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Creatine supplementation in health and disease. Effects of chronic creatine ingestion *in vivo*: Down-regulation of the expression of creatine transporter isoforms in skeletal muscle

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Abstract

Interest in creatine (Cr) as a nutritional supplement and ergogenic aid for athletes has surged over recent years. After cellular uptake, Cr is phosphorylated to phosphocreatine (PCr) by the creatine kinase (CK) reaction using ATP. At subcellular sites with high energy requirements, e.g. at the myofibrillar apparatus during muscle contraction, CK catalyzes the trans-phosphorylation of PCr to ADP to regenerate ATP, thus preventing a depletion of ATP levels. PCr is thus available as an immediate energy source, serving not only as an energy buffer but also as an energy transport vehicle. Ingestion of creatine increases intramuscular Cr, as well as PCr concentrations, and leads to exercise enhancement, especially in sprint performance. Additional benefits of Cr supplementation have also been noticed for high-intensity long-endurance tasks, e.g. shortening of recovery periods after physical exercise.

The present article summarizes recent findings on the influence of Cr supplementation on energy metabolism, and introduces the Cr transporter protein (CreaT), responsible for uptake of Cr into cells, as one of the key-players for the multi-faceted regulation of cellular Cr homeostasis. Furthermore, it is suggested that patients with disturbances in Cr metabolism or with different neuro-muscular diseases may benefit from Cr supplementation as an adjuvant therapy to relieve or delay the onset of symptoms. Although it is still unclear how Cr biosynthesis and transport are regulated in health and disease, so far there are no reports of harmful side effects of Cr loading in humans. However, in this study, we report that chronic Cr supplementation in rats down-regulates *in vivo* the expression of the CreaT. In addition, we describe the presence of CreaT isoforms most likely generated by alternative splicing. (Mol Cell Biochem **184**: 427–437, 1998)

Key words: creatine supplementation, creatine transporter, creatine transporter isoenzymes, differential splicing, creatine kinase, creatine metabolism, muscle diseases, bioenergetics, phosphocreatine

Abbreviations: Cr – creatine; PCr – phosphocreatine; CK – creatine kinase; CreaT – creatine transporter or carrier; Crn – creatinine; β-GPA=GPA – 3-guanidinopropionate; β-GBA – 4-guanidinobutyrate; GABA – guanidino γ-aminobutyric acid; IGF-1 – insulin-like growth factor I; CHO – carbohydrate; Mi-CK – mitochondrial CK isoenzyme

Introduction

Creatine and creatine kinase

Creatine (Cr) is a naturally occurring guanidino compound first described in 1832 by Chevreul [1]. For a 70 kg man, the total body content of Cr is approximately 120 g, most of which is present in muscle and brain. The estimated daily requirement of Cr for an average individual is about 2 g [2]. Cr is ingested with food and is also endogenously synthesized from arginine, glycine and methionine in the liver, pancreas and, in humans, primarily in the kidney [2, 3]. After its synthesis, Cr is released into the blood stream, from where it is taken up by cells via the creatine transporter (CreaT) [4].

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Without a continuous supply of muscle cells with creatine, either synthesized by the body or supplemented externally, Cr and thus also phosphocreatine (PCr) would be depleted in muscle by continuous irreversible non-enzymatic conversion of Cr into creatinine (Crn), the latter being released from muscle cells and excreted by the kidney [5, 6] (for review see Walker 1979). The total intracellular Cr concentration [PCr + Cr_{free}] is an essential component of energy metabolism of skeletal, cardiac and smooth muscle and brain, since it determines the concentration of available PCr [7]. PCr serves as a dynamic reservoir of 'high-energy' phosphate during rapid utilization of ATP, delays and dampens fluctuations in adenosine nucleotides, modulates glycolysis and oxidative phosphorylation, and, through the Cr/PCr interconversion by the CK reaction ($PCr^{2-} + MgADP^{1-} + H^+ \leftrightarrow MgATP^{2-} + Cr$), also buffers intracellular changes in pH and, in conjunction with ATP hydrolysis, influences inorganic phosphate levels ($PCr + ADP \rightarrow ATP + Cr \rightarrow ADP + P_i$, net PCr breakdown followed by hydrolysis of the ATP formed leads to an increase in P_i) (for reviews see [8, 9]).

