ABSTRACT: Total creatine or phosphocreatine, or both, are reduced in the skeletal muscle of patients with inflammatory myopathy, mitochondrial myopathy, and muscular dystrophy/congenital myopathy. We used Western blotting techniques to measure skeletal muscle creatine transporter protein and sarcomeric mitochondrial creatine kinase (mtCK) protein content in patients with inflammatory myopathy ($N = 8$), mitochondrial myopathy ($N = 5$), muscular dystrophy ($N = 7$), and congenital myopathy ($N = 3$), as compared to a control group without a neuromuscular diagnosis ($N = 8$). Creatine transporter protein content was lower for all groups compared to control subjects ($P < 0.05$; $P < 0.01$ for congenital myopathy). Mitochondrial CK (mtCK) was lower for inflammatory myopathy ($P < 0.05$), higher for mitochondrial myopathy ($P < 0.05$), not different for muscular dystrophy, and markedly lower for the congenital myopathy group ($P < 0.01$), compared to control subjects. Together, these data suggest that the reduction in total creatine or phosphocreatine in patients with certain myopathies is correlated with creatine transporter and not mtCK protein content. This further supports the belief that creatine monohydrate supplementation may benefit patients with low muscle creatine stores, although the reduction in creatine transporter protein may have implications for dosing.
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CREATINE TRANSPORTER AND MITOCHONDRIAL CREATINE KINASE PROTEIN CONTENT IN MYOPATHIES

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Using direct biochemical analysis, we have previously reported that patients with dystrophy/congenital myopathy and inflammatory myopathy have lower muscle phosphocreatine or total creatine concentrations.[34] Reductions in phosphocreatine have also been found in patients with mitochondrial cytopathy, muscular dystrophy, and inflammatory myopathies using magnetic resonance spectroscopy.[1,2,20,34] We hypothesized that the reduction in total creatine and/or phosphocreatine was due to secondary energy deficiency, alterations in the creatine kinase enzyme system,[34] or an alteration of creatine uptake.[3]

Recently a creatine transporter has been cloned and sequenced, and its tissue distribution has been characterized.[10] Antibodies have been raised to the creatine transporter protein.[9] There appear to be two distinct creatine transporter isoforms (55 and 70 kDa) expressed in skeletal muscle, heart, and brain.[9] These two isoforms are likely the result of alternative splicing of the mRNA or glycosylation.[9] The protein content of skeletal muscle creatine transporter can be altered by physiological stimuli, as evidenced by the reduction and augmentation of creatine transporter content seen after long-term high-dose creatine monohydrate supplementation and creatine depletion (with guanidinopropionic acid administration) in the rat, respectively.[9] It is known that both the total creatine and phosphocreatine concen-

Abbreviations: ATP, adenosine triphosphate; CK, creatine kinase; CPEO, chronic progressive external opthalmoplegia; Cr, creatine; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes; mtCK, sarcomeric mitochondrial creatine kinase; PCr, phosphocreatine; TTBS, Tween-20 and Tris-buffered saline

Key words: creatine monohydrate; dystrophy; inflammatory myopathy; mitochondrial disease

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tration are reduced in cardiomyopathy,[14] and a recent study has also found a reduction in creatine transporter protein content in this patient population.[23]

In addition to the potential for a lower creatine transporter protein content to explain the lower total creatine and phosphocreatine concentration seen in certain neuromuscular disorders,[1,2,34] an alteration in the creatine–phosphocreatine circuit may also play a role.[37] The purpose of the creatine–phosphocreatine circuit is to shuttle adenosine triphosphate (ATP) equivalents from the mitochondria to the cytosol, and to stimulate mitochondrial respiration[16] in response to a reduction in energy charge in the cytosol via cytosolic and mitochondrial (mtCK) creatine kinase enzymes.[37] Animal knockouts of mtCK,[30] and free radical–induced mtCK damage,[18,28] provide evidence that structural or catalytic alterations of mtCK are linked to a decrease in cytosolic phosphocreatine concentrations and thus local ATP availability. However, it is unlikely that damage, and consequently a decrease of functional mtCK protein, can explain a reduction in total cellular creatine content. In mitochondrial cytopathies, there is a reduction in phosphocreatine concentration,[20,34] and a simultaneous increase in mtCK protein[24] and mRNA[12] accumulation. This pathological increase in mtCK may be paralleled by the appearance of intramitochondrial mtCK paracrystalline inclusions, such as those seen in patients with certain mitochondrial cytopathies.[33]

