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Creatine Amides: Perspectives for Neuroprotection

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The neuroprotective activity of creatine amides – amino acid derivatives of creatine – was studied in a model of cerebral ischemia created by transient occlusion of the middle cerebral artery (OMCA) in rats. The neuroprotective effect of treatment with glycine (creatinylglycine ethyl ester acetate), phenylalanine (creatinyl-L-phenylalaninamide acetate), tyrosine (creatinyl-L-tyrosinamide acetate), and gamma-aminobutyric acid (creatinyl-γ-aminobutyric acid ethyl ester acetate) derivatives were studied after administration 1 h before 30-min cerebral ischemia. Brain damage was assessed by staining brain slices with triphenyltetrazolium chloride 48 h after ischemia. As compared with the control group, all the creatine amides studied significantly decreased the sizes of areas of brain damage ($p < 0.05$). However, the amides of creatine with tyrosine, phenylalanine, and GABA showed marked toxicity after intravenous administration and could only be used intraperitoneally. The amide of creatine and glycine was not toxic on administration of intravenous boluses at a dose of 1 mmol/kg. Conclusions: amides of creatine and amino acids decrease ischemic damage after transient occlusion of the middle cerebral artery in rats.

Keywords: creatine derivatives, cerebral ischemia, neuroprotection.

The search for and development of pharmacological agents with neuroprotective actions have acquired a particular relevance in recent years. Ischemic brain damage is one of the most common and severe diseases of the cardiovascular system and leads to high levels of disability and death. The multifactorial nature of the ischemic cascade [12] means that there are several biological targets where influences might have neuroprotective effects. Thus, one of the strongest mechanisms damaging cells is ischemic damage leading to insufficient oxygen supply to the tissues, energy deficit, and cell death. Energy deficit is directly linked with decreases in the quantity of ATP in neuron mitochondria, which is one of the key causes of damage and subsequent death.

Increases in ATP content in brain cells can be achieved using creatine, which is converted to creatine phosphate by the specific enzyme creatine kinase, and this compound is directly involved in ATP formation – an important component of the process of oxidative phosphorylation of glucose [21].

The literature contains few data on the use of creatine in experimental cerebral ischemia (hypoxia) [20].

Experimental studies have demonstrated that creatine increases the formation of creatine phosphate in nerve cells and has significant neuroprotective actions in ischemic (hypoxic) damage to brain tissue [5]. However, data from the same authors indicate that creatine shows inadequate penetration of the blood-brain barrier, such that only high doses used over long periods have effective neuroprotective actions in vivo.

Thus, the development of agents able to increase cell survival in conditions of energy deficit is of great importance. The use of such agents has potential to decrease the mortality of ischemic strokes, myocardial infarcts, and other diseases associated with ischemic damage to various organs.

Thus, the search for creatine derivatives able to cross the blood-brain barrier and exert neuroprotective effects in cerebral ischemia is a very relevant task.

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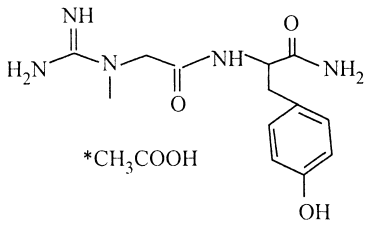
The aim of the present work was to evaluate the neuroprotective effects of new creatine derivatives – creatine amide derivatives of natural L-amino acids [1] in experimental cerebral ischemia in rats.

Methods

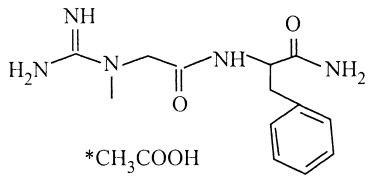
All experiments were performed in compliance with the "Guidelines for the Care and Use of Laboratory Animals" (National Institutes of Health publication No. 85-23, USA) and the "Guidelines for Experimental (preclinical) Study of New Pharmacological Substances," chief editor R. U. Khabriev, 2nd edition (2005).

Studies were performed on male Wistar rats aged 14–16 weeks and weighing 220–260 g (Russian Academy of Sciences, animal house of the I. P. Pavlov Institute of Physiology, Koltushi). Animals were kept on standard lab rat diet K-120 (Inform-Korm, Russia) with and water ad libitum.

