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Acute and moderate-term creatine monohydrate supplementation does not affect creatine transporter mRNA or protein content in either young or elderly humans

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Abstract

Animal studies have shown that supra-physiological creatine monohydrate (Cr-mH) supplementation for 3 months reduced skeletal muscle creatine transporter (CRT) content. The doses of Cr-mH (1–2 g/kg/day) used in these studies were between 5 and 10 times those usually used in human studies, and it is unclear whether a down-regulation of CRT would occur in humans at the recommended doses of 0.1–0.2 g/kg/day. We measured CRT, and citrate synthase (CS) protein content using Western blotting before and after 2 months of Cr-mH supplementation and weight training in young men (N = 11 Cr-mH (0.125 g/kg/day); N = 8 placebo). CRT and CS were also measured before and after 4 months of Cr-mH supplementation and weight training in elderly (> 65 years) men and women (N = 14 Cr-mH (0.075 g/kg/day); N = 14 placebo). Finally, CRT mRNA was measured using competitive RT-PCR before and after 8–9 days of Cr-mH loading in young men and women (N = 14, CR-mH (mean = 0.18 g/kg/day); N = 13, PL). Total creatine content was significantly elevated after the Cr-mH supplementation period as compared to placebo in each of the studies. Neither Cr-mH supplementation, nor exercise training resulted in measurable alterations in CRT protein content and acute Cr-mH loading did not alter CRT mRNA. There were no gender differences in CRT mRNA or total creatine content in the young subjects and no gender differences in total creatine content or CRT protein content in the elderly subjects. Weight training in young men did not increase CS protein content, however, in the elderly there was a significant increase in CS protein content after exercise training ($p < 0.05$). These results demonstrated that Cr-mH supplementation during weight training resulted in increases in skeletal muscle total creatine without reductions in CRT protein and acute Cr-mH loading did not decrease CRT mRNA content. (Mol Cell Biochem **244**: 159–166, 2003)

Key words: creatine transport, dietary supplements, drug safety, transporter regulation

Introduction

The transport of creatine into tissues is mediated predominantly by a sodium dependant creatine transporter (CRT, SLC6A8) that has been sequenced, mapped, and heterologously expressed [1, 2]. The SLC6A8 gene is on the X chromosome with an open reading frame of 1,905 bp, with 13 exons and 12 transmembrane spanning regions [1, 2]. There are two main protein species in skeletal muscle with apparent molecular weights of 52- and 70-kDa, which appear to arise from alternative splicing of the mRNA [3]. Prior to the identification of the CRT, it was known that creatine uptake into muscle was predominantly a saturable process [4], and that extracellular creatine regulated creatine transport in muscle by a protein synthesis dependant mechanism [5]. This process was later linked to the CRT from the results of ex-

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periments where the transient expression of CRT in COS-7 cells showed sodium dependence and attenuation by exogenous creatine [1]. More recent *in vivo* evidence demonstrated that the administration of high dose Cr-mH (4% diet; ~ 1 g/kg/day) to rats over a 3 month period resulted in a decrease in CRT protein content [3].

Creatine monohydrate (Cr-mH) supplementation has become a popular practice among athletes wishing to increase muscle mass and power [6]. Oral Cr-mH supplementation at dosages of 20 g per day (~ 0.2–0.3 g/kg/day) over 3–5 days resulted in an increase in muscle total creatine and phosphocreatine by 12–20% [6–8]. Other studies have shown a maintenance of elevated total creatine and phosphocreatine concentration following an acute load with the continued consumption of 2 g per day (~ 0.03 g/kg/day) for a month and similar increases were observed without the initial load at 3 g per day (~ 0.04 g/kg/day) for a month [7]. Several studies have shown that this increased high intensity power output particularly with repetitive bouts of activity [6, 9, 10]. Other studies have demonstrated that the longer-term ingestion of Cr-mH over weeks to months resulted in a greater increase in fat free mass, muscle power and muscle fiber area during resistance exercise training [11–13].