Given the growing interest in creatine monohydrate as a potential therapeutic intervention in neuromuscular disease,[6,9,17,21,22,32,35,40] together with the decreases in total creatine and phosphocreatine levels in myopathies,[1,2,20,34] a better understanding of the creatine transporter and mtCK in these disorders is warranted. Therefore, the purpose of the current study was to determine the expression and accumulation levels of creatine transporter and mtCK proteins and to investigate whether the results could provide a partial explanation for the observed reductions in total creatine and phosphocreatine in patients with neuromuscular disorders.[1,2,20,34]

MATERIALS AND METHODS

Patients. Muscle biopsy samples (~50 mg) were taken at the time of diagnostic biopsy over a 1-year period (January 1999 to January 2000) for analysis. Samples were immersed into liquid nitrogen within 1 min of removal and stored at −80°C until analysis (see later). All diagnostic biopsies were taken using a suction-modified Bergström needle. All samples were taken from the distal third of the vastus lateralis muscle. All patients gave informed, written consent for the diagnostic and biochemical analyses in accordance with the McMaster University research ethics board.

Based on our previous results showing alterations in high-energy phosphate compounds in certain patient groups,[34] we obtained samples from the following similar groups: a control group of subjects who, after biopsy and detailed evaluations, were deemed not to have a neuromuscular diagnosis ($N = 8$); a mitochondrial myopathy group consisting of non–carnitine-related mitochondrial myopathy subjects ($N = 5$); a muscle dystrophy group ($N = 7$); a congenital myopathy group ($N = 3$); and an inflammatory myopathy group ($N = 8$) (Table 1). The categorization was based on clinical history and

Table 1. Subject characteristics.

Group	Number	Age (years)	Diagnoses	Relative protein expression (% of control)	
				Creatine transporter	mtCK
Control	8	45.5 ± 13.5	Nonneurological diagnoses	100	100
Mitochondrial myopathy	5	41.0 ± 13.5	Mitochondrial myopathy (ragged red fibers, lactic acidosis) (3); MELAS-3271 (1); CPEO (1).	63 ± 12*	272 ± 146†
Inflammatory myopathy	8	47.1 ± 12.5	Polymyositis (6)/dermatomyositis (1); inclusion body myositis (1)	65 ± 22*	63 ± 46*
Muscular dystrophy	7	35.7 ± 16.2	Limb girdle (3); Duchenne (1); fascioscapulohumeral (1); Emery-Dreifuss (1); oculopharyngeal (1)	73 ± 34*	99 ± 54
Congenital myopathy	3	59.3 ± 11.8	Centronuclear (1); nemaline rod (2)	10 ± 6†	5.7 ± 4.8†

Values expressed as mean ± SD. Group definitions given in text; MELAS-3271, mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes; mtCK, mitochondrial creatine kinase; CPEO, chronic progressive external opthalmoplegia.
*$P < 0.05$ vs. control.
†$P < 0.01$ vs. control.

examination, plasma creatine kinase activity, electromyography and nerve conduction studies, forearm ischemic testing, muscle electron and light microscopic evaluation, muscle enzyme analysis, and genetic testing (where appropriate). Each biopsy was examined using NADH-tetrazolium reductase, actomyosin ATPase, modified Gomori trichrome, succinate dehydrogenase, cytochrome oxidase, alkaline and acid phosphatase, Congo red, oil-red O, elastic Van Giesson, myoadenylate deaminase, phosphorylase, hematoxylin and eosin, and periodic acid Schiff staining, and immunohistochemistry was performed for specific proteins (where appropriate). For all groups we examined the area of fat and connective tissue using oil-red O and Van Giesson stains, respectively. We had to eliminate one case of Duchenne dystrophy from analysis because his total area of nonmuscle constituents amounted to >5% of the total myofibrillar cross-sectional area.

