Neuroprotective Effects of Some Creatine Derivative Compounds

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Abstract

Some derivatives more lipophylic than creatine, thus theoretically being capable to better cross the blood-brain barrier, were studied for their neuroprotective effect in mouse hippocampal slices. In mouse hippocampal slices we found that EM21 is ineffective, EM22 weakly increased the latency to population spike disappearance during anoxia. Creatine, Creatine-Mg-complex (acetate) and Phosphocreatine-Mg-complex (acetate) increased more effectively the latency to population spike disappearance during anoxia. Moreover, Phosphocreatine-Mg-complex (acetate) significantly reduced neuronal hyperexcitability during anoxia, an effect that no other compound (including creatine itself) showed.
Summing up, EM21 is not useful for brain protection, while EM22 and chelates of both creatine and phosphocreatine do replicate some of the known protective effects of creatine. In addition, Phosphocreatine-Mg-complex (acetate) also reduced neuronal hyperexcitability during anoxia.

Introduction

A decrease of ATP has been shown after anoxia or ischemia both in vivo and in vitro because the lack of oxygen or glucose prevents or limits the ability of neurons to synthesize ATP.
The creatine/phosphocreatine system can compensate for the lack of oxygen or glucose. In normal brain creatine (Cr) is synthesized in the brain and is used to form phosphocreatine (PCr) with a reaction catalized by creatine kinase (CK) (Cr+ ATP → PCr + ADP + H+). Under conditions of ATP shortage (such as anoxia or ischemia) PCr can donate its phosphate group to ADP to resynthesize ATP, allowing ATP synthesis even in the absence of oxygen and glucose. The content of PCr is related to the maintenance of tissue polarization during oxygen deprivation. Brain PCr is substantially increased by treatment with Cr [1-2], and it has been repeatedly shown that Cr pretreatment protects against anoxic damage in vitro [3-4].
Three hereditary diseases characterized by lack of brain Cr have been described, two of them being at least partially ameliorated by Cr supplementation. Two of them cause decreased Cr synthesis (guanidinoacetate-methyltransferase deficiency [5] and arginine: glycine amidinotransferase deficiency [6], and can be treated by Cr supplementation. The third one (Cr transporter deficiency) [7] is characterized by lack of cerebral uptake of blood Cr, and is not corrected by Cr supplementation. In the latter case improvement might be afforded by Cr-derived compounds that cross biological membranes in a transporter-independent way.
Cr is a very promising molecule in the treatment of both brain ischemia and Cr transporter deficiency, but its limited crossing of the blood-brain barrier prevents its satisfactory use in those conditions. A possible way of improving Cr crossing of the blood-brain barrier is to modify its molecule in such a way as to make it less polar while maintaining its biological activity. In the present paper we investigated the possible neuroprotective effects of some Cr-derived molecules in an in vitro model of brain anoxia. We tried to verify if these Cr-derived compounds improve tissue resistance to anoxia like Cr does, if they increase the tissue content of Cr and PCr and if such increase occurs even after functional inactivation of the Cr transporter.

Materials and methods

Mouse hippocampal slices

Male ICRCD1 mice, 4 weeks old, were anaesthetised with ether and decapitated. The left hippocampus was dissected free and cut in 400 μm thick transversal slices. All dissection procedures were done under ice-cold artificial cerebrospinal fluid (ACSF, see below). Slices were immediately transferred into one or more beakers (see

below) containing ACSF composed of: NaCl 130 mM, KCl 3.5 mM, NaH2PO4 1.25 mM, NaHCO3 24 mM, CaCl2 2.4 mM, MgSO4 1.2 mM, glucose 10 mM. The concentration of K^+ in this medium is 3.5 mM, corresponding to that found *in vivo* [2]. This medium was continuously bubbled with 95% O2 / 5% CO2, resulting in a pH of 7.35-7.40. The beaker was immersed in a constant-temperature bath at 30°C (for subsequent electrophysiological measurement) or 36°C (for subsequent High Pressure Liquid Chromatography – HPLC - measurement). Incubation was carried out in the absence of added compounds ("controls") or with the addition of 1 mM (for subsequent electrophysiological experiments) or 2 mM (for subsequent HPLC measurements) of one of the following compounds: Cr, Cr-Mg-complex (acetate), PCr, PCr-Mg-complex (acetate), EM21, EM22. The chemical formula and the synthesis of EM21 and EM22 are not explained because they could be patented shortly. Incubation lasted 3 hr [2]. After this time, slices for electrophysiological measurements were transferred and incubated at 35 ± 1°C into an "interface" recording chamber with ACSF perfused at 2 ml/min. Anoxia was induced by replacing oxygen with nitrogen in the gas phase for 20 minutes. During anoxia, the composition of ACSF was not changed from that used before anoxia. Although experimental factors like, for example, methods of tissue preparation, ether administered before decapitation, slice handling and others may have influenced outcome after anoxia, the procedure we followed was absolutely identical in all experimental groups, including the control one. Thus, we believe that none of the above factors had any influence on the results we obtained. Incubation medium in the chamber was identical for each slice to that in the beaker. Specifically, when the slice had been previously incubated in the presence of an experimental compound (Cr or its derivatives), the same compound was added to the ACSF flowing into the chamber. By contrast, after the 3 hr incubation slices for HPLC measurement of Cr and PCr were processed as described below.

