Molecular and Cellular Endocrinology 317 (2010) 25–30

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Effects of oral creatine and resistance training on serum myostatin and GASP-1

A. Saremi[a], R. Gharakhanloo[b], S. Sharghi[c], M.R. Gharaati[d], B. Larijani[c], K. Omidfar[c,*]

[a] Department of Sport Science, Arak University, Arak, Iran
[b] Department of Sport Science, Tarbiat Modares University, Iran
[c] Endocrinology and Metabolism Research Center, Tehran University of Medical Sciences, Tehran, Iran
[d] Department of Biochemistry, School of Basic Sciences, Tarbiat Modares University, Iran

ARTICLE INFO

Article history:
Received 11 June 2009
Received in revised form
16 September 2009
Accepted 15 December 2009

Keywords:
Myostatin
GASP-1
Creatine
Skeletal muscle

ABSTRACT

Myostatin is a catabolic regulator of skeletal muscle mass. The purpose of this study was to determine the effect of resistance training for 8 weeks in conjunction with creatine supplementation on muscle strength, lean body mass, and serum levels of myostatin and growth and differentiation factor-associated serum protein-1 (GASP-1). In a double-blinded design 27 healthy male subjects (23.42 ± 2.2 years) were assigned to control (CON), resistance training + placebo (RT + PL) and resistance training + creatine supplementation (RT + CR) groups. The protocol consisted of 3 days per week of training for 8 weeks, each session including three sets of 8–10 repetitions at 60–70% of 1 RM for whole-body exercise. Blood sampling, muscular strength testing and body composition analysis (full body DEXA) were performed at 0, 4th and 8th weeks. Myostatin and GASP-1 was measured. Resistance training caused significant decrease in serum levels of myostatin and increase in that of GASP-1. Creatine supplementation in conjunction with resistance training lead to greater decreases in serum myostatin ($p < 0.05$), but had not additional effect on GASP-1 ($p > 0.05$). The effects of resistance training on serum levels of myostatin and GASP-1, may explain the increased muscle mass that is amplified by creatine supplementation.
© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Myostatin is a member of TGF-β superfamily and acts on skeletal muscle as a growth inhibitor (McPherron et al., 1997). In skeletal muscle, myostatin lack, caused by gene mutation leads to muscular hypertrophy (Schuelke et al., 2004) and increased myostatin is accompanied by muscle atrophy (Zimmers et al., 2002). Myostatin mediates its actions through binding to activin IIb receptors (Yang et al., 2007). It seems that the target cells are primarily satellite cells whose proliferation and differentiation are inhibited by myostatin. This is accomplished by downregulation of myogenic regulatory factors, MyoD and myogenin, and also cyclins and cyclin dependant kinases (cdk) (Craig et al., 2008; Joulia et al., 2003; Langley et al., 2002; Rios et al., 2002).

Some binding proteins in serum and in skeletal muscle may be bounded to myostatin and modulate its secretion, receptor binding, and activity (Joulia-Ekaza and Cabello, 2007). GASP-1, a recently introduced protease inhibitor in human and mouse sera, binds directly to both mature myostatin and its propeptide. It is also capable of blocking myostatin proprotein processing through inhibition of furin-like protease (Hill et al., 2003). So, GASP-1 may have a critical role in controlling myostatin function in skeletal muscle cell and serum.

Resistance training can cause muscle fiber hypertrophy. Also, nutritional factors, inducing a net positive protein balance have great impact on muscle fiber size (Kerksick et al., 2007). Creatine monohydrate is a popular dietary supplement used by athletes to enhance muscle mass and strength and improve sport performance. Several studies have shown the effects of creatine supplementation on muscle strength, body composition and exercise performance, the majority reporting an ergogenic effect (Candow and Chilibeck, 2008). The exact mechanism of action of creatine in conjunction with resistance training on muscle strength and mass has not been identified. Willoughby and Rosen demonstrated that creatine supplementation plus resistance training results in a greater increase in myosin heavy chain mRNA expression (Willoughby and Rosene, 2001). In a subsequent study, these researchers demonstrated that creatine ingestion plus resistance training increased myogenic regulatory factors such as myogenin and MRF-4 compare with resistance training alone (Willoughby and Rosene, 2003). Some studies have shown that creatine poses some effects on satellite cell proliferation and differentiation in cell culture (Vierck et al., 2003). Furthermore, creatine supplementations in conjunction with resistance training amplifies the training-induced increase in satellite cell number and myonuclei concentration in skeletal muscle fibers (Olsen et al., 2006). In addi-

* Corresponding author at: The Laboratory of Biochemistry, Endocrinology and Metabolism Research Center, Medical Sciences/Tehran University, P.O. Box 14395/1179, Tehran, Iran. Tel.: +98 21 8820037/8820038; fax: +98 21 88220052.
E-mail address: omidfar@tums.ac.ir (K. Omidfar).

0303-7207/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2009.12.019

Table 1
Subject characteristics at baseline.

