the older population since aging slows down the recovery of the muscle from exercise (Brooks and Faulkner 1990) and it has been speculated that decreased satellite cell proliferation response to a RE bout may have a role in this difference between young and the old (Dreyer et al. 2006).

Most of the mRNA expressions increased due to a RE bout in this study. There were more than 20 significant correlations between the RE-induced eight genes of which expression was examined. Interestingly, all the significant ( $P \leq 0.05$ ) or also almost significant correlations ( $P < 0.20$ ) between the mRNA changes from pre to post-1 h and from pre to post-48 h in response to RE (myostatin, Acvr1Ib, FLRG, p21, p27, cdk2, myogenin, MyoD) were positive correlations. In other words, the more one up-regulated expression of some gene, the higher was also the increase in at least one of the other studied gene's expression. Thus, there is something downstream to RE-stimulus that affects the response in mRNA abundance of these genes that are all

related to muscle mass regulation and all related to each other based on many in vivo and in vitro studies (Bickel et al. 2003; Joulia et al. 2003; Kim et al. 2005; Langley et al. 2002; McCroskery et al. 2003; Rios et al. 2002; Taylor et al. 2001; Yang et al. 2007). This stimulus may be some factor that affects transcription of the mRNAs studied or additionally some factor that affects the mRNA half-life.

Cribb and Hayes (2006) showed recently that nutrient intake before and immediately after a RE session is more beneficial in terms of muscle protein anabolism than nutrient ingestion at other times. Another study demonstrated that only the group that consumed the nutrition immediately post-RE demonstrated significant increases in muscle hypertrophy after 12 weeks of strength training in older men (Esmarck et al. 2001). While not directly comparing the protein ingested at different time of a day, the effects of protein ingestion on cell cycle protein cdk2 mRNA and therefore possibly satellite cell proliferation observed in the present study may have a role since satellite cells are important in the muscle growth (e.g., Adams et al. 2002). Other mechanistic explanations for the importance of nutrient timing may be the protein induced hyperaminoacidemia with an increase in insulin at a time when blood flow is increased that appears to offer the maximum stimulation of muscle protein synthesis (Bohe et al. 2003, Tipton et al. 2001). It has been speculated that serum insulin response to nutrient ingestion after RE session could be important permitting maximal muscle anabolism and growth (Manninen 2006). In the current study, serum insulin was not significantly affected by 15 g of whey protein ingested both immediately before and after a RE bout in older or middle-aged men. Previously we found that ingestion of 25 g of protein 30 min prior to a heavy RE session in young men increased serum insulin levels (Hulmi et al. 2005). The reason for not seen significant effect in the present study may be, at least in part due to using older subjects in the present study because aging may lower the serum insulin response to acute nutrition ingestion (Paddon-Jones et al. 2004).

It was interesting to observe that RE bout with or without protein nutrition seem to have in addition to acute changes seen in many RE studies (e.g., Raue et al. 2006) also a delayed response in a transcription of a genes relating to muscle growth and recovery from RE. We and others have seen long-term changes in the gene expression due to a RE bout also earlier (Hulmi et al. 2007; Kim et al. 2005). Moreover, many responses to a single RE bout are rather long lasting and/or delayed: for example, protein synthesis (Phillips et al. 1997) and satellite cell proliferation (Cramer et al. 2004). Protein or amino acid ingestion per se may not have such a long-lasting effect. However, protein or amino acid intake has been shown to affect some delayed responses to a RE bout, for example muscle soreness and damage (Nosaka et al. 2006).

In the present study we did not measure muscle protein levels of the muscle gene transcripts due to the lack of muscle samples. It has been shown for many proteins that the transcription step is extremely important in the protein synthesis. For example, recent finding with short hairpin interfering RNA (shRNA) technology showed that even minor reduction in myostatin mRNA leads to an increase in the satellite cell number and muscle size in mice (Magee et al. 2006). While increased transcription of a gene or a decrease in mRNA degradation does not always lead to an increase in the protein levels, the present study provides novel results of the effects of protein ingestion close to a RE bout on muscle-growth specific gene expression in men. More specifically, protein ingestion seems to prevent resistance exercise induced decrease in myostatin mRNA expression, while increasing FLRG and cdk2 mRNA expression. This study also shows important evidence for the future exercise loading studies that nutrition is important to standardize if gene expression effects of exercise per se are investigated.