Based upon the above data, there has been some concern that the longer term administration of Cr-mH in athletes and patients may down-regulate creatine uptake [3, 6]. Ultimately, if Cr-mH supplementation were stopped, there could be an 'undershoot' of muscle creatine stores. In one longer-term study, in young men, there was a significant increase in muscle total creatine at 1 week of supplementation, yet this was not significantly elevated with 12 weeks of continued supplementation (~ 0.075 g/kg/day) [13]. Conversely, the muscle phospho-creatine content remained elevated above baseline following 10 weeks of supplementation and following 6 weeks of discontinuing the supplementation there was no 'undershoot' [11]. To date there have been no determinations of muscle CRT protein content in humans following short or longer Cr-mH supplementation. Given that the dose of Cr-mH given to rats in the aforementioned study, showing a reduction in CRT content [3], was approximately 4 fold higher than the acute loading dose in humans, and 30 fold higher than the dose shown to increased total creatine content after 30 days of continuous dosing [7], it is likely that the stimulus for down regulating the CRT would be much less or even non-existent in humans following creatine supplementation at recommended doses used in human studies.

Therefore, the purpose of the current study was to determine whether a moderate-term (i.e. months) Cr-mH supplementation protocol would down-regulate the total amount of CRT protein in young and elderly individuals participating in a resistance exercise training protocol. We also evaluated whether an acute Cr-mH supplementation strategy would down regulate the CRT mRNA content.

Materials and methods

Subjects/design

The data for the current study came from muscle samples obtained from 3 separate studies that were all approved by the McMaster University Ethics Committee and conformed to the principles of the Declaration of Helsinki.

Study #1 – acute creatine loading in young men and women

This study was a randomized, double-blind study designed to examine the effect of an acute 'loading' dose of Cr-mH compared to placebo (PL-glucose polymer) on protein turnover in moderately active young men (N = 13) and women (N = 14). The protein turnover data, phosphocreatine and total creatine concentrations have been reported previously [14]. Each subject had a muscle biopsy taken from the *vastus lateralis* under local anaesthesia (which was immediately quenched in liquid nitrogen) before and after 8–9 days of each intervention. The Cr-MH group consumed 4 × 5 g·day^{-1} of Cr-mH (99% pure, ISA, Hamilton, ON, Canada) for 5 days, and then consumed a maintenance dose of 5 g·day^{-1} for 3–4 days (mean = ~ 0.18 g/kg/day over the 8–9 days) and the PL group consumed an equivalent amount of glucose polymer. Subjects were instructed to consume their supplement dissolved in juice, chocolate milk, or a carbohydrate-containing soda beverage and refrained from leg exercise for 3 days prior to the testing session.

Study #2 – creatine supplementation during 2 months of resistance exercise training in young men

This study was a randomized, double-blind study designed to examine the effect of Cr-mH supplementation as compared to PL when consumed during an intensive 2 month resistance exercise training program in moderately active young men (N = 19). The strength gains, total and fat-free mass gains and muscle fiber characteristics, protein turnover data, phosphocreatine and total creatine concentrations have been previously reported [15]. Each subject had a muscle biopsy taken from the *vastus lateralis* under local anaesthesia (which was immediately quenched in liquid nitrogen) before and after the two month resistance exercise program. The Cr-CHO group consumed 10 g of Cr-mH and 75 g of dextrose within 30 min of completing each of the exercise sessions, (N = 11; mean = ~ 0.125 g/kg/day, Cell Tech, Muscle Tech. Research and Development, Mississauga, ON, Canada), and the PL group consumed 10 g of casein and 75 g of dextrose (N = 8) at the same time. The 8 weeks of exercise training was performed 6 days

per week under direct supervision with each subject completing a split-routine weight training, 1 h·day^{-1} @ 80% of one repetition maximum, 3 sets, 6 days·week^{-1}, on a 3day rotating schedule.

