Dietary Supplement Creatine Protects against Traumatic Brain Injury

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Creatine, one of the most common food supplements used by individuals at almost every level of athleticism, promote gains in performance, strength, and fat-free mass. Recent experimental findings have demonstrated that creatine affords significant neuroprotection against ischemic and oxidative insults. The present experiments investigated the possible effect of creatine dietary supplementation on brain tissue damage after experimental traumatic brain injury. Results demonstrate that chronic administration of creatine ameliorated the extent of cortical damage by as much as 36% in mice and 50% in rats. Protection seems to be related to creatine-induced maintenance of mitochondrial bioenergetics. Mitochondrial membrane potential was significantly increased, intramitochondrial levels of reactive oxygen species and calcium were significantly decreased, and adenosine triphosphate levels were maintained. Induction of mitochondrial permeability transition was significantly inhibited in animals fed creatine. This food supplement may provide clues to the mechanisms responsible for neuronal loss after traumatic brain injury and may find use as a neuroprotective agent against acute and delayed neurodegenerative processes.

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There has been an enormous focus on the discovery and development of neuroprotective agents that might have clinical relevance after traumatic brain injury (TBI).[1–3] Motor vehicle accidents, falls, assaults, and various sports-related activities result in TBI that affects about 7 million individuals each year in North America.[4,5] The cost to treat these individuals is estimated at approximately $20 to $25 billion annually.[6] Athletes, particularly those participating in full-contact sports such as boxing, football, hockey, and soccer, are exposed to single and repeated concussions, which may result in subdural hematomas, loss of cognitive function, or death.[7] Regardless of rule changes, improvements in training methods, better equipment, and conditioning, approximately 300,000 people still experience sport-related TBI annually.[8,9] Despite its prevalence and devastating consequences, little in the way of therapeutics is available to prevent or treat this type of TBI.

Functional deficits result from primary and secondary mechanisms after TBI. The primary damage occurs at the time of impact and results from the mechanical insult itself. The secondary injury, defined as cellular damage not immediately apparent after the trauma but developing within minutes, hours, or even days, seems to be amenable to treatment. Much of the thrust of TBI research is directed toward prevention and treatment of secondary damage. The exact cause of the secondary injury is poorly understand but seems to be related to mitochondrial dysfunction associated with the disruption in cellular calcium homeostasis that is known to occur after TBI.[10–14] Maintenance of cellular calcium homeostasis is intimately related to adenosine triphosphate (ATP) use and synthesis, which are key to proper brain functioning.[15] Enhanced neuronal survival may be obtained by providing an adequate supply of ATP immediately after trauma.

Creatine (*N*-[aminoiminomethyl]-*N*-methyl glycine) is an amino acid endogenously produced from glycine, methionine, and arginine in the liver, kidney, and pancreas. Dietary supplementation of creatine monohydrate is used by many athletes to enhance performance.[16] In addition to the pool of creatine contained in muscle, high levels of creatine are found in the brain.[17] Recent experimental findings have demonstrated that creatine affords significant neuroprotection against ischemic and oxidative insults.[18–23] This neuroprotective effect may result from inhibition of the

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mitochondrial permeability transition pore (MPTP) as a result of structural and functional interactions between creatine kinase (CK) and the mitochondrial adenine nucleotide translocator.[23,24] In the present study, we demonstrate that creatine supplementation protects against neuropathology of TBI through mechanisms involving maintenance of mitochondrial bioenergetics and preservation of ATP levels. Furthermore, we demonstrate for the first time in vivo that creatine supplementation inhibits Ca^{2+}-induced activation of the MPTP after TBI.

Materials and Methods

Animals

Adult ICR mice (n = 40) and adult Sprague-Dawley rats (n = 24) (Harlan Laboratories, Indianapolis, IN) were housed 3 per cage in rooms maintained on 12-hour light/dark cycles, and animals were allowed free access to water and food. All animal procedures were approved by the Animal Care and Use Committee at the University of Kentucky.

