Muscle creatine uptake and creatine transporter expression in response to creatine supplementation and depletion

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Brault, Jeffrey J., Kirk A. Abraham, and Ronald L. Terjung. Muscle creatine uptake and creatine transporter expression in response to creatine supplementation and depletion. J Appl Physiol 94: 2173–2180, 2003. First published February 28, 2003; 10.1152/japplphysiol.01171.2002.—The total creatine pool size \( [\text{Cr}]_{\text{total}} \); creatine (Cr) + phosphocreatine (PCr) is crucial for optimal energy utilization in skeletal muscle, especially at the onset of exercise and during intense contractions. The \( [\text{Cr}]_{\text{total}} \) likely is controlled by long-term modulation of Cr uptake via the sodium-dependent Cr transporter (CrT). To test this hypothesis, adult male Sprague-Dawley rats were fed 1% Cr, their muscle CrT total was reduced by ~85% [1% \( \beta \)-guanidinopropionic acid (\( \beta \)-GPA)], or their muscle CrT total was depleted (1% Cr after \( \beta \)-GPA depletion). Cr uptake was assessed by skeletal muscle \( ^{14} \text{C-Cr} \) accumulation to Cr and PCr by using hindlimb perfusion, and CrT protein content was assessed by Western blot. Cr uptake rate decreased with dietary Cr supplementation in the white gastrocnemius (WG; 45%) only. Depletion of muscle CrT total to ~15% of normal increased Cr uptake in the soleus (21%) and red gastrocnemius (22%), corresponding to 70–150% increases in muscle CrT content. In contrast, the inherently lower Cr uptake rate in the WG was unchanged with depletion of muscle CrT total even though CrT band density was increased by 230%. Thus there was no direct relationship between apparent muscle CrT abundance and Cr uptake rates. However, Cr uptake rates scaled inversely with decreases in muscle CrT total in the high-oxidative muscle types but not in the WG. This implies that factors controlling Cr uptake are different among fiber types. These observations may help explain the influence of initial muscle CrT total, time dependency, and variations in muscle CrT total accumulation during Cr supplementation.

phosphocreatine; \( \beta \)-guanidinopropionic acid; muscle fiber types

THE TOTAL CREATINE CONTENT \( [\text{Cr}]_{\text{total}} \); phosphocreatine (PCr) + creatine (Cr) of skeletal muscle differs more than twofold across species and among fiber types (10). Furthermore, this \( [\text{Cr}]_{\text{total}} \) is variable to some degree within a particular muscle. For example, PCr or CrT total content may be decreased in muscle because of neuromuscular disease (36), heart failure (29), or by Cr analog feeding (13, 38). Conversely, PCr and/or CrT total content may be increased in humans and animals by dietary Cr supplementation (19, 23, 26) or in myocyte culture by increasing extracellular Cr concentration (24, 30). Despite these observations and the integral role that Cr and PCr play in energy management, it is still unclear how the \( [\text{Cr}]_{\text{total}} \) of skeletal muscle is controlled.

One obvious means to maintain the intracellular CrT total content is control of Cr uptake by the sodium-dependent Cr transporter (CrT), the major route of Cr entry into muscle cells. Differences in CrT protein expression (7, 27) and activity (7) have been observed among skeletal muscle fiber types of the rat. CrT protein expression has been shown to be downregulated in skeletal muscle of some myopathies (37) and in cardiac muscle during heart failure (29). Similarly, CrT protein expression is lower in skeletal muscle of rats when dietary Cr supplementation was begun at 3 wk of age (18). Furthermore, cultured L6 myoblasts incubated with 5 mM Cr for 24 h decrease the maximal velocity but not the Michaelis-Menten constant of Cr uptake suggesting that the number of available transporters decreases but the affinity of the transporter for Cr does not change (24). Therefore, it appears that the CrT expression is regulated. It has not been tested whether the Cr uptake rate and CrT protein expression in adult skeletal muscle can be modified and, if so, what may signal these changes.