Study #3 – four months of resistance exercise training in men and women over 65 years of age

Fifteen men (67.8 ± 4.0 years) and 15 women (69.3 ± 6.3 years, none taking hormone replacement therapy) volunteered to participate in a 14-week resistance exercise training program. The subjects were randomly assigned in a double blind manner to receive either a Cr-mH (5 g Cr-mH (~ 0.075 g/kg/day) + 2 g dextrose/day (Neotine, Avicena, Cambridge, MA, USA) or a placebo (PL; 7 g dextrose/day) supplement during the training program. Training was conducted 3 times weekly on nonconsecutive days for 14 weeks. Each training session was preceded by a 5-min warm-up and followed by a cool down consisting of stretching of the muscle groups involved in the resistance exercises. Using weight training machines, twelve exercises were used to train the major muscle groups of the upper and lower body in a circuit set system: seated chest press, latissimus pull-down, leg press, military press, calf raise, arm extension, arm flexion, back extension, abdominal crunch, upright row, knee extension, and knee flexion. Subjects performed 10 repetitions of each arm exercise and 12 repetitions of the remaining exercises. Training progressed from one set of each exercise at 50% of the initial 1 repetition maximum (1 RM) to three sets at 80% of 1 RM over the training period. Each subject had a muscle biopsy taken from the *vastus lateralis* under local anaesthesia (which was immediately quenched in liquid nitrogen) before and after the 14-week resistance exercise program.

Muscle analyses

Phosphocreatine, creatine and ATP

Muscle samples were lyophylized and powdered and the metabolites were extracted using a perchloric acid method as previously described [16]. The resultant extract was assayed for phosphocreatine, free creatine and ATP using enzymatic methods as previously described [16].

Western blotting

Muscle samples were homogenized and prepared for electrophoresis using methods previously described [17]. A membrane preparation was also made from fresh human erythrocytes. The muscle samples from the training studies in the young males (Study #2) and from the elderly men and women (Study #3) using polyclonal rabbit antibodies directed against the C-terminus of the rat CRT-1 (synthetic 15mer peptide corresponding to amino acids 621–635 derived from the known sequence of CRT-1, SwissProt P48029). This antibody recognizes two major polypeptides with apparent molecular weights of ~ 52 kDa (CRT-52) and 70 kDa (CRT-70) [3, 17, 18]. In addition, we had enough muscle homogenate left from Study #3 that we were able to probe using a newer antibody a new sheep antibody against the sequence motive, GSAWERRGESTMSAH, of a synthetic peptide of CRT-2 (Swiss Prot P53796) [2]. By using this antibody, we obtained a more robust and consistent band at 52 kDa and we found a polypeptide band at ~ 56 kDa (Walzel *et al*., 2001 (in this issue); [19]). We also probed the blots from Study #2 and #3 using a polyclonal rabbit, anti-human antibody raised against purified human citrate synthase protein (Roche Chemicals) which recognized one polypeptide with an apparent molecular weight of ~ 65 kDa (a kind gift of Dr. B.H. Robinson).

RT-PCR

Total RNA was extracted from frozen human muscle by using TRIZOL Reagent according to the manufacturer's instructions (GIBCO-BRL, Life Technologies). Tissue was hand homogenized (glass-Teflon) in TRIZOL Reagent (25–30 mg/ml). The final RNA pellet was re-suspended in 12 µL of RNase-free water. Total RNA concentration was determined spectrophotometrically at 260 nm, and samples were treated with DNaseI using the DNA-free™ Kit (Ambion). Primers complementary to selected regions of the gene encoding for the CRT (GenBank accession No. L31409) were designed to produce a single 86 base pair (bp) product using Primer Express software (PE Biosystems, Foster City, CA, USA). The forward primer sequence (5′-3′) was GCCGGCAGCATCAATGTC, and the reverse primer sequence (5′-3′) was GGTGTTGCAGTAGAAGACGATCAC. 18S rRNA was used as an internal control according to the manufacturer's instructions using a QuantumRNA™ 18S Internal Standards Kit (Ambion). The Alternate 18S Internal Standards were used, which produced a 324 bp PCR product.

