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Original Contribution

REDUCED CREATINE KINASE ACTIVITY IN TRANSGENIC AMYOTROPHIC LATERAL SCLEROSIS MICE

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Abstract—Creatine (Cr), the substrate of the creatine kinase (CK) isoenzymes, was shown to be neuroprotective in several models of neurodegeneration, including amyotrophic lateral sclerosis (ALS). In order to investigate the mechanism of this beneficial effect, we determined CK activities and mitochondrial respiration rates in tissues from G93A transgenic mice, which overexpress a mutant form of human superoxide dismutase associated with familial ALS (FALS). While respiration rates of mitochondria from G93A transgenic or wild-type control mice isolated from spinal cord showed no difference, a significant and dramatic loss of CK activity could be detected in these tissues. In homogenates from spinal cord of G93A transgenic mice, CK activity decreased to 49% and in mitochondrial fractions to 67% compared to CK activities in wild-type control mice. Feeding the G93A transgenic mice with 2% Cr, the same tissues showed no statistically significant increase of CK activity compared to regular fed G93A transgenic mice. Experiments with isolated mitochondria, however, showed that Cr and adenosine triphosphate (ATP) protected mitochondrial CK activity against peroxynitrite-induced inactivation, which may play a role in tissue damage in neurodegeneration. Our data provide evidence for oxidative damage to the CK system in ALS, which may contribute to impaired energy metabolism and neurodegeneration. © 2002 Elsevier Science Inc.

Keywords—Creatine kinase, Creatine, Neuroprotection, Neurodegeneration, Amyotrophic lateral sclerosis, Oxidative damage, Free radicals

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disorder characterized by motor neuron loss, rapidly progressive motor weakness, and early death [1]. The disease exists in a sporadic and in an autosomal dominant familial form (FALS), the latter constitutes 10% of all ALS cases. The finding that about 20% of the patients with FALS carry a point mutation in the superoxide dismutase 1 (SOD1) gene, which encodes for the cytosolic copper- and zinc-dependent SOD [2], has focused interest on the involvement of oxidative damage in the pathogenesis of the disease, and lead to the development of several animal models of FALS [3]. Interestingly, most of the mutations, found in FALS patients retain full or partial specific SOD activity [4]. This, together with the dominant inheritance pattern in FALS, suggests that the onset of the disease is rather dependent on a gain-of-function of SOD than a loss-of-function. Evidence supporting this includes (i) the finding that SOD-knockout mice do not develop symptoms of ALS, (ii) the report of a lack of correlation between disease severity and enzyme activity, and (iii) the finding that overexpression of the mutant enzyme in transgenic mice leads to motor neuron degeneration [5–7]. An increased ability to generate superoxide radicals from hydrogen peroxide was found in mutant SOD in which glycine 93 has been replaced by alanine, corresponding to one mutation found in FALS patients [8]. Transgenic mice overexpressing this G93A SOD mutant (G93A transgenic mice) develop symptoms similar to those seen in ALS patients [5].

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An elevated level of superoxide will contribute to enhanced oxidative stress, which is thought to play a role in neurodegenerative diseases (for review see [9]). Among the toxic agents contributing to this oxidative stress, peroxynitrite, the reaction product of superoxide and nitric oxide, produces damage to proteins, lipids, and DNA [10,11]. The pathogenesis of ALS, however, may involve an interplay between oxidative stress and mitochondrial dysfunction, the latter having been shown to lead to adenosine triphosphate (ATP) depletion and selective motoneuron death [12]. Therefore, buffering intracellular energy levels could protect against ATP depletion and thus help the cell to cope with energy deficiency.

The creatine kinase (CK, EC 2.7.3.2) system, consisting of a cytosolic and a mitochondrial isoform together with their substrates creatine (Cr) and phosphocreatine (PCr), is the cell's most important immediate energy buffering and transport system [13,14], especially in muscle and neuronal tissues [15]. Cr is phosphorylated to PCr in the intermembrane space of mitochondria, where mitochondrial creatine kinase (MtCK) is located, and is then transported into the cytosol [16]. There, the energy pool can be regenerated by transphosphorylation of PCr to ATP, which is catalyzed by cytosolic CK (B-CK in brain) located in close vicinity of cellular ATPases. Increased Cr levels in the cell, paralleled by increased PCr levels, can enhance ATP regeneration and thus improve the cellular energy status of brain and muscle cells. Cr protects against neuronal death in vitro [17], and supplementation with Cr was shown to be neuroprotective in several animal models of neurodegenerative diseases [18,19], including the G93A transgenic mouse model of ALS [20]. This study showed that life span, as well as the motor performance of G93A transgenic mice fed with a Cr diet, was significantly improved and that motor neuron loss was decreased.

