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Role of creatine and phosphocreatine in neuronal protection from anoxic and ischemic damage

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Summary. Phosphocreatine can to some extent compensate for the lack of ATP synthesis that is caused in the brain by deprivation of oxygen or glucose. Treatment of in vitro rat hippocampal slices with creatine increases the neuronal store of phosphocreatine. In this way it increases the resistance of the tissue to anoxic or ischemic damage. In in vitro brain slices pretreatment with creatine delays anoxic depolarization (AD) and prevents the irreversible loss of evoked potentials that is caused by transient anoxia, although it seems so far not to be active against milder, not AD-mediated, damage. Although creatine crosses poorly the blood-brain barrier, its administration in vivo at high doses through the intracerebroventricular or the intraperitoneal way causes an increase of cerebral phosphocreatine that has been shown to be of therapeutic value in vitro. Accordingly, preliminary data show that creatine pretreatment decreases ischemic damage in vivo.

Keywords: Creatine – Phosphocreatine – Ischemia – Anoxia – Stroke – Guanidinoacetate methyltransferase deficiency – Hyperornithinemia – Protection

Background

Phosphocreatine can compensate lack of oxygen and glucose, and is increased by administration of creatine

Lack of oxygen or glucose prevents or limits the ability of neurons to synthesize ATP. Thus, decrease of ATP has been shown after anoxia or ischemia both in vivo (Obrenovitch et al., 1988) and in vitro (Lipton, 1982). The creatine/phosphocreatine system can to some extent compensate for the lack of oxygen or glucose. In normal brain functioning, creatine (Cr) is synthesized in the brain and is used to form phosphocreatine (PCr) with a reaction catalized by creatine kinase (CK) (Wyss and Kaddurah-Daouk, 2000)[1]. When the phosphate group is detached from PCr, 45 kJ/mol of free energy become available, compared with only 31.8 for ATP (Wyss and Kaddurah-Daouk, 2000). Thus, under conditions of ATP exhaustion (such as anoxia or ischemia) PCr can donate its phosphate group to ADP to resynthesize ATP, a reaction that is also catalized by CK (Clarke, 1999; Wyss and Kaddurah-Daouk, 2000). In this way PCr allows ATP synthesis (from ADP) even in the absence of oxygen and glucose. Accordingly, it was shown long time ago (Krivánek, 1958) that the content of PCr was related to the maintenance of tissue polarization during oxygen deprivation. However, neuronal PCr content is limited, and soon after complete anoxia or ischemia PCr is depleted, too (Lipton, 1982; Obrenovitch et al., 1988). Decrease in PCr precedes the fall of ATP (Lowry et al., 1964; Norberg et al., 1975), thus showing that PCr is used up by the cell to maintain as long as possible ATP concentration. Such a mechanism has been known for a long time to occur in the skeletal muscle (Hohorst et al., 1962).

Brain PCr is substantially increased by treatment with creatine (Cr). In fact, the CK reaction can shift from left to right and viceversa according to the gradient of concentration of its products. When excess Cr is available, the reaction is shifted toward the synthesis of PCr. Twenty years ago Whittingham and Lipton

[1] $Cr + ATP \rightarrow PCr + ADP + H^+$

(Whittingham and Lipton, 1981) showed that by incubating in vitro rat hippocampal slices with a high Cr concentration (25 mM), their PCr content increased four-fold, from 40 μmol/g protein to approximately 150 μmol/g protein. Accordingly, the same group showed that this treatment was able to prevent the irreversible loss of synaptic transmission in rat hippocampal slices after a 10 minutes' deprivation of oxygen (Kass and Lipton, 1982). This protection was accompanied by a partial conservation of ATP in anoxic slices: in Cr-treated slices ATP after anoxia fell to 7.9 μmol/g protein, while in control slices it fell to 3.6 μmol/g protein (control pre-anoxic content: 13.9 μmol/g protein) (Kass and Lipton, 1982). Subsequently, it was shown that pretreatment with creatine also prevented anoxia-induced decrease in protein synthesis in rat hippocampal slices (Carter et al., 1995).