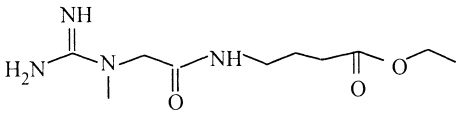
Study agents. The following creatine derivatives (agents) were studied:



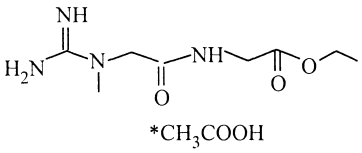
creatinyl-L-tyrosinamide acetate (VR-I)



creatinyl-L-phenylalaninamide acetate (VR-II)



creatinyl-γ-aminobutyric acid ethyl ester acetate (VR-III)



creatinyl-L-glycine ethyl ester acetate (VR-VI)

Creatine derivatives were synthesized using standard methods of peptide synthesis as described previously [1]; derivative identities were confirmed by NMR spectroscopy. The contents of substances in formulations were assessed by anhydrous titration and were at least 98%.

Test compound solutions were prepared using water for injection. Doses were determined by tolerance. All agents were given intraperitoneally (i.p.); if tolerance was good, agents were also given as intravenous (i.v.) boluses. Compounds were dissolved immediately before injections. Injections were given i.p. or i.v. 60 min before ischemia or during the last 5 min of 30-min ischemia and the first 10 min of postischemic reperfusion (during onset of ischemia).

Modeling of cerebral ischemia. All operative interventions were performed under sodium thiopental anesthesia (60 mg/kg, i.p.). Endovascular occlusion of the middle cerebral artery was performed as described by Koizumi et al. [10] as modified by Longa et al. [13] and Belayev et al. [7]. Midline incisions were made on the rats' necks to expose the anterior belly of the digastric muscle, which was resected to expose the bifurcation of the common carotid artery. The external carotid artery was carefully separated and its distal segment was ligated; a ligature was placed at the proximal part close to the bifurcation; the lingual artery, running from the external carotid artery, was ligated and transected. A clip was placed on the common carotid artery and the isolated segment of the external carotid artery was punctured. A 3-cm 4-0 propylene monofilament with an end treated with silicone and poly-L-lysine was inserted through the opening. The filament was passed from the external carotid artery through the bifurcation into the internal carotid artery to a distance of 20 mm until resistance was felt. Using this method, the side of the introduced filament covers the opening of the middle cerebral artery, producing cerebral ischemia. After ischemia, the filament was withdrawn, the proximal ligature on the external carotid artery was tightened to prevent bleeding from the opening, and the wound was sutured. A relative disadvantage of this method is the 20% probability of subarachnoid hemorrhage. Further assessment of brain damage excluded animals with subarachnoid hemorrhage.

Experiments used standard durations of ischemia (30 min) and reperfusion (48 h).

Evaluation of the extent of damage. Animals were sacrificed, brains were extracted, and, after separation from the cerebellum and olfactory bulbs, were cut into five parts to prepare standard frontal slices of thickness 2 mm. The areas of damage zones were identified using standard methods. Frontal brain slices were incubated in 0.1% triphenyltetrazolium chloride solution (TTC, MP Biomed, USA) at 37°C for 15 min. A special apparatus was then used to obtain digital photographs of the anterior and posterior surfaces of the slices, and these were processed in ImageJ to identify the relative area of damage for each side of each slice; the mean for each slice was then calculated, along with the mean damage area for the whole brain.

Because of significant decreases in the activity of NAD-dependent respiratory mitochondrial enzymes (including dehydrogenases) in the damage (necrosis) zone, these areas