Cytosolic, as well as MtCK isoenzymes, are prime targets for oxidative damage leading to inactivation of CK, which can be partly protected if both substrates, Cr and MgATP, are present [21–23]. Additionally, reactive oxygen species lead to dimerization of MtCK [24]. Inactivation of CK and dimerization of MtCK are both expected to contribute to a deterioration of the cellular energy state [25]. In the present study we therefore examined whether CK activity in G93A transgenic mice was altered in the spinal cord or cortex, and whether succinate-stimulated respiration of mitochondria isolated from these regions of diseased brains was affected. Furthermore, we investigated the effect of Cr supplementation on these parameters.

MATERIALS AND METHODS

Mice

Transgenic male mice with the G93A human SOD1 (G1H/+) mutation (the high copy number mice: B6SJL-TgN (SOD1-G93A)1 Gur; Jackson Laboratories, Bar Harbor, ME, USA) were bred with female B6SJL mice (Jackson Laboratories). The F1 generation was genotyped with polymerase chain reaction (PCR) on tail DNA and used in the experiments. All animal experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the local animal care committee.

Treatment/protocol

Cr (Avicena Group, Cambridge, MA, USA) was mixed into the mouse food (Purina Test Diet, Richmond, IN, USA) at 2% (w/w) concentration. Treatment started at 4 weeks of age. At 84 or 120 d of age, five to seven animals in each group were decapitated, the spinal cord and brain were rapidly removed, and the cortex was immediately dissected on a chilled surface. The tissue was placed in a special preservation medium (mixture of 1 unit B27 and 49 units Hibernate A; Life Technologies, Gaithersburg, MD, USA [26]) for transport purposes and kept cold for 24 h until it was processed for biochemical assays.

Isolation of mitochondria from mouse brain and spinal cord

Mitochondria were isolated from spinal cord and cortex by differential centrifugation according to a protocol described earlier [27]. Briefly, brain sections from five to eight mice were washed with buffer A (225 mM mannitol, 75 mM sucrose, 10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] [HEPES], 1 mM EGTA, pH 7.1) and incubated in 2.5 ml of buffer C (0.6 mg/ml subtilisin and 3 mg/ml bovine serum albumin [BSA], dissolved in buffer A) for 1 min on ice. Then, 7.5 ml of buffer B (1 mg/ml fatty acid-free BSA in buffer A) was added and the brain sections were homogenized with a glass/Teflon potter. The resulting homogenate was centrifuged for 3 min at 2500 $\times$ *g* and the supernatant was centrifuged for 8 min at 12,000 $\times$ *g*, resulting in a crude mitochondrial fraction, which was further purified by ultracentrifugation (35 min at 100,000 g) on a 20% Percoll gradient. Percoll was removed from the purified mitochondrial fractions by washing twice with buffer A.

Peroxynitrite administration

The concentration of peroxynitrite stock solution, synthesized from the reaction of gaseous nitric oxide with solid potassium superoxide [28], was determined photometrically at 302 nm in 10 mM NaOH (ϵ_{mM} =

1.67) prior to use. 135 μM (final concentration) peroxynitrite was added to inactive mitochondria (0.2 mg/ml mitochondrial protein), mitochondria respiring on 5 mM succinate or mitochondria respiring on 5 mM succinate and stimulated with 1 mM ATP, in the presence or absence of 20 mM Cr. After an incubation time of about 5 min, mitochondria were centrifuged for 20 min at 12,000 $\times$ *g* and resuspended in CK assay buffer to remove Cr present in solution. Then the solutions were kept at -20°C until CK activity determination.

Protein concentration

Protein contents of homogenates or mitochondria were determined according to Bradford using the commercially available Bio-Rad protein assay with BSA as a standard.

CK-activity determination

CK activity was assayed photometrically in the reverse reaction (ATP production from PCr), using the glucose-6-phosphate-dehydrogenase/hexokinase—coupled enzyme assay as described [29] at room temperature (22°C). For the calculation of activity, a blank of each sample, measured without PCr, was subtracted from the determined CK activity values. Specific CK activity was expressed as IU/mg protein whereby 1 IU corresponds to 1 μmol PCr hydrolyzed per min at 22°C.