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## References

- Adams GR, Caiozzo VJ, Haddad F, Baldwin KM (2002) Cellular and molecular responses to increased skeletal muscle loading after irradiation. *Am J Physiol Cell Physiol* 283(4):C1182–C1195
- Adams GR, Haddad F, Baldwin KM (1999) Time course of changes in markers of myogenesis in overloaded rat skeletal muscles. *J Appl Physiol* 87(5):1705–1712
- Allen DL, Roy RR, Edgerton VR (1999) Myonuclear domains in muscle adaptation and disease. *Muscle Nerve* 22(10):1350–1360
- Andersen LL, Tufekovic G, Zebis MK, Crameri RM, Verlaan G, Kjaer M, Suetta C, Magnusson P, Aagaard P (2005) The effect of resistance training combined with timed ingestion of protein on muscle fiber size and muscle strength. *Metabolism* 54(2):151–156
- Atkinson G (2001) Analysis of repeated measurements in physical therapy research. *Phys Ther Sport* 2:194–208
- Atkinson G (2002) Analysis of repeated measurements in physical therapy research: multiple comparisons amongst level means and multifactorial designs. *Phys Ther Sport* 3:191–203
- Bickel CS, Slade JM, Haddad F, Adams GR, Dudley GA (2003) Acute molecular responses of skeletal muscle to resistance exercise in able-bodied and spinal cord-injured subjects. *J Appl Physiol* 94(6):2255–2262
- Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS (2002) Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 420(6914):418–421
- Bohe J, Low A, Wolfe RR, Rennie MJ (2003) Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study. *J Physiol* 552(Pt 1):315–324
- Brooks SV, Faulkner JA (1990) Contraction-induced injury: recovery of skeletal muscles in young and old mice. *Am J Physiol* 258(3 Pt 1):C436–C442
- Burke DG, Chilibeck PD, Davidson KS, Candow DG, Farthing J, Smith-Palmer T (2001) The effect of whey protein supplementation with and without creatine monohydrate combined with resistance training on lean tissue mass and muscle strength. *Int J Sport Nutr Exerc Metab* 11(3):349–364
- Chauvigne F, Gabillard JC, Weil C, Rescan PY (2003) Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. *Gen Comp Endocrinol* 132(2):209–215
- Crameri RM, Langberg H, Magnusson P, Jensen CH, Schroder HD, Olesen JL, Suetta C, Teisner B, Kjaer M (2004) Changes in satellite cells in human skeletal muscle after a single bout of high intensity exercise. *J Physiol* 558:333–340
- Cribb PJ, Hayes A (2006) Effects of supplement timing and resistance exercise on skeletal muscle hypertrophy. *Med Sci Sports Exerc* 38(11):1918–1925
- Dreyer HC, Blanco CE, Sattler FR, Schroeder ET, Wiswell RA (2006) Satellite cell numbers in young and older men 24 hours after eccentric exercise. *Muscle Nerve* 33(2):242–253
- Esmarck B, Andersen JL, Olsen S, Richter EA, Mizuno M, Kjaer M (2001) Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans. *J Physiol* 535(Pt 1):301–311
- Gibala MJ, MacDougall JD, Tarnopolsky MA, Stauber WT, Elorriaga A (1995) Changes in human skeletal muscle ultrastructure and force production after acute resistance exercise. *J Appl Physiol* 78(2):702–708
- Greenlund LJS, Nair KS (2003) Sarcopenia—consequences, mechanisms, and potential therapies. *Mech Ageing Dev* 124(3):287–299
- Guernec A, Chevalier B, Duclos MJ (2004) Nutrient supply enhances both IGF-I and MSTN mRNA levels in chicken skeletal muscle. *Domest Anim Endocrinol* 26(2):143–154
- Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y (2002) The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *J Biol Chem* 277(43):40735–40741
- Hulmi JJ, Volek JS, Selänne H, Mero AA (2005) Protein ingestion prior to strength exercise affects blood hormones and metabolism. *Med Sci Sports Exerc* 37(11):1990–1997
- Hulmi JJ, Ahtiainen JP, Kaasalainen T, Pollanen E, Häkkinen K, Alen M, Selänne H, Kovanen V, Mero AA (2007) Postexercise myostatin and activin IIb mRNA levels: effects of strength training. *Med Sci Sports Exerc* 39(2):289–297
- Häkkinen K, Kallinen M, Izquierdo M, Jokelainen K, Lassila H, Malkia E, Kraemer WJ, Newton RU, Alen M (1998) Changes in agonist-antagonist EMG, muscle CSA, and force during strength training in middle-aged and older people. *J Appl Physiol* 84(4):1341–1349
- Jeanplong F, Bass JJ, Smith HK, Kirk SP, Kambadur R, Sharma M, Oldham JM (2003) Prolonged underfeeding of sheep increases myostatin and myogenic regulatory factor Myf-5 in skeletal muscle while IGF-I and myogenin are repressed. *J Endocrinol* 176(3):425–437
- Jouliá-Ekaza D, Cabello G (2007) The myostatin gene: physiology and pharmacological relevance. *Curr Opin Pharmacol* 7(3):310–315
- Jouliá D, Bernardi H, Garandel V, Rabenoelina F, Vernus B, Cabello G (2003) Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. *Exp Cell Res* 286(2):263–275
- Kadi F, Schjerling P, Andersen LL, Charifi N, Madsen JL, Christensen LR, Andersen JL (2004) The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. *J Physiol* 558(Pt 3):1005–1012
- Kim JS, Cross JM, Bamman MM (2005) Impact of resistance loading on myostatin expression and cell cycle regulation in young and