	Bench press (kg)	Leg press (kg)	Weight (kg)	Height (cm)	Age (year)
CON	51.50 ± 4.1	139.50 ± 14.2	76.85 ± 1.45	179.10 ± 6.7	24.10 ± 1.9
RT + PL	52.37 ± 7.6	143.87 ± 10.5	77.20 ± 2.01	178.82 ± 5.7	22.28 ± 2.4
RT + CR	52.86 ± 5.1	140.31 ± 12.6	77.59 ± 1.31	179.50 ± 4.5	23.85 ± 2.8

Values are means ± SD.

tion, it has been shown that creatine supplementation increased IGFI-I expression (Deldicque et al., 2005; Louis et al., 2004). Interestingly, it has been reported myostatin expression decreased in creatine supplemented pig (Young et al., 2007). Evidence exists to suggest that a homeostatic balance likely exists between positive (e.g., IGF-I) and negative (e.g., myostatin) growth regulators in skeletal muscle, and this balance shift during loading or atrophy conditions (Heinemeier et al., 2007; Lalani et al., 2000). However, it is not previously investigated whether creatine supplementation in association with resistance training affect myostatin pathway (as a negative growth regulator in skeletal muscle).

Therefore, the aim of the present study was to investigate the effects of resistance training and creatine supplementation on serum levels of myostatin, GASP-1 and lean body mass. We hypothesized that following resistance training serum myostatin would be decreased, and GASP-1 increased, and these changes would be accompanied by gains in muscle mass and strength. We further hypothesized creatine supplementation during resistance training would result greater changes in myostatin, and increases in muscle mass and strength.

2. Methods

2.1. Subjects

Twenty-seven healthy non-resistance-trained young men, from the university population, volunteered to take part in the study (Table 1). Three subjects dropped out of the study during the training period for unrelated reasons. No subject had a history of cardiovascular or respiratory disease, and none had any subjective evidence of musculoskeletal injury. They all were physically active but not engaged in a structured weight-training program for at least 6 months prior to the study and had not ingested any ergogenic supplement in 12 weeks before the study. The subjects' eligibility was assessed by interview before participation in the study. The subjects were carefully informed about the design of the study with special information regarding possible risks. Thereafter, all subjects signed the written informed consent to participate in the study. The study protocol was approved by the ethics committee of Endocrinology and Metabolism Research Center of Tehran University of Medical Sciences. The study was conducted in accordance with Helsinki declaration and guideline of Iranian Ministry of Health and Medical education.

2.2. Experimental design

Subjects were randomly assigned, in a double-blind fashion, to-either a control group [CON (N = 8)], with no resistance training, placebo, or cr; a resistance training plus cr group [RT + CR (N = 8)]; and a resistance training plus placebo group [RT + PL (N = 8)]. The randomization was performed using envelopes. Before commencing the first training, each participant had to choose a randomization envelope. The message inside state that they were in group 1 (CON), 2 (RT + CR) or 3 (RT + PL). The measurements were done by masked assessors. They examined the subjects without being aware of the program followed by each individual.

All the subjects were analyzed at 0, 4, and 8 weeks to find out any change in criterion variables. The total duration of the study was 8 weeks.

2.3. Supplementation protocol

Subjects assigned to the RT + CR group received creatine monohydrate in capsule form (Gensan Abiogen Pharama; Italy) at a dose 0.3 g kg^{-1} day^{-1} (divided into three equal doses) for the 1st week (loading period) and 0.05 g kg^{-1} day^{-1} (once daily) for the remaining 7 weeks. This supplementation protocol was anticipated to increase muscle creatine levels by 14–28% (Volek et al., 1999). Subjects in the RT + PL group were offered the same number of capsules identical in appearance (powdered cellulose). All of the supplements were administered by a registered dietician who calculated each serving size and distributed them in marked plastic bags. All subjects consumed their drink (creatine or placebo) immediately after the training sessions.

There were no side effects of the supplementation protocol reported by the subjects at any time during the study.

Dietary intakes were registered during the study period using 3-day dietary records. The food records were analyzed using the Food Processor II Nutrition System-Analysis Software Version 3.1 (Tehran University of Medical Sciences).

2.4. Training protocol

The subjects participated in a resistance training program, designed to target both the upper and lower body muscle groups. They were trained three times a week on non-consecutive days for 8 weeks. All sessions were supervised by a member of the research team. In brief, the program was designed specifically to increase muscle mass. It consisted of moderate-intensity workouts using mostly compound exercises. Training intensity for the program was determined using one-repetition maximum (1-RM). The format and relative intensity for the training protocol involved three sets of 8–10 repetitions at 60–70% of 1-RM (at moderate velocity) with six exercises per workout (bench press, late pull down, biceps curl, leg press, knee extension, and knee flexion). Approximately 2 min of rest between the sets were allowed (Kraemer and Ratamess, 2004). Exercises involving large muscle groups (i.e., bench press, late pull down, and leg press) were performed before those of small muscles (i.e., knee extension/flexion, and arm curl). Upper and lower body exercises were performed in an alternating order to provide additional recovery. To assess muscle strength, 1-RM was examined at weeks 0, 2, 4, 6, and 8, so adjustments could be made to accommodate for increases in strength and ensure that subject continued to train at an intensity of 60–70% of their 1-RM based on the repetition continuum. Each workout was performed with flexibility exercises combined with calisthenics during 10–15 min.

