Neuroprotective Activity of Creatylglycine Ethyl Ester Fumarate

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Background: We have recently shown neuroprotective activity of the creatine amides in the focal cerebral ischemia in rats on the 280 mg/kg administration. In the present study, neuroprotective properties of creatylglycine ethyl ester fumarate (CrGEt) in rats with focal cerebral ischemia were explored in a wide dosage range (30-280 mg/kg, intravenous and intragastric). *Methods:* Focal cerebral ischemia was induced by the middle cerebral artery occlusion (MCAO). *Results:* The CrGEt administration 30 minutes before and at the last 5 minutes of MCAO dose dependently attenuated cerebral ischemic damage on 35%-65%, reduced neurobehavioral deficits, led to high neuronal survival in ischemic rat brains. The neuroprotective activity of CrGEt was mediated by its following abilities: (1) normalize the energy metabolism in the ischemic brains, maintaining adenosine triphosphate levels, and reducing lactate concentration; (2) inhibit the ischemia-reperfusion–related oxidative stress as evidenced by the increased activity of superoxide dismutase and the reduced levels of malondialdehyde. CrGEt served as a substrate for creatine kinase and a partial agonist of N-methyl-D-aspartate receptors; this partly explains mechanism of its neuroprotective action. *Conclusions:* In view of the previously mentioned results, CrGEt holds a promise as a compound for treatment of ischemic brain disorders. **Key Words:** Creatine derivatives—cerebral ischemia—neuroprotection—neuroprotective activity.

Primary brain damage during ischemia is characterized by energy failure, overload of intracellular Ca^{2+} and Na^{+} ions, membrane depolarization, glutamate excitotoxicity, activation of anaerobic glycolysis, lactic acidosis, and activation of free radical processes.[1] Subsequent pathologic processes cause cell death. The interval between the primary changes and the secondary events provides an opportunity for effective therapeutic intervention.[2-4]

Significant progress has been made in the prevention of ischemic stroke and in the postischemic treatment to restore the functions of brain cells.[5] In contrast, therapeutic possibilities for acute ischemic stroke are very limited.[6] In this regard, modern pharmacology seeks to develop drugs that can be used for both the preventative and for the acute phase of the disease. From this point of view, substances that enhance brain energy metabolism, restore the cerebral blood flow, and possess antioxidant activity are of critical importance.

Our recent studies showed neuroprotective activity of the creatine amides in the focal cerebral ischemia in rats on the 280 mg/kg administration.[7-8] In the present

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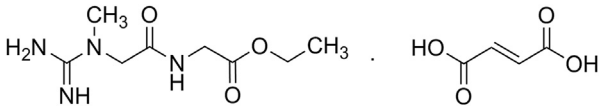

Figure 1. *Structure of creatylglycine ethyl ester fumarate (CrGEt).*

study, we explored the neuroprotective activity of the creatylglycine ethyl ester fumarate (CrGEt; Fig 1) in a wide dosage range (30-280 mg/kg, intravenous [i.v.] and intragastric) and considered possible mechanisms of the pharmacologic action.

Materials and Methods

Animals

Adult Wistar male rats (13-14 weeks) were obtained from the kennel "Rappolovo" RAS, St Petersburg. All rats were housed in cages (4 rats per cage) under 12-hour light–12-hour dark conditions at 18°C-20°C, relative humidity 50%-70%, with food and water ad libitum. All protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals[9] and in agreement with the national regulations on animal research in the Russian Federation.

After 14-day quarantine, animals were randomly assigned to treatment groups. The animals were weighted (250 ± 20 g) before the drug administration. The exclusion criterion was the low blood flow in the cortical branch of the left middle cerebral artery (systolic <20 cm/second, diastolic least 10 cm/second). The cerebral blood flow was monitored by MiniMax-Doppler-K (MiniMax, St. Petersburg, Russia).

Drugs

CrGEt and creatylglycine were synthesized according to the procedure described previously.[10] The purity of CrGEt and creatylglycine was determined by high performance liquid chromatography (HPLC) and was found to be greater than 98.0% (.05% creatine, .66% creatinine, .82% creatylglycine). Phosphocreatine disodium salt was purchased from Sigma (#P6502; St. Louis, MO) and used as a reference compound.[11] The compounds were dissolved in sterile water.

Drug Administration

Intravenous injections were administered into the lateral tail vein according to the standard procedures. The injection volume was 5 mL/kg of body weight and did not exceed the recommended values for rats.[12] Neither adverse effects, nor pathophysiologic changes, nor deaths of animals were observed in the all CrGEt-treated groups.