Electrophysiological Methods

For stimulation a tungsten microelectrode was inserted among the Schaffer collaterals, and for extracellular recording a glass micropipette of 2 to 10 mV tip resistance was inserted into the CA1 cell body layer. Slices that did not display a stable compound action potential (population spike, PS) in CA1 upon Schaffer collaterals stimulation were discarded. To evaluate hypoxic damage we used the extracellular compound action potential population spike recorded in the CA1 cell body layer after stimulation of the Schaffer collateral. It indicates functioning of the monosynaptic pathway between Schaffer collaterals and CA1 pyramidal neurons. Data were recorded on an IBM-compatible PC using the Axotape software (Axons Instruments, Foster City, CA, USA). After baseline recording to confirm stability of the preparation, anoxia was induced (see above), and electrophysiological monitoring was carried out. We delivered a stimulus to the Schaffer collaterals and recorded PS once a minute, noting its characteristics and its time to disappearance.

Determination of tissue content of Cr and PCr

Slices from each beaker were removed after 3 hr incubation, washed two times in physiological solution and dried on the wall of an Eppendorf vial. The vial was dipped in liquid nitrogen, then stored at -80°C until measurements. For HPLC measurement, the frozen slices were rapidly homogenized directly in the Eppendorf vials with 200µ l ice-cold $HClO_4$ (0.3 M) to neutralize Cr-kinase. The pestel was washed three times with 100 µl of $HClO_4$ (0.3 M). Samples of the homogenate were centrifuged at 12,550 *g* for 5 min and the supernatant was neutralized with 70 µl of $KHCO_3$ (3M) and again centrifuged in the same way. The resulting supernatant was used for HPLC determination of Cr and PCr (see below). The pellet left from the former centrifugation was stored at -80°C for tissue protein measurements. To this aim, the pellet was washed two times with 1 ml of Hank's Salt Solution (Bio-Rad Laboratories, Hercules, CA, USA) and the protein content was quantified with the method of Bradford [8] using a commercial protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The levels of Cr and PCr were determined by HPLC. Briefly, a model Series 200 apparatus made up of binary pump, refrigerated autosampler and UV/Vis detector (Perkin-Elmer, Boston, MA, USA), was used for the HPLC analyses. Chromatograms were monitored by Totalchrom data analysis software (Perkin-Elmer, Boston, MA, USA) loaded on an IBM PC. Concentrations were determined by comparing sample peak areas with those of external standards. Chromatograms were performed using an Atlantis C_{18}-3µm particle size, 4.6x150 mm column protected by a guard column of the same material. The mobile phase was an aqueous solution of 25 mM NaH_2PO_4, 1 mM tetrabutylammonium hydrogen sulfate, adjusted to pH 7.0 with NaOH 2N aqueous solution. All reagents were of analytical grade (Sigma, St. Louis, MO, USA) and water was of HPLC grade. Flow rate was 1.0 ml/min and wavelength was set to 220 nm. In these conditions, GPA had a much lower extinction coefficient than Cr and PCr. Therefore, when GPA was present in the samples, it only very little interfered with the assay. Standards of Cr were freshly prepared in water at concentrations from 50 to 0.5 µg/ml each day prior to a set of analyses. Samples of 10 µl were injected into the HPLC system. A clear separation was obtained for Cr and peak purity appeared good without co-elution of interfering compounds. Linear regression analysis between standard concentrations and chromatographic responses provided a good linearity over the range 50–0.5 µg/ml (r^2>0.997). Within- and between-day accuracy and precision evaluated as relative mean error (RME%) and coefficient of variation (CV%), respectively, were always lower than 10% [9].

Inactivation of Cr transporter in brain slices

The Cr transporter is functionally inactivated by 3-guanidinopropionic acid (GPA) [10] and incubation with chloride-free medium. We previously showed [11] that these two treatments prevent uptake of Cr in mouse

hippocampal slices, too. Thus, in the present paper we functionally inactivated the Cr transporter with either one of the following treatments: (a) adding to the incubation medium 10 mM 3-guanidinopropionic acid (GPA) or (b) replacing NaCl in the medium with an equimolar amount of Na-acetate to obtain an almost total chloride-free incubation.