Decrease in brain ATP leads to anoxic depolarization, which is a cause of irreversible damage

As for the mechanism through which decrease of ATP leads to irreversible damage, we believe that it is mainly related to the generation of anoxic depolarization (AD). The latter (Fig. 1) is a depolarization that occurs in brain tissue soon after anoxia, and is reminiscent of Leão's "spreading depression" (Bureš et al., 1974; Leão, 1944). Anoxic depolarization (AD) is primarily an electrophysiological event. Specifically, it consists of an 8 to 15 mV (or sometimes higher) decrease in brain extracellular voltage that occurs after a few minutes of brain anoxia or ischemia (Bureš et al., 1974; Leão, 1947). Nevertheless it marks a dramatic metabolic change in neuronal metabolism and environment. At the time of AD neurons depolarize (Hansen, 1985) showing a large, abnormal, inward current (Czéh et al., 1992). Permeability of membrane to ion movements increases dramatically, allowing a rather free flow of ions across the cell membrane. Extracellular Na^+ falls from 154 to 63 mM, and extracellular Cl^- falls from 129 to 75 mM, implying massive entry of both ions into neuron and glial cells (Hansen, 1985). Intracellular K^+, by contrast, massively leaves neurons, so that its extracellular concentration increases from 3.1 to 58 mM (Hansen, 1985). Ca^{2+} massively leaves the extracellular space entering neurons and glial cells, so that its extracellular concentration at the time of AD falls from 1.3 to 0.06 mM (Hansen and Zeuthen, 1981). Tissue pH shows a transient alkaline shift of 0.10 pH units coincident with the negative deflection in voltage, which in turn is followed by a lasting acidic shift to 6.75 (normal pH is 7.4) (Mutch and Hansen, 1984).

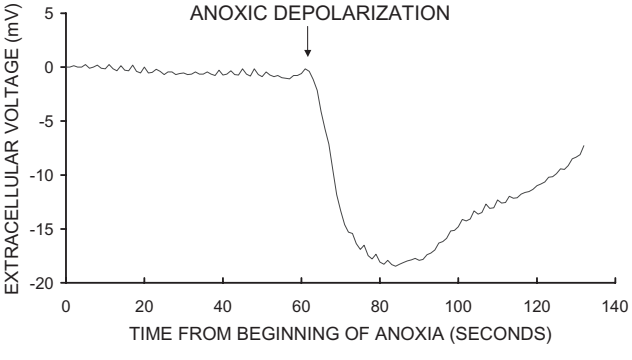


Fig. 1. Rat hippocampal slice. Extracellular voltage from a micropipette in the cell body layer of the CA1 region. Oxygen is replaced by nitrogen at time = 0 (beginning of anoxia). Note that the voltage remains almost stable for about 1 minute, then falls abruptly (arrow). This sudden deflection is called anoxic depolarization. See text for more explanations

The mechanism leading to AD is not fully known. It is certainly reasonable to assume that AD is caused by decrease of ATP. Lack of ATP blocks the (Na^+,K^+)ATPase, the "sodium pump" which actively extrudes Na^+ from the neuron and causes K^+ to enter the intracellular space. Thus, neurons slowly depolarize, then lose their membrane potential causing anoxic depolarization. Although a decrease in ATP had been detected around the time of AD in hypoglycemic rats (a condition similar if not identical to anoxia or ischemia), it was debated whether or not it was the fall in ATP that caused the depolarization (Harris et al., 1984).

Experimental demonstration of a cause-and-effect relationship between inactivation of (Na^+,K^+)ATPase and AD has been actually given (Bureš et al., 1974) by showing that application to the intact cortex of ouabain, the glycoside that inhibits (Na^+,K^+)ATPase, caused a depolarization that was similar to that of AD. More recently, one of us and others confirmed and expanded those earlier findings (Balestrino et al., 1999b). They perfused in vitro rat hippocampal slices with 100 μM ouabain, and were able to reproduce the voltage and $[K^+]_o$ changes that are typical of AD (Balestrino et al., 1999b). Those experiments showed that inactivation of (Na^+,K^+)ATPase is sufficient to reproduce the electrical and ionic changes that are the hallmark of AD. Since during anoxia or ischemia

(Na^+,K^+)ATPase is obviously inactivated by lack of ATP, these findings strengthened the belief that AD is caused by functional inactivation of (Na^+,K^+)ATPase due to lack of ATP.