The purpose of this study is to measure CrT expression and the rate of Cr uptake among skeletal muscle fiber types of the rat in response to a long-term altered Cr environment designed to supplement, deplete, and replete intracellular \( [\text{Cr}]_{\text{total}} \). Dietary Cr supplementation has been shown previously to increase plasma Cr concentration and intracellular \( [\text{Cr}]_{\text{total}} \) in some muscle fiber type sections of the rat (6, 26, 31), whereas dietary provision of the Cr analog \( \beta \)-guanidinopropionic acid (\( \beta \)-GPA) produces a substantial decrease in intramuscular \( [\text{Cr}]_{\text{total}} \) content (13, 38). We hypothesize that dietary Cr supplementation will cause a decrease in Cr uptake and transporter expression, whereas \( \beta \)-GPA feeding will cause an increase in uptake and CrT expression.

METHODS

Animal Care

Male Sprague-Dawley rats (Taconic, Germantown, NY) weighing 150–325 g were housed two per cage with a 12:12-h
light-dark cycle in a temperature-controlled (20–22°C) environment. All animals were provided unrestricted access to water and powdered rat chow (5001 Rodent Diet, PMI Nutrition International, Brentwood, MO). Rats were divided into three feeding groups: 1) Cr supplementation (1% Cr diet (w/wt)) for up to 7 wk, 2) Cr depletion (1% β-GPA diet) for up to 7 wk, and 3) Cr repletion (1% Cr diet) for up to 4 wk after an initial 7 wk of the β-GPA diet. Data from control-fed animals were similar to those presented in a companion study (7) performed coincident with this study. To avoid potential complications due to age or size, the youngest animals were assigned the longest feeding protocols. Therefore, all animals were approximately the same age and weighed 325–357 g at the time of death. Creatine monohydrate was purchased from Sigma Chemical (St. Louis, MO). The Cr analog β-GPA was synthesized from β-alanine and cyanamide (33), and purity was verified by high-performance liquid chromatography (HPLC) (44). This study was approved by the University of Missouri-Columbia Animal Care and Use Committee.

Hindquarter Perfusion

**Perfusion system.** The perfusion medium consisted of 5% bovine serum albumin in Krebs-Henseleit buffer, 5 mM glucose, 100 μU/ml bovine insulin, and typical plasma concentrations of amino acids (3). Immediately before use, the perfusate was filtered (0.45 μm), warmed to 37°C, and adjusted to a pH of 7.40. A portion was used to prime the perfusion apparatus, which included, in series, a peristaltic pump, a filter, a heating and oxygenating chamber supplied with 95% O2-5% CO2, and a bubble trap. The entire apparatus was located inside a Plexiglas cabinet maintained at 37°C. Perfusion pressure and temperature were monitored continuously throughout the experiment.

**Surgical preparation.** Rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and administered 100% oxygen during surgical preparation as described previously (16). Blood was drawn from the aorta via a heparinized syringe. The sample was immediately spun and plasma removed for determination of plasma Cr. Then catheters were secured in the abdominal aorta and inferior vena cava, and flow was begun, after which the rats were humanely killed with an overdose of pentobarbital into the carotid artery. The hind feet and tail were tied with umbilical tape to concentrate blood flow to the hindlimbs.

**Perfusion protocol.** The flow rate was increased gradually over ~20 min. The initial 150 ml was discarded to clear the system of essentially all the rat red cells, after which the perfusate was recirculated. During this time, a fresh 300-ml volume of perfusate was prepared with 0.2 μCi [4-14C]-Cr/ml perfusate (Moravek Biochemicals, Brea, CA) at a Cr concentration of 1 mM. This Cr concentration was selected because it represents a high physiological dose and would be representative of Cr uptake capacity (7). The perfusate was switched to this radiolabeled volume after the aortic perfusion pressure (~45 Torr) was stable at 50 ml/min. After the initial void was cleared, the perfusate was recirculated throughout the experiment. Perfusion samples were collected every 15 min.

After 58 min, the perfusate was switched to medium without Cr and was not recirculated. Timed samples were initially collected to verify the effective removal of perfusate radioactivity as described recently (7). The left lower hindlimb muscle sections were quick frozen at 66 min by using aluminum tongs cooled in liquid nitrogen. Sections included the soleus (predominantly slow-twitch red fibers), deep lateral red gastrocnemius (predominantly fast-twitch red fibers), superficial medial white gastrocnemius (predominantly fast-twitch white fibers), and the remainder of the gastrocnemius (mixed fast-twitch fibers) (1). Frozen tissue samples were stored at −80°C until analyzed.