2.5. Strength testing

Prior to 1-RM assessments, a familiarization session was conducted in order to minimize learning effects on the strength testing protocols. In these sessions specific exercise techniques were toughed and sub-maximal practice for each exercise session determined. Before training and at the end of weeks 2, 4, 6 and 8 the subjects were tested to determine muscular strength using the 1-RM for bench press and leg press to assess absolute upper and lower body strength, respectively (Willoughby and Rosene, 2001). The 1-RM for each lift was reached within five attempts, after a brief warm-up of five to eight repetitions with loads of approximately 50% of the anticipated 1-RM. During the 1-RM process, the attempts were separated by 3–5 min breaks. All strength assessments (pre- and post-testing sessions) followed the same direction (bench press and leg press), and each test was followed by an adequate recovery period of at least 10 min (Bemben et al., 2001). All lifts were performed according to standardized procedures and were monitored by the staff.

2.6. Blood collection and analysis

Blood samples were drawn after an overnight fast between 06:30 and 07:30 (baseline, week 4, and week 8). In addition, to avoid the effects of the previous training session, the subjects were not exposed to any strenuous exercise on the day before blood sampling, and each subject was ensured a minimum of 14 h of rest. Blood samples were drawn from a superficial arm vein and were immediately iced and centrifuged at 1500 rpm for 15 min (at 4 °C). Serum samples were then aliquated into separate tubes and frozen (−80 °C).

2.7. Urinary creatinine

Subjects completed 24-h urine collections at 0 (baseline), week 1 (the final day of loading) and week 8 of supplementation. Urine volume was measured and a representative sample was stored frozen at −20 °C for subsequent analysis of creatinine concentrations using a spectrophotometric enzymatic Cr kit (Pars Azmoon – kit no. 86001-S, IR).

2.8. Body composition

Lean body mass, fat mass, and body fat percentage were determined using a lunar DPX-L (Lunar Co, Waukesha, WI, USA) dual energy X-ray absorptiometer, software version 4.6 days, at baseline, 4 and 8 weeks. Whole-body scans were performed by the same device, and the same licensed operator. Quality assurance was assessed by analyzing a phantom spine and daily calibrations were performed prior to all scans using a calibration block provided by the manufacturer. Intra-class correlation coefficients ($R \geq 0.99$) for bone mineral content, lean body mass, and fat mass

were obtained from repeated scans on a group of ten men and women who were tested on 2 consecutive days.

2.9. Myostatin and GASP-1 assessment

2.9.1. Myostatin assay method

Serum myostatin concentrations were assessed using a competitive enzyme-linked immunosorbent assay (ELISA) method. At first, the wells of microtiter plates were coated with recombinant human myostatin (Santa Cruz Biotechnology, SC-6884P) (300 ng/ml) dissolved in phosphate-buffered saline (PBS, pH 7.2, 0.01 M) for 12 h at room temperature. The plates were washed four times with PBS. The wells were blocked with 300 μl of 3% bovine serum albumin (BSA) in PBS to prevent non-specific binding and the plate incubated for 1 h. Sera were mixed with equal volume of mouse anti-human myostatin monoclonal antibody (Santa Cruz Biotechnology, SC-6884) (500 ng/ml) in PBS containing 0.1% BSA for 1 h. Standard curves were constructed by mixing serial dilutions of recombinant human myostatin with equal volume of the same anti-human myostatin antibody (500 ng/ml) in PBS containing 0.1% BSA for 1 h. The mixtures were transferred to the coated well and incubated for 1 h at room temperature. After washing the wells with PBS containing 0.05% Tween 20 (PBST), peroxidase-conjugated rabbit anti-mouse polyclonal antibody was added to the wells and the plate was incubated for 1 h at room temperature. Finally, it was washed six times with PBST and then 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) reagent was added and incubated at room temperature for about 10 min. Peroxidase reaction was stopped by adding 100 μl of 200 mM H_2SO_4, and the optical density at 450 nm was determined by a microplate reader. All the standards and samples were assayed in duplicate. The data for the standard curve were fitted to a logistic plot and the levels of myostatin were calculated.