Mitochondrial respiration measurements

Mitochondrial respiration was measured at room temperature by using the two-channel high resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria) in 1 ml of buffer A containing 5 mM $MgCl_2$, 10 mM NaP_i, and 5 μM Rotenone and a final mitochondrial protein content of 0.2 mg/ml. Succinate was added (5 mM final concentration) to initiate state 4 respiration, followed by administration of ADP (1 mM final concentration) to stimulate oxidative phosphorylation to the maximum state 3 respiration. Subsequently, atractyloside was added to a final concentration of 25 μM to check whether the mitochondria were still coupled to ATP-production.

Western blot analysis

Sodium dodecyl sulfate (SDS)-gel chromatography was performed according to Laemmli, loading 10 μg of protein per lane. Following electrophoresis, proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 4% fat-free milk powder in phosphate buffered saline (PBS) for 1 h at room temperature, then incubated with a polyclonal rabbit anti-MtCK antibody, generated in our laboratory, diluted 1:1000 in PBS/milk powder for 1 h at room temperature. After three washing steps with PBS, membranes were incubated with anti-rabbit-antibody, horseradish peroxidase-conjugated (Nordic), diluted 1:5000 in PBS/milk powder for 1 h at room temperature, then washed three times and developed using enhanced Luminol reagent from NEN (Boston, MA, USA).

Statistical analysis

Statistical comparisons were by analysis of variance with post hoc Fisher's PLSD test or Student's *t*-test in the case of Fig. 3.

RESULTS AND DISCUSSION

Mitochondrial respiration

Mitochondrial respiration was measured using mitochondria isolated from spinal cord of 120 d old G93A transgenic mice and their littermate wild-type controls, both either fed a regular or a Cr-supplemented diet. In these mice the initial sign of the disease is a resting tremor, which progresses to gait abnormalities, paralysis of the hind limbs and impaired motor performance. At the end stage, there is a complete paralysis, and the average survival in our colony is 129.9 $\pm$ 0.8 d of age, with Cr increasing survival by 15–18% [20,30]. The performance on the rotarod test starts to decline at 100 d of age in this transgenic mouse colony [30,31], and 2% Cr in the diet delayed the onset of motor disease by approximately 15 d [20,30]. The mice from our G93A colony stopped to gain weight at age 90–100 d [30,31], and we showed that 2% Cr in the feed delayed the weight loss by approximately 2 weeks [20,30].

Despite the very small amounts of spinal cord tissue and consequently the minute final yield of mitochondria, we were nevertheless able to successfully measure some important respiratory parameters. Because earlier studies showed increased complex I (NADH-ubiquinone oxidoreductase) activity in FALS patients [32], as well as in G93A transgenic mice [33], we decided to measure mitochondrial oxygen consumption mediated by succinate, the substrate of complex II (succinate-ubiquinone reductase). Succinate was added to induce state 4 respiration. By adding Rotenone, complex I was inhibited so that only complex II-mediated oxygen consumption was determined. To stimulate respiration to maximal state 3 levels, ADP was added to a final concentration of 1 mM. Atractyloside, a specific inhibitor of the adenine nucleotide translocator, reduced the respiration rates to state 4