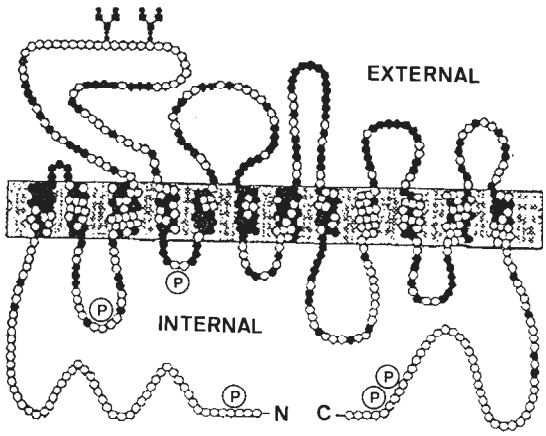

Fig. 1. Schematic representation of the creatine transporter demonstrating its hypothetical orientation within the plasma membrane. Amino acids which are conserved among members of the GAT/NET family (Na^+- and Cl^--dependent, twelve transmembrane spanning domain transporters; see Introduction) are indicated by filled circles. The location of two consensus N-linked glycosylation sites are marked with stylized drawings of a polysaccharide side chain. The canonical phosphorylation sites (circled P) are also indicated. The figure was adopted from Nash *et al.* [16].

The creatine transporter

A saturable sodium- and chloride dependent, high affinity uptake process, which moves Cr against a concentration gradient, has been studied in a number of tissues and cell types [4, 10–13]. The CreaT has recently been cloned [14–16]. Based on sequence homology, it belongs to the γ-aminobutyric acid/norepinephrine (GAT/NET) transporter gene family. The members of this family are Na^+ and Cl^--dependent twelve transmembrane-helix-spanning transporters responsible for the uptake of certain neurotransmitters (e.g. dopamine, guanidino γ-aminobutyric acid (GABA), serotonin and norepinephrine), and amino acids (e.g. glycine and taurine) [15–18]. Northern blot analysis indicated the expression of CreaT mRNA in kidney, heart, skeletal muscle, brain, testis and colon, but not in liver, pancreas, spleen, uterus or intestine [15,16,19, 20], essentially reproducing the expression pattern of CK (see [21]). *In situ* hybridization showed high CreaT transcript levels in cerebellum, hippocampus and other regions of the brain, as well as in muscle tissues [17]. From analysis of the CreaT cDNA one predicts an Asn-linked plasma membrane glycoprotein of 635 amino acids (70.5 KDa) with an isoelectric point of 6.38 and five putative phosphorylation sites [14] (Fig. 1).

The CreaT gene (SLC6A8 solute carrier class 6, member 8) was mapped to human chromosome Xq28 telomeric to G6PD [22]. Iyer *et al.* [23] confirmed these findings, and additionally showed the existence of a novel autosomal, testis-specific form of the human CreaT gene located on chromosome 16p11.2. The Xq28 locus has been linked to the genes for several neuromuscular disorders such as Emery-Dreifuss muscular distrophy [24, 25], Barth syndrome [26, 27], infantile cardiomyopathy [28], and myotubular myopathy [29]. Since CreaT plays an important role in Cr homeostasis and muscular physiology, it was postulated as a potential gene responsible for one of these muscular disorders.

Regulation and inhibition of the creatine transporter

Several types of animal cells from heart, brain, skeletal and smooth muscle, adipose tissue, as well as fibroblasts, and macrophages in isolated tissues or in culture take up Cr [4, 10–13, 30]. These cells transport Cr against a concentration gradient (200:1 in skeletal muscle) [31] through a saturable, sodium dependent process, whose K_m value varies between 25–110 μM [4, 10–13, 15–17, 32]. Under physiological conditions, the CreaT is working at about its half maximal rate since resting levels of Cr in plasma amount to 25–100 μM only [33, 34]. The sodium Cr cotransporter uses the energy of the Na^+ gradient and the membrane potential maintained by the Na^+,K^+-ATPase, which itself depends on the function of CK being in close vicinity of and functionally coupled to this ion pump [35]. Kinetic data of the Na^+ dependence of Cr transport in glial cells suggest a stoichiometry of 2 Na^+ per Cr transported [13].

The total cellular Cr content depends on the balance of uptake, retention and efflux of Cr. However, the mechanisms by which cells regulate their intracellular Cr stores are

poorly understood. A variety of potential regulatory mechanisms have been suggested (for an extensive review see [2]). One of the identified sites for regulation of intra- and extracellular Cr occurs at the level of Cr biosynthesis in the liver, pancreas and kidney. Here, Cr exerts a negative feedback control on the arginine glycine amidinotransferase (AGAT) [36], which is the enzyme catalyzing the first step in *de novo* Cr biosynthesis, the formation of guanidinoacetate from L-arginine and glycine.