Surgical Procedures and Experimental Design

Controlled cortical contusions were performed as described previously.[13,25] The moderate cortical contusion used in these experiments results in severe behavioral deficits, significant loss of cortical tissue, blood–brain barrier disruption, and loss of hippocampal neurons,[25–28] which represents a sequela that mimics human closed-head injury.

Mice were randomly assigned to treatment groups and received daily intraperitoneal injections (0.1 ml per 10 g of body weight) of either creatine monohydrate (Sigma Chemical Company, St Louis, MO) suspended in olive oil (3 mg/g of body weight) or vehicle alone for 1, 3, or 5 days before injury.[17] Seven days after injury, mice were killed, and brain lesion volumes were assessed as described below. Because of the limited aqueous solubility of creatine monohydrate and its viscosity in suspension with olive oil, it was impossible to inject the rats with a dose comparable to that administered to the mice. Rats were thus fed either Harlan Teklad Standard Rodent Diet #7012 (Harlan Teklad, Madison, WI) or the same food custom-enriched (Harlan Teklad) with 1% creatine monohydrate (Sigma Chemical Company) for 4 weeks before injury. Rats were age and weight matched at the onset of the experiments and weighed twice a week to ensure that any differences observed were not due to age or weight differences. One hour after injury, one half of the rats (n = 6 per diet) were killed. Synaptoneurosomes and nonsynaptosomal mitochondria were prepared from cerebral cortex ipsilateral and contralateral to the cortical impact. The remaining rats (n = 6 per diet) were fed diets an additional 7 days after TBI (5 weeks total); they were then killed, and lesion volumes were assessed.

Tissue Handling and Measurement of Lesion Volume

Seven days after controlled cortical contusion, rats and mice were anesthetized with sodium pentobarbital (95 mg/kg of body weight; Nembutal, Abbott Laboratories, Chicago, IL) and perfused transcardially with physiological saline followed by phosphate-buffered saline (pH 7.4) containing 10% formalin (wt/vol). Brains were removed, postfixed for 24 hours in 10% formalin in phosphate-buffered saline, and subsequently placed in a solution containing 10% formalin and 15% sucrose for an additional 24 hours. Coronal sections (40 μm for the mouse model and 50 μm for the rat model) were cut with a cryostat throughout the rostral-caudal extent of the damaged cortex extending from the septal area to the most posterior aspect of the hippocampus. Sections were stained with cresyl violet and subjected to image analysis (BIOQUANT; R & M Biometrics, Nashville, TN). Quantitative assessment of cortical damage employed the Cavalieri method as described previously.[25,29] The amount of cortical damage was expressed as a percentage of the total cortical volume of the damaged hemisphere. These methods obviate a need to adjust values due to possible differential shrinkage resulting from fixation and tissue processing. All slides were ranked blindly with respect to treatment group. Differences in group means were assessed with an ANOVA for the mice and a two-tailed *t* test for the rats.

Synaptoneurosome and Mitochondria Preparation

Rats were killed by decapitation 1 hour after injury, and brains were dissected on ice. Cerebral cortical hemispheres ipsilateral and contralateral to the injury were separated, and mitochondria (synaptosomes and nonsynaptosomal) were prepared (n = 6 per group, with tissue from 2 animals pooled for each assay) as described previously.[30] A minimum of five replicates were used for each assay, and the means were used for analyses. Synaptosomal protein concentrations were determined using the Pierce BCA kit (Rockford, IL), and equivalent amounts of synaptosomal protein were used in all experiments. All experiments were carried out in Locke's solution (154 mM of NaCl, 5.6 mM of KCl, 2.3 mM of $CaCl_2$, 1.0 mM of $MgCl_2$, 3.6 mM of $NaHCO_3$, 5 mM of glucose, 5 mM of HEPES, pH 7.2) unless noted otherwise. For all variables examined, mean values from ipsilateral cortex were obtained and expressed as a percentage of mean contralateral levels, and the data were analyzed using a two-tailed *t* test.

