Anoxic ATP depletion in neonatal mice brainstem is prevented by creatine supplementation

B Wilken, J M Ramirez, I Probst, D W Richter, F Hanefeld

Abstract

Background—Sufficient ATP concentrations maintain physiological processes and protect tissue from hypoxic damage. With decreasing oxygen concentration, ATP synthesis relies increasingly on the presence of phosphocreatine.

Aim—The effect of exogenously applied creatine on phosphocreatine and ATP concentrations was studied under control and anoxic conditions.

Methods—Pregnant mice were fed orally with creatine monohydrate (2 g/kg body weight/day). Brainstem slices from these mice pups were compared with those from pups of non-creatine supplemented pregnant mice. Measurements were performed under normoxic and anoxic conditions. In addition, brainstem slices from non-creatine treated mice pups were incubated for 3 hours in control artificial cerebrospinal fluid (CSF) (n = 10) or in artificial CSF containing 200 µM creatine (n = 10). ATP and phosphocreatine contents were determined enzymatically in single brainstem slices.

Results—ATP concentrations were in the same range in all preparations. However, there was a significant increase of phosphocreatine in the brainstems from pups of creatine fed mice when compared with the brainstems of pups from non-creatine treated mice or in non-incubated brainstems of control animals. After 30 minutes anoxia, ATP as well as phosphocreatine concentrations remained significantly higher in creatine pretreated slices compared with controls.

Conclusion—The data indicate that exogenous application of creatine is effective in neuroprotection.

The brain is one of the most metabolically active organs and depends on a continuous supply of energy to stabilise ionic homeostasis, energy consuming biochemical reactions, and physiological processes. The primary energy source is ATP generated via oxidative phosphorylation of NADH within mitochondria, which requires a sufficient oxygen supply. Under hypoxic conditions, ATP is primarily supplemented by the phosphocreatine pool, before anaerobic glycolysis is activated, leading to increased production of lactate and H+.

Experimental setup

ATP and phosphocreatine contents in brainstem slices obtained from neonatal animals (P2) (n = 60) were determined enzymatically. In a first experimental setup, pregnant mice were fed orally with creatine monohydrate (2 g/kg body weight/day) (Sigma, Deisenhofen, Germany) throughout pregnancy (20 ± 1 day). Brainstem slices (600 µM thick) from these mice pups (n = 10) were compared with those from pups born to non-creatine supplemented pregnant mice (n = 10). Thereafter, mice pups from controls (n = 10) as well as from creatine treated animals (n = 10) were exposed to anoxia for 30 minutes.

In a second series of experiments, brainstem slices from non-creatine treated mice pups were incubated for three hours in control artificial cerebrospinal fluid (CSF) (n = 10) or in artificial CSF containing 200 µM creatine (n = 10).

Preparation

Technical details of the transverse brainstem slice preparations have been described previously. The brainstem was isolated in ice cold artificial CSF and secured in a vibratome with its rostral end directed upwards. Slices were sectioned serially until the rostral
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Table 1 ATP concentrations for controls and supplemented pups under normoxic and anoxic conditions

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Creatine fed mothers</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute</td>
<td>Relative</td>
<td>Absolute</td>
</tr>
<tr>
<td>Normoxic</td>
<td>9.4 (0.1)</td>
<td>9.7 (0.4)</td>
<td>+0.3</td>
</tr>
<tr>
<td>Anoxic</td>
<td>5.1 (0.2)</td>
<td>9.0 (0.1)</td>
<td>+3.9</td>
</tr>
<tr>
<td>Difference</td>
<td>−4.3</td>
<td>54%</td>
<td>−0.7</td>
</tr>
</tbody>
</table>

Values are mean (SE). Absolute values in µmol/wet weight.

Results

Using enzymatic tests, ATP values did not differ significantly between normal brainstem slices and those from mice pups of animals supplemented with creatine during pregnancy. Table 1 gives the ATP concentrations. There were also no significant changes in ATP concentrations when slices were incubated for three hours in artificial CSF or artificial CSF containing 200 µM creatine: mean (SE) ATP concentrations were 7.2 (0.2) µmol/g in control slices and 7.5 (0.2) µmol/g in slices incubated in creatine supplemented artificial CSF. Both sets of values were significantly lower than in the group receiving oral supplementation via the mother animals.

After 30 minutes anoxia, there was a 54% decrease in ATP values in neonatal control slices, whereas there was only an 8% decrease in ATP values in the creatine pretreated slices (p < 0.05) (fig 1).

The mean (SE) phosphocreatine concentration was 0.8 (0.1) µmol/g in control slices and thus significantly different from the values found in slices obtained from pups of mother animals fed with creatine (mean, 1.9; SE, 0.1 µmol/g; p < 0.05). After three hours creatine incubation, the mean (SE) concentration of phosphocreatine increased from 2.4 (0.3) µmol/g to 3.2 (0.4) µmol/g (p < 0.05; fig 2).