Cr homeostasis is also regulated through control of CreaT expression and activity. The saturable component of Cr uptake is affected by Cr structural analogs and metabolic inhibitors [4, 10]. The sensitivity of the CreaT to several guanidinocarboxylic acids such as 3-guanidinopropionate (β-GPA), 2-amino-3-guanidinopropionate, 4-guanidinobutyrate (β-GBA) and guanidinoacetate, has been widely investigated [4, 10–13, 15, 16, 20, 32]. Other guanidino compounds like guanidinosuccinate, GABA, taurine and taurocyamine (or β-guanidinoethanesulfonic acid) as well as several amino acids (e.g. β-alanine, glycine, arginine), and Cr derivatives (PCr and creatinine) were also tested for their ability to compete for Cr at the uptake site. The most efficient blocking agent in all mammalian Cr transport systems studied so far ([10–13, 15, 20] is β-GPA, which acts as a competitive inhibitor of Cr uptake systems and also of CK [4]. Inhibition of Cr entry by β-GPA varies from 45–96% in the different cells and tissues measured and the inhibition constant values range between 15–450 μM depending on the tissue [4,10–13, 30]. Other compounds exerting weaker inhibition on CreaT include γ-guanidinbutyrate and guanidinoacetate in *Torpedo* and humans, N-ethylguanidinoacetic acid or guanidinoacetate in rat skeletal muscle, and taurocyamine in astroglia cells [4, 13, 16, 20, 32]. The competing guanidino compounds as well as Cr interact reversibly with the transporter protein. Compounds lacking an amidine group fail to inhibit Cr transport. Certain mono- and N,N-disubstituted guanidines can compete with higher affinity for uptake [4].

Oddom *et al.* [37] have identified a series of hormones which influence net Cr uptake into a skeletal muscle cell line. They have shown that catecholamines (noradrenalin, isoproterenol and clenbuterol) can stimulate net Cr uptake preferentially through β_2 receptors, probably via a cyclic AMP-dependent mechanism. In addition, they demonstrated that insulin (at supraphysiological concentrations) and IGF-I can also stimulate net Cr uptake. Furthermore, they have identified a modulation of total Cr content by ouabain that inhibits – as well as by agonists that stimulate the Na^+,K^+-ATPase.

Creatine supplementation comes of age: creatine loading in man

Although studies with creatine supplementation have been already performed at the end of the nineteenth century [1], (for references see [34]), it would appear that only recently the implications of Cr metabolism in bioenergetics, physiology and human pathology are attracting considerable attention and are actively being investigated in many research groups.

A century ago, studies with dietary Cr intake performed in both humans and animals concluded that some of the ingested Cr was retained in the body [1]. Recent studies have shown that administration of different Cr doses for variable time periods (typically 20 g daily for 5 days) result in an ≈20% (20 mmol/kg dry muscle) increase in total muscle Cr. As a consequence, Cr loading has a positive effect on exercise performance during single and repeated bouts of exhaustive, short-lasting exercise and increases the rate of PCr resynthesis [7, 38–41].

The extent of Cr retention during Cr supplementation is highly variable between subjects. This variations suggested that Cr uptake is dependent on several factors including differences in the composition of the diet, gender, muscle fiber composition and on the initial total muscle Cr concentration 13, 31, 41]. The positive effect of Cr loading on PCr resynthesis and improvement in exercise performance, however, is basically only observed in those individuals who show an increase of more than 20 mmol/kg dry muscle in total Cr concentration upon dietary Cr-loading [42].

Improvement of the accumulation of creatine in muscle

Koszalka and Andrew [43] as well as Haugland and Chang [44] reported the effect of insulin on Cr transport in rat skeletal muscle. They have found that insulin enhances the transfer of Cr from the circulation into skeletal muscle. Based on this result, Green *et al.* [45] investigated the effect of carbohydrate (CHO) ingestion on muscle Cr accumulation during Cr supplementation in humans. Administration of both Cr and CHO resulted in an increase of the skeletal muscle Cr retention. This appeared to be mediated by insulin, since upon Cr supplementation in conjunction with CHO, the concentration of insulin augmented considerably compared to dietary Cr loading alone. Insulin stimulates the Na^+,K^+-ATPase, in turn promoting the Na^+-Cr-cotransport by maintaining or restoring the normal transmembrane Na^+ gradient and membrane potential [19, 37, 46, 47]. These findings suggested a potential mechanism for optimising muscle Cr accumulation in humans, which can have important implications for improving exercise performance and for the treatment of some neuromuscular diseases related to deficiencies in Cr metabolism and/or chronically lowered Cr levels.