Results

We observed the population spike disappearance during anoxia in the hippocampal slices treated with the different compounds. We showed that PCr did not have any effect. By contrast, Cr, Cr-Mg-complex and PCr-Mg-complex all were different from controls, showing a longer maintenance of PS during anoxia. EM21 did not have any effect while EM22 showed a limited maintenance of PS during anoxia. We reported that PCr-Mg-complex (acetate) increases tissue content of Cr, albeit not that of PCr [11]. Cr and Cr-Mg-complex (acetate) caused an increase in intracellular compounds, EM22 a low increase of tissue content of Cr, while EM21 did not have any effect on the Cr and PCr content in the brain (Fig.1-2). We also showed that the increase in tissue Cr obtained with Cr-Mg-complex is abolished by blocking the Cr transporter, either by adding GPA 10mM or by incubating the tissue in a Cl-free medium. It means that the uptake of Cr-Mg-complex (acetate) is mediated by the Cr transporter, as well as the uptake of Cr. This result is different with what we obtained with PCr-Mg-complex (acetate), whose uptake was not blocked by functional inactivation of the Cr transporter [11]. Our studies about EM21 and EM22 showed that the uptake of both the compounds is mediated by the Cr carrier.

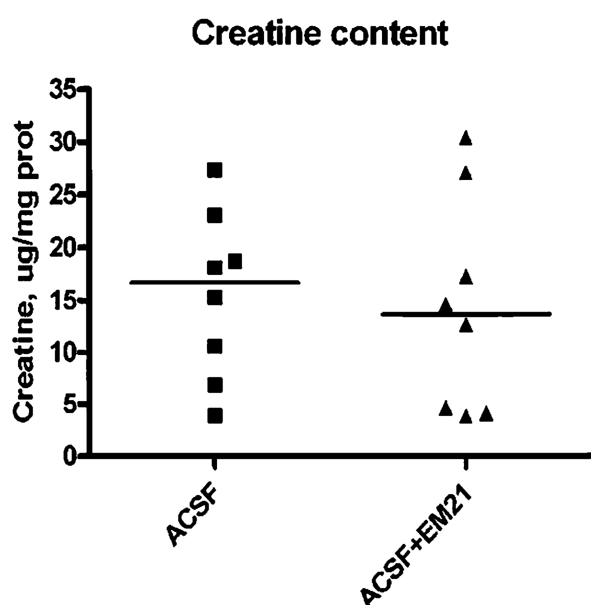

Fig. 1 - Cr content in slices treated with EM21: Black squares indicate slices incubate in ACSF without drug, black triangles the slices incubated with EM21. EM21 doesn't increase the Cr content in the cells.

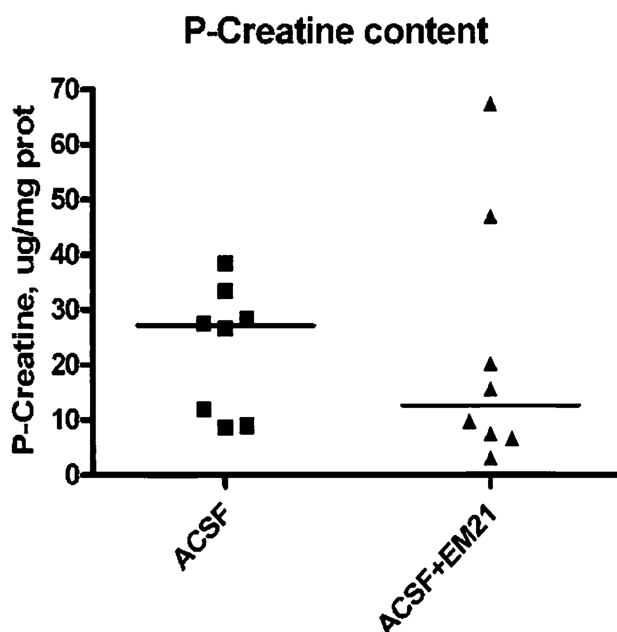

Fig. 2 - PCr content in slices treated with EM21: Black squares indicate slices incubate in ACSF without drug, black triangles the slices incubated with EM21. EM21 doesn't increase the PCr content in the cells.

Discussion

We could confirm the known neuroprotective actions of Cr against disappearance of population spike during anoxia [1]. Delay evoked potential disappearance are caused by Cr-induced sparing of energy charge during anoxia [2, 12]. Of the compounds we synthetized and investigated both Cr-Mg-complex (acetate) and PCr-Mg-complex (acetate) did show neuroprotective action by delaying PS disappearance during anoxia. EM22 did show a limited neuroprotective action by delaying PS disappearance during anoxia and EM21 did not show a neuroprotective activity. We showed that not only Cr, but also two other Cr-derived compounds are neuroprotective against in vitro anoxia. We previously showed [11] that one of these compounds (PCr-Mg-complex acetate) crosses biological membranes in a transporter-independent way. Thus, it might be useful in the therapy of hereditary Cr transporter deficieny, a condition presently incurable [13]. Moreover, this compound could be more rapidly available to the brain than Cr, thus providing neuroprotection in syndromes of cerebral ischemia, where Cr has some efficacy even when administered early after the event [14].

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