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with the use of Titan One Tube RT-PCR Kit (Roche Molecular Biochemicals, Germany) according to the instruction manual with the following minor modifications; 10 µL reaction volumes of 1 × RT-PCR buffer, 0.5 µM of each dNTP, 0.5 µM forward CRT primer, 0.5 µM reverse CRT primer, 1.2 µM Alternate 18S Internal Standards primer mixture (18S PCR Primer Pair to 18S PCR Competimers, 1:18), 3.0 mM $MgCl_2$, 0.4 µl enzyme mixture, and 0.2 µg total RNA template per tube. Reverse transcription was carried out at 50°C for 30 min. RT-PCR was run for 1 cycle @ 94°C for 2 min, 10 cycles (94°C for 30 sec, 53°C for 30 sec, 68°C for 45 sec), 18 cycles (94°C for 30 sec, 53°C for 30 sec, 68°C for 50 sec with additional 5 sec to each successive cycle), and 1 final annealing cycle @ 68°C for 7 min. Control experiments were performed under identical conditions to deter-

mine the ratio of the density of the CRT PCR product:18S rRNA PCR product was within the linear range at PCR cycle 28. At 28 cycles, the coefficient of variation with replicate samples was 13.8%. For each gel, male and female, pre- and post-supplementation samples were run simultaneously. Products were run on a 2% agarose gel, stained with ethidium bromide. Image was acquired and analyzed with the use of UVP UV Darkroom and LabWorks™ Image Acquisition and Analysis Software.

Statistical analyses

A two-way analysis of variance (ANOVA) was used to analyze the mRNA data from Study #1 and the protein data from Study #3, with gender as a within variable and Cr-mH vs. placebo as the other between variable. When no gender differences were found, the genders were collapsed and the data was run as a between-within split-plot design with Cr-mH and placebo as the between variable and PRE/POST treatment as the within (repeated measure) variable. The protein data from Study #2 was analyzed with a between-within split-plot design with CR-CHO and PRO-CHO as the between variables and PRE/POST treatment as the within (repeated measure) variable. A p value of ≤ 0.05 was considered to be statistically significant.

Results

Study #1

There were no gender differences in CRT mRNA/18-S ribosomal RNA content between men and women (p = N.S.). CRT/18-S mRNA content did not change as a result of the acute Cr-mH supplementation protocol (P = N.S.) (Fig. 1B). Furthermore, men and women showed similar increases in muscle total creatine in response to the Cr-mH supplementation and had similar baseline total creatine concentrations (Table 1).

Study #2

Two months of supplementation with Cr-mH and carbohydrate (CR-CHO) resulted in a significant increase in total muscle creatine concentration, whereas there was no increase in response to protein and carbohydrate (PRO-CHO) (Table 1). Neither supplementation nor resistance exercise training altered either the citrate synthase (not shown) or CRT-70 content (Fig. 1A). The CRT-52 band was not reproducibly apparent in all of the blots using the CRT-1 antibody, hence, we did not include this analysis (with the limited number of homogenate available for probing with the newer CRT-2 antibody, we found that pre and post-supplementation analysis was possible on only 5 subjects and there was no evidence for down-regulation of the CRT protein, however with such a limited sample size, we feel that it is best to interpret these results as preliminary).