Nonsynaptosomal mitochondrial were obtained by resuspending the mitochondrial pellet in isolation buffer and washing by centrifugation at 14,500 and 7,500*g*. To assess mitochondrial swelling, 250 μg of isolated mitochondrial protein was suspended in respiration buffer (250 mM of sucrose, 20 mM of HEPES, 2 mM of $MgCl_2$, 2.5 mM of inorganic phosphates, pH 7.2) and 10 mM of succinate in a final volume of 1 ml. After a 5-minute preincubation at 30°C and baseline measurement, $CaCl_2$ (40 or 100 nM/mg) was added to the cuvette.

Mitochondrial Permeability Transition (Mitochondrial Swelling)

Isolated mitochondria were measured as a loss of absorbance (540 nm) using a spectrometer after the addition of Ca^{2+} employing standard methodology.[14] All data are expressed relative to an initial absorbance of 1.

Synaptosomal Mitochondrial Transmembrane Potential

Synaptosomal mitochondrial transmembrane potential was measured as previously described using the dye 5,5′,6,6′-

tetrachloro-1,1′,3,3′-tetraethylbenzimidazolycarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR). The relative amount of mitochondrial polarization per milligram of synaptosomal protein was quantified by exciting samples at 500 nm and measuring the 590- to 535-nm emission ratios. Mitochondrial transmembrane potential values in ipsilateral cortex were expressed as a percentage of contralateral cortex.

Synaptosomal Intramitochondrial Calcium Levels

Levels of synaptosomal intramitochondrial levels of calcium ($[Ca^{2+}]_s$) were determined as described previously.[14,31] Using a CERES 2200 fluorescence plate reader (Foster City, CA) (549-nm excitation and 581-nm emission), fluorescence in the synaptosomes from ipsilateral regions was expressed as a percentage of contralateral samples.

Synaptosomal Mitochondrial Reactive Oxygen Intermediate Formation

Reactive oxygen intermediate (ROI) production was measured using dichlorodihydrofluorescein diacetate (Molecular Probes) as described previously.[14,31] Using a CERES 2200 fluorescence plate reader (490-nm excitation and 526-nm emission), fluorescence intensity, a measure of the relative amounts of H_2O_2 and free radical production in the synaptosomes, from ipsilateral regions was expressed as a percentage of contralateral samples. Addition of H_2O_2 as a positive control increased dichlorodihydrofluorescein diacetate fluorescence in a linear fashion.

Quantification of ATP Levels

ATP was measured as described previously.[14] ATP levels were determined using an ATP Bioluminescence Assay Kit CH II (Boehringer Mannheim, Indianapolis, IN), and samples were compared with a standard curve generated using known concentrations of ATP.

Results

Creatine Reduces TBI-Related Tissue Damage

We pretreated adult ICR mice with creatine (3 mg/g of body weight) for 1, 3, or 5 days and subjected them to brain injury employing the controlled cortical impact model. At 7 days after injury, all mice were assessed for cortical tissue damage immediately below the site of impact (Fig 1a). ANOVA revealed a significant group difference (F [5,34] = 4.16; $p < 0.01$) in cortical damage after TBI. Animals treated with creatine for a single day revealed nonsignificant ($p > 0.10$) 3% protection, although significant protection was observed with 3 days (21%; $p < 0.05$) and 5 days of creatine pretreatment (36%: $p < 0.01$) (see Fig 1b).

Similar significant neuroprotection was observed in rats fed a standard rodent diet formulated to contain 1% creatine monohydrate for a period of 4 weeks. All rats demonstrated obvious injury to the cortex immediately below the area of impact at 7 days after TBI (see Fig 1c). The extent of cortical damage was analyzed using a two-tailed t test (t [10] = −2.89; $p < 0.01$) and revealed that rats fed the creatine-enriched diet for 4 weeks before TBI demonstrated a significant (50%) reduction in cortical damage compared with animals fed the standard diet (see Fig 1d).