Despite their drama, most of the AD-induced changes (see above) are reversible upon reoxygenation. Therefore there has been for a while considerable debate concerning whether or not AD is in fact a cause of irreversible anoxic damage. One of us then contributed to show (Balestrino and Somjen, 1986) that it is the *duration* of AD which is related to irreversible damage. That is, AD of short duration is compatible with functional brain recovery, while if AD lasts longer than a critical time irreversible loss of synaptic transmission occurs. In that work one of us and Somjen showed that slices pretreated with chlorpromazine (7 or 70 μM) were dose-dependently protected from anoxic damage compared to control slices. Specifically, they subjected rat hippocampal slices to 9 minutes of anoxia by replacing oxygen with nitrogen in the incubating chamber. They found that extracellular compound action potential returned after anoxia in 0 out of 8 (0/8) untreated controls, in 5/8 slices treated with 7 μM chlorpromazine and in 5/5 slices treated with 70 μM chlorpromazine. Those slices that did not recover from anoxia had had a duration of AD during anoxia comparable to that of controls, despite chlorpromazine treatment. Moreover, the Authors deliberately induced AD early during the 9-minute anoxia in slices treated with 70 μM chlorpromazine (by dropping onto them a microdrop of 130 mM K^+), so to subject them to a long AD duration despite the protective treatment. In 5 slices this high-K^+ was administered 4 minutes after beginning of anoxia, thus causing them to remain in the depolarized state for 5 minutes despite chlorpromazine (total anoxia time was 9 minutes). In 3 slices it was administered 7 minutes after beginning of anoxia, thus causing them to remain in the depolarized state for only 2 minutes (total anoxia time was 9 minutes). In all early-AD slices (5 out of 5) population spike did not recover after reoxygenation, while in all the late-AD slices (3 out of 3) it did recover after reoxygenation (Balestrino and Somjen, 1986). It was later shown that exposure to a depolarized state of long duration is harmful in normoxic slices, too (Kawasaki et al., 1988). The reader is also referred to an earlier review for a further discussion of the role of AD in generating irreversible damage (Somjen et al., 1990). These findings led to the belief that if during *transient* anoxia AD could be delayed long enough, then its duration would have been short enough to be compatible with recovery. A positive correlation between time spent in the depolarized state and irreversible brain damage was since then confirmed in vivo (Chen et al., 1993). Those Authors investigated middle cerebral artery occlusion in rats, and found a significant correlation ($r = 0.90$, $p < 0.001$) between the number of depolarizations that in any given rat occurred at the periphery of the infarct and the final volume of the infarct itself.

Pretreatment with creatine delays anoxic depolarization and protects against irreversible damage

Thus, treatments (specifically, Cr-induced increase of neuronal PCr) that delay ATP depletion during anoxia were expected to delay AD as well, thus limiting or preventing irreversible damage.

That this was the case was indeed confirmed by one of us, who showed that pretreatment of rat hippocampal slices with 25 mM Cr caused both delay of AD during anoxia and protection against irreversible loss of synaptic transmission (Balestrino, 1995) (similar data are shown in Fig. 2 and 3).

Alhough these data were quite encouraging, their use for human therapy faced two main obstacles. First, the concentration of Cr that was used in the original papers was quite high (25 mM). Second, Cr is a highly polar molecule that does not easily cross the blood-brain barrier (Chanutin, 1927).

As for the first problem, it had been showed that lower Cr concentrations (0.03–3 mM) are effective in preventing anoxia-induced block of protein synthesis (Carter et al., 1995). Thus, one of us and others set out

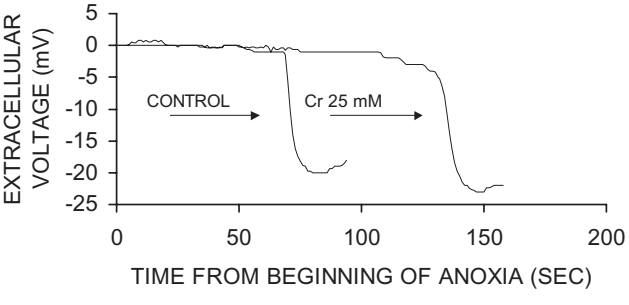


Fig. 2. Anoxic depolarization in a control slice and in a slice pretreated with 25 mM creatine. In both cases anoxia begins at time = 0. In the treated slice anoxic depolarization occurs later than in the control one, an effect that was statistically significant (see text). Modified from (Balestrino et al., 1999a)