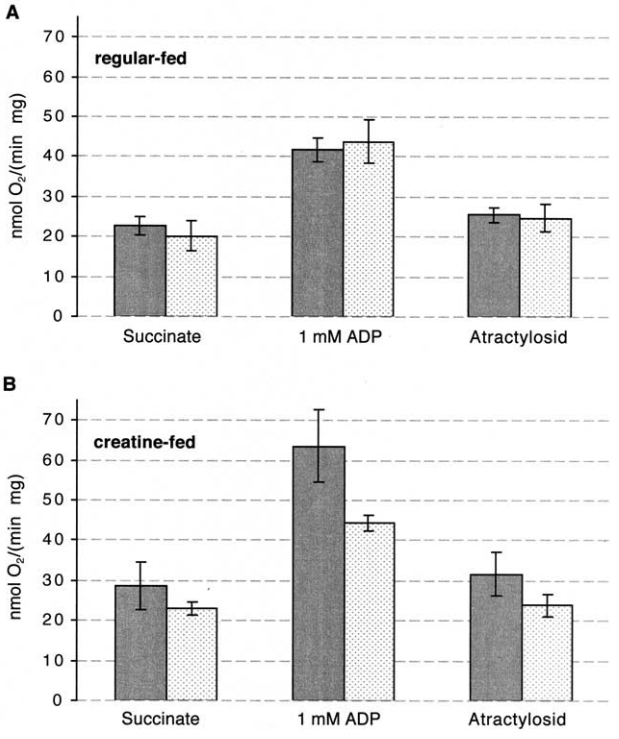

Fig. 1. Mitochondrial respiration rates in spinal cord from (A) regular fed mice and (B) creatine fed mice. The dark bars represent wild-type control, the light bars G93A transgenic animals. Values are the mean from four independent experiments with five to seven animals. No differences in respiration rates between wild-type control and G93A transgenic animals could be detected. Data are mean ± SEM.

levels, indicating that in these mitochondria respiration was still coupled to ATP production.

The respiration rates are summarized in Fig. 1. Mitochondria from wild-type control mice fed with Cr showed increased oxygen consumption in ADP-stimulated state 3 respiration (Fig. 1B middle, dark bar) as compared to wild-type control mice on regular diet (Fig. 1A, middle, dark bar, $p = .02$). Mitochondria from diseased spinal cord of G93A transgenic mice fed with Cr no longer showed this increase in oxygen consumption (Fig. 1B, middle, light bar) compared to regularly fed G93A transgenic mice (Fig. 1A, middle, light bar). Interestingly, this was the only change we could detect in respiration rates from wild-type and FALS mice. Mitochondria from regularly fed mice, both wild-type control and G93A transgenic mice did not show any difference in oxygen consumption rates at any state. This finding is somewhat surprising because mitochondrial swelling and vacuolization are prominent pathological features of the G93A transgenic animals [34], that should result in reduced mitochondrial function in ALS. It is, however, possible that degenerating mitochondria may be lost specifically during the isolation procedure, or that cells containing damaged mitochondria may have undergone degeneration, although we did not detect an observable alteration in mitochondrial yield.

CK activity in G93A transgenic mice

There is strong evidence that neurodegeneration in ALS is at least partly mediated by oxidative stress. It is also well known that CK, cytosolic as well as mitochondrial isoforms, are exquisitely susceptible to oxidative damage, especially when exposed to peroxynitrite [21, 22]. We previously found evidence for increased oxidative damage in G93A transgenic mice as early as 6 weeks of age [35]. To investigate the question of whether CK may be involved in the development of ALS, we measured CK activities in spinal cord and cerebral cortex of G93A transgenic mice and their littermate wild-type controls.

In homogenates from cortex of regular fed 120 d old mice, total CK activity levels did not differ between G93A transgenic and littermate wild-type control brains, but total CK activity levels were decreased to 49% in the homogenates of spinal cord from regular fed G93A transgenic mice compared to littermate wild-type controls (Fig. 2A). In mitochondrial fractions, a similar picture was noted. MtCK activity in cerebral cortex was not changed in G93A transgenic as compared to wild-type control brains, whereas MtCK activity in spinal cord from G93A transgenic mice was significantly decreased to 67% of wild-type control values (Fig. 2B). To rule out that this decrease in MtCK activity in 120 day old G93A transgenic mice was due to decreased expression levels of MtCK protein as a consequence of motor neuron loss, Western blot analysis of mitochondrial fractions was performed. This revealed very similar amounts of immunoreactive CK protein in spinal cord of wild-type control and G93A transgenic animals (data not shown).

Together with the unchanged respiration rates in isolated mitochondria, these results suggest free radical-induced inactivation of MtCK rather than a decreased expression level of MtCK or a general alteration in the amount of mitochondria present in the cells. Further support for this theory comes from a previous study where G93A transgenic mice showed enhanced free radical content in spinal cord but not in brain, which correlated with tissue-specific differences in the level of transgene expression [36]. This would explain why CK activity decreases tissue-specifically in spinal cord but not in cortex.