Another attempt to improve muscle Cr trapping was made

by Vandenberghe *et al.* [48]. These authors expected that oral Cr uptake, combined with a physiological degree of adrenergic stimulation by caffeine, might facilitate muscle Cr accumulation. Caffeine has been shown to directly stimulate muscle Na^+,K^+-ATPase activity and to enhance plasma epinephrine levels, another direct stimulus of the muscle Na^+,K^+-pump [19, 37, 46–48]. Surprisingly, however, their results demonstrated that caffeine does not improve the efficiency of oral Cr supplementation, neither increasing muscle PCr levels nor improving exercise performance. Caffeine fully abolished the ergogenic effect of muscle Cr loading. Additional work is needed on the effects of Cr intake together with caffeine on the systems that regulate muscle energetics and improve performance during intermittent exercise, e.g. the PCr-CK energy shuttle between energy providing and energy consuming sites in the cell [8].

Creatine supplementation and exercise performance

Cr loading improves the ability to maintain power output during exhaustive, high intensity tasks, especially when repeated exercise bouts are carried out [3, 38–41, 49–52]. This appears to be due to a rise in the total Cr concentration and thus to an increase in pre-exercise PCr availability and to an increasing rate of PCr resynthesis during episodes of muscle relaxation. In addition, an enhanced fatigue resistance is also observed [39–41, 49, 50].

Some results related to metabolic response after Cr supplementation are controversial. It appears, however, that post-exercise plasma ammonia and hypoxanthine concentration [40, 41], and in some cases muscle lactate [49, 53], are lowered after ingestion of creatine. Low levels of ammonia may indicate that enhanced Cr and PCr concentrations prevent a loss of adenine nucleotides via adenylate kinase and AMP-deaminase, by keeping, via the CK reaction, [ADP] and [AMP] low (see [8]).

For long endurance exercise, however, one cannot yet clearly rationalize how a higher concentration of total Cr could improve exercise performance. The enhancement of intramuscular Cr and PCr levels upon dietary Cr intake causes an increase in anaerobic capacity [41]. In addition, increased Cr stimulates oxidative phosphorylation via activation of mitochondrial creatine kinase (Mi-CK) [9]. Phosphorylation of Cr through Mi-CK increases the ADP levels. Green *et al.* [45] reported higher ADP concentration following Cr supplementation. This ADP, in turn, could enter into the mitochondrial matrix through the adenine nucleotide translocator and facilitate ATP resynthesis by stimulating mitochondrial respiration [45]. By this line of arguments, one could explain the advantages provided by Cr ingestion for enhancing aerobic output in a continuously working muscle. In addition, although there is no hard evidence yet, chronic elevation of Cr levels could lead to alterations in expression and/or activity of enzymes such as phosphofructokinase, hexokinase or adenylate kinase, possibly in an opposite way than observed with the Cr analogue β-GPA [54]. Cr may also delay fatigue by the higher availability and rate of PCr resynthesis observed in athletes after ingestion of Cr [50, 53].

Creatine levels in disease

Extensive studies on the PCr-CK system and its involvement in regulation of energy metabolism have been performed in the past. In contrast, only little is known about Cr metabolism in disease, e.g. whether there are alterations in the regulation of Cr synthesis, uptake or retention in different tissues and in the whole organism compared to the healthy state [55]. Consequently, research concerned with the effects of Cr ingestion on disease is also scarce. It has been demonstrated that several pathologic states such as ischemia, anoxia, hypoxia, toxic cardiomyopathies, muscular dystrophies or atrophies and mitochondrial myopathies are related to energy deficiencies [56–60]. Some muscle diseases were shown to be associated with disturbances in Cr metabolism, low intracellular levels of Cr and phosphocreatine and derangements of the CK system. In this section, we shortly describe some reports related to the effects of Cr supplementation both on animal and human disease states and its potential role as an adjuvant therapeutic aid.

Recently, Cr treatment was tried with a patient with MELAS, a maternally inherited disease characterized by mitochondrial **m**yopathy, **e**ncephalopathy with seizures, and/or dementia, **l**actic **a**cidosis, and **s**troke-like episodes [61], who was given Cr in addition to his normal therapy [62]. His symptoms included exercise-induced muscle pain, headache, chronic lactic acidosis, and episodes of cortical blindness, visual hallucinations, dysphasia and arm paresis. Cr was given orally at 10 g daily for 2 weeks and 4 g daily, thereafter. The patient reported reduced headache, less weakness, better appetite, and an improved work performance during Cr treatment. Interestingly enough, a connection of CK with mitochondrial myopathies has been clearly elaborated in that mitochondrial CK (Mi-CK) has been shown to be the major component of the crystalline intramitochondrial inclusions seen in patients with these diseases [63], indicating that a lowering of the cellular energy status, e.g. by a defect in enzymes of oxidative phosphorylation or by Cr depletion, leads to compensatory overexpression and finally a crystalline deposition of Mi-CK between mitochondrial membranes [54, 64, 85, 86].