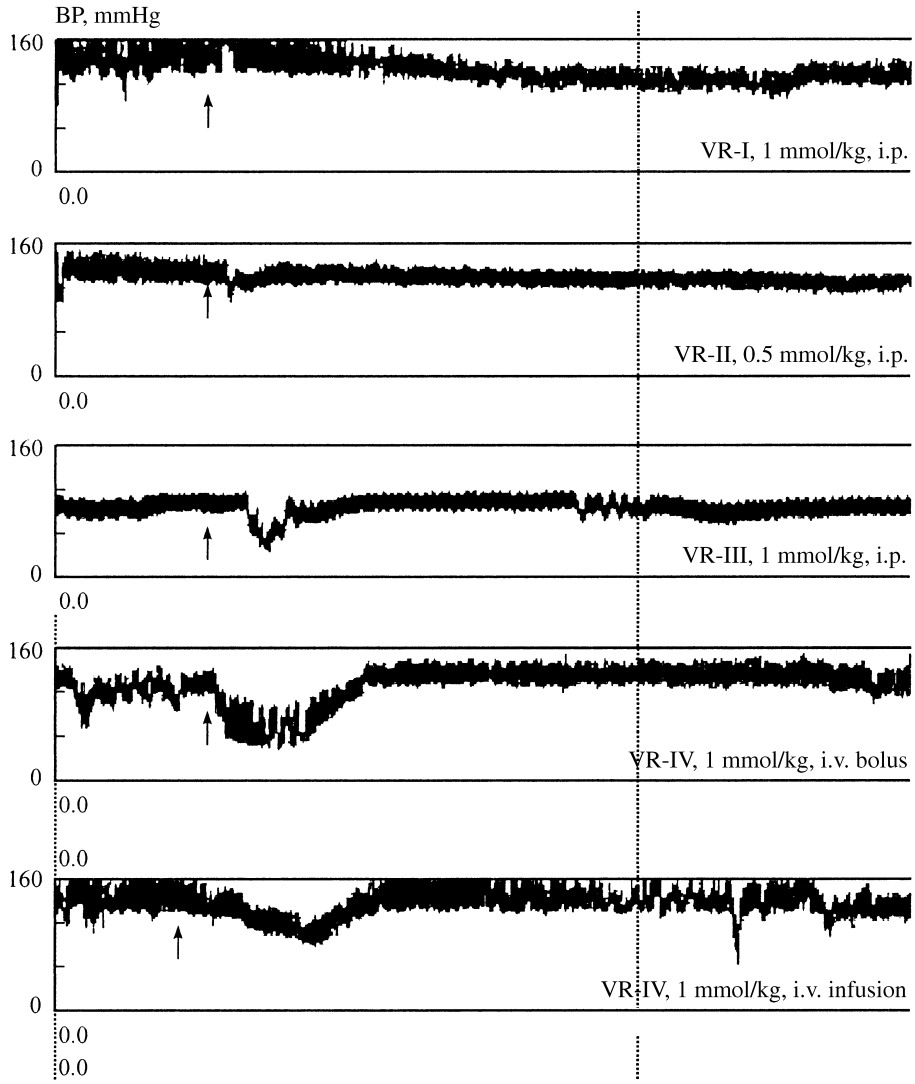


Fig. 1. Changes in arterial blood pressure after administration of study agents.

remained colorless while unaltered tissue acquired a red color as a result of conversion of TTC to the colored product formazan.

The extent of brain damage was also assessed indirectly, in terms of the coefficient of hemisphere asymmetry. Images of brain slices were displayed on the computer screen and the percentage ratio of the area of the damaged hemisphere (S_{dh}) to the total area of the whole frontal brain slice (S_{total}) was determined. In healthy animals, this coefficient is 50%.

Effects of agents on systemic hemodynamics. Rats were placed on a thermostatted bench and a cannula connected to an arterial blood pressure probe (Baxter, USA) was inserted into the carotid artery for display of arterial pressure on the computer and subsequent processing using the program PhysExp 3.0 (St. Petersburg). Agents were given after stabilization of arterial pressure and changes in arterial pressure were recorded over the following 40–60 min.

Statistical analysis. Results were analyzed statistically in StatSoft Statistica 6.0 Multilingual. Significant differences in parameter values were identified using the nonparametric Mann–Whitney test for independent sets. All values are presented as mean ± standard deviation. Results for which p was less than 0.05 were taken as significant.

Results

The following groups of animals were studied:

Controls. Animals were given i.p. ($n = 2$) or i.v. ($n = 4$) 0.9% NaCl solution (1 ml) with 30-min cerebral ischemia being produced 1 h later as described above.

1. VR-I. At a dose of 1 mmol/kg, this was not tolerated by animals when given i.v. (100% mortality in the first minutes after administration ($n = 3$)), but was well tolerated when given i.p. (0% mortality 1 h post-treatment) and was studied using this route of administration.