One experiment with five to seven G93A transgenic and littermate control mice of age 80 d, which is a time point preceding the development of ALS symptoms, revealed no decreased CK activity, neither in homogenates nor in mitochondrial fractions from spinal cord or cortex of G93A transgenic mice (data not shown). Thus,

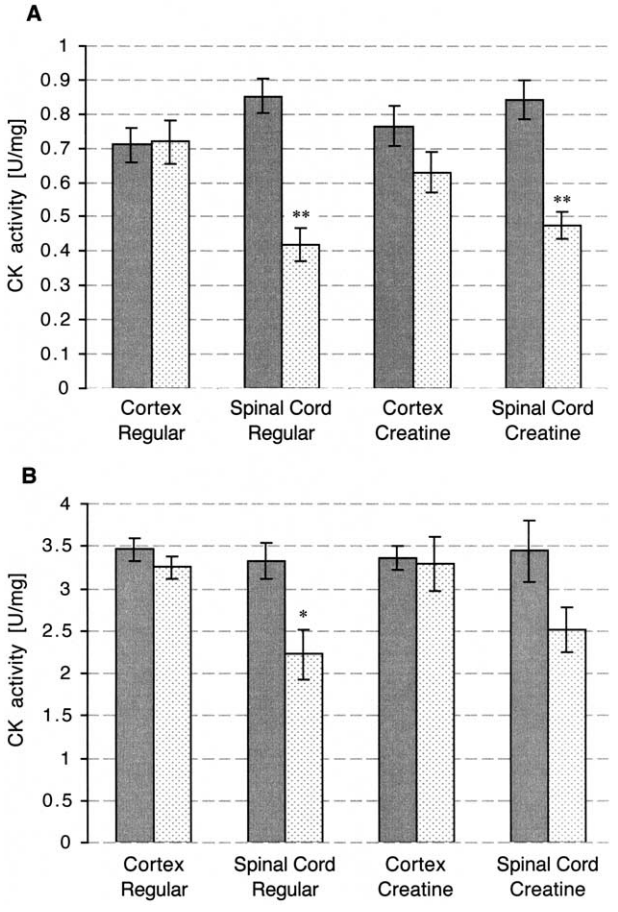

Fig. 2. Creatine kinase (CK) activities in tissues from mice with and without 2% creatine supplementation (A) in homogenates and (B) in mitochondrial fractions. The dark bars represent wild-type control, the light bars G93A transgenic animals. Values are the mean from four to five independent experiments with five to seven animals. Note the decreased CK activities in both homogenates and mitochondrial fractions prepared from spinal cord of G93A transgenic mice. Data are mean ± SEM; $*p < .05$; $**p < .005$.

the decrease in CK activity in G93A transgenic mice is related to the duration of the disease but this inactivation of the enzyme occurs at a time point where the mitochondrial respiration chain is not yet affected.

About 80% of total brain CK activity is derived from cytosolic BB-CK, which is present as a prominent enzyme in the brain as well as in spinal cord. Because we detected a 50% decrease in total CK activity in the homogenate of FALS mice, we conclude that cytosolic BB-CK in spinal cord is also affected to a significant extent.

Effect of creatine on CK activity

We previously showed that feeding G93A transgenic mice with 2% Cr in the diet from 4 weeks of age on protected against motor neuron loss and increased life

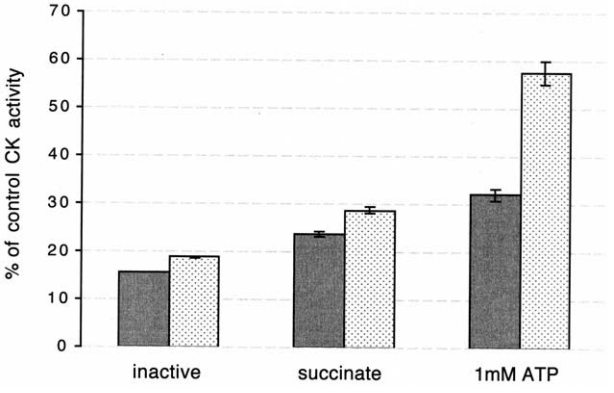

Fig. 3. The effect of peroxynitrite on mitochondrial creatine kinase (MtCK)-activity in brain mitochondria with or without creatine (Cr). Peroxynitrite (135 μM, final concentration) was added at different states of respiration to inactive mitochondria (inactive), to mitochondria respiring on succinate (succinate) and mitochondria, respiring on succinate and stimulated with ATP (1 mM ATP), in the absence (dark bars) or presence (light bars) of 20 mM Cr. Activities are expressed in percent of control CK activity, which was measured in mitochondria under the same conditions but not exposed to peroxynitrite. In a control experiment, degraded peroxynitrite showed no effect on CK activity under these conditions. Note the protective effect of Cr on peroxynitrite-induced inactivation of MtCK. Data are mean ± SEM.

span by 15–18% [20,30]. One possible reason for this could be a direct protective effect by Cr against radical induced damage of CK. We therefore determined CK activities in these tissues from control, as well as Cr-supplemented G93A transgenic mice. However, CK activities did not show a significant increase compared to unsupplemented G93A transgenic mice (Fig. 2A, B).