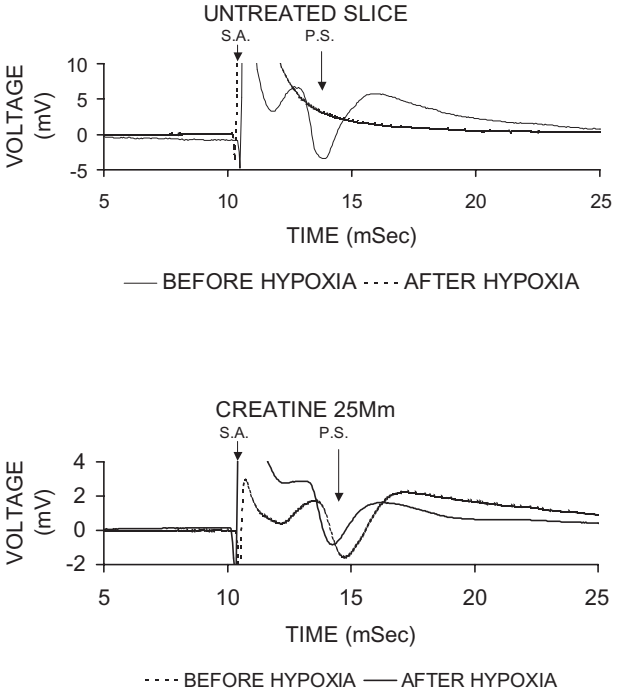


Fig. 3. Extracellular compound action potential (population spike, *PS*) from the CA1 cell body layer of a rat hippocampal slice. In a control slice (above) PS disappears after anoxia. In a creatine-treated slice (below) PS is not changed. This effect was statistically significant. See text for more explanations. *S.A.* Stimulus artifact

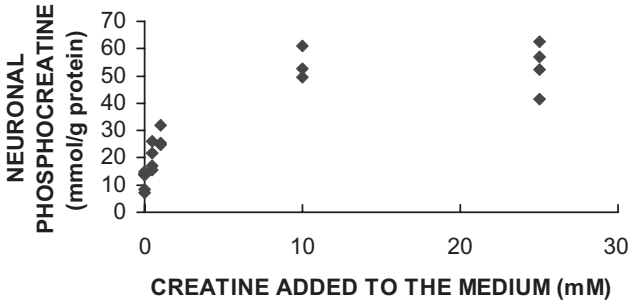
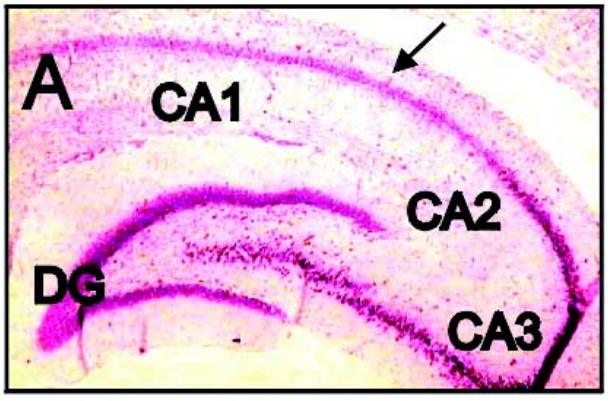
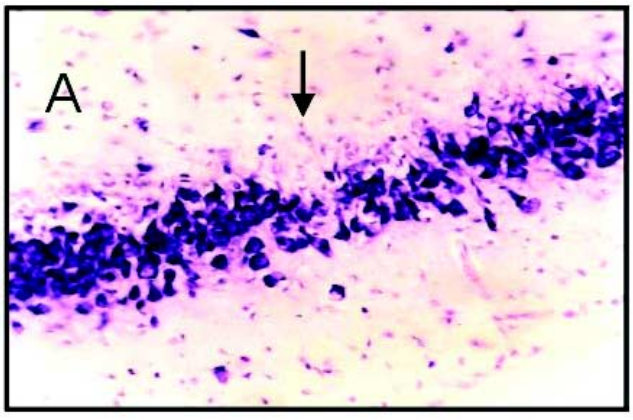