**Metabolite and Uptake Rate Analyses**

Metabolites from muscle sections were extracted in cold ethanolic (20% vol/vol) perchloric acid (PCA; 3.5% wt/vol) and neutralized with tri-n-octylamine and 1,1,2-trichloro-trifluoroethane (9). Perfusate, plasma, and powdered food samples were similarly extracted using PCA. Extracts were stored at −80°C until analyzed.

Cr, PCR, β-GPA, and phosphorylated β-GPA (β-GPA P) were determined by using ion-exchange HPLC as described by Wiseman et al. (44). PCR- and Cr-specific fractions were collected, and radioactivity was counted by dual-channel liquid scintillation counting [quench corrected to disintegrations/min (DPM)]. Adenine nucleotides were determined by reverse-phase HPLC (39).

Cr uptake rates were calculated from the total radioactivity (DPM/g) found in the muscle PCR- and Cr-specific pools divided by the average perfusate Cr-specific activity for that animal (DPM/nmol). The net amount of radiolabel found in the muscle Cr and PCR pools likely represents the actual uptake of Cr since the turnover of the bulk intracellular pool is only a few percent per day (4, 11).

Metabolite concentrations and uptake rates were calculated to a common water content of 76%, typical for rested rat skeletal muscle (20). The muscle water content was determined by drying a 150- to 250-mg portion of each gastrocnemius mixed fiber section at 60°C to a stable weight.

**Protein Analysis**

Frozen muscle sections were homogenized in 100 mM KCl and 50 mM imidazole (pH 7.0). After centrifugation, the precipitate was solubilized in sodium dodecyl sulfate and urea by incubation at 37°C for 2 h. Total protein concentrations were determined against bovine serum albumin standards by using a bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL).

Samples (40 μg of total protein) were separated by electrophoresis on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. After the membrane was blocked with milk, it was incubated overnight with a 1:2,000 dilution of the CrT protein (55- and 58-kDa bands) per milligram of protein. Although it has yet to be completely defined, the protein represented by the 55-kDa band likely resides only intracellularly, whereas that found at 58 kDa is considered to be at the sarcolemma (41).

**Statistics**

Completely randomized analysis of variance was used to compare differences over time within a fiber type with sig-
significance accepted at $P < 0.05$. Tukey’s procedure was used to calculate critical differences among means, if necessary. Values are given as means ± SE.

RESULTS

Creatine Supplementation Group

Rats fed standard rat chow have a plasma Cr concentration of $0.23 ± 0.02$ mM, despite the fact that normal chow is essentially Cr-free ($<0.001\%$ by weight as measured by HPLC). Dietary Cr supplementation resulted in a threefold higher plasma Cr concentration after 1 wk ($0.66 ± 0.12$ mM), and it remained elevated for the remainder of the time course (Fig. 1).

Although plasma Cr concentration was elevated for up to 7 wk, there were no significant changes in intracellular $Cr_{\text{total}}$ among the muscle fiber type sections examined (Fig. 2). Similarly, total adenine nucleotide (ATP + ADP + AMP) contents were not altered over time, having values of $6.13 ± 0.20$, $8.37 ± 0.39$, and $9.12 ± 0.33 \mu$mol/g in the soleus, red gastrocnemius, and white gastrocnemius, respectively.

Seven weeks of Cr supplementation also did not significantly alter CrT protein content as defined by the 55- or 58-kDa band (Fig. 3). On the other hand, Cr uptake rate significantly decreased in the fast-twitch white gastrocnemius section (Fig. 4).

Creatine Depletion and Repletion Groups

Creatine depletion, caused by dietary provision of $\beta$-GPA, resulted in an initial drop in plasma Cr concentration that returned to near the control levels after 5 wk (Fig. 5). During Cr repletion, plasma Cr increased appreciably over the initial 2 wk of Cr feeding.