Effects of peroxynitrite on isolated mitochondria

The enzymatic activity of purified MtCK is partly protected against peroxynitrite-induced oxidative damage by the presence of the full set of substrates [22]. Possibly, this may be one of the reasons why MtCK activities tend to be elevated in Cr-fed as compared to unsupplemented G93A transgenic animals. To further corroborate this hypothesis, peroxynitrite was administrated to isolated mitochondria at different states of respiration in the presence or absence of exogenous Cr.

After exposure to as low as 135 μM peroxynitrite, CK activities were measured in the mitochondrial fractions (Fig. 3). Addition of $ONOO^-$ to inactive mitochondria resulted in a decrease to 15% of CK activity compared to control CK activity, where mitochondria have not been treated with ONOO-. When adding ONOO- to respiring mitochondria there was a slight increase in residual activity to 23%. However, in the presence of 1 mM ATP the residual activity in the absence of Cr is 32% whereas the presence of 20 mM Cr protects $ONOO^-$-mediated CK inactivation dramatically with approximately 58% of

original activity still preserved. The conditions with the presence of 1 mM ATP plus 20 mM Cr, under which maximal protection was obtained in vitro, correspond to the conditions in vivo where the full set of substrates is present.

An increased level of cellular Cr can lead to higher saturation and/or substrate turnover of the enzyme and therefore, binding of substrates to CK at a given time point seems statistically more probable. If both ATP and Cr are bound to CK, the active site cysteine is no longer accessible to radical damage and thus protected [22]. We therefore propose that Cr supplementation of G93A transgenic mice contributes to the observed neuroprotective effects of Cr by protecting MtCK, as well as cytosolic CK, from inactivation by oxygen radicals.

CONCLUSIONS

There is substantial evidence implicating mitochondrial dysfunction and free radical generation as major mechanisms of neuronal death in neurodegenerative diseases including ALS [33]. For the first time, we report that CK activity is significantly reduced in a tissue-specific manner in the spinal cord but not in the cortex of the G93A transgenic mice at a time point when mitochondrial respiration chain was not yet affected. This supports the notion that both impaired energy production and oxidative damage contribute to the development of ALS.

Even though a marked protection by Cr against inactivation of CK by peroxynitrite could be observed earlier in vitro with isolated CK enzyme [22] and in the present study with isolated intact mitochondria (see Fig. 3), it remains to be elucidated whether the significant neuroprotection by Cr that has been observed in ALS animal models in vivo [20] relies mainly on a mechanism of protection by Cr of the CK active site, or whether it is rather a combination of effects, including a general improvement of cellular energetics by Cr [13,17] and/or protection by Cr of mitochondrial permeability pore opening [37], an early event in apoptosis.

Nevertheless, in light of these combined results, treatment of ALS patients with Cr as a food supplement within the framework of clinical studies seems to offer a sensible adjuvant therapeutic approach for this incurable disease.

ADDENDUM

Most recently, direct antioxidant properties of creatine have been described that may also be relevant mechanistically to explain some of the neuroprotective actions of creatine [38].

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REFERENCES

[1] Brown, R. H. Jr. Amyotrophic lateral sclerosis: recent insights from genetics and transgenic mice. *Cell* **80:**687–692; 1995.

[2] Rosen, D. R.; Siddique, T.; Patterson, D.; Figlewicz, D. A.; Sapp, P.; Hentati, A.; Donaldson, D.; Goto, J.; O'Regan, J. P.; Deng, H. X.; et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**(6415):59–62; 1993.

[3] Elliott, J. L. Experimental models of amyotrophic lateral sclerosis. *Neurobiol. Dis.* **6:**310–320; 1999.