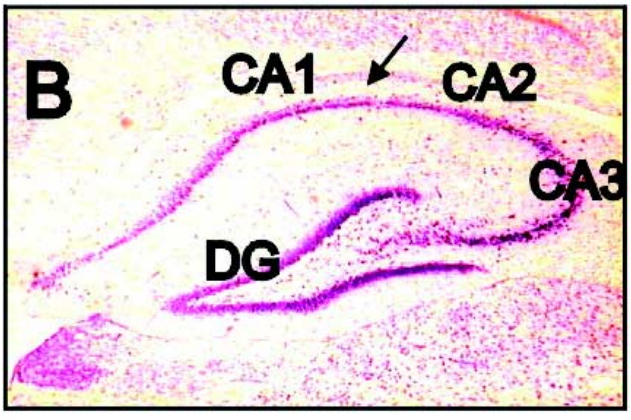
Fig. 4. Relationship between concentration of creatine added to the medium and neuronal phosphocreatine. See text for details and comments. Modified from Balestrino et al. (1999a)

to investigate how concentrations of Cr lower than had so far been used could delay AD and prevent irreversible damage in rat hippocampal slices. They found that concentrations of Cr in the range 1–25 mM significantly increased PCr content of the slices, significantly delayed AD during anoxia, and protected against anoxia-induced loss of synaptic transmission (Balestrino et al., 1999a). In those experiments it was shown that PCr continued to increase between baseline and 10 mM creatine (from 12.70 ± 2.97 to 54.40 ± 5.93 μmol/g protein, mean ± SD), then it reached a plateau and seemed not to increase further even if Cr concentration was increased to 25 mM (Fig. 4). In the lower part of this range, preincubation with 1 mM Cr showed a PCr increase that was statistically significant (27.35 ± 3.96 vs. 12.70 ± 2.97 μmol/g protein, mean ± SD; $p < 0.003$, t-test). In those experiments a statistically significant increase with 0.5 mM creatine was not obtained, probably due to the small number of observations ($N = 3$). However, a small increase was still observed (from 12.70 ± 2.97 to 18.13 ± 3.26 μmol/g protein, mean ± SD) that was of borderline significance ($p < 0.07$, t-test). In later experiments one of us contributed to administer the latter Cr concentration (0.5 mM) in vivo. Rats were implanted in the lateral ventricle with osmotic pumps (Alzet, Paolo Alto, CA) loaded with 0.5 mM Cr. Three days after, the animals were sacrificed, their brains frozen in liquid nitrogen, then stored at −80°C before processing for HPLC determination of PCr (Rebaudo et al., 2000). Thus, the 0.5 mM Cr was administered to the brain (by intracerebroventricular injection) for a much longer time than was possible in vitro (3 days vs. 3 hours). Those experiments demonstrated a substantial increase in tissue PCr even with this lower Cr concentration. In fact, brain PCr rose from a control value (in pump-implanted but saline-perfused rats) of 33 ± 17 μmol/g protein (mean ± SD, $N = 9$) to a value in Cr-treated rats of 55 ± 17 μmol/g protein (mean ± SD, $N = 7$; $p = 0.02$, t-test). Thus, even 0.5 mM Cr can substantially increase brain PCr when tissue exposure is sufficiently protracted.

This increase is probably sufficient to afford neuronal protection in vivo. In preliminary experiments rats were implanted with an intracerebroventricular osmotic pump, loaded with 0.5 mM Cr, according to our above described, published protocol (Rebaudo et al., 2000). Thus, they were intracerebroventricularly infused for 3 days with 0.5 mM Cr, according to the protocol that caused the above reported increase in brain PCr. Control rats were similarly implanted and infused with saline solution. After this 3-day infusion they were subjected to 10-minute transient global cerebral ischemia clamping of carotid arteries and controlled arterial hypotension (Payan et al., 1965). Two or four days after ischemia, they were sacrificed. After sacrificing the animals brains were removed and fixed in freshly prepared paraformaldehyde. Thin coronal slices from the level of the mid-dorsal hippocampus