As a second case, gyrate atrophy of the choroid and retina is an autosomal recessive dystrophy characterized by constriction of visual field, myopia, and posterior cataract

and total blindness. Concomitantly with the eye disease, clinically mild but marked morphological atrophy of type II skeletal muscle fibres is observed [65, 66]. Patients affected by gyrate atrophy have 10–20 fold increased ornithine concentrations in body fluids and significantly reduced activity of ornithine aminotransferase [67–69]. High ornithine concentrations inhibit arginine-glycine transamidinase, the rate limiting enzyme in Cr biosynthesis. The latter leads to deficient guanidinoacetate formation and subsequent reduction of Cr and PCr production. Deficiency of 'high-energy' phosphates cause the atrophy in the muscles and eyes. Sipilä *et al.* [70] treated patients suffering gyrate atrophy with 1.5 g Cr daily during a year. The treatment showed a reduction of the type II muscle fiber atrophy but the chorioretinal degeneration continued. They suggested that the latter might be due to poor entry of Cr into the retina [66].

Additionally, Stöckler *et al.* [59] described a case in which extrapyramidal movement disorder and extremely low creatinine concentrations in serum and urine were observed. This was caused by a generalized depletion of Cr in the brain. This patient with an inborn error of Cr biosynthesis at the level of guanidinoacetatemethyltransferase (GAMT), was treated with Cr-monohydrate. Oral substitution of Cr-monohydrate led to a significant increase of brain Cr, a decrease of brain guanidinoacetate, a normalization of creatinine in serum and urine, and a decrease in plasma ammonia. Partial restoration of cerebral Cr concentrations was accompanied by improvement of the patient's neurologic symptoms [59].

Finally, based on reports on the amazing effects of creatine supplementation on exercise performance in top athletes, a growing number of muscle patients, especially in Germany, started to take creatine as self-medication. Some of these patients with different neuromuscular disorders reported a marked improvement of their subjective status as well as of objective parameters (see [71]); One of us (T.W.) receives encouraging letters from a growing number of different patients taking creatine (letters may be obtained on request [87, 88]). This movement from the basis prompted physicians to take creatine supplementation seriously and to start with controlled studies [62, 89].

In an animal experiment, the effects of anoxia and hypoxia on synaptic transmission, protein synthesis and levels of energy metabolites were investigated in rat brain or guinea pig hyppocampal slices. Anoxia or hypoxia were found to abolish electrical activity as a result of membrane depolarization caused by a decline in neuronal energy-rich metabolites PCr and ATP and a subsequent increase in intracellular Ca^{2+} [58, 72]. Preincubation of hippocampal slices with Cr before exposing them to anoxia and allowing recovery increased the levels of free- and phosphorylated Cr, slowed down the rate of decline in ATP and, in parallel, enhanced electrophysiological recovery, as well as protein synthesis [58].

The reported findings suggest that Cr is a substance with therapeutic properties. If it is administered together with established treatments for a specific disease, significant improvements in a growing number of patients with different diseases affecting cellular energetics in one way or another may be achieved (for additional information and analysis of the therapeutic role of Cr and PCr see the recent book by Conway and Clark [73]. We propose that the possibility of Cr treatment as an adjuvant therapy for a whole variety of diseases of the central and peripheral nervous system, the skeletomuscular and cardiovascular systems should be seriously considered and studied in double-blinded tests in the future.

A potential problem of chronic Cr supplementation, however, could be a downregulation of the CreaT expression which would antagonize such a treatment. To study the role of CreaT in the regulation of intracellular creatine levels and to answer the question of whether CreaT synthesis is influenced by long-term creatine ingestion, as well as by the Cr analogue 3-GPA, we decided to use an immunochemical approach rising specific antibodies against the CreaT protein. Despite the homologous transmembrane topology which the CreaT shares with other neurotransmitter transporters, its N- and C-terminal regions are clearly divergent and unique. Therefore, we have chosen the latter amino acid sequences as immunogens for the production of specific anti-CreaT antibodies as a tool for the detection and quantification of CreaT in different tissues under different experimental conditions. The antibodies against synthetic peptides (15-mers) corresponding to the N- and C-terminal regions of the cDNA-derived CreaT amino acid sequence reacted on Western blots with two distinct polypeptides of equal prevalence in all CreaT positive tissues (see Results).

The study of the CreaT could help us to understand its role in Cr metabolism, its regulation upon chronic Cr supplementation, as well as its potential involvement in a variety of neuromuscular diseases.