The tail was warmed before performing the procedure either by massaging it with warm, wet gauze or by placing the animal under a heating lamp. A rat was transferred into a holding device, and the lateral tail vein was identified on either side. A fine gage needle was inserted into the vein at about a 15°-30° angle to the tail skin. The i.v. drug injections were bolus over 1 minute and infusive over 10 minutes. After removing the needle, gentle pressure was applied to the injection site with gauze for 1-3 minutes or until bleeding stops. Intravenous administration was performed once.

Intragastric administrations were performed according to the Manual of Stroke Model in Rats.[12] A single-handed restraint technique was applied to hold the rat. The nose, head, and spine were aligned to keep the esophagus straight. The needle bulb went over the base of the tongue and gently down without resistance into the esophagus. Once the desired position was achieved, the drugs were administered in a volume of 4 mL per kg of body weight. The procedure was repeated twice a day, during 10 days.

Focal Cerebral Ischemia

Endovascular middle cerebral artery occlusion (MCAO) was induced by the Koizumi method[13] with modifications according to Longa[14] and Belayev.[15] Operational interventions were made under anesthesia with thiopental sodium (60 mg/kg, intraperitoneal). The induced period of ischemia was 30 minutes, resulted in more than 80% decline in cerebral blood flow. Period of reperfusion was 48 hours.

Animals were excluded from the statistical analysis of ischemic brain damage based on the following criteria: (1) the blood flow in middle cerebral artery after ischemia recovered to less than 80% of the prestanding baseline; (2) subarachnoid hemorrhage; (3) carotid artery was damaged during the surgery; and (4) severe inflammation in the wound after the surgery.

Quantification of Ischemic Brain Damage

After 48 hours of the reperfusion period, animals were decapitated. The brains were removed, after clipping of the cerebrum cerebellum and olfactory bulbs, and dissected into 5 parts to obtain standard front sections with a thickness of 2 mm. Areas of the ischemic injury were determined by staining with triphenyltetrazolium chloride (MP Biomed; Santa Ana, CA)[16] and were expressed as a percentage of necrotic area to the area of damaged hemisphere.

Neuronal survival was evaluated by histologic examinations of the brains 48 hours after ischemia. Frontal sections of rat brains performed at bregma (±.5 mm) were stained with toluidine blue according to the Nissl method and used for immunocytochemical reactions for neuronal nuclear antigen NeuN[17] and glial fibrillary acidic protein.[18] The reagents were

purchased from Chemicon-Millipore (Billerica, MA), Dako (Glostrup, Denmark), Biogenex (Fremont, CA).

Evaluation of Neurologic Deficits

Neurologic status of rats was evaluated by Garcia scale.[19] Maximum severity of defects corresponded to the minimum score of this scale—3 points, the absence of the damage to 18 points.

Quantification of Malondialdehyde

Malondialdehyde (MDA) content in brain tissue was determined 30 minutes after MCAO according to the Uchiyama method[20] based on using thiobarbituric acid (Sigma, #T5500).

Quantification of Superoxide Dismutase Activity

The superoxide dismutase (SOD) activity was assayed 30 minutes after MCAO spectrophotometrically (Cary 50; Varian, Mulgrave, Australia) at 505 nm using the xanthine/xanthine oxidase method. The method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye.

Incomplete Cerebral Ischemia. Quantification of Adenine Nucleotides in Brain Tissues

Bilateral occlusion of the common carotid arteries in rats has been established as a procedure to investigate the effects of CrGEt on content of adenosine phosphates in brain.[21] Bilateral occlusion of the common carotid arteries of rats was induced by the standard methods.[21] Briefly, in anesthetized rats (sodium pentobarbital, 45 mg/kg, intraperitoneally), both common carotid arteries were isolated, and a surgical silk loop was placed below the bifurcation for subsequent occlusion of the arteries. Both common carotid arteries were exposed through a ventral cervical U-shaped incision. The arteries were separated from their sheaths and vagal nerves and permanently ligated with 4-0 silk sutures. The induced period of ischemia was 30 minutes.

Extraction of nucleotides from the brain tissue was performed 30 minutes after ischemia, according to the method described by Grob et al.[22] Briefly, immediately after the occlusion, animals were decapitated, and brains were placed in liquid nitrogen. After 30 minutes, the brains were homogenized in 5 mL of an acetonitrile/water mixture (4:6), precooled to −10°C. The homogenate was centrifuged at −15°C for 15 minutes