TABLE 1. Measures of Brain Damage in Rats with Experimental Ischemia/Postischemic Reperfusion on the background of Administration of Agents 60 min before Ischemia (groups 1–4) and during Its Development (group 4a)

Group of animals	Agent	Parameter, %		
		S_{dh}/S_{total}	S_{dz}/S_{total}	S_{dz}/S_{dh}
Control	Control ($n = 6$)	51.8 ± 0.6	17.0 ± 1.9	32.8 ± 3.3
1	VR-I, 1 mmol/kg, i.p. ($n = 5$)	50.6 ± 1.8	10.3 ± 1.0**	20.3 ± 1.7**
2	VR-II, 0.5 mmol/kg, i.p. ($n = 5$)	49.5 ± 2.3	11.9 ± 2.7**	23.9 ± 4.9*
3	VR-III, 1 mmol/kg, i.p. ($n = 5$)	52.3 ± 1.1	12.5 ± 2.4*	23.8 ± 4.3*
4	VR-IV, 1 mmol/kg, i.v. ($n = 5$)	50.4 ± 1.2	8.4 ± 0.7**	16.7 ± 1.0**
4a	VR-IV, 1 mmol/kg, i.v. ($n = 5$)	50.3 ± 1.6	8.8 ± 1.1**	17.6 ± 2.4**

Note. Significance: $*p < 0.05$; $**p < 0.01$. S_{dh}/S_{total} – area of damaged hemisphere (S_{dh}) as percentage of total frontal brain slice area; S_{dz}/S_{total} – area of damaged zone (S_{dz}) as percentage of total frontal brain slice area; S_{dz}/S_{dh} – area of damage (S_{dz}) as percentage of area of damaged hemisphere (S_{dh}).

2. VR-II. A dose of 1 mmol/kg was not tolerated when given i.v. (100% mortality in the first minutes after administration ($n = 3$)), and was poorly tolerated when given i.p. at a dose of 1 mmol/kg (66% mortality ($n = 3$)). This agent was therefore studied at a dose of 0.5 mmol/kg given i.p. This dose was well tolerated (0% mortality 1 h post-treatment).

3. VR-III. A dose of 1 mmol/kg was not tolerated when given i.v. (100% mortality in the first minutes after administration ($n = 3$)) but was well tolerated at a dose of 1 mmol/kg given i.p. (0% mortality 1 h post-treatment).

4. VR-VI. A dose of 1 mmol/kg was well tolerated (0% mortality 1 h after administration) when given i.v. This agent was therefore studied after i.v. bolus administration.

Effects of agents on systemic hemodynamics. Agents VR-I, VR-II, and VR-III. Arterial pressure was measured following i.p. administration of VR-I at a dose of 1 mmol/kg, VR-II at a dose of 0.5 mmol/kg, and VR-III at a dose of 1 mmol/kg. No significant influences of agents on systemic hemodynamics in rats were found and their arterial pressure remained stable for 40 min after administration (Fig. 1).

Agent VR-VI. Both i.v. boluses and slow i.v. infusions at a dose of 1 mmol/kg were accompanied by marked transient hypotensive effects, with reductions in blood pressure by 40–50 mmHg and complete normalization of arterial pressure by 2–4 min after the end of administration and maintenance of normal BP to the end of the experiment.

Sizes of zones of ischemic brain damage. The results from this series of experts are shown in Table 1.

The control group showed no differences in any parameters after i.v. ($n = 4$) and i.p. ($n = 3$) administration of physiological saline, so the results presented in Table 1 and Fig. 2 are combined controls ($n = 6$). All animals showed significant brain damage. The areas of lesions constituted 32.8 ± 3.3% of the area of the damaged hemisphere and 17.0 ± 1.9% of the total area of the frontal slice (damaged and healthy hemispheres). In addition, there were increases in the size of the damaged hemisphere, apparent as a change in the coefficient of asymmetry, which was 51.8 ± 0.6% versus 50% in intact rats. This provides evidence of cerebral edema on development of ischemic damage.