Materials and methods

Generation of antipeptide antibodies against C- and N-terminal peptides of the creatine transporter

The 15-mer amino acid residue peptides corresponding to the NH_2- and COOH-terminal sequences, 1–15 and 621–635 respectively, (NH_2-M-A-K-K-S-A-E-N-G-I-Y-S-V-S-G; P-V-S-E-S-S-K-V-V-V-V-E-S-V-M-COOH) of the creatine transporter were synthesized by ANAWA Trading SA, Zürich according to standard solid phase procedures, followed by HPLC purification. The CreaT peptides were

coupled separately to keyhole limpet hemocyanin as follows [74]: hemocyanin (80 µl, 100 mg/ml) in 50% glycerol was mixed with 420 µl of buffer A (140 mM NaCl, 1.6 mM KCl, 1.1 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) and supplemented with 20 µl of a freshly prepared solution of 0.1 M succinimidyl suberate. Then, 6.3 mg of solid peptide was added, the mixture stirred, and finally diluted with 500 µl of buffer A. The material was divided into 160 µl aliquots and stored at –20°C. Rabbits were injected intracutaneously with a mixture of 160 µl of coupled peptide, 340 µl of buffer A and 500 µl of either Freund's complete adjuvant (day 0) or incomplete adjuvant (days 15, 29, 41, 66, 98 and 170). Sera were analysed by Western blots (see below) and stored at –20°C.

Preparation of rat skeletal muscle extracts

Skeletal muscle tissue (Quadriceps muscle) from rats fed creatine, 3-GPA, as well as control animals fed a creatine-free diet, was excised and proteins were extracted by hypo-osmotic swelling of the tissue followed by phosphate extraction at high pH. In short, the tissue was minced, exposed to 2–3 vols. of bidistilled water for 15 min, then incubated with 2–3 vols of phosphate buffer (50 mM NaH_2PO_4, 10 mM 2-mercaptoethanol, plus 0.05–1% Triton X-100 (depending on the tissues), freshly added, at pH > 8.75) for another 60–90 min, and finally centrifuged at 10,000 × g for 10 min. The supernatant served as a tissue extract and was kept at –20°C. The protein concentration was determined by the method of Bradford [75] using bovine serum albumin as a standard.

Electrophoretic techniques and immunoblotting

SDS polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [76] on an 8% gel, with 40 µg of tissue extract proteins being loaded per lane. After electrophoresis, separated proteins were either stained with Coomasie Blue or semy-dry blotted onto nitro-cellulose paper (Schleicher and Schüll, Dassel Germany). Unspecific sites were blocked by a solution containing 3% fat-free milk powder in phosphate-buffered saline. Papers were labelled with polyclonal anti-C- or anti-N-termini CreaT antibodies (at 1:500 dilution in blocking solution) for 2–3 h at 22°C. After 3 washes with the above blocking buffer, the membranes were incubated with the secondary antibody (goat anti-rabbit IgG-conjugated with horseradish peroxidase and diluted 1:2000 in blocking buffer). For detection, the peroxidase reaction was carried out using luminol (2.5 mM luminol, 0.5 mM *p*-iodophenol, 50 mM Tris-HCl, pH 7.5, and 0.15% H_2O_2) [77] and exposure to X-ray film for 1–10 scc.

Materials and animal care and feeding

β-GPA was synthesised in the laboratory using the method of Rowley *et al.* [78]. Purity was checked by TLC and ^{1}H-NMR. Raw materials were purchased from Fluka. Creatine monohydrate was kindly provided by Chemie Linz, Austria. Female Sprague-Dawley rats were fed at three weeks of age either on control diet free of creatine, on a diet containing 4% creatine plus 50 mM creatine in their water supply, or a diet containing 2.5% β-GPA plus 1% β-GPA in water, for 3–6 months. Rats were killed by exposure to CO_2 gas and then exsanguinated.

Results

Identification of two highly related creatine transporter isoforms

We have produced specific antibodies against synthetic peptides corresponding to the amino acid sequence of 15 residues at either the N- or C-termini of the cDNA-derived CreaT amino acid sequence. Two proteins with a molecular mass of 70 and 55 kDa were consistently recognized by any of the above antipeptide antibodies (see Fig. 2). As one can see in the same figure (control sample lane 1 of B), the 70 and 55 kDa proteins are coexpressed nearly at a 1:1 ratio in skeletal muscle tissue extracts as well as in a number of other tissues. Both polypeptides are minor and almost ubiquitously expressed in all the rat tissues tested so far (data not shown). The highest levels of expression of these two proteins occur in rat kidney, heart, brain, and skeletal muscle extracts. This agrees with the distribution of mRNA in rat and human tissues [15, 16, 20]. Based on the cDNA analyses, the CreaT has an expected molecular weight of 70 kDa. Since antibodies against the N- as well as the C-terminal always reacted with the same two polypeptides in all positive tissues, it is inferred that the 55 kDa polypeptide must be highly related to the 70 kDa protein, representing most likely a novel CreaT isoform arising by differential splicing of the original CreaT mRNA. Preliminary results suggest that the 55 kDa putative novel CreaT isoenzyme is not generated by de-glycosylation, since incubation with glycosidases does not convert the 70 kDa glycoprotein into a 55 kDa polypeptide species (data not shown). Also, the 55 kDa polypeptide is unlikely a degradation product of the 70 kDa species, since both bands were always detected, although sometimes varying in staining intensity, with both, the anti-N as well as the anti-C-terminal antibody. In addition, there was never a smaller difference polypeptide, expected to run at approximately 15 kDa, detected on Western blots. These data supports the hypothesis of the existence of at least two CreaT isoforms.