2.9.2. GASP-1 assay method

GASP-1 concentrations in sera were determined using a sandwich ELISA. At first, 100 μl of the mouse anti-human GASP-1 monoclonal antibody (R&D, MAB20701), dissolved in PBS was added into the wells of the microtiter plate and incubated for 12 h at room temperature. Assay plates were washed three times with PBS and blocked with 300 μl of 3% BSA in PBS to prevent non-specific binding and incubated for one more hour. Then the sera were diluted three times with PBS containing 0.1% BSA and 100 μl of diluted samples were added into the wells and incubated for 1 h at room temperature. Serial dilutions of recombinant human GASP-1 (R&D, 2070GS) in PBS containing 0.1% BSA were prepared and added into the wells and incubated for 1 h at room temperature to construct a standard curve. Thereafter the wells were washed four times with 400 μl PBST. Then, 100 μl of the goat anti-human GASP1 polyclonal antibody (R&D, AF2070) (400 ng/ml) diluted in PBS containing 0.1% BSA, were added to the wells, and the plates were incubated for 1 h at room temperature. They were washed four times with 400 μl PBST, and subsequently 100 μl of HRP-conjugated rabbit anti-goat antibody diluted with PBS containing 0.1% BSA was added. The plates were incubated for 1 h and washed six times with PBST. Then, 100 μl of TMB reagent was added to the wells. The reaction was stopped by adding 100 μl of 200 mM H_2SO_4, and color intensity was measured at 450 nm. All measurements were performed in duplicate and data for the standard curve were fitted to a logistic plot and the levels of GASP-1 calculated.

2.9.3. Intra- and inter-assay imprecision

Three samples of known concentration (low moderate and high) were tested for both myostatin and GASP-1 in eight independent analytical runs to assess precision within and between assays.

2.10. Statistical analysis

Data were analyzed by analysis of variance for repeated measures (treatment group × time). Significant group × time interactions were determined using the Student Neumann–Keuls Post Hoc test. The linearity of the ELISA standard curves was determined with linear regression analysis. All statistical procedures were performed using SPSS 11.0 software and data were considered significant when the probability of type I error was 0.05 or less. Data are shown as means ± standard deviation.

3. Results

3.1. Baseline characteristics

Three participants did not complete the whole program, so their data were not included. This reduced the population to eight people in each group. Their Baseline characteristics are shown in Table 1. There were no differences between the groups in any variable at the beginning of the study ($p > 0.05$).

Table 2
Dietary analysis.

	CON	RT + PL	RT + CR
Energy intake (kcal kg^{-1} day^{-1})			
Week 0	35.8 ± 7.3	36.5 ± 6.7	34.8 ± 7.5
Week 4	36.00 ± 7.6	36.8 ± 5.9	34.7 ± 8.9
Week 8	35.9 ± 8.3	36.6 ± 8.1	34.9 ± 5.2
Carbohydrate (g kg^{-1} day^{-1})			
Week 0	2.8 ± 0.7	3.8 ± 1.4	4.0 ± 0.6
Week 4	2.9 ± 0.7	3.9 ± 1.4	3.7 ± 0.7
Week 8	2.7 ± 0.8	4.7 ± 1.8	4.0 ± 1.3
Protein (g kg^{-1} day^{-1})			
Week 0	1.6 ± 0.5	2.1 ± 1.00	1.6 ± 0.4
Week 4	1.7 ± 0.4	1.9 ± 0.9	1.7 ± 0.5
Week 8	1.6 ± 0.8	1.7 ± 0.8	1.6 ± 0.6
Fat (g kg^{-1} day^{-1})			
Week 0	2.1 ± 0.5	2.1 ± 0.8	2.2 ± 0.3
Week 4	2.2 ± 0.6	1.9 ± 0.9	2.2 ± 0.5
Week 8	2.2 ± 0.8	1.8 ± 0.8	2.0 ± 0.6

Values are means ± SD.

3.2. Diet analysis

There were no significant differences ($p > 0.05$) between the groups with respect to total calorie, carbohydrate, protein, and fat intakes (Table 2). The presented data do not include supplementation.

3.3. Body composition

There were a significant effect of time and group on lean body mass ($p < 0.05$), increasing in RT + CR group from baseline to week 4 and 8 ($p < 0.05$). Likewise, time effects were observed in RT + PL at week 8 ($p < 0.05$). A significant treatment effect was observed in RT + CR, which had higher lean mass than RT + PL group at week 4 and 8 ($p < 0.05$). No significant changes were observed in CON (Table 3).

3.4. Muscle strength

A significant group and time ($p < 0.05$) effect was seen on lower and upper-body strength. It was increased in RT + CR group at week 4 and 8 ($p < 0.05$) (Table 3). Likewise, a significant time effect was observed in RT + PL at week 4 and 8 ($p < 0.05$). A significant treatment effect was observed for RT + CR, which was elevated compare to RT + PL at week 4 and 8 ($p < 0.05$). No changes were observed for CON.

3.5. Urine creatinine

Urine creatinine showed a significant group and time ($p < 0.05$) effect. Baseline urine creatinine concentrations (mg/24 h) were similar in RT + CR (1339.6 ± 374.1), RT + PL (1576.2 ± 411) and CON (1415.1 ± 386) groups. Furthermore, at end of the week 1 (1916.2 ± 384.7) and 8 (1896.1 ± 396.5) were significantly increased in RT + CR group. No changes were observed for RT + PL and CON.