The results presented in Table 1 show that all study agents led to significant decreases in the area of brain damage in experimental ischemia. The most marked neuroprotective effect was seen with VR-VI, administration of which led to a two-fold reduction in damage area from that in the control group (Fig. 2).

The degree of edema of the lesioned hemisphere, assessed in terms of the coefficient of asymmetry, also varied significantly. Thus, significant reductions in edema in the lesioned hemisphere were seen only after administration of VR-I, VR-II, and VR-VI. VR-III produced no significant change in this parameter.

The severity of overall cerebral edema was assessed as the percentage water content of brain tissue after drying for five days at 80°C.

No significant differences were seen in the total water content of the left and right hemispheres as compared with controls. The spread of data appeared to be associated with the formation of damage in this model of cerebral ischemia and the development of general edema.

Discussion

Many agents with different spectra of action have been proposed as neuroprotectors in ischemic brain damage: glutamate receptor antagonists [3], erythropoietin [23], and others, including agents whose actions are directed to restoring cellular energy potential.

One approach to neuroprotection consists of using macroergic molecules such as creatine phosphate, which published data indicate have cytoprotective actions in ischemia/reperfusion of the myocardium [2, 19]. The main aim of this action is to increase the cellular energy potential resulting from the accumulation of intracellular phosphocreatine, which is relevant in conditions of ischemic energy deficit. However, addressing the efficacy of delivering creatine to the ischemic damage zone is a very "narrow" view of its use as a neuroprotector. Creatine molecules are hydrophilic, so passage of creatine through the blood-brain barrier and neuron membrane requires a specific creatine transporter [14]. Despite endogenous creatine synthesis in glial cells and its

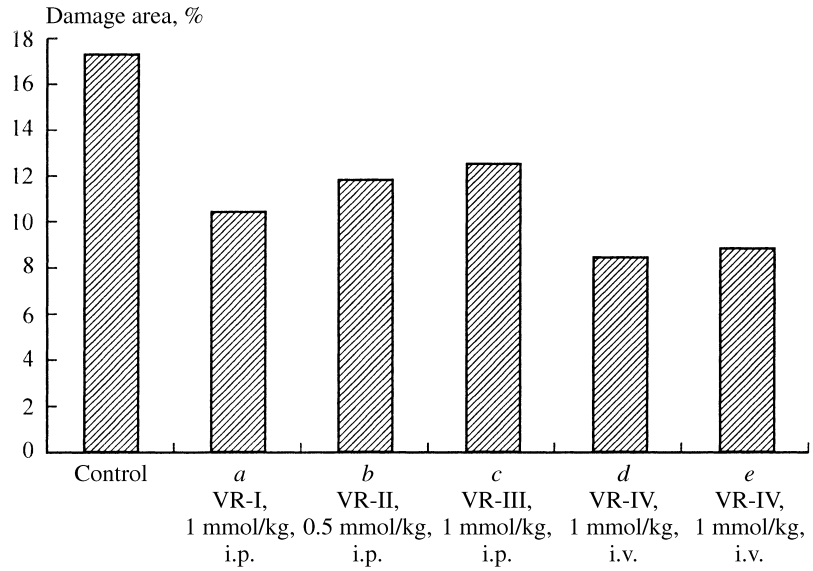


Fig. 2. Ratios of the areas of ischemic brain damage in rats to the total areas of frontal slices (damage area, %). Administration of agents: *a–d*) 60 min before ischemia; *e*) during the onset of ischemia. Differences between each of the experimental groups and the control group were significant ($p < 0.05$).

subsequent delivery to neurons [25], the transport of creatine into the brain is, according to some data, its most important source for neurons [16]. Notwithstanding the existence of a specific mechanism for creatine transport into the brain, its delivery from the blood is limited [18]. Thus, it is important to find physiologically active creatine derivatives better able to cross the blood-brain barrier. The structures of creatine derivatives are important in this regard. Thus, the creatine transporter (CRT) has been shown not to be able to transport creatine benzyl ester or a phosphocreatine-magnesium acetate complex [14].

The present report describes studies of new creatine derivatives – amides with natural L-amino acid derivatives. The results suggest that these compounds can penetrate into brain tissue because of their hydrophobicity and by means of different types of transporter. Natural amino acids are known to cross barriers, including cell membranes, using special transporters: phenylalanine and tyrosine using a common neutral L-type amino acid transporter (LAT) and GABA and glycine using specific transporters – GAT [22] and GlyT [9], respectively.