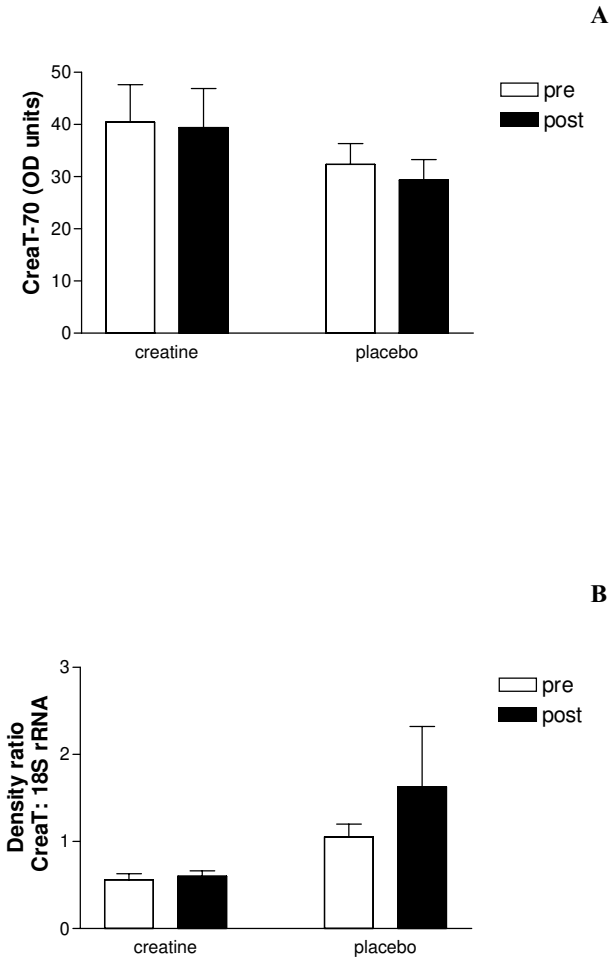

Fig. 1. (A) CreaT-70 protein content before (pre) and after (post) 2 months of resistance exercise training in young men consuming Cr-mH (10 g/day) + dextrose (70 g/day) or casein (10 g/day) + dextrose (70 g/day; 'placebo') (Study #2). (B) CreaT mRNA expressed relative to 18-S rRNA mRNA content in young men and women (collapsed for there were no gender differences) before (pre) and after (post) ~ 9 days of Cr-mH 'loading' (Study #1; see text for more details; values are mean ± S.D.).

Study #3

Four months of Cr-mH supplementation (5 g/day, ~ 0.075 g/kg/day) in elderly subjects who were performing resistance exercise training, resulted in an increase in muscle total creatine concentration for both males and females (Table 1). CRT-70 protein content was similar in males and post-menopausal females and there was no effect of training (PRE/POST) or

Table 1. Total creatine concentration

	Total creatine (mmol × kg^{-1} dm)			
	Males		Females	
	Cr-mH	Placebo	Cr-mH	Placebo
Study 1				
Pre	125.5 ± 8.0 (n = 7)	128.0 ± 16.0 (n = 7)	134.0 ± 14.0 (n = 7)	131.3 ± 13.0 (n = 7)
Post	141.2 ± 12.0*	130.0 ± 8.0	151.5 ± 25.0*	134.7 ± 13.0
Study 2				
Pre	134.8 ± 12.0 (n = 11)	125.6 ± 6.0 (n = 8)	N/A	N/A
Post	142.9 ± 8.0*	124.1 ± 7.0	N/A	N/A
Study 3				
Pre	116.8 ± 15.0 (n = 8)	140.8 ± 21.0 (n = 7)	129.7 ± 25.0 (n = 6)	138.5 ± 14.0 (n = 7)
Post	159.3 ± 24.0*	125.5 ± 25.0	151.7 ± 19.0*	147.5 ± 20.0
Basal		130.1 ± 13.0 (n = 47)		127.2 ± 19.2 (n = 30)

Values are mean ± S.D. *Denotes a significant change ($p < 0.05$) after supplementation. Study #1. Acute (9 day) creatine loading in young men and women. Study #2. Two months of resistance exercise training in young men with Cr-mH (10 g) + dextrose (70 g) or caseine (10 g) + dextrose (70 g). Study #3. Four months of resistance exercise training in elderly (> 65 years) men and women with Cr-mH (5 g), or placebo (dextrose). (see text for further details). Basal refers to resting, non-supplemented values in males and females.