3.6. Serum myostatin and GASP-1

The mean intra- and inter-assay imprecision was determined from five analytical runs for each of the 11 calibrators (0.01–300 ng/ml) in the standard curve.

Serum myostatin showed significant time and group ($p < 0.05$) effect, decreasing in RT + CR at week 4 and 8 ($p < 0.05$). Similar, time effects were observed in RT + PL at week 4 and 8 ($p < 0.05$).

Table 3
Body composition and muscle strength.

	CON	RT + PL	RT + CR
Body mass (kg)			
Week 0	76.74 ± 2.1	77.0 ± 2.2	77.71 ± 2.0
Week 4	76.34 ± 2.1	77.79 ± 2.1	78.71 ± 1.8
Week 8	76.77 ± 1.9	78.58 ± 2.3	79.83 ± 2.2
Lean body mass (kg)			
Week 0	59.75 ± 1.1	60.28 ± 1.5	60.40 ± 1.0
Week 4	59.82 ± 1.1	61.28 ± 1.1	61.50 ± 1.3[a,c]
Week 8	59.81 ± 1.3	62.31 ± 1.3[a]	63.00 ± 1.3[b]
Fat mass (kg)			
Week 0	13.86 ± 2.8	13.64 ± 1.9	14.51 ± 2.6
Week 4	13.40 ± 2.1	13.40 ± 1.6	14.31 ± 2.1
Week 8	13.82 ± 2.9	13.15 ± 1.8	14.03 ± 2.3
%Fat			
Week 0	17.55 ± 2.5	17.21 ± 1.8	18.33 ± 2.3
Week 4	17.03 ± 2.1	16.73 ± 1.7	17.66 ± 2.0
Week 8	17.50 ± 2.5	16.23 ± 1.7	17.05 ± 2.1
Bench press (kg)			
Week 0	52.25 ± 7.1	53.37 ± 8.0	54.5 ± 7.1
Week 4	52.75 ± 8.0	59.5 ± 9.3[a]	64.87 ± 5.9[a,c]
Week 8	52.62 ± 8.0	63.02 ± 10.1[b]	71.75 ± 7.7[b,c]
Leg press (kg)			
Week 0	155.25 ± 13.6	156.37 ± 14.2	145.62 ± 12.9
Week 4	155.87 ± 13.8	165.25 ± 13.9[a]	160.62 ± 14.2[a,c]
Week 8	155.62 ± 15.2	172.12 ± 14.5[b]	166.87 ± 12.5[b,c]

Values are mean ± SD.
[a] Significantly different from pre-training (week 0).
[b] From week 4.
[c] From RT + PL and CON (p < 0.05).

A significant treatment effect was observed for RT + CR, which was decreased compare to RT + PL at week 4 and 8 (p < 0.05). No changes were observed for CON. The measured intra-assay CV for low, moderate and high range of myostatin was 4.5, 5.1 and 7.2%, respectively, and the inter-assay was 3.2, 2.5 and 5.5%, respectively.

Serum GASP-1 showed significant time (p < 0.05) effect, increasing in RT + CR and RT + PL from baseline to week 8 (p < 0.05). No significant treatment effect was observed for RT + CR compare RT + PL. No changes were observed for CON. The measured intra-assay CV for low, moderate and high range of GASP-1 was 5, 5.3 and 6%, respectively, and the inter-assay was 4.2, 2 and 5.1%, respectively (Fig. 1).

4. Discussion

This is the first study to demonstrate that creatine supplementation added to resistance training program amplifies the training-induced decrease in serum levels of myostatin, increasing the effects of exercise on muscle strength and mass. In addition, it was shown that 8 weeks of resistance training resulted in significant elevations in serum GASP-1.