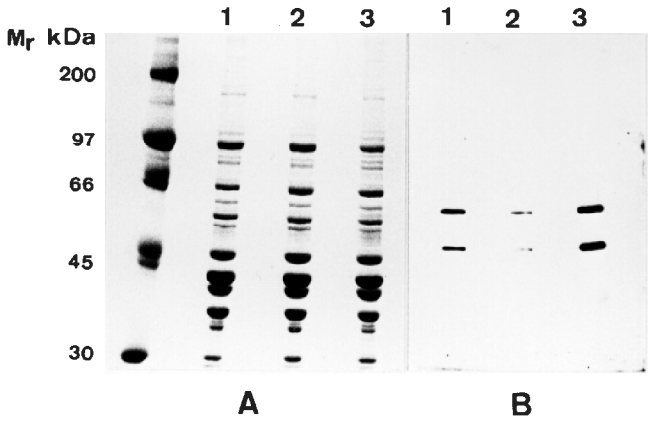

Fig. 2. Effect of creatine and its analogue 3-guanidinopropionic acid (3-GPA) on creatine transporter expression. Skeletal muscle proteins from rats fed for 3 months with creatine (lane 2); 3-GPA (lane 3); as well as proteins from control animals on a creatine free diet (lane 1), were extracted as described, subjected to electrophoresis on an 8% polyacrylamide SDS gels and were either stained with Coomasie Blue to visualize total protein (A) or semy-dry-blotted onto nitrocellulose membrane. The transfer was labelled with 500 diluted anti-C-terminus peptide antibody to stain the creatine transporter protein (B). The same immunostaining of both the 70 and 55 kDa polypeptides were also obtained with the anti-N-terminal antibody (not shown). Therefore, the 55 kDa protein species cannot be a degradation product of the 70 kDa polypeptide but must be a highly related CreaT isoform most likely generated by alternative splicing at internal site(s) of CreaT mRNA. Note that the creatine carrier synthesis is down-regulated *in vivo* by its substrate creatine (lane 2) whereas 3-GPA prevented this effect of creatine.

Down-regulation in vivo *of the expression of both creatine transporter isoforms upon chronic creatine supplementation*

Extracellular creatine regulates creatine transport in L6 rat muscle cells as measured by isotope studies [30]. Down-regulation of Cr uptake in this cells can be partially reversed when cells are maintained in medium lacking creatine. The creatine analogue 3-GPA is a well known competitive inhibitor of creatine entry. Long-term feeding of rats with 3-GPA decreases creatine levels in skeletal muscle. Therefore, we studied the *in vivo* effect of dietary creatine as well as of 3-GPA on the CreaT expression in skeletal muscle (quadriceps) of rats chronically fed either 4% creatine or 2.5% GPA. Dietary creatine, administered for 3–6 months, significantly lowers the levels of both the 70 and 55 kDa polypeptides as shown in Fig. 2 (lane 2B). In contrast, in rats fed with the substrate analogue 3-GPA, the expression levels of both proteins remain similar or are slightly increased compared to those of control rats (Fig. 2, lane 3B).

Both the 70 and 55 kDa polypeptide bands respond in a similar manner to creatine (down-regulation) and 3-GPA (up-regulation) in skeletal muscle. Therefore, it is very likely that the two proteins must be related to creatine transport *per se*.

Further studies are needed to characterize the two CreaT isoforms on a molecular level and to document the regulation of the CreaT by creatine supplementation in other tissues than muscle.

Discussion

Regulation of creatine homeostasis: role of the creatine transporter

In this preliminary study, we have presented evidence for the existence of two highly related CreaT isoforms which are coexpressed in all tissues where the CreaT, and incidentally also CK, is found, except for the liver which according to our results also contains CreaT but no or very little CK. In addition, we show that both of these highly homologous proteins are down-regulated after chronic administration of external Cr and that this down-regulation is prevented or even counteracted by GPA.