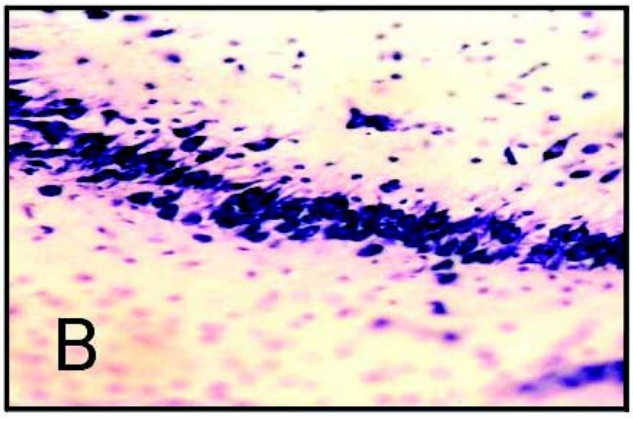


Fig. 5. Haematoxyline-eosine staining of rat hippocampus 5 days after transient ischemia (see text). Enlargement was 40×. **A** is a control rat, infused intracerebroventricularly with saline solution 3 days before ischemia. **B** is a rat similarly infused with 0.5 mM creatine (Rebaudo et al., 2000). Letters indicate the CA1, CA2 and CA3 regions, and the dentate gyrus (*DG*). The most interesting regions are the cell body layers of CA1–CA2 (arrows) where damage is selectively suffered in this model (Pulsinelli and Buchan, 1988). The low density of normal neurons of CA1 and CA2 regions can be clearly seen in control rat (rat A) in contrast to the rather large proportion of normal cells in the creatine-treated (Rat B) animal. Rat A, receiving saline infusion, had severe neuronal necrosis in hippocampus; 100% pyramidal neurons of CA1 and CA2 region had ischemic cell change (ICC). The number of neurons with ICC in CA3 region was 42%. Rat B, receiving creatine infusion, had a moderate degree of necrosis, that represented 70% in CA1, 52% in CA2 and 32% in CA3 regions. See text for further details and discussion

Fig. 6. Enlargement, from Fig. 5, of the CA2 cell body layer, to better show absence (**A**, control rat) and presence (**B**, creatine-treated rat) of surviving neurons

were dehydrated, embedded in paraffin, and stained either with hematoxylin-eosin or according to Nissl. The number of neurons showing ischemic cell change (ICC) was evaluated in one 200× microscopic field of the CA1, CA2 and CA3 region of mid-dorsal hippocampus. The term "ischemic cell change" was defined as indicating neurons with shrunken cell body, focal cytoplasmic condensations and pyknotic nuclei. Examples are shown in Figs. 5, 6 and 7. Cr pretreatment decreases the number of necrotic neurons in hippocampal CA1 area after transient ischemia from 21 ± 14% (controls) to 5 ± 9 (creatine-treated) two days after ischemia, and from 100% (control) to 70% (creatine-treated) four days after ischemia (Fig. 8). While the latter data are too few to demonstrate effectiveness, it is clear that they do suggest an effect of creatine.

The above results are quite encouraging, since they show (1) that Cr pretreatment does indeed afford protection in the nervous tissue and that (2) when Cr is administered in an appropriate fashion, brain PCr can increase, in vivo as well as in vitro, to such an extent as to possibly afford protection. Of course, a tempting development will be administering creatine to patients suffering from brain ischemia in order to limit their brain tissue damage. To do so, one of the important questions to ask is whether or not a sufficient increase in PCr can be obtained by administering Cr via more usual ways (e.g., intravenously). We are currently car-

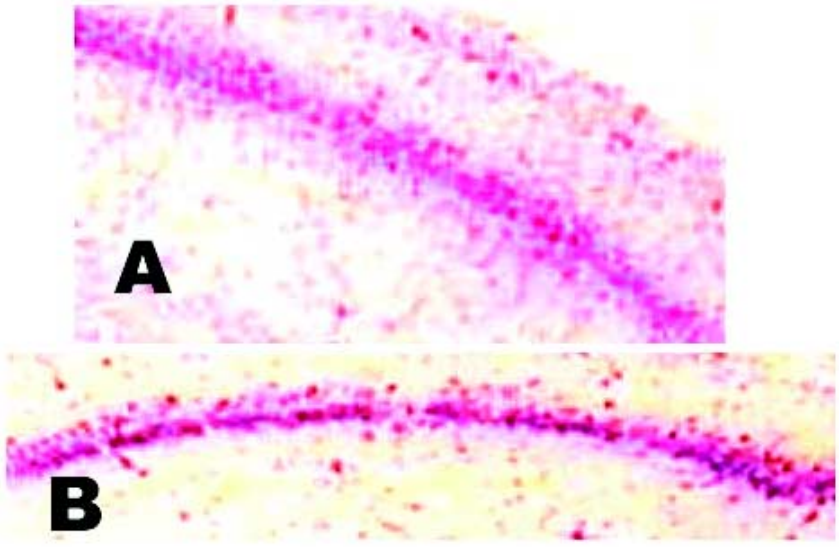


Fig. 7. Haematoxyline-eosine staining of rat hippocampus (CA1 cell body layer) 5 days after transient ischemia (see text). Enlargement was 200×. The much larger number of neurons with shrunken cell bodies, focal cytoplasmic condensations and pyknotic nuclei can be seen in the control rat (**A**), as compared to the creatine-treated one (**B**). See text for further details and discussion