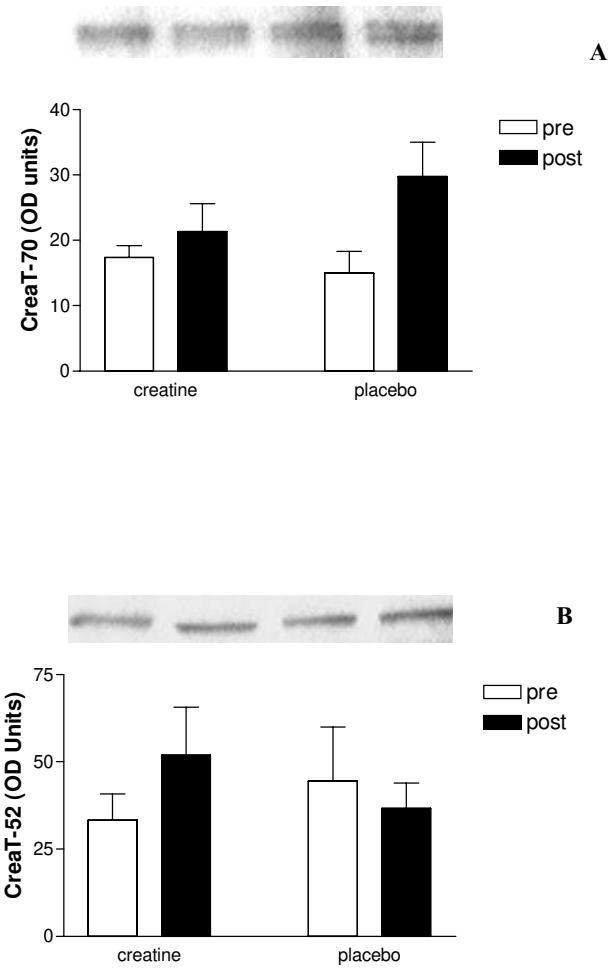

Fig. 2. (A) CreaT-70 protein content measured using the CRT-1 antibody before (pre) and after (post) 4 months of resistance exercise training in elderly (> 65 years) men and women (collapsed across gender for their were no gender differences) consuming Cr-mH (5 g/day) or placebo (dextrose 7 g/day) (Study #3). (B) Identical set-up but using the CRT-2 antibody to emphasize the 52 kDa band (Study #3).

supplementation (Cr-mH vs. PL) (Fig. 2A). In contrast to the younger subjects performing resistance exercise, the elderly subjects showed an increase in citrate synthase protein content ($p < 0.05$), with no effect of supplementation (N.S., data not shown). There was a significant correlation ($r = 0.72$, $p < 0.01$) between the change in citrate synthase and CreaT (CRT-70 + CRT-52) before and after exercise training. Using the original polyclonal rabbit antibody directed against the COOH-terminus of CRT-1 (SwissProt P48029), we also found low signal and inconsistent bands for the CRT-52 band in the elderly, however, the CRT-52 band was consistently apparent using the newer CRT-2 sheep antibody (Swiss Prot P53796), and there was no effect of supplementation or exercise training (Fig. 2B). In about 50% of the samples a faint band was also observed with an apparent MW of ~ 56 kDa using the CRT-2 antibody, that has been proposed as the actual sarcolemmal transporter (Walzel *et al*. (abstract in this issue) and Walzel *et al*. 2002 (in press). To confirm this, we made a human red blood cell preparation (no mitochondria) and found that the CRT-2 antibody recognized a single band with an apparent MW of ~ 56 kDa (Fig. 3, upper). When human muscle samples were overloaded on the gels, this same band was observed at the same MW of ~ 56 kDa (Fig. 3, lower). At this point however, it would be too speculative to report on the data concerning the quantitation of this latter polypeptide from the limited number of observations before and after supplementation in each of the groups.