- older men and women. *Am J Physiol Endocrinol Metab* 288(6):E1110–E1119
- Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R (2002) Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *J Biol Chem* 277(51):49831–49840
- Lee SJ (2007) Quadrupling muscle mass in mice by targeting TGF- $\beta$  signaling pathways. *PLoS ONE* 2(8):1–7:(e789)
- Lee SJ, McPherron AC (2001) Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci USA* 98(16):9306–9311
- Liu W, Saint DA (2002) Validation of a quantitative method for real time PCR kinetics. *Biochem Biophys Res Commun* 294:347–353
- Magee TR, Artaza JN, Ferrini MG, Vernet D, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J, Gonzalez-Cadavid NF (2006) Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. *J Gene Med* 8(9):1171–1181
- Malumbres M, Ortega S, Barbacid M (2000) Genetic analysis of mammalian cyclin-dependent kinases and their inhibitors. *Biol Chem* 381(9, 10):827–838
- Manninen AH (2006) Hyperinsulinaemia, hyperaminoacidaemia and post-exercise muscle anabolism: the search for the optimal recovery drink. *Br J Sports Med* 40(11):900–905
- McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R (2003) Myostatin negatively regulates satellite cell activation and self-renewal. *J Cell Biol* 162(6):1135–1147
- McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF- $\beta$  superfamily member. *Nature* 387(6628):83–90
- Nakazato K, Hirose T, Song H (2006) Increased myostatin synthesis in rat gastrocnemius muscles under high-protein diet. *Int J Sport Nutr Exerc Metab* 16(2):153–165
- Nosaka K, Sacco P, Maeatari K (2006) Effects of Amino Acid Supplementation on Muscle Soreness and Damage. *Int J Sport Nutr Exerc Metab* 16(6):620–635
- Olsen S, Aagaard P, Kadi F, Tufekovic G, Verney J, Olesen JL, Suetta C, Kjaer M (2006) Creatine supplementation augments the increase in satellite cell and myonuclei number in human skeletal muscle induced by strength training. *J Physiol* 573:525–534
- Paddon-Jones D, Sheffield-Moore M, Zhang XJ, Volpi E, Wolf SE, Aarsland A, Ferrando AA, Wolfe RR (2004) Amino acid ingestion improves muscle protein synthesis in the young and elderly. *Am J Physiol Endocrinol Metab* 286(3):E321–E328
- Phillips SM, Tipton KD, Aarsland A, Wolf SE, Wolfe RR (1997) Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am J Physiol* 273(1):E99–107
- Raue U, Slivka D, Jemiolo B, Hollon C, Trappe S (2006) Myogenic gene expression at rest and after a bout of resistance exercise in young (18–30 yr) and old (80–89 yr) women. *J Appl Physiol* 101(1):53–59