Effects of Creatine Dietary Supplementation on Synaptic Homeostasis after TBI

In attempting to determine underlying mechanism(s) responsible for the neuroprotection, various parameters of synaptosomal homeostasis were assessed in rats fed standard or 1% creatine-supplemented diets for 4 weeks before injury. One hour after TBI, control rats demonstrated a decline in several aspects of mitochondrial function as described previously.[11,14,32,33] Mitochondrial membrane potential assessed using the dye JC-1 was significantly lower in the control rats compared with the creatine-fed rats (t [4] = 4.02; $p > 0.05$). This maintenance of homeostasis was also reflected in significantly decreased levels of ROI production, which were assessed by measuring the oxidation of the dye 2,7-dichlorofluorescin diacetate in synaptosomes isolated from creatine-fed rats. The levels of ROIs were significantly reduced (t [4] = −7.63; $p > 0.01$) compared with those of control rats (Fig 2). The intramitochondrial Ca^{2+} levels were also significantly reduced (t [4] = −2.79; $p > 0.05$), although ATP levels were increased (t [4] = 5.54; $p > 0.01$). Finally, activation of the MPTP in ipsilateral nonsynaptosomal mitochondria was blocked in animals supplemented with 1% creatine compared with nonsupplemented animals.

Induction of mitochondrial permeability transition was monitored by following absorbance decreases, which has previously been shown to be associated with mitochondrial swelling and opening of the MPTP (Fig 3). Calcium ions (40 nM) initiated rapid swelling (ie, opening of the MPTP) in nonsynaptosomal mitochondria isolated from nonsupplemented animals but had no such effect on creatine-supplemented animals after TBI. Preincubation with 1 μM of cyclosporin A (Sigma Chemical Company) blocked the Ca^{2+}-induced mitochondrial permeability transition in the isolated nonsynaptosomal mitochondria.

Discussion

Important contributors to TBI-induced neuronal cell damage include glutamate-mediated excitotoxicity, dysregulation of intracellular calcium levels, formation of ROIs, and lipid peroxidation.[25,34–36] Mitochondrial dysfunction and reduction of cellular bioenergetics seem to be common factors related to all these contributors to TBI-related neuronal damage. Reduced levels of cellular ATP and increased levels of ROIs observed after TBI are believed to be the cause of TBI-induced neuronal cell death.[11,14,37,38]

Levels of ATP, the primary source of energy within cells, are carefully regulated in neurons.[15] Impaired en-