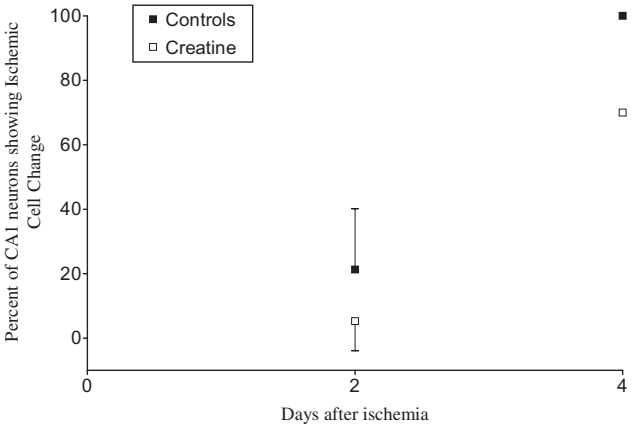


Fig. 8. Percent of CA1 neurons showing ischemic damage (Ischemic Cell Change) after transient ischemia. A protective effect of creatine is clearly suggested, even if not yet statistically demonstrated in these preliminary data. At Day 2, mean and S.D. are shown (N = 3 for each treatment). At Day 4, we have only one data point for each treatment (shown)

rying out experiments testing brain PCr increase after i.p. administration of Cr in the rat. In fact, even if Cr crosses poorly the blood-brain barrier, it is possible to hypothesize that, if one injects directly into the blood a sufficiently high amount of Cr, enough of it will reach the brain to possibly exert a therapeutic effect.

We injected a quite high amount (160 mg/Kg) of ^{14}C-Cr to rats by i.p. way, and measured radioactivity in the brain after various times. The results obtained so far show that after 6 hours exogenous labeled Cr reached a brain concentration of 74.7 ± 4.6 μM (mean ± SD, N = 3). Although this must be confirmed by further studies (e.g., HPLC determination of brain Cr and PCr) it is quite reasonable to assume that the radioactivity we find in the brain represents Cr which has crossed the blood-brain barrier and has been transformed into PCr. If this will indeed prove to be the case, it will mean that parenteral injection of high-dose Cr causes, in vivo as well as in vitro, a PCr increase of possible therapeutic value. Thus, acute parenteral treatment of patients may be possible.

Does creatine affect anoxic damage that does not involve anoxic depolarization?

We asked whether or not Cr can protect against anoxic or ischemic damage that does not involve AD.

If anoxia of rat hippocampal slices in vitro is maintained within a mild degree (either by decreasing incubation temperature in the chamber (Schiff and Somjen, 1987) or by limiting to a minimum the time spent under oxygen deprivation (Melani et al., 1999)) evoked potentials are not abolished, but are increased in amplitude, often with double spikes ("bursting"). This hyperexcitability has been likened to long-term potentiation, a lasting potentiation that is supposed to be a mechanism of learning and memory (Hammond et al., 1994). However, it is more reasonable to assume that it represents not a physiological phenomenon, akin to learning and memory, but a pathologic one, more similar to stroke-induced epilepsy (Daniele et al., 1996; Ferracci et al., 2000). We asked whether or not creatine can prevent this type of anoxia-induced damage. We subjected rat hippocampal slices to 3

minutes anoxia, then reoxygenated them. Preliminary results show that 30 minutes after anoxia the evoked compound action potential ("population spike") is 140 ± 37% of pre-anoxia value (mean ± SD; $p < 0.03$, t-test for paired data). In three slices that had been pretreated with 1 mM Cr for 3 hours, population spike was 105%, 134% and 306%, respectively, of pre-anoxia value. Although these results are too preliminary to warrant a firm conclusion, they clearly suggest that Cr pretreatment can not prevent anoxia-induced hyperexcitability.