[4] Borchelt, D. R.; Lee, M. K.; Slunt, H. S.; Guarnieri, M.; Xu, Z. S.; Wong, P. C.; Brown, R. H. Jr.; Price, D. L.; Sisodia, S. S.; Cleveland, D. W. Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Proc. Natl. Acad. Sci. USA* **91:**8292–8296; 1994.

[5] Gurney, M. E.; Pu, H.; Chiu, A. Y.; Dalcanto, M. C.; Polchow, C. Y.; Alexander, D. D.; Caliendo, J.; Hentati, A.; Kwon, Y. W.; Deng, H. X.; Chen, W. J.; Zhai, P.; Sufit, R. L.; Siddique, T. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* **264**(5166):1772–1775; 1994.

[6] Reaume, A. G.; Elliott, J. L.; Hoffman, E. K.; Kowall, N. W.; Ferrante, R. J.; Siwek, D. F.; Wilcox, H. M.; Flood, D. G.; Beal, M. F.; Brown, R. H. Jr.; Scott, R. W.; Snider, W. D. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet.* **13:**43–47; 1996.

[7] Ripps, M. E.; Huntley, G. W.; Hof, P. R.; Morrison, J. H.; Gordon, J. W. Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. USA* **92:**689–693; 1995.

[8] Yim, M. B.; Kang, J. H.; Yim, H. S.; Kwak, H. S.; Chock, P. B.; Stadtman, E. R. A gain-of-function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: an enhancement of free radical formation due to a decrease in Km for hydrogen peroxide. *Proc. Natl. Acad. Sci. USA* **93:**5709–5714; 1996.

[9] Torreilles, F.; Salman-Tabcheh, S.; Guerin, M.; Torreilles, J. Neurodegenerative disorders: the role of peroxynitrite. *Brain Res. Brain Res. Rev.* **30:**153–163; 1999.

[10] Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288:**481–487; 1991.

[11] Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266:**4244–4250; 1991.

[12] Kaal, E. C.; Vlug, A. S.; Versleijen, M. W.; Kuilman, M.; Joosten, E. A.; Bar, P. R. Chronic mitochondrial inhibition induces selective motoneuron death in vitro: a new model for amyotrophic lateral sclerosis. *J. Neurochem.* **74:**1158–1165; 2000.

[13] Wallimann, T.; Wyss, M.; Brdiczka, D.; Nicolay, K.; Eppenberger, H. M. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem. J.* **281**(Pt. 1):21–40; 1992.

[14] Wallimann, T.; Dolder, M.; Schlattner, U.; Eder, M.; Hornemann, T.; O'Gorman, E.; Ruck, A.; Brdiczka, D. Some new aspects of creatine kinase (CK): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetics and physiology. *Biofactors* **8:**229–234; 1998.