Sample Analysis. Wet muscle samples (35–50 mg) were homogenized (Kontes Co., Vineland, NJ) with 100 passes in buffer containing 250 mmol/L sucrose, 100 mmol/L KCl, 5 mmol/L ethylene-diamine tetraacetic acid (EDTA; 1:14 w/v). Following this step, an additional 100 μL of the homogenizing buffer was added and the sample was centrifuged at 1200*g* for 10 min. The pellet was resuspended in 1 mL of wash buffer (175 mmol/L KCl and 0.5% Triton X-100). This was recentrifuged at 1200*g* for 10 min. The protein content of the second supernatant was determined using a Bradford reagent kit and bovine serum albumin as the standard (Bio-Rad, Hercules, CA). The second supernatant was added to a reducing buffer (62.5 mol/L Tris-HCl, 20% glycerol, 2% sodium dodecylsulfate [SDS], 5% β-mercaptoethanol, 0.5% bromophenol blue, pH 6.8) at a ratio of 2:1 and heated at 100°C for 7 min. From each sample, 20 μg of protein (this was held constant between and within blots) was then loaded onto a 4% polyacrylamide stacking gel and separated using an 8% polyacrylamide separating gel of 1.0-mm thickness at 200 V with a running time of approximately 35 min in Tris-glycine running buffer. We performed several experiments loading incremental amounts of protein onto the gels and found that the densitometric analysis of Western blots was linear in the range from 15 to 60 μg for both the anti-mtCK and anti-creatine transporter antibodies. The blots were optimized using 20 μg of protein, which was within the linear range.

The gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes in transfer buffer (0.1% SDS, 2.5 mmol/L Tris, 192 mmol/L glycine, 24% methanol) for 1 h at 100 V. Following this, the membranes were incubated in 5% blotting grade nonfat dry milk (Bio-Rad) for 1 h and then 6% teleostin gelatin for 30 min (Sigma, St. Louis, MO) in Tris-buffered saline (TTBS; 20 mmol/L Tris, 500 mmol/L NaCl, 0.1% Tween-20). The membranes were then washed with TTBS for 15 s × 3 and 7.5 min × 3, and then incubated with a rabbit-derived antibody directed toward the carboxyterminal end of the human creatine transporter protein at a 1:2000 dilution in TTBS overnight (about 15 h). Details of the antibody preparation have been described elsewhere.[9] The protocol was identical for mtCK protein, using a primary antibody directed against heterologously expressed and highly purified human sarcomeric mtCK,[27] which, under the conditions used, is very specific for mtCK (~Mr = 45 kDa) and does not recognize cytosolic muscle-type MM-CK migrating as a separate lower molecular weight band (~Mr = 43 kDa) (Schlattner and Wallimann, unpublished observations, 2000). Following incubation with the primary antibody, the membranes were rewashed as described earlier, and then incubated with a goat, anti-rabbit (Bio-Rad) antibody (1:3000 dilution) avidin-alkaline phosphatase conjugate (Bio-Rad) in TTBS for 1 h. Membranes were exposed to imaging film (Kodak, X-Omat-AR) in a dark room for 30 s using a chemiluminescent method (Immuno-Star Protein Detection System, Bio-Rad). The x-ray films were scanned into a computer and the density was determined using image-analysis software (UVP Laboratory Imaging and Analysis System, Upland, CA). Care was taken to ensure that the intensity of the scanned bands were within the linear dose response of the x-ray film.

The gels were loaded such that patients of similar age and gender were compared directly on the same blot. Duplicate lanes were run for each patient and control group, and a control sample was run with each blot. To ensure equal loading of myofibrillar proteins between groups we also ran samples from all groups using the preparation and running the conditions described earlier, but staining the gels with Coomassie brilliant blue. These gels were also scanned for the density of the polypeptide bands corresponding to the molecular weights of actin (~Mr = 43,000 kDa) as described earlier.

Statistics. We compared the control sample band density run on the same gel to that of the patient run on the same gel using a nonpaired *t*-test. We considered $P < 0.05$ statistically significant. All data are presented as mean ± standard deviation.