Discussion

The main findings from the current study are that two and four months of Cr-mH supplementation at doses of ~ 0.075–0.125 g/kg/day, did not result in a decrease in the amount of immunodetectable CRT protein. Furthermore, an acute Cr-mH 'loading' protocol for 8–9 days at a dose of 0.18 g/kg/day did not decrease muscle CRT/18-S mRNA content. Additionally, there does not appear to be a gender difference in basal CRT/18-S mRNA or CRT protein content. Finally, the increase in muscle total creatine in response to acute Cr-mH

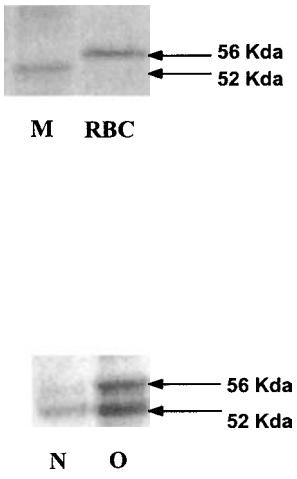

Fig. 3. (Upper) CreaT-52 protein in human skeletal muscle (M) using the CRT-2 antibody and the corresponding results in a purified human red blood cell (RBC) preparation highlighting the presumed plasma membrane isoform (56 kDa). (Lower) CreaT-52 protein in human skeletal muscle using the CRT-2 antibody loaded with the 'normal' (N) amount of protein and the same skeletal muscle sample with and overloaded amount of protein (O, 3 × the usual amount), highlighting that the 56 kDa band is visible in human skeletal muscle, however, to consistently observe the band ~ 3 times more protein must be loaded to obtain comparable optical density values to the 70 and 52 kDa bands.

loading or lower dose supplementation for four months was similar in men and women.

The addition of exogenous creatine (5 mM) to rat L6 myoblasts resulted in 33% of the creatine transport activity as compared to incubation in a creatine-free medium [5]. Furthermore, these changes were apparent within 24 h of incubation and 24 h of cyclohexamide treatment (inhibitor of protein synthesis) partially blocked the decrease in creatine transport induced by 5 mM of exogenous creatine [5]. Earlier research demonstrated that creatine transport into skeletal muscle was reduced with anaerobiosis, uncoupling of oxidative phosphorylation (2,4 dinitophenol), and cooling [4]. Together, these results demonstrate that creatine transport is modulated by an energy dependant process that involves functional protein synthetic capacity. The transport of creatine and it's attenuation by exogenous creatine was subsequently shown to involve the human CRT from the results of studies where the human CRT was transiently expressed in COS-7 cells [1]. Interestingly, the transport of creatine into COS-7 cells transfected with the CRT, was also acutely inhibited by β-guanidinopropionic acid [1]. Medium-term creatine feeding studies showed that skeletal muscle total CRT protein content was reduced that when rats received a diet containing 4% creatine and 50 mM in drinking water for 3 months [3]. In addition, the inhibition of creatine transport with β-guanidinopropionic acid in rats for 3–6 months resulted in a significant increase in CRT protein in skeletal muscle [3]. Therefore, the medium-term administration of exogenous creatine to rats in doses that were an order of magnitude higher than the doses used in humans [7], can decrease CRT protein content. Given the divergent effects of β-guanidinopropionic in the acute *in vitro* experiments (decrease creatine transport [1]), as compared to the longer-term administration studies *in vivo* (increase CRT protein [3]), it would appear that there are likely different regulatory mechanisms involved in the processes.