[15] Wallimann, T.; Hemmer, W. Creatine kinase in non-muscle tissues and cells. *Mol. Cell. Biochem.* **133–134:**193–220; 1994.
[16] Schlattner, U.; Forstner, M.; Eder, M.; Stachowiak, O.; Fritz-Wolf, K.; Wallimann, T. Functional aspects of the X-ray structure of mitochondrial creatine kinase: a molecular physiology approach. *Mol. Cell. Biochem.* **184:**125–140; 1998.
[17] Brewer, G. J.; Wallimann, T. W. Protective effect of the energy precursor creatine against toxicity of glutamate and beta-amyloid in rat hippocampal neurons. *J. Neurochem.* **74:**1968–1978; 2000.
[18] Ferrante, R. J.; Andreassen, O. A.; Jenkins, B. G.; Dedeoglu, A.; Kuemmerle, S.; Kubilus, J. K.; Kaddurah-Daouk, R.; Hersch, S. M.; Beal, M. F. Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. *J. Neurosci.* **20:** 4389–4397; 2000.
[19] Matthews, R. T.; Yang, L.; Jenkins, B. G.; Ferrante, R. J.; Rosen, B. R.; Kaddurah-Daouk, R.; Beal, M. F. Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. *J. Neurosci.* **18:**156–163; 1998.
[20] Klivenyi, P.; Ferrante, R. J.; Matthews, R. T.; Bogdanov, M. B.; Klein, A. M.; Andreassen, O. A.; Mueller, G.; Wermer, M.; Kaddurah-Daouk, R.; Beal, M. F. Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nat. Med.* **5:**347–350; 1999.
[21] Konorev, E. A.; Hogg, N.; Kalyanaraman, B. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. *FEBS Lett.* **427:**171–174; 1998.
[22] Stachowiak, O.; Dolder, M.; Wallimann, T.; Richter, C. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. *J. Biol. Chem.* **273:**16694–16699; 1998.
[23] Yuan, G.; Kaneko, M.; Masuda, H.; Hon, R. B.; Kobayashi, A.; Yamazaki, N. Decrease in heart mitochondrial creatine kinase activity due to oxygen free radicals. *Biochim. Biophys. Acta* **1140:**78–84; 1992.
[24] Soboll, S.; Brdiczka, D.; Jahnke, D.; Schmidt, A.; Schlattner, U.; Wendt, S.; Wyss, M.; Wallimann, T. Octamer-dimer transitions of mitochondrial creatine kinase in heart disease. *J. Mol. Cell. Cardiol.* **31:**857–866; 1999.
[25] Stachowiak, O.; Schlattner, U.; Dolder, M.; Wallimann, T. Oligomeric state and membrane binding behaviour of creatine kinase isoenzymes: implications for cellular function and mitochondrial structure. *Mol. Cell. Biochem.* **184:**141–151; 1998.
[26] Brewer, G. J.; Torricelli, J. R.; Evege, E. K.; Price, P. J. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35:**567–576; 1993.
[27] Rehncrona, S.; Mela, L.; Siesjo, B. K. Recovery of brain mitochondrial function in the rat after complete and incomplete cerebral ischemia. *Stroke* **10:**437–446; 1979.
[28] Koppenol, W. H.; Kissner, R.; Beckman, J. S. Synteses of peroxynitrite: to go with the flow or on solid grounds. *Methods Enzymol.* **269:**296–302; 1996.
[29] Eder, M.; Stolz, M.; Wallimann, T.; Schlattner, U. A conserved negatively charged cluster in the active site of creatine kinase is critical for enzymatic activity. *J. Biol. Chem.* **275:**27094–27099; 2000.
[30] Andreassen, O. A.; Jenkins, B. G.; Dedeoglu, A.; Ferrante, K. L.; Bogdanov, M. B.; Kaddurah-Daouk, R.; Beal, M. F. Increases in cortical glutamate concentrations in transgenic amyotrophic lateral sclerosis mice are attenuated by creatine supplementation. *J. Neurochem.* **77:**383–390; 2001.
[31] Andreassen, O. A.; Dedeoglu, A.; Friedlich, A.; Ferrante, K. L.; Hughes, D.; Szabo, C.; Beal, M. F. Effects of an inhibitor of poly(ADP-ribose) polymerase, desmethylselegiline, trientine, and lipoic acid in transgenic ALS mice. *Exp. Neurol.* **168:**419–424; 2001.
[32] Bowling, A. C.; Schulz, J. B.; Brown, R. H. Jr.; Beal, M. F. Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. *J. Neurochem.* **61:**2322–2325; 1993.
[33] Beal, M. F. Mitochondrial dysfunction in neurodegenerative diseases. *Biochim. Biophys. Acta* **1366:**211–223; 1998.
[34] Kong, J.; Xu, Z. Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1. *J. Neurosci.* **18:**3241–3250; 1998.
[35] Bruijn, L. I.; Houseweart, M. K.; Kato, S.; Anderson, K. L.; Anderson, S. D.; Ohama, E.; Reaume, A. G.; Scott, R. W.; Cleveland, D. W. Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. *Science* **281**(5384):1851–1854; 1998.
[36] Liu, R.; Althaus, J. S.; Ellerbrock, B. R.; Becker, D. A.; Gurney, M. E. Enhanced oxygen radical production in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Ann. Neurol.* **44:**763–770; 1998.
[37] O'Gorman, E.; Beutner, G.; Dolder, M.; Koretsky, A. P.; Brdiczka, D.; Wallimann, T. The role of creatine kinase in inhibition of mitochondrial permeability transition. *FEBS Lett.* **414:**253–257; 1997.
[38] Lawler, J. M.; Barnes, W. S.; Wu, G.; Song, W.; Demaree, S. Direct antioxidant properties of creatine. *Biochem. Biophys. Res. Commun.* **290:**47–52; 2002.

ABBREVIATIONS

ALS—amyotrophic lateral sclerosis
CK—creatine kinase
Cr—creatine
FALS—familial amyotrophic lateral sclerosis
MtCK—mitochondrial creatine kinase
PCr—phosphocreatine
SOD—superoxide dismutase