RESULTS

Creatine Transporter. Creatine transporter protein content was significantly lower for the mitochondrial (63 ± 12%), inflammatory (65 ± 22%), and dystrophy (73 ± 34%) groups as compared to the control group ($P < 0.05$) (Fig. 1). For the congenital myopathy group, there was a striking reduction in the protein content to 10 ± 6% of control, which was significant despite the small sample size ($N = 3$; $P < 0.01$) (Table 1 and Fig. 2). The aforementioned results were based on the analysis of the 55-kDa isoform of the creatine transporter, but the directional change in this isoform was identical to that of the 70-kDa isoform in all cases.

Mitochondrial Creatine Kinase. Mitochondrial creatine kinase (mtCK) protein content was higher for the mitochondrial myopathy group (272 ± 146%; $P < 0.01$), lower for the inflammatory myopathy group (63 ± 46%; $P < 0.05$), and not different for the dystrophy group (99 ± 54%; P = NS), compared to the control group (Fig. 1). Similar to the results with the creatine transporter, the congenital myopathy group showed a severe reduction in mtCK content to 5.7 ± 4.8% of the control group ($P < 0.01$) (Fig. 2).

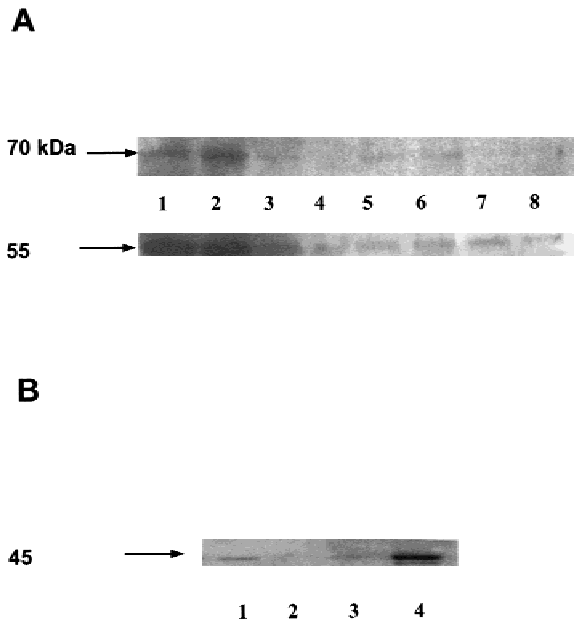

FIGURE 1. Creatine transporter **(A)** and mitochondrial creatine kinase **(B)** protein in patients with various myopathies. **(A)** Creatine transporter content was significantly ($P < 0.05$) lower in mitochondrial (lanes 3 and 4), inflammatory (lanes 5 and 6), and muscular dystrophy (lanes 7 and 8) groups compared to controls (lanes 1 and 2) for both the 70-kDa and 55-kDa isoforms. **(B)** Mitochondrial creatine kinase content was significantly lower ($P < 0.05$) in the inflammatory group (lane 2) and higher ($P < 0.01$) in the mitochondrial group (lane 4) with no difference in the muscular dystrophy group (lane 3) compared to the control group (lane 1) (45-kDa isoform).

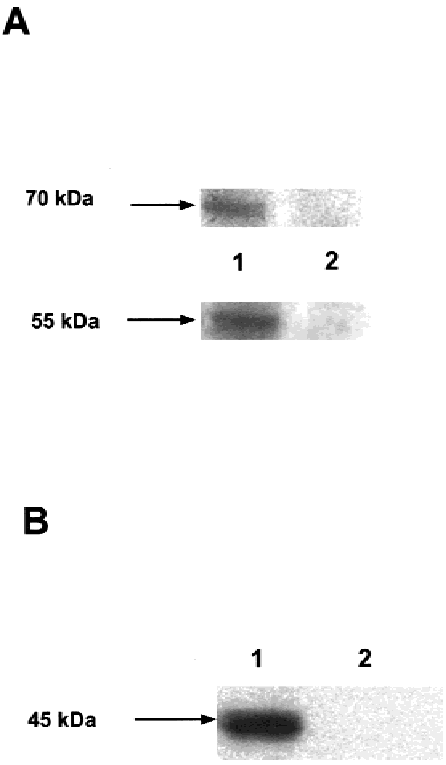

FIGURE 2. Creatine transporter **(A)** and mitochondrial creatine kinase **(B)** protein in patients with congenital myopathy. **(A)** Creatine transporter content for 70-kDa and 55-kDa isoforms were significantly lower ($P < 0.01$) for the congenital myopathy (lane 2) group compared to controls (lane 1). **(B)** Mitochondrial creatine kinase (45-kDa isoform) content was significantly lower ($P < 0.01$) in the congenital myopathy group (lane 2) compared to controls (lane 1).