As expected of the Cr-depletion group, $\beta$-GPA supplementation resulted in a substantial $\beta$-GPA and $\beta$-GPA P accumulation in all three muscle fiber sections examined (Fig. 6B) and a concomitant decrease in $Cr_{\text{total}}$ to $\sim 15\%$ of control values after 7 wk (Fig. 6A). Interestingly, the total $\beta$-GPA accumulated was in excess of the $Cr_{\text{total}}$ lost in all fiber types after 7 wk. For example, in the soleus, $Cr_{\text{total}}$ decreased from 24 to 4 $\mu$mol/g, whereas $\beta$-GPA rose from 0 to 32 $\mu$mol/g. Total adenine nucleotides were lower as well, being $3.51 ±$
0.23, 5.01 ± 0.25, and 5.88 ± 0.20 μmol/g in the soleus, red gastrocnemius, and white gastrocnemius, respectively, after β-GPA feeding.

During Cr repletion, Cr_total accumulated at the same rate among fiber-type sections (~1.7 μmol·day⁻¹·g⁻¹ or ~70 nmol·h⁻¹·g⁻¹) over the first 6 days. By 3.5 wk, Cr_total was near control values (Fig. 6A). On the other hand, β-GPA decreased in a first-order fashion (r = 0.99 for each fiber type) with a half time of ~4.5 days for the soleus and red gastrocnemius and ~7.2 days for the white section of the gastrocnemius. Adenine nucleotide content returned toward normal during the Cr repletion phase, which was 4.98 ± 0.16, 6.90 ± 0.46, and 7.22 ± 0.20 μmol/g in the soleus, red gastrocnemius, and white gastrocnemius, respectively, after 3.5 wk.

CrT protein expression of the 55- and 58-kDa bands were markedly increased by Cr depletion (55-kDa band: 1.9 ± 0.15-, 2.1 ± 0.28-, and 2.4 ± 0.18-fold, relative to the normal diet control muscle, in the soleus, red, and white gastrocnemius, respectively; 58-kDa band: 2.5 ± 0.44-, 1.7 ± 0.34-, and 2.3 ± 0.39-fold, relative to the normal diet control muscle). As illustrated in Fig. 7, subsequent repletion of muscle Cr_total by Cr supplementation did not alter the abundance of the 55- and 58-kDa bands of the CrT protein over the 3.5 wk of observation. Cr uptake rates were significantly elevated (P < 0.05) after 7 wk of β-GPA feeding in the soleus and red gastrocnemius muscle sections (Fig. 8). These elevated rates of Cr uptake decreased over the 3.5 wk of Cr feeding. Unlike the high-oxidative fiber types of the soleus and red gastrocnemius muscle, Cr uptake was not elevated in the white gastrocnemius nor was there any change in Cr uptake over time of Cr repletion. Thus changes in Cr uptake with Cr depletion and subsequent muscle Cr repletion were specific to the muscle fiber section.

**DISCUSSION**

The major finding of this study is that Cr uptake rates of the high-oxidative fiber muscle sections are affected by changes in the intracellular Cr concentration ([Cr]ᵢ). As illustrated in Fig. 9, this was observed in the slow-twitch red soleus and fast-twitch red gastrocnemius when [Cr]ᵢ was decreased below “normal” by β-GPA feeding. Cr uptake was measured during conditions of high extracellular Cr concentration ([Cr]ₑ) of 1 mM, which is well above the apparent Kᵢ for the Cr uptake into skeletal muscle (7, 14, 43). Thus variations in Cr uptake rate are not confounded by issues of extracellular Cr supply but should reflect changes in CrT protein content (12) and/or CrT activity. On the basis of the observation that CrT protein content is reduced with elevated [Cr]ᵢ (18), we expected that CrT protein content would be elevated as [Cr]ᵢ was reduced. This could be a means to compensate by increasing Cr uptake and helping return [Cr]ᵢ to nor-
This was not the case. This is most evident for the soleus and red gastrocnemius during Cr repletion, where Cr uptake rates decline (Fig. 8) with no systematic change in CrT protein content (Fig. 7). We have evaluated this relationship by using the 58-kDa CrT protein band, based on the recent evidence by Walzel and coworkers (40, 41) showing that only this band is associated with the sarcolemmal membrane fraction. The more dominant 55-kDa CrT band is associated with the mitochondrial fraction of the muscle (40, 41). This lack of correlation between CrT uptake and Cr content over an 10-fold range of [Cr] below normal strongly argues for regulation of Cr uptake by means other than CrT protein expression.