It is interesting to note that in the mild anoxia that causes postanoxic hyperexcitability AD does not occur. This was reported in the original paper (Schiff and Somjen, 1987), where incubation temperature was lowered to minimize the effects of anoxia. It has also been confirmed in our observations (unpublished). In fact, in order to obtain post-anoxic hyperexcitability we subjected brain slices to only 3-minute anoxia, and we never observed AD within this time period. Thus, postanoxic hyperexcitability is due to a mechanism which is different from inactivation of (Na^+,K^+)ATPase. If it will be confirmed that Cr pretreatment does not improve this type of damage, it will be reasonable to assume that postanoxic hyperexcitability is not caused by a large decrease of ATP. It may in fact be caused by other mechanisms, such as anoxia-induced release of excitatory amino acids (Hammond et al., 1994).

Also, one of us has contributed to show that Cr pretreatment is not effective against 24-hour anoxia of rat neurons in culture, a model that is, by contrast, protected by the NMDA-receptor antagonist APV (Di Loreto and Balestrino, 1997). This result might be explained by the fact that CK, the enzyme that transforms Cr into PCr, is not fully developed in the perinatal rats from which those cultured neurons are obtained. However, it might also be hypothesized that this represents another example of mild anoxic damage that, just like post-anoxic hyperexcitability, is not prevented by Cr pretreatment.

By contrast, it should be noted that in several animal models human diseases are mimicked by impairment of energy metabolism. In these models AD is not expected to occur, yet Cr pretreatment has been shown to be protective (Tarnopolsky and Beal, 2001).

Thus, Cr pretreatment protects against AD-induced anoxic or ischemic damage. It probably does not protect against post-anoxic hyperexcitability, a condition where AD and profound decrease of ATP do not occur. By contrast, it may be useful in other abnormal conditions (Tarnopolsky and Beal, 2001) where profound decrease of ATP is involved, disregarding whether or not AD is a factor.

Creatine and the blood-brain barrier

Creatine is a very polar (hydrophilic) molecule, therefore it is expected to cross poorly the blood-brain barrier. It has been known for a very long time (Chanutin, 1927) that *oral* treatment with creatine does not increase brain creatine content in *normal* animals, thus suggesting a difficulty in blood-brain barrier crossing. A more recent study found no increase in phosphocreatine after oral creatine treatment in normal rats, only an increase in rats that very likely had a damaged blood-brain barrier due to the experimental lesion that was under study (see table I of reference (Matthews et al., 1998)). Human studies show that in healthy volunteers cerebral PCr can be increased only by 10% by oral creatine administration (Dechent et al., 1999). However, oral Cr treatment was effective in replenishing cerebral Cr store in a child completely lacking brain Cr due to guanidinoacetate methyltransferase (GAMT) deficiency (Stockler et al., 1996). Nevertheless, as expected due to the poor penetration of creatine through the blood-brain barrier, the replenishment took months to complete (Stockler et al., 1996). Although biochemical recovery was near complete, clinical recovery was not. It is quite reasonable to hypothesise that a faster creatine replenishment would have improved clinical outcome. Also, in the brain of children with hyperornithinemia a decreased creatine content was found, which was only partially corrected by oral creatine supplementation (Nanto-Salonen et al., 1999).

We already summarized, above, our results with intracerebroventricular and parenteral (i.p.) administation of Cr.

Thus, although oral and parenteral administration of Cr does probably have some therapeutic usefulness, a modified Cr molecule that more easily crosses the blood-brain barrier would probably have a better therapeutic value. Some of us are currently involved in a multicenter effort to identify such a molecule.

Conclusions

Pretreatment with creatine is effective in improving severe anoxic or ischemic damage that is caused by

anoxic depolarization. Milder anoxic damage, such as post-anoxic hyperexcitability, is probably not affected in a significant way by creatine pretreatment. However, literature data suggest that creatine is effective in improving other types of damage which are caused by impairment of energy metabolism.

Creatine is a very hydrophilic molecule, therefore it crosses poorly the blood-brain barrier. It has been administered p.o. with some success, and its administration by intracerebroventricular or intra-peritoneal way does afford brain concentrations of phosphocreatine in the therapeutic range. However, it would be useful to obtain creatine-derived molecules that cross the blood-brain barrier in a better way. A search for these molecules is currently in progress.

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