Several lines of evidence support the hypothesis that more than one CreaT exist. Two CreaT mRNA species of 4.0–4.3 and 2.2–3.0 kb, whose tissue expression patterns differ from one another, were detected by Northern blot analysis [20, 79]. Barnwell *et al.* [80] cloned and sequenced two cDNAs: one, CreaT1, homologous throughout its length with the rat CreaT sequence and a second, CreaT2, encoding a new protein containing regions of perfect homology with the CreaT amino-acid sequence and four segments of unique sequences. Although no evidence was presented, the authors suggest that CreaT2 mRNA may be transcribed from the CreaT1 gene, possibly by alternative splicing [80]. Other evidence is provided by genomic localization studies. These reveal the existence of an autosomal, 'testis-specific' form of the human CreaT gene besides the previously identified and mapped X-linked CreaT gene. This is reminiscent in the sense that there is also evidence for a sperm-specific CK isoenzyme expressed in fish and rooster testis [81–83].

In conclusion, the above experimental findings clearly support the existence of two different CreaT mRNA species, as well as of two CreaT transporter polypeptides with identical N- and C-termini, as judged by anti-peptide-specific antibodies. We believe that the two mRNA, most likely being generated by alternative splicing, correspond to the 70 and 55 kDa proteins detected by both the anti-N- as well as the anti-C- terminal antipeptide antibodies. The differential splicing, therefore, must take place in a region of the mRNA coding for the amino acid sequence between the N- and C-termini. This interpretation is consistent both with the results from molecular biology, as well as with our

protein and immunological data.

The Cr analogue, β-GPA, competitively inhibits CreaT activity. Long-term feeding (6–10 weeks) of rats with β-GPA results in a marked decrease in PCr, Cr and ATP levels in skeletal muscle [84]. On the other hand, Cr supplementation augments Cr uptake and accumulation. Therefore, it is not surprising that depending on the substrate and dosis used, and the time of ingestion, different metabolic adaptations will occur. Concerning the CreaT, it was suggested that extracellular Cr may down-regulate the level of the CreaT expression and its activity in a rat skeletal muscle cell line [30]. In the present study, we have directly shown that, using our anti-NH_2- and anti-COOH-terminal CreaT antipeptide antibodies, the expression of both, the 70 and 55 kDa proteins, is indeed down-regulated *in vivo* by long-term Cr-feeding in rat skeletal muscle. The Cr-analogue β-GPA, however, prevented this effect or even slightly up-regulated the CreaT.

These results can most likely also be extrapolated to human athletes who chronically ingest Cr. Human muscle appears to have an upper limit for its Cr content of 150–160 mmol/kg of dry muscle. [50]. The latter suggests that long-term Cr intake influences the synthesis of the CreaT in order to prevent the accumulation of excessive intramuscular Cr. On the other hand, the down-regulation of the CreaT could be interpreted as an undesirable side effect of Cr supplementation. Therefore, it is unadvisable to consume Cr continuously for longer periods of time, e.g. over 3 months or so, or to abuse Cr ingestion in order to improve performance at any cost. A creatine-free period of one month, after 3 months of Cr ingestion, is thus advisable.

In the section devoted to Cr levels in disease, we discussed the benefits of Cr ingestion and point out that Cr supplementation may be a promising adjuvant therapy for patients with a variety of neuromuscular diseases.

Our results suggest that the effect of extracellular Cr on the transporter protein is to control, by a negative feedback repression mechanism, the synthesis of the CreaT itself. Thus, down-regulation of CreaT expression is a mean to control intracellular Cr homeostasis. The study of the CreaT could also help us to understand its potential role in neuromuscular diseases. Utilization of our antibodies on biopsy material of patients with different neuromuscular diseases, mitochondrial myopathies and dystrophies may reveal altered expression of CreaT, defects in its regulation or of mutations in the CreaT protein.

Note added in proof

Recently, Kekelidze *et al.* [90] reported that by using a polyclonal antiserum against a CreaT fusion protein on Western blots, also two distinct polypeptide bands with an apparent M_r of 70 and 50 kDa could be observed in lysates from HeLa cells transfected with rat CreaT cDNA, as well as in homegenates of rat brain and muscle. These results obtained by a different strategy are fully in line with our observation that two CreaT species, which need further characterization, are found in different tissues. Very recently, neuroprotective effects of creatine and cyclo-creatine were reported in animal models of Huntington disease [91] and a remarkable protection of brain metabolism by creatine [92] and GPA [93] was demonstrated in mice that showed stabilized brain ATP and significantly enhanced survival during hypoxia. Finally, a protective effect of creatine and cyclo-creatine preventing calcium (40 μm)- plus atractyloside (5 μm)-induced mitochondrial destabilization by the so-called permeability transition have been demonstrated [94], linking the CK system and its substrates to early events of apoptosis. These results are bound to encourage further investigations. For a recent review on the nutritional biochemistry of creatine see also Greenhaff [95].

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