Although it is presently unclear what process may modulate Cr uptake with low [Cr], several aspects may be suggested. First, it is unlikely that a significant change in energy driving Cr uptake is a factor, because the electrochemical gradient for two Na⁺ ions (~30 kJ), which is thought to provide the energy to move Cr up its concentration gradient, is well in excess of that needed to achieve Cr transport even at high [Cr]. Second, the reduction in [Cr] might be expected to accelerate the transport process by enhancing the re-
lease step of Cr from the CrT inside the sarcolemma membrane. This, of course, presumes that the Cr “off” rate is much slower than the internalization process once Cr is bound to the external side of the transporter. This is consistent with greater relative Cr accumulation in tissues with low initial [Cr], such as the liver and kidney, compared with tissues with high [Cr], such as skeletal and cardiac muscle (21, 23). A third potential explanation is acute modulation of CrT by posttranslational modification. The CrT shares a 50% homology with GABA/taurine transporters, whose activity is modified by protein phosphorylation (28). Control of Cr uptake by phosphorylation has been suggested recently, because the amount of CrT phosphorylation in mixed fast-twitch muscle can be modified (42, 45). This could be relevant, because Zhao et al. (45) observed a 38% increase in Cr uptake in skeletal muscle membrane vesicles corresponding to a 30% reduction in serine phosphorylation of the CrT. Finally, there could be an acute modulation of the number of CrT present in the sarcolemmal membrane, much like that described for GLUT-4 (15). Recruitment of additional CrT from an internalized pool would increase transport capacity at the membrane that should be manifest as an increase in Cr uptake. Precedent for this occurring in skeletal muscle comes from the recent work of Boehm et al. (5) showing that plasma membrane CrT abundance in rat heart could be varied in a manner corresponding to variations in Cr transport. Thus Cr uptake may be modulated by the content of CrT from an internalized pool would increase transport capacity at the membrane that should be manifest as an increase in Cr uptake. Precedent for this occurring in skeletal muscle comes from the recent work of Boehm et al. (5) showing that plasma membrane CrT abundance in rat heart could be varied in a manner corresponding to variations in Cr transport. Thus Cr uptake may be modulated by the content of CrT.

The relationship between Cr uptake rate and the [Cr], in the high-oxidative fiber-type sections may explain some intriguing results seen previously with dietary Cr supplementation if our results are applied to humans. First, during dietary Cr supplementation, Cr accumulation is most pronounced in those individuals with the lowest initial [Cr], (8, 17, 19). Assuming that all subjects achieved similar elevations in plasma Cr concentration during Cr supplementation, individuals with the lowest starting [Cr], would be expected to have the highest initial Cr uptake rates. These high rates should rapidly expand the intracellular Cr pool. As this expansion progresses, the elevation in [Cr], from below normal would temper the Cr uptake rate, as illustrated in Fig. 9, even with plasma Cr remaining elevated. Because Cr degradation to creatinine is ostensibly a simple fractional process, an increased [Cr], will also result in a greater absolute loss of Cr from the muscle per day. The new steady state is achieved when this creatinine loss matches the greater Cr uptake rate evident with Cr supplementation. On the other hand, individuals with initially high [Cr], would not be expected to exhibit the marked increase in Cr uptake rate as for the individuals with low initial [Cr]. In addition, the Cr uptake rate could be downregulated with a sustained extracellular Cr load (Fig. 4) when [Cr], is normal or elevated, although this was suggested only in the white gastrocnemius. Second, intramuscular Cr accumulation occurs primarily during the initial few days of the loading regimen (19, 22). Despite a sustained elevated plasma Cr concentration of many times normal during prolonged Cr supplementation (19, 35), muscle Cr content remains relatively constant thereafter. Accelerated uptake of Cr appears to occur initially, followed by a decline, possibly related to a de-