Quantitation of Myofibrillar Muscle Protein. There were no statistically significant differences between the groups with respect to the protein band density for actin. Thus, the biopsy samples, and hence the aliquots loaded onto the gels, contained equal amounts of myofibrillar proteins. The data are supported by histological examination of muscle sections from the patients, showing that nonmuscle tissue was less than 5% of all biopsies examined.

DISCUSSION

The current study demonstrated that creatine transporter protein content was generally lower in a variety of neuromuscular disorders as compared with otherwise healthy controls. Importantly, this did not represent a global reduction in proteins involved in creatine metabolism, for the mtCK protein was reduced (inflammatory and congenital myopathy

groups), increased (mitochondrial myopathy group), or unchanged (dystrophy group) compared with healthy controls. The lower creatine transporter protein content may partially explain the observed reductions in creatine and phosphocreatine concentration in these patient groups.

We previously demonstrated that total creatine was 13%, 14%, and 15% lower in patients with mitochondrial cytopathy, inflammatory myopathy, and dystrophy/congenital myopathy, respectively.[34] Our group,[34] and others,[2,20] have also reported a reduction in phosphocreatine concentration. Phosphocreatine concentration was 22%, 29%, and 32% lower for mitochondrial cytopathy, inflammatory myopathy, and dystrophy/congenital myopathy groups, respectively, compared with healthy controls in our previous study.[34] The results of the current study demonstrate that creatine transporter protein content was generally lower (~36%) in these patient groups compared to control subjects with nonneuromuscular disorders. These results imply that a reduction in creatine transporter protein can occur in neuromuscular disorders with markedly different pathophysiologies and cellular effects.

Given the obvious differences between the four groups of neuromuscular disorders, one unifying mechanism for the observed decrease in creatine transporter protein is unlikely. Creatine transporter protein reductions could occur at a number of levels, including a reduction in gene transcription, instability of the mRNA transcript, an impairment of translation, or an increase in creatine transporter proteolysis.[39] It is possible that, for inflammatory myopathies and muscular dystrophies, there is an increase in creatine transporter proteolysis.[39] In muscular dystrophy, an alteration in cytoskeletal plasma-membrane stability could alter creatine transporter incorporation into the sarcolemma, whereas, in mitochondrial cytopathy, a reduction in energy charge could impair overall translational activity, due to its energy dependence. Future studies, especially on mRNA content and cellular creatine transporter localization, will be necessary to establish the molecular events taking place in the diseased muscle that alter creatine transporter and mtCK protein levels or activity. Irrespective of the mechanism(s), the current results are consistent with, and provide some explanation for, the observation that ^{15}N-glycine tracer uptake and incorporation into creatine is impaired in progressive muscular dystrophies.[3]

Changes in mtCK protein content were not directionally similar to those of creatine transporter protein or to the total creatine and phosphocreatine concentrations reported earlier in similar patient populations.[34] For example, based on muscle biopsy samples, we have demonstrated that, in mitochondrial cytopathies, there is a significant increase in mtCK protein content, which eventually forms paracrystalline inclusions,[29] paralleled by an increase in mtCK mRNA.[12] The increased amount of mtCK in mitochondrial cytopathies likely represents a coordinated compensatory induction of genes involved in energy transduction.[12] We did not find a reduction in the mtCK protein content for the muscular dystrophy group, but others have reported that mtCK activity is reduced in muscular dystrophy.[19] These findings may suggest that the enzyme activity could be reduced without a parallel reduction in protein expression.