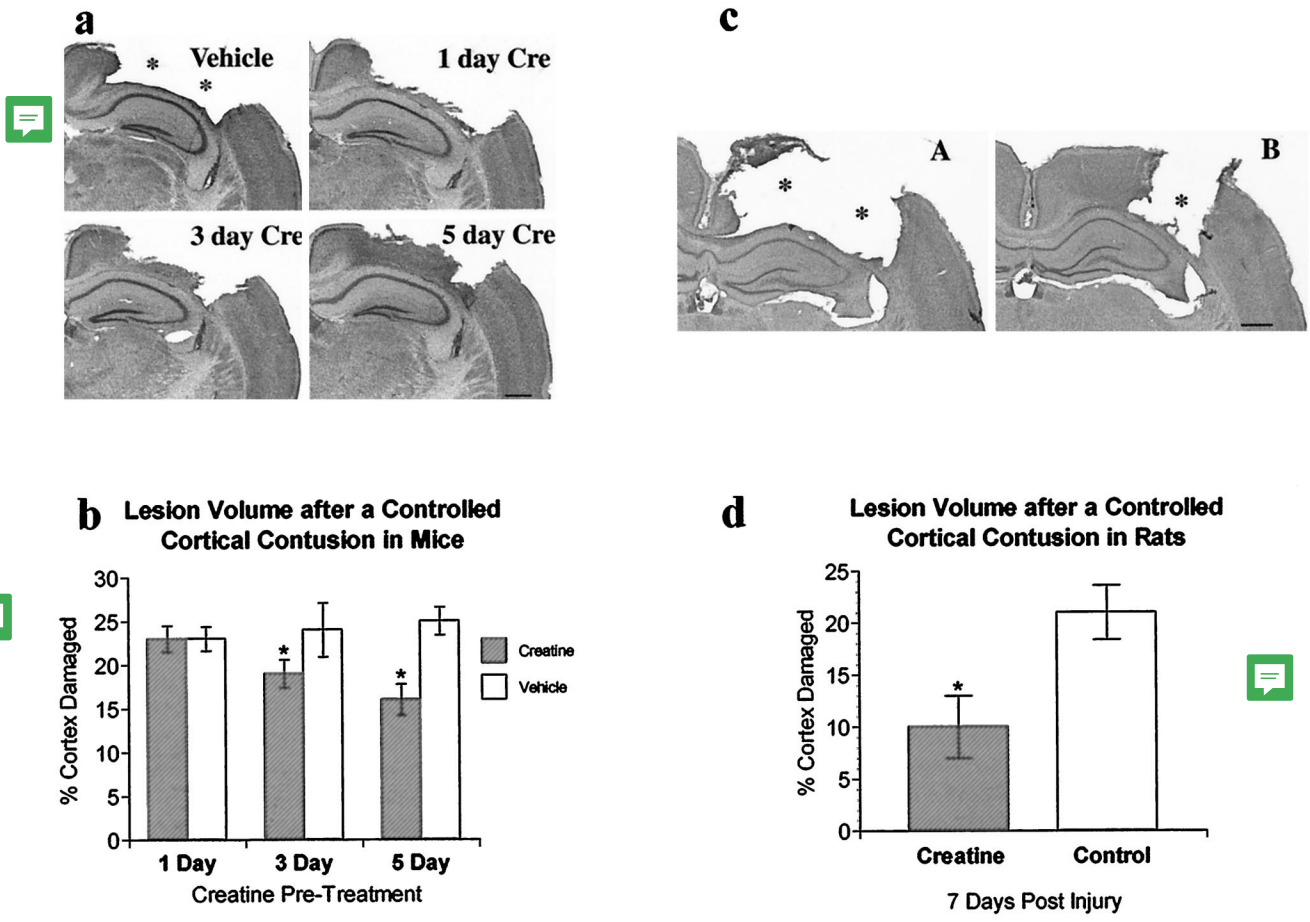

*Fig 1. (a) Cresyl violet–stained coronal sections through traumatic brain injury (TBI)–damaged cerebral hemispheres of adult mice. Mice were administered vehicle or creatine (3 g/kg of body weight) for 1 (1 day Cre), 3 (3 day Cre), or 5 (5 day Cre) days before injury. The impact results in an obvious cavitation (indicated by asterisks) in the cortex immediately below the impact site 7 days after injury. Calibration bar = 0.5 mm. (b) Pretreatment of mice with creatine for 5 days significantly decreased cortical lesions 7 days after TBI. Creatine or vehicle was administered to mice for 1 (n = 6 per group), 3 (n = 6 per group), or 5 days (n = 8 per group) before TBI. Cortical damage was significantly reduced by 21% and 36% in mice administered creatine for 3 and 5 days, respectively, before injury compared with vehicle-treated mice or mice receiving creatine 1 day before injury. No significant differences were observed in mice receiving creatine for 1 day before injury. Bars represent means (± SEM). *p < 0.01. (c) Cresyl violet–stained coronal sections through TBI-damaged cerebral hemispheres of rats. The impact results in an obvious cavitation (indicated by asterisks) in the cortex immediately below the impact site 7 days after injury. The size of the injury was significantly reduced in animals supplemented with creatine. Control animals (a) and animals fed a creatine-supplemented diet for 4 weeks before injury (b) were killed 7 days after injury. Calibration bar = 1 mm. (d) Creatine supplementation is neuroprotective after TBI in rats. Animals were fed a creatine-supplemented diet for 4 weeks before TBI and were killed 7 days after injury. Cortical damage was reduced by 50% in rats fed a creatine-supplemented diet compared with rats fed a regular diet. Bars represent means (±SEM). *p < 0.01.*