Our study was also designed to increase [Cr], above normal by 1% dietary Cr supplementation. This dose (1% of diet by weight = 0.01 × 30 g/day per 350 g rat = −0.85 g Cr·kg⁻¹·day⁻¹) is in excess of that routinely used by humans during a Cr-loading regimen (20 g/day per 70 kg person = 0.3 g·kg⁻¹·day⁻¹). Unfortunately, we were not able to reproduce the even modest 6–20% increase in [Cr], of rat muscle observed previously by some (26, 31, 32) but not by others (21). The reasons for the lack of response are unclear but may be related to the extremely high doses (up to 5% in the diet) used previously. Thus we are not able to evaluate whether the decrease in CrT protein content observed by Guerrero-Ontiveros and Wallimann (18), who fed rats 4% Cr for 3 mo from a young age, results in the predictable decrease in Cr uptake. On the other hand, there was a response during prolonged Cr feeding that warrants consideration. The white gastrocnemius section exhibited a decrease in Cr uptake rate as the duration of Cr feeding progressed (cf. Fig. 4). This was not evident in the soleus or red gastrocnemius muscle sections. At the same time, CrT protein content (defined as the 58-kDa band) decreased (P < 0.05) by the end of the Cr feeding period (cf. Fig. 3) even though [Cr], was not significantly elevated. This decrease in Cr uptake with prolonged Cr feeding could be related to reduced CrT protein. Unfortunately, our results are not definitive in establishing this matter.

By contrast with the response in the high-oxidative fiber sections, similar decreases in [Cr], did not result in any change in Cr uptake in the low-oxidative white gastrocnemius section. Although [Cr], decreased to only ~15% of normal, Cr uptake rate remained unchanged. This marked difference in behavior of Cr uptake between fiber-type sections cannot be easily attributed to an inadequate Cr supply to this low vascular conductance white gastrocnemius muscle section (16, 25), because the rate of Cr delivery via hindlimb perfusion was well in excess (>300-fold) of the rate of Cr uptake. Rather, there appears to be some fundamental difference among the high- and low-oxidative muscle sections. Differences in CrT activity due to differential protein phosphorylation and/or externalization of CrT to the sarcolemmal membrane as discussed above could contribute to this distinction in Cr uptake as [Cr], was reduced. Curiously, the increase in Cr uptake was observed in the high-oxidative fiber sections that possess relatively high mitochondrial contents (2) and the cell fraction identified by Walzel and coworkers (40, 41) that contains the vast majority of the CrT protein. How the mitochondrial fraction CrT is related to whole cell Cr uptake is presently unknown.

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cline in CrT number (18, 45) and the modulation of CrT activity associated with an increased [Cr]i, if the relationship illustrated in Fig. 9 (soleus and red gastrocnemius) can be extended to above-normal [Cr]. This latter expectation is supported by the findings of Dodd et al. (12) showing that the initial high Cr uptake rate, observed with cells that had over expressed the CrT protein, declined as the [Cr]i increased. Thus the magnitude of the increase in [Cr]i appears to be self limiting. Finally, if applicable to humans, the nonresponsiveness of the Cr uptake rate to [Cr]i in the low-oxidative fast-twitch muscle section may also explain some previous results. Whereas an average increase of 40% (8, 17, 19). The variations in muscle fiber composition among subjects could contribute to this variability associated with Cr supplementation.

Direct chemical measurements of muscle Cr_total and β-GPA during the Cr repletion phase can provide further insight into the cellular management of the Cr pool. Because the absolute rate of Cr degradation is expected to be quite low because of the low [Cr]i; content and [Cr]i is high because of oral supplementation, Cr_total accumulation during repletion should approximate the uptake rate. Indeed, the rate calculated herein for the three fiber types (~70 μmol·h⁻¹·g⁻¹) for the first 6 days of repletion agrees with the uptake rates measured in vivo at a similar Cr concentration (75–5 μmol·h⁻¹·g⁻¹ among fiber types) (7). Conversely, total β-GPA loss differs among the fiber types with the lowest fractional rate (9% per day) found in the white gastrocnemius compared with the soleus (14% per day) and red gastrocnemius (16% per day). If the loss of β-GPA from the cell is representative of Cr loss, our work implies that the cellular conditions that influence degradation of Cr are different among the fiber types, a conclusion made recently from Cr uptake results (7).

In summary, Cr uptake rates were significantly elevated by Cr depletion in the high-oxidative muscle fiber-type sections. These uptake rates correlate inversely with intracellular Cr content but not with the 58-kDa CrT protein band in the high-oxidative fiber types. On the other hand, Cr uptake to low-oxidative fast-twitch fiber type did not change with intracellular Cr or 58-kDa CrT protein content. Therefore, long-term management of Cr uptake is different depending on muscle fiber type.

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