Many binding proteins for myostatin have been described but their specific role in regulating myostatin activation and receptor binding is not clear (Joulia-Ekaza and Cabello, 2007). One of the binding proteins and inhibitors of myostatin is GASP-1. It has been shown to be expressed in human skeletal muscle, circulates in human blood, and inhibits myostatin activity (Hill et al., 2003). We observed an increase in GASP-1 in both training groups, although no significant changes were observed at initial phase of the training (at week 4). Thus, a relatively long training course is required to increase GASP-1. This finding is unique and may be related to the specific type of training program, however, the exact reasons and the physiological mechanism(s) mediating this adaptation remain speculative. To our knowledge, our results are the first reported findings on the behavior of GASP-1 after resistance training. It is likely that the increased GASP-1 may have inhibited myostatin from binding to the activin IIb receptor. This is conceivable because serum myostatin is bound to, inhibited, and negatively regulated by GASP-1 (Hill et al., 2003). Therefore, based on our and previous (Hill et al., 2003) results, we submit that in young, apparently healthy males participating in resistance exercise the increases in serum GASP-1 that accompany decreased serum myostatin may serve to inhibit myostatin signaling and muscle catabolism that could conceivably accompany heavy resistance exercise. The finding that serum myostatin decreased after resistance training when no extra-nutrition supplementation was provided and was accompanied with gains in lean body mass and muscle strength are in agreement with Walker et al. (2004), who reported decreased plasma myostatin after 10 week of resistance training (Walker et al., 2004). In contrast, Willoughby (2004) demonstrated increases in serum myostatin following resistance exercise (Willoughby, 2004). These inconsistent findings may be due to differences in subjects, blood sampling time, exercise mode, intensity, and duration making comparisons between the studies difficult. For example, in our study, blood sampling was obtained 48 h after the last resistance training session, thus minimizing the short-term effects from this last bout, whereas in the aforementioned study by Willoughby (2004), blood samples were taken immediately after the exercise session. However, the present study shows that 8 weeks resistance training (hypertrophic model) decreases serum myostatin, and there is inverse relationship between changes of myostatin and lean body mass during training period. This inverse relationship consistent with the theoretical negative role of myostatin in regulating muscle mass (McPherron et al., 1997; Zimmers et al., 2002; Yang et al., 2007).

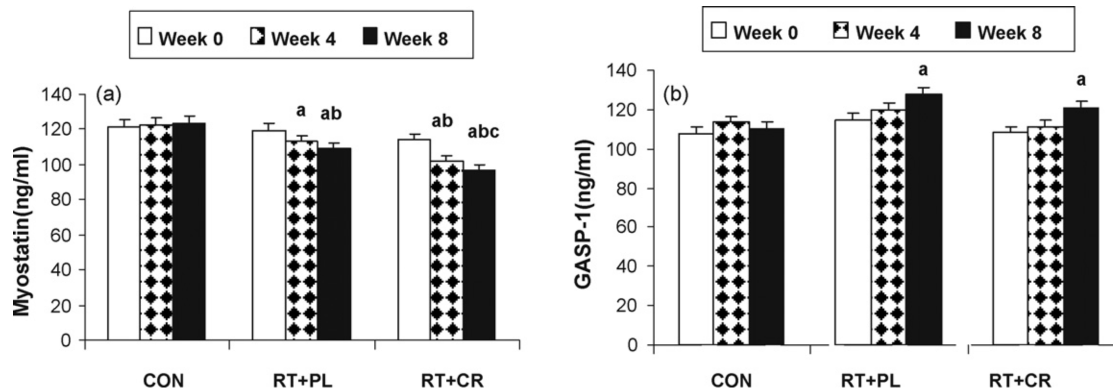

Fig. 1. Serum myostatin (a) and GASP-1 (b) levels between groups following 8 weeks resistance training and supplementation. [a]Significant different from pre-training (week 0), [b]from week 4, and [c]from RT + PL and CON (p < 0.05).