- Rios R, Carneiro I, Arce VM, Devesa J (2002) Myostatin is an inhibitor of myogenic differentiation. *Am J Physiol Cell Physiol* 282:C993–C999
- Salehian B, Mahabadi V, Bilas J, Taylor WE, Ma K (2006) The effect of glutamine on prevention of glucocorticoid-induced skeletal muscle atrophy is associated with myostatin suppression. *Metabolism* 55(9):1239–1247
- Schuelke M, Wagner KR, Stolz LE, Hubner C, Riebel T, Komen W, Braun T, Tobin JF, Lee SJ (2004) Myostatin mutation associated with gross muscle hypertrophy in a child. *N Engl J Med* 350(26):2682–2688
- Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH Jr, Kull FC Jr, Gonzalez-Cadavid N (2001) Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. *Am J Physiol Endocrinol Metab* 280(2):E221–E228
- Terova G, Bernardini G, Binelli G, Gornati R, Saroglia M (2006) cDNA encoding sequences for myostatin and FGF6 in sea bass (*Dicentrarchus labrax*, L.) and the effect of fasting and refeeding on their abundance levels. *Domest Anim Endocrinol* 30(4):304–319
- Thalacker-Mercer AE, Fleet JC, Craig BA, Carnell NS, Campbell WW (2007) Inadequate protein intake affects skeletal muscle transcript profiles in older humans. *Am J Clin Nutr* 85(5):1344–1352
- Tipton KD, Wolfe RR (2001) Exercise, protein metabolism, and muscle growth (2001) *Int J Sport Nutr Exerc Metab* 11(1):109–132
- Tipton KD, Rasmussen BB, Miller SL, Wolf SE, Owens-Stovall SK, Petrini BE, Wolfe RR (2001) Timing of amino acid-carbohydrate ingestion alters anabolic response of muscle to resistance exercise. *Am J Physiol Endocrinol Metab* 281:E197–E206
- Tipton KD, Elliott TA, Cree MG, Aarsland AA, Sanford AP, Wolfe RR (2007) Stimulation of net muscle protein synthesis by whey protein ingestion before and after exercise. *Am J Physiol Endocrinol Metab* 292:E71–E76
- Wagner KR, Liu X, Chang X, Allen RE (2005) Muscle regeneration in the prolonged absence of myostatin. *Proc Natl Acad Sci USA* 102(7):2519–2524
- Wang J, Walsh K (1996) Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. *Science* 273(5273):359–361
- Welle S, Bhatt K, Pinkert CA (2006) Myofibrillar protein synthesis in myostatin-deficient mice. *Am J Physiol Endocrinol Metab* 290(3):E409–E415
- Willoughby DS (2004) Effects of an alleged myostatin-binding supplement and heavy resistance training on serum myostatin, muscle strength and mass, and body composition. *Int J Sport Nutr Exerc Metab* 14(4):461–472
- Yang W, Zhang Y, Li Y, Wu Z, Zhu D (2007) Myostatin induces cyclin D1 degradation to cause cell cycle arrest through a phosphatidylinositol 3-kinase/AKT/GSK-3  $\beta$  pathway and is antagonized by insulin-like growth factor 1. *J Biol Chem* 282(6):3799–3808