The experiments reported here show that relatively high doses (0.5–1 mmol/kg of creatine-tyrosinamide acetate (VR-I), creatinyl-phenylalaninamide acetate (VR-II), and creatinyl-γ-aminobutyric acid ethyl ester acetate (VR-III) have marked toxic actions when given as i.v. boluses. This suggests that penetration of these creatine derivatives through the blood-brain barrier on rapid creation of high blood concentrations is unhindered, indirectly supporting the involvement of transporter systems other than CRT operating with creatine amides. Such compounds may either be direct substrates of creatine kinase or be cleaved to form free creatine and the corresponding amino acids both in the bloodstream and in the brain. The amino acids are either neurotransmitters themselves (γ-aminobutyric acid, glycine) or serve as substrates for the formation of neurotransmitters (tyrosine, phenylalanine). Sharp increases in the concentrations of these amino acids in the brain can lead to the formation of the corresponding neurotransmitters (dopamine) or produce direct effects on receptors (GABA, glycine). This suggestion is consistent with data reported by other authors. Thus, Sved et al. [24] reported that i.p. tyrosine led to rapid decreases in arterial blood pressure in spontaneously hypertensive rats, which the authors linked with changes in neurotransmitter activity. The experimental results also showed that s.c. administration of tyrosine to rats led to rapid increases in dopamine formation in the brain [17]. Excess aromatic amino acids are known to lead to sharp increases in their transport, as demonstrated in [4]. These data are consistent with our experimental results obtained after administration of creatinyl-tyrosinamide acetate (VR-I) and creatinyl-phenylalaninamide acetate (VR-II). The toxic effects obtained on i.v. administration of creatinyl-γ-aminobutyric acid ethyl ester acetate may be associated with the fact that even minor overdosage of GABA receptor agonists leads to CNS impairment, reaching the level of coma [15], as seen in our case. Only creatinyl-glycine ethyl ester acetate (VR-VI), given both i.v. and i.p. at a dose of 1 mmol/kg, had no apparent toxic effects. The side effects of overdosage with free glycine, described in the literature, such as reductions in arterial blood pressure, vomiting, and impairments to consciousness [6], were not seen using CrGlyOEt.

All the creatine amides studied had marked neuroprotective actions. In general, the effects of creatine derivatives were consistent with results obtained from studies of free creatine as a neuroprotector in cerebral ischemia [11, 19]. Creatine derivatives entering the rat brain immediately before ischemia or at the moment ischemia ended and reperfusion started (group 4a) appear to have acted as a substrate for creatine kinase or to have formed, via hydrolysis, free amino acids and creatine, stimulating the formation of phosphocreatine and hence ATP, thus promoting increases in neuron survival.

Of all the agents tested, the most marked cytoprotective activity was obtained with creatinylglycine ethyl ester acetate (VR-VI). It is impossible to exclude the possibility that glycine had an additional positive influence, this being formed as a result of the metabolism of the study compound, as glycine is known to correct NMDA receptor dysfunction with a resultant neuroprotective effect [8].

Further study of the properties of creatine derivatives is of interest in relation to developing agents effective in the treatment of ischemia, as well as post- and pre-ischemic disorders.

Conclusions

Intraperitoneal administration of creatinyl-L-tyrosinamide acetate, creatinyl-L-phenylalaninamide acetate, and creatinyl-γ-aminobutyric acid ethyl ester acetate 1 h before cerebral ischemia produced significant neuroprotective effects.

Intravenous administration of creatinyl-L-tyrosinamide acetate, creatinyl-L-phenylalaninamide acetate, and creatinyl-γ-aminobutyric acid ethyl ester acetate led to instant severe systemic toxicity, which may be evidence for the rapid penetration of creatine amides into brain tissue. This effect was virtually absent on i.p. administration.

Creatinylglycine ethyl ester acetate had significant neuroprotective effects on both i.v. administration 1 h before ischemia and when given at the end of ischemia and the beginning of the reperfusion period. This agent had no appreciable toxic effect when given as i.v. boluses.

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