Our data shows that in response to Cr-mH supplementation strategies that increased muscle total creatine content in humans there was no decrease in CRT protein content. Clearly, the fact that we found no reduction in CRT protein or mRNA content does not exclude that creatine transport could have been inhibited by a different process such as post-translational modification of the protein (phosphorylation, glycosylation, etc.), or differences in the intra-cellular localization of the transporter such as the transmigration and recruitment seen in with the GLUT-4 glucose transporter [20]. Recently, it was shown that cyclosporin A by inhibiting, among others, the mitochondrial protein cyclophilin also inhibits creatine uptake by apparently altering the targeting of CRT to the plasma membrane by inhibiting the mitochondrial protein cyclophilin [21]. Furthermore, modification of CRT transport activity has been shown by site directed mutagenesis of a critical cysteine-144 residue [22]. In spite of the evidence that alterations in CRT transport can occur by mechanisms unrelated to the total CRT protein content, there did not appear to be an attenuation of muscle total creatine accumulation after acute, two month or four month Cr-mH supplementation. These observations are supported by the fact that the cessation of Cr-mH supplementation after 10 weeks (~ 0.125 g/kg/day), did not result in an 'undershoot' of muscle phosphocreatine concentrations 5 weeks later [11]. Thus, it appears that elevations of skeletal muscle total creatine concentration can be maintained for 2 months in men at a dose of 0.125 g/kg/day, for 4 months in elderly men and women at a dose of 0.075 g/kg/day, and for 10 weeks in women at a dose of ~ 0.0125 g/kg/day [11]. Whether or not these elevated concentrations can be maintained for longer periods of time awaits further investigation. These results are of importance for the long-term treatment of patients in whom creatine supplementation may prove to be effective.

Another issue raised by the results of the current study is the location of the CRT protein that is being detected by the antibodies. The first antibody (CRT-1) that was generated appears to recognize two distinct bands of apparent MW 52 and 70 kDa [3, 23], whereas, a newer antibody shows a faint band inconsistently in skeletal muscle crude homogenates at an apparent MW of ~ 56 kDa. The fact that there was only a single band in human erythrocytes at ~ 56 kDa suggests that

this represents the true sarcolemmal transporter. Furthermore, the results of Murphy and colleagues who demonstrated that the ~ 52 and 70 kDa isoforms were in higher abundance in type I muscle fibers, yet the total creatine content was higher in type II muscle fibers, suggested that the 52 and 70 kDa isoforms are mitochondrial, in spite of the fact that immunofluoresence staining suggested that the transporter was located at the sarcolemma [23]. A difficulty with the immunofluorescence data is the fact that an apparent sarcolemmal localization may be due to the presence of sub-sarcolemmal mitochondria. Our data support that these isoforms are mitochondrially located for there was a positive correlation between the increase in the citrate synthase protein content (mitochondrial) and the total CRT protein (52 + 70 kDa) content in the 4 month training study in the elderly. By biochemical fractionation and immuno-localization, using the same COOH-terminal anti-CRT-1 antibody, both of these polypeptides were shown recently to be highly enriched in mitochondrial preparations and specifically localized in mitochondria, respectively (Walzel *et al*. this issue; [19]).

Finally, our group has found that there are gender differences in the increase in fat-free mass [24] and in the regulation of protein turnover [14] in response to Cr-mH supplementation. These findings are consistent with the results of Murphy and colleagues (in the current issue) and together, provide very strong evidence against a gender difference in total creatine concentration, CRT protein, or CRT mRNA content. Therefore, the apparent gender differences in FFM accumulation and protein breakdown and amino acid oxidation, must be attributable to processes distal to the accumulation of creatine in skeletal muscle.

Given the discovery of genetic defects in the CRT [25] and in creatine biosynthesis [26], the understanding of the control of the CRT in response to Cr-mH supplementation is of great importance. Furthermore, the potential for Cr-mH to be a therapeutic agent in neurological disorders [27–30], provides further reason to evaluate the long-term effects of Cr-mH on the regulation of the CRT. A major issue that is still not resolved is the exact intra-cellular localization of the various CreaT isoforms and their role in health and disease states. The recent characterization of a genetic defect in the SLC6A8 gene highlights the importance of this transporter to cellular function [25].

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