In the 3 patients with congenital myopathy, both creatine transporter and mtCK proteins were nearly completely absent, with residual protein levels of 5–10% of controls. This was not disease-specific in that 2 of the patients had nemaline rod myopathy (α-tropomyosin defect), whereas the remaining patient had centronuclear myopathy (cause unknown). A reduction in mtCK enzyme activity has been reported in an 11-month-old boy with congenital muscular dystrophy.[5] It is possible that the presence of the defect, presumably in utero, and its persistence for between five and seven decades (age range of patients in our study), accounted for the extreme reductions observed. Reductions in creatine transporter and mtCK content in congenital myopathy could therefore be an attenuation of the ontogenic development of protein expression. Given the importance of the CK system and the substrates involved (creatine and phosphocreatine) to both skeletal and cardiac muscle, as well as brain function,[37] high-grade primary reductions in creatine transporter expression or function should result in a severe phenotype. A recent case report found profound reductions in brain creatine with normal plasma creatine concentrations in 2 sisters with mental retardation and language delays.[4] The phenotype and biochemical abnormalities in these cases were improved by creatine supplementation, and a creatine transporter defect was postulated as the cause.[4] It will be of interest to study such cases using the current antibody and methods described in this article.

It is not clear whether the observed reduction in creatine transporter protein has practical implications for patients with neuromuscular disorders who supplement with dietary creatine.[32,35,38] For example, in healthy young males there is an increase in muscle creatine and phosphocreatine following a loading dose of 20 g/day for 4–5 days[11] or 3 g/day

for 30 days.[13] In patients with neuromuscular disorders, the creatine dose may need to be adjusted upward to achieve the same intracellular increases occurring in young healthy subjects.[11,13]

The data from this study and our previous study, showing reductions in muscle total creatine and phosphocreatine in patients with neuromuscular disorders,[34] provide theoretical support for the use of creatine monohydrate in the treatment of neuromuscular disorders.[9,26,32,35,38,40] For example, the increase in muscle phosphocreatine in response to creatine loading is greater in those with lower initial muscle creatine concentrations.[11] Thus, the potential to increase muscle total creatine and phosphocreatine stores may be greater for a patient with a neuromuscular disorder, as compared to a healthy person with already normal stores. The fact that the increase in muscle performance for patients with neuromuscular disorders is greater that that measured in young healthy subjects,[7,15,31,36] provides some indirect evidence that the response to creatine supplementation is enhanced in patients with neuromuscular disorders[32,35,38] ("replacement of a deficiency state" [i.e., neuromuscular disease] compared to "supplementation in a sufficient state" [i.e., healthy young subjects]).

We have shown increases in muscle strength following acute supplementation with creatine monohydrate in 81 patients with a variety of neuromuscular disorders (mean dose = 7.5 g day^{-1} × 10 days),[32] and 7 patients with mitochondrial cytopathy (mean dose = 8.0 g day^{-1} × 21 days).[35] Recently, a randomized, controlled trial found improvements in both strength and activities of daily living in patients with a variety of muscular dystrophies following 8 weeks of creatine supplementation at 10 g/day.[38] Based on the beneficial effects of creatine supplementation upon intracellular calcium handling and cell survival in dystrophic myoblasts,[26] and a recent finding that *mdx* mice fed a 10% diet of creatine monohydrate (weight of creatine relative to the total diet weight) from 14 days of life experienced less skeletal muscle degeneration and enhanced mitochondrial function,[25] it is possible that adjunctive creatine monohydrate treatment early after the diagnosis of a neuromuscular disorder could confer greater and longer lasting effects. Furthermore, recent studies have found that creatine supplementation can increase satellite-cell mitotic activity during compensatory hypertrophy,[8] which may partially explain enhanced muscle function following creatine supplementation.

In summary, we have found that creatine transporter protein content is lower in a variety of neuromuscular conditions that are known to have lower concentrations of phosphocreatine and total creatine. This observation may have practical clinical implications for patients treated with creatine monohydrate in that the dose and duration of supplementation may have to be optimized. If the loss of creatine transporter protein is a progressive phenomenon, treatment should be initiated as soon as possible after diagnosis. Clearly, longer term randomized, double-blind trials of creatine monohydrate with measures of intracellular high-energy phosphates, impact upon activities of daily living, and safety will have to be employed in the future.

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