Furthermore, we found out that serum myostatin decrease after resistance training was augmented with creatine supplementation, and this also resulted in greater lean body mass and strength gain. Creatine monohydrate is an extremely popular nutritional supplement, which has been widely reported to have ergogenic and anabolic effects in individuals undergoing resistance training (Candow and Chilibeck, 2008; Rawson and Persky, 2007). The dosage of creatine used in the present study was similar to dosages used in other studies which increased intramuscular creatine levels and FFM, and improved muscle strength (Volek et al., 1999). Considering the issue of responders and non-responders to creatine supplementation (Kilduff et al., 2003), most subjects in the RT + CR group was likely responders because these group had greater gains in LBM compared with the RT + PL group. Measurement of muscle creatine concentrations could be used to confirm whether these subjects were creatine responsive (Greenhaff et al., 1994), but that was not done in the present study. Creatinine formation is directly related to creatine phosphate concentration in skeletal muscle. Harris et al. (1992) showed that creatine saturation in muscle was associated with elevated levels of urinary creatinine. This finding suggests that the muscle cannot phosphorylate excess free creatine, therefore, excessive supplementation yields elevated urinary creatinine. Alternatively, urinary creatinine could be used as a non-invasive method for measuring creatine muscle retention during supplementation (Mendes et al., 2004). In our study, urinary creatinine concentrations were measured throughout 8 weeks, and only RT + CR group showed a significant increase. It is possible that creatine ingestion did increase muscle creatine levels, and our subjects were probably responders. Although the mechanisms governing the ergogenic and anabolic effects of creatine are not entirely understood, several theories have been proposed. Recent findings have supported the idea of facilitating effect of creatine on skeletal muscle growth with resistance training. It has been shown in rats that creatine supplementation during increased functional loading and compensatory hypertrophy (synergist ablation) induced increased satellite cell mitotic activity (Dangott et al., 2000), or creatine supplementation in combination with strength training augment the training-induced increase in number of satellite cells and myonuclei in human skeletal muscle (Olsen et al., 2006). Furthermore, myogenin and MRF-4 mRNA and protein expression increase more after creatine supplementation compared to training alone after 12 weeks of resistance training (Willoughby and Rosene, 2003). These myogenic regulatory factors (MRFs) are thought to regulate muscle heavy chain (MHC) expression at the translational level, and therefore up-regulation of MRF may lead to muscle accretion (Willoughby and Rosene, 2001). The observed decrease in serum levels of myostatin in the present study supports a role for creatine in activating myogenic pathway (MRFs and satellite cells), thereby augmenting the training-induced accretion of muscle mass, especially in the early part of the time course of training. Interestingly, in the present study during creatine supplementation the largest decreases in myostatin levels were observed at week 4. Recently, Olsen et al. (2006) reported that the largest increases in satellite cell content after creatine supplementation were occurred in the initial phase of training (at week 4), also, Volek et al. (1999) reported that treatment with creatine during early phase of resistance training resulted in significantly greater hypertrophy of type II fiber. Since the inhibitory effects of myostatin primarily occur in type II muscle fiber (Girgenrath et al., 2005), it is probable that creatine supplementation during resistance training (initial weeks) leads to greater increases in satellite cells activation (Olsen et al., 2006) and skeletal muscle hypertrophy via decreases in myostatin production in type II muscle fibers (Willoughby and Rosene, 2001). Although we presently do not know the specific physiological means by which creatine affects myostatin, it is possible that, as an osmotically active substance, it can cause water retention in the muscle fiber (Ziegenfuss et al., 1998), and increased osmotic pressure and resultant cell swelling due to increased creatine concentration and muscle glycogen content (Op't Eijnde et al., 2001) may represent an anabolic stimulus for downregulation of myostatin and further it may stimulate myogenic regulatory factors expression (Willoughby and Rosene, 2001) and then satellite cells to proliferate and fuse with enlarging myofibers (Dangott et al., 2000). Also, it has been suggested that the enhanced muscle size gain observed when strength training is combined with creatine supplementation could be caused by a rise in training quality and/or greater total training load (Volek et al., 1999) which was supported by a higher total resistance load lifted by creatine supplemented subjects as reported by Steenge (1999). The effect of increased work output during creatine supplementation could cause a greater than normal stimulus to muscle anabolism (Louis et al., 2003). To eliminate this effect, in the present study total training load was not greater in subjects supplemented by creatine compared to the placebo group.

We explored a potential myogenic pathway to explain the anabolic action of creatine supplementation. However, possible direct action of creatine within the muscle cells to stimulate net protein synthesis must also be considered. With this respect, some early observations in muscle cell culture have suggested that creatine may directly stimulate the rate of muscle protein synthesis (Vierck et al., 2003). The possible impact of creatine intake on the intracellular signaling pathways involved in the regulation of muscle protein synthesis and breakdown needs to be addressed in future studies.

In conclusion, the present study is the first to demonstrate that decreasing myostatin and inhibiting its function by GASP-1 may play important role in increasing muscle strength and mass by resistance training, and supplementation with creatine resulted in greater increases in muscle mass and strength, and this improvements were accompanied by more decreased myostatin levels. Although we presently do not know the specific physiological reason(s) by which creatine affects myostatin, part of the enhancements in muscle mass induced by creatine supplementation seen in human clinical trials could be explained.

References

Bemben, M.J., Bemben, D.A., et al., 2001. Creatine supplementation during resistance training in college football athletes. Med. Sci. Sports Exerc. 33, 1667–1673.

Candow, D.G., Chilibeck, P.D., 2008. Timing of creatine or protein supplementation and resistance training in the elderly. Appl. Physiol. Nutr. Metab. 33, 184–190.

Craig, M., Alex, H., et al., 2008. Myostatin signals through Pax7 to regulate satellite cell self-renewal. Exp. Cell Res. 314, 317–329.

Dangott, B., Schultz, E., et al., 2000. Dietary creatine monohydrate supplementation increases satellite cell mitotic activity during compensatory hypertrophy. Int. J. Sports Med. 21, 13–16.

Deldicque, L., Louis, M., et al., 2005. Increased IGF mRNA in human skeletal muscle after creatine supplementation. Med. Sci. Sports Exerc. 37, 731–736.

Girgenrath, S., Song, K., et al., 2005. Loss of myostatin expression alters fiber-type distribution and expression of myosin heavy chain isoforms in slow and fast-type skeletal muscle. Nerve 31, 34–40.

Greenhaff, P.L., Bodin, K., et al., 1994. Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. Am. J. Physiol. 66, 730–745.

Harris, R.C., Soderlund, K., et al., 1992. Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. Clin Sci. 83, 367–374.

Heinemeier, K.M., Olesen, J.L., et al., 2007. Short-term strength training and the expression of myostatin and IGF-I isoforms in rat muscle and tendon: differential effects of specific contraction types. J. Appl. Physiol. 102, 573–581.