ergy production results in the activation of excitatory amino acid receptors, increased intracellular Ca^{2+}, and the generation of free radicals,[39] which are events that are directly involved in necrosis and apoptosis. The key to maintaining the correct levels of ATP in the brain is the interaction between phosphocreatine (PCr) and the enzyme CK.[40] Mitochondrial CK catalyzes the reversible conversion of creatine and ATP to adenosine diphosphate and PCr to generate energy reserves in the cytoplasm. A cytosolic CK catalyzes the reversible conversion of PCr and adenosine diphosphate to ATP and creatine. The mitochondrial CK seems to be part of the MPTP and is associated with porin, an integral component of the MPTP,[41,42] and the adenine nucleotide translocator, linking mitochondrial CK to oxidative phosphorylation and consequently to both necrosis and apoptosis.[23,43]

The mechanistic basis for the neuroprotective effects of creatine may involve alterations of the insult-induced depletions of cellular ATP[18–23] and the fact

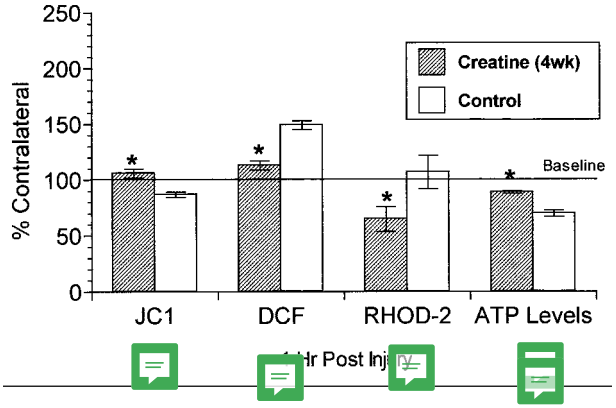

*Fig 2. Dietary supplementation with 1% creatine for 4 weeks maintains synaptic homeostasis after traumatic brain injury (TBI) in rats. Synaptic homeostasis was determined in synaptosomes prepared from rats maintained on a regular or creatine-supplemented diet for 4 weeks before TBI by analyzing mitochondrial membrane potentials, reactive oxygen intermediate (ROI) production, and mitochondrial Ca^{2+} and adenosine triphosphate (ATP) levels. Data from ipsilateral cortex were expressed as a percentage of contralateral cortex. Significantly increased mitochondrial membrane potentials, significantly decreased levels of ROIs, and significantly increased levels of mitochondrial Ca^{2+} and ATP were noted after TBI in rats fed a creatine-supplemented diet. Bars represent means (±SEM). *$p < 0.05$ as compared to control animals.*

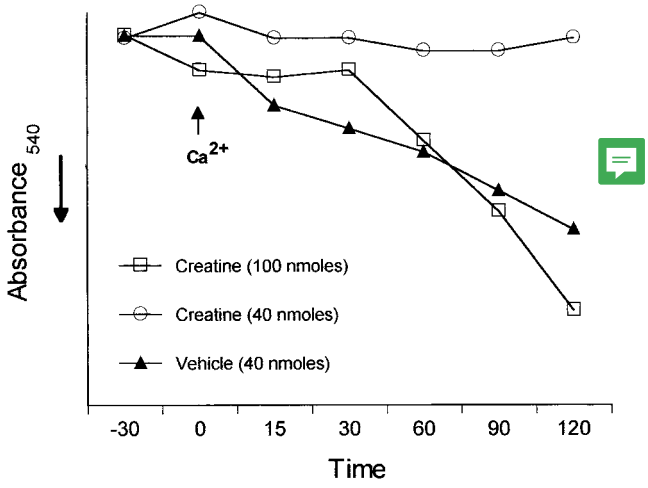

Fig 3. Calcium ion-induced swelling of de-energized nonsynaptosomal mitochondria isolated from the ipsilateral cortex of animals after traumatic brain injury was measured as a decrease in light scattering at 540 nm. The addition of 40 nM of calcium ions initiated rapid swelling (ie, opening of the mitochondrial permeability transition pore [MPTP]) in mitochondria isolated from nonsupplemented animals but had no such effect on creatine-supplemented animals. The addition of 100 nM of calcium ions initiated a rapid swelling in the creatine-supplemented animals, however, indicating that the threshold for MPTP opening is raised in creatine-supplemented animals. This could be due in part to the lowered basal levels of intramitochondrial calcium or to the direct inhibition of the MPTP by creatine kinase interaction with the adenine nucleotide translocator. The addition of 1 μM of cyclosporin A inhibits the calcium-induced swelling observed (data not shown).

that chronic ingestion of creatine results in increased brain levels of PCr.[22] The present findings demonstrate maintenance of cellular ATP levels, which is analogous to earlier findings with chronic creatine.[22] The creatine-induced maintenance of ATP levels may represent the first and most important event that helps to explain why we and other investigators demonstrated decreased levels of ROIs,[22] decreased levels of intramitochondrial calcium, increased maintenance of mitochondrial $\Delta\Psi$, and increased uptake of glutamate and glucose with chronic creatine ingestion.

The present results also suggest the possible involvement of the MPTP. Creatine can stabilize CK that is associated with the mitochondrial adenine nucleotide translocator. Through structural and functional interactions, the adenine nucleotide translocator can inhibit the MPTP.[24,44,45] The protection against TBI by creatine may involve the MPTP, because we and other investigators have shown that cyclosporin A inhibits MPTPs and also significantly protects against TBI-induced neuronal loss.[14,32,46,47] Our data showing that creatine helps to maintain mitochondrial membrane potentials, reduces free radical production, and lowers levels of intramitochondrial Ca^{2+} are consistent with our previous findings. Furthermore, the present results are the first to demonstrate that in vivo creatine supplementation blocks the Ca^{2+}-induced opening of the MPTP in isolated mitochondria after TBI. Mechanistically, our data and those of others suggest that the neuroprotection afforded by creatine may involve preservation of normal levels of mitochondrial ATP, membrane potential, and calcium, conceivably via inhibition of the MPTP. Together, these actions of creatine may help to prevent decrements in synaptic homeostasis and loss of brain parenchyma after TBI.

As a group, athletes have an increased risk of experiencing single and repeated blows to the head, with consequences ranging from minor concussion to death. Starting as young as 11 years of age, athletes at every competitive level ingest drugs and food-based supplements in efforts to enhance athletic performance or improve "body image."[48] Currently, the food-based supplement most widely ingested by athletes is creatine; as many as 75% of players on some professional teams routinely use creatine.[49] For athletes, creatine is used to increase levels of PCr, which serves as a phosphate donor to generate ATP and thereby decrease muscle fatigue during and enhance recovery after repeated bouts of high-intensity exercise.[17] In addition to its effects on skeletal muscle function, chronic ingestion of creatine

may have other important actions, including acting as a temporal or spatial energy buffer. The intermittently extensive and variable energy demands of excitable cells such as neurons require that the energy currency of the cell (ATP) be tightly regulated. Thus, the PCr-CK circuit allows the transport and buffering of high-energy phosphate production to locales of utilization in the form of phosphagens (ie, PCr).[42] The present experimental findings demonstrate that creatine provided significant protection against synaptic dysfunction and loss of cerebral cortical tissue in mice and rats subjected to TBI. Our data suggest that athletes may be afforded neuroprotective benefit, inadvertently, through the chronic ingestion of creatine.

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