Biochemical analysis

The rationale of the biochemical analysis was to determine the ATP, ADP, phosphocreatine, and creatine contents of brainstem slices to obtain information about the creatine kinase regulated transfer of phosphate from phosphocreatine to ADP, which results in de novo production of ATP.

Immediately after withdrawal from the recording chamber all slices were homogenised by ultrasonic in 250 µl 8% perchloric acid and centrifuged for 15 minutes at 15 500 g. The supernatant fluid was neutralised by the addition of KHCO₃, and recentrifuged. ATP and phosphocreatine was measured enzymatically by adding 30 µl of a 100 mM triethanolamin hydrochloride stem solution (containing 7 mM MgCl₂, 2 nmol (4 µl of 0.5 mM) NADP solution, 12 nmol ADP (4 µl of 3 mM) solution, 8 nmol glucose (4 µl of 2 mM), and 1 µl G6P-DH) to 100 µl of the homogenised brainstem slices. The enzymatic reaction was started with 0.5 µl hexokinase. ATP was measured using a photometer with light absorption at 366 nm. After the end of the biochemical reaction, 20 µl creatine kinase was added to measure phosphocreatine contents. All ATP and phosphocreatine values are given in µmol/g wet weight. Significant differences were determined by the Student’s t test.
The most pronounced preservation of phosphocreatine values was seen in incubated slices. After 30 minutes anoxia, the mean (SE) phosphocreatine concentration was 1.4 (0.1) µmol/g in creatine pretreated slices, whereas the phosphocreatine was almost completely depleted (mean, 0.1; SE, 0.1 µmol/g) in control slices (p < 0.05; fig 2).

**Discussion**

Using biochemical analysis, we found that oral treatment of pregnant mother animals with creatine or incubation of brainstem slices in creatine containing artificial CSF increased phosphocreatine concentrations and stabilised ATP values during anoxia in brain tissue of neonatal mice. ATP concentrations were lower, but not significantly different, in slices incubated for three hours either in normal or in creatine containing artificial CSF. However, phosphocreatine concentrations were significantly higher after slices were incubated for three hours either in normal or in creatine containing artificial CSF. ATP concentrations were decreased in both groups after incubation for three hours. We interpret this finding to be the result of the low metabolic activity of slices held at a temperature of 27°C.

After 30 minutes anoxia, ATP was significantly reduced (54%) in control brainstem slices, while there was only a slight decrease of ATP values (8%) in slices that were pretreated with creatine. In addition, phosphocreatine was only slightly decreased in creatine pretreated slices, whereas it was almost completely depleted after 30 minutes anoxia in control tissue.

Physiological processes and life rescuing mechanisms—for example, the highly energy demanding hypoxic augmentation of respiratory activity depend on a sufficient energy supply and hence a sufficient phosphocreatine pool. Under normal conditions, phosphocreatine decreases rapidly to 40–50% during hypoxic conditions, which is accompanied by a comparable fall in ATP values, indicating failure of ATP synthesis. Such energy depletion is correlated with the onset of hypoxic depression of the central respiratory network, as Pierard and co-workers have shown by magnetic resonance spectroscopy. This neural response can easily be explained by the blockade of synaptic interaction within the network. Inhibitory synaptic transmission seems to be more sensitive to hypoxic stress, which leads to the danger of excitotoxicity of excitatory amino acids. Excessive excitation could lead to the massive influx of cations, which can lead to severe damage to neurones. This is particularly threatening to neonatal animals in which compensatory rescue mechanisms are underpowered. The immature isof orm of the NMDA (N-methyl-D-aspartate) receptor reveals a higher open probability, and its deactivation is slower than that of adult NMDA receptors, resulting in an enhanced Ca²⁺ influx. This may be potentiated by a Ca²⁺ influx through depolarisation activated L-type Ca²⁺ channels. Immature brain tissue might not be able to cope with such a Ca²⁺ overload. The reason is that creatine kinase is three to six times less active in immature than in mature animals, which results in a limited energy pool. The responsible cellular mechanisms for this observation are summarised in the scheme shown in fig 3.

In immature as well as in mature animals, cell death does not occur as long as the ATP content remains above 25% of control. This has important clinical implications, because stabilisation of a sufficient ATP content could be neuroprotective. ATP is needed for energy consuming processes such as 3Na⁺/2K⁺ and Ca²⁺ pumping, which are the key processes for ionic homeostasis determining neuronal excitability and synaptic transmission. Inhibitory and excitatory synaptic transmission should also be protected by increasing ATP supply. This is consistent with the finding that preincubation of neocortical cells with creatine also had a pronounced protective effect against hypoxic depression of synaptic transmission.

In conclusion, our data demonstrate that hypoxic energy failure in neonatal mice can be prevented by exogenous creatine applied before the hypoxic event. From this observation, the working hypothesis is derived that creatine might also be neuroprotective in humans.
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7 Whittingham TS, Lipton P. Cerebral synaptic transmission during anoxia is protected by creatine. *J Neurochem* 1985;43:1618–21.
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