Hill, J.J., Qiu, Y., et al., 2003. Regulation of myostatin in vivo by growth and differentiation factor-associated serum protein-1: a novel protein with protease inhibitor and follistatin domains. Mol. Endocrinol. 17, 1144–1154.

Joulia, D., Bernardi, H., et al., 2003. Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin. Exp. Cell Res. 286, 263–275.

Joulia-Ekaza, D., Cabello, G., 2007. The myostatin gene: physiology and pharmacological relevance. Cur. Opin. Pharmacol. 7, 310–315.

Kerksick, C.M., Rasmussen, C., et al., 2007. Impact of differing protein sources and a creatine containing nutritional formula after 12 weeks of resistance training nutrition. Nutrition 23, 647–656.

Kilduff, L.P., Pitsiladis, Y.P., et al., 2003. Effects of creatine on body composition and strength gains after 4 weeks of resistance training in previously nonresistance-training humans. Int. J. Sport Nutr. Exerc. Metab. 13, 504–520.
Kraemer, W.J., Ratamess, N.A., 2004. Fundamental of resistance training: progression and exercise prescription. Med. Sci. Sports Exerc. 36, 674–688.
Lalani, R., Bhasin, S., et al., 2000. Myostatin and insulin-like growth factor-I and -II expression in the muscle of rats exposed to the microgravity environment of the Neurolab space shuttle flight. J. Endocrinol. 167, 417–428.
Langley, B., Thomas, M., et al., 2002. Myostatin inhibits myoblast differentiation by down-regulation myoD expression. J. Biol. Chem. 282, 993–999.
Louis, M., Beneden, R.V., et al., 2004. Creatine increases IGF-I and myogenic regulatory factor mRNA in C2C12 cells. FEBS Lett. 557, 243–247.
Louis, M., Poortmans, J.R., et al., 2003. No effect of creatine supplementation on human myofibrillar and sarcoplasmic protein synthesis after resistance training. Am. J. Physiol. Endocrinol. Metab. 285, 1089–1094.
McPherron, A.C., Lawler, A.M., et al., 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387, 83–90.
Mendes, R.R., Pires, I., et al., 2004. Effects of creatine supplementation on the performance and body composition of competitive swimmers. J. Nutr. Biochem. 15, 473–478.
Olsen, S., Aagaard, P., et al., 2006. Creatine supplementations augments the increase in satellite cell and myonuclei number in human skeletal muscle induced by strength training. J. Physiol. 573, 525–534.
Op't Eijnde, B., Richter, E.A., et al., 2001. Effects of creatine supplementation on creatine and glycogen content in rat skeletal muscle. Acta Physiol. Scand. 171, 169–176.
Rawson, E.S., Persky, A.M., 2007. Mechanisms of muscular adaptations to creatine supplementation. Int. Sport Med. J. 8, 43–53.
Rios, R., Carneiro, I., et al., 2002. Myostatin is an inhibitor of myogenic differentiation. Am. J. Physiol.: Cell Physiol. 282, 993–999.
Schuelke, M., Wanger, K.R., et al., 2004. Myostatin mutation associated with gross muscle hypertrophy in a child. N. Engl. J. Med. 350, 2682–2688.
Steenge, G., 1999. Factors affecting creatine accumulation in human skeletal muscle. PhD Thesis, University of Wottingham, UK.
Vierck, J.L., Icenoggle, D.L., et al., 2003. The effects of ergogenic compounds on myogenic satellite cells. Med. Sci. Sports Exerc. 35, 769–776.
Volek, J.S., Duncan, N.D., et al., 1999. Performance and muscle fiber adaptation to creatine supplementation and heavy resistance training. Med. Sci. Sports Exerc. 31, 1147–1156.
Walker, K.S., Kambadur, R., et al., 2004. Resistance training alters plasma myostatin but not IGF-I in healthy men. Med. Sci. Sports Exerc. 36, 787–793.
Willoughby, D.S., Rosene, J., 2001. Effects of oral creatine and resistance training on myosin heavy chain expression. Med. Sci. Sports Exerc. 33, 1674–1681.
Willoughby, D.S., 2004. Effects of heavy resistance training on myostatin mRNA and protein expression. Med. Sci. Sports Exerc. 36, 574–582.
Willoughby, D.S., Rosene, J.M., 2003. Effects of oral creatine and resistance training on myogenic regulatory factor expression. Med. Sci. Sports Exerc. 35, 923–929.
Yang, W., Zhang, Y., et al., 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, 3799–3808.
Young, J.F., Bertram, H.C., et al., 2007. In vitro and in vivo studies of creatine monohydrate supplementation to durco and landrace pigs. Meat. Sci. 76, 342–351.
Ziegenfuss, T.N., Lowery, L.M., et al., 1998. Acute fluid volume changes in men during three days of creatine supplementation. J. Exerc. Physiol. Online 1, 10.
Zimmers, T.A., Davies, M.V., et al., 2002. Induction of cachexia in mice systemically administered myostatin. Science 296, 1486–1488.