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Short Communication

Neurochemical correlates of differential neuroprotection by long-term dietary creatine supplementation

Emiliano Peña-Altamira[1], Christophe Crochemore[1], Marco Virgili, Antonio Contestabile*

Department of Biology, University of Bologna, Via Selmi 3, 40126 Bologna, Italy

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Abstract

Dietary supplementation with creatine has proven to be beneficial in models of acute and chronic neurodegeneration. We report here data on the neurochemical correlates of differential protection of long-term creatine supplementation in two models of excitotoxicity in rats, as well as in the mouse model for ALS (G93A mice). In rats, the fall in cholinergic and GABAergic markers due to the excitotoxic death of intrinsic neurons caused by intrastriatal infusion of the neurotoxin, ibotenic acid, was significantly prevented by long-term dietary supplementation with creatine. On the contrary, creatine was unable to recover a cholinergic marker in the cortex of rats subjected to the excitotoxic death of the cholinergic basal forebrain neurons. In G93A mice, long-term creatine supplementation marginally but significantly increased mean lifespan, as previously observed by others, and reverted the cholinergic deficit present in some forebrain areas at an intermediate stage of the disease. In both rats and mice, creatine supplementation increased the activity of the GABAergic enzyme, glutamate decarboxylase, in the striatum but not in other brain regions. The present data point at alterations of neurochemical parameters marking specific neuronal populations, as a useful way to evaluate neuroprotective effects of long-term creatine supplementation in animal models of neurodegeneration.

Theme: Disorders of the nervous system
Topic: Neurotoxicity

Keywords: Rat; Transgenic mice; Neurodegeneration; Neuroprotection; Cholinergic neuron; GABAergic neuron

Long-term dietary supplementation with creatine has been proposed to be neuroprotective in both acute and chronic neurodegenerative diseases. Neuroprotective effects of creatine have been demonstrated in some animal models of neurotoxicity/excitotoxicity but not in others [23–25], in traumatic brain and spinal cord injury [17,31] and in ischemia [38]. Furthermore, attenuation of disease symptoms and increased lifespan through dietary creatine supplementation has been described in animal models of human neurodegenerative diseases [3]. In addition to recent results obtained in a mouse model of Huntington disease in which dietary creatine supplementation delayed motor symptoms and neuronal atrophy [8], several reports have dealt with mouse models of amyotrophic lateral sclerosis (ALS). In these mice (G93A strain), long-term dietary supplementation with creatine delayed motor neuron death and onset of motor symptoms and increased survival [19,37], even if no significant beneficial effects were reported regarding muscle function [9]. Clinical trials based on creatine administration to ALS patients have not demonstrated, so far, any significant beneficial effect [3,10,29]. In these trials, however, the human equivalent dose extrapolated from animal studies was likely underestimated and new trials, based on increased daily creatine administration, are awaited.

Surprisingly, reports on dietary creatine supplementation in animal models of neurodegenerative injuries and diseases have not dealt, so far, with the derangement of neuro-

* Corresponding author.
E-mail address: acontest@alma.unibo.it (A. Contestabile).
[1] The first two authors have equally contributed to the present work.

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chemical parameters related to degenerating neurons and with their rescue through the dietary manipulation. This information is very relevant, because specific alterations of neurochemical parameters are one of the most reliable markers to evaluate the extent of neural damage. Furthermore, this approach may be potentially able to put in evidence differential responses to insults and protection of different neuronal populations present in the same brain region. We report here data on the neurochemical correlates of differential protection of long-term creatine supplementation in two models of excitotoxicity in rats, as well as in the mouse model for ALS (G93A mice). To quantitatively evaluate neuronal damage and protection granted by the dietary supplementation, we have measured neurochemical parameters, whose alterations are established markers for the degeneration of specific types of neurons in the experimental models adopted for the present study [5–7].

Male Wistar rats form Harlan Italy were divided into two groups at 40 days of age. The first group was fed ad libitum with standard diet. The second group received the same diet supplemented with 2% creatine. Rats underwent surgical operation at 70 days of age and were then allowed to survive for additional 30 days, continuing to be fed with the normal or the supplemented diet during all these periods. For surgery, rats were anesthetized with ether, fixed to a David Kopf stereotaxic instrument and holes were drilled in the skull at the appropriate stereotaxic coordinates. Two different models of excitotoxic neurodegeneration were adopted to test possible neuroprotection from creatine supplementation. In the first model, rats received unilateral injections of the neurotoxin, ibotenic acid (IBO, 7 μg/1 μl saline), into the left striatum at the following coordinates [28]: 1 mm in front of bregma, 3 mm lateral, 5 mm below the dura. This treatment results in widespread degeneration of striatal neurons and in particular of the cholinergic and GABAergic neuronal population present in this brain region [5]. In the second model, IBO (5 μg/1 μl saline) was unilaterally injected in the area of the nucleus basalis magnocellularis, where cholinergic neurons providing most of the cholinergic input to the neocortex are localized [26,35], at the following coordinates: 1 mm behind bregma, 2.7 mm lateral, 7.8 mm below the dura. The excitotoxic lesion of these neurons results in fall of cholinergic parameters in cortical areas as a consequence of the degeneration of cholinergic terminals [4,6]. Injections were performed slowly (about 3 min followed by 2 min during which the needle was left in place), using a 10 μl Hamilton syringe operated by a micrometric device. In both cases, the contralateral (right) side of the brain received an equivalent injection of saline and served as control for the excitotoxic lesion. After 30 days of recovery, rats were killed by decapitation, the brains were rapidly removed and sliced with a Sorvall tissue chopper. Samples of the striata (for striatum-injected animals) obtained from two consecutive slices comprised between levels bregma 1.2 and bregma 0.2 [28], or the fronto-parietal cortex (for nucleus basalis magnocellularis-injected animals) obtained from three slices comprised between levels bregma 1.2 and bregma −1.3 [28], from the two brain sides were separately microdissected under the stereomicroscope, frozen in dry ice and stored at −80 °C until assayed. This procedure, which has been used for many years in our lab [5–7], allows reproducible dissections of equivalent samples from different animals without compromising reliability of the neurochemical determinations indicated below. Samples were homogenized in 0.32 M sucrose, added with 0.5% Triton X-100 (final concentration) and the whole homogenate was used to assay the activity of the cholinergic marker, choline acetyltransferase (ChAT) [12] and of the GABAergic marker, glutamate decarboxylase (GAD) [13], as well as sample protein content [22]. ChAT activity was assayed by incubating aliquots of the homogenates containing known amount of protein, for 10–30 min at 37 °C in the presence of choline (8 mM) and ^{14}C-acetylCoA (0.2 mM, NEN, specific activity 51.6 mCi/mmol) and adding eserine (0.1 mM) to block acetylcholinesterase. The labeled acetylcholine formed through the enzymatic reaction was extracted by Kalignost (0.5% in acetonitrile), brought to the organic phase of a scintillation cocktail (Instafluor, Packard), counted and expressed as μmol formed/unit protein weight. GAD activity was assayed by incubating aliquots of the same samples for 1 h at 37 °C in the presence of ^{14}C-glutamate (21.6 mM, NEN, specific activity 45 mCi/mmol), trapping the CO_2 evolved by the enzymatic reaction with hyamine hydroxyde, counting and expressing the activity as μmol of CO_2 formed/unit protein weight. We knew from previous experiments that glial proliferation consequent to the lesion does not alter the protein content of the affected regions [33].

Transgenic mice, G93A, carrying a high number of copies of the mutated human Cu/Zn superoxide dismutase (SOD1) gene (glycine/alanine substitution at codon 93) [16] were purchased from Jackson Laboratories under the strain designation B6SJL-TgN (SOD1)^{1GUR}. The colony was maintained by crossing male transgenic mice with females of the B6EiC3Sn strain. This allows to maintain both transgenic and wild type mice on the same genetic background and, therefore, to avoid effects not directly related to the specific mutation investigated. To identify transgenic mice, genotyping was performed at 30 days of age by extracting DNA from tails with Dneasy Tissue kit (Qiagen, GmbH, Germany) and using the PCR protocol suggested by Jackson Laboratories. Starting from 40 days of age, wild type and G93A transgenic mice were divided in groups, composed of approximately the same number of males and females, which received standard diet or diet supplemented with 2% creatine. When 110 days old, an age at which disease symptoms are manifested in G93A mice and cholinergic deficit is evident not only in the degenerating segments of the spinal cord, but also in some forebrain regions [7], wild type mice and part of the G93A ones were killed by decapitation. Samples from the lumbar spinal cord, the hippocampus, the olfactory cortex and the striatum [14] were rapidly collected in parallel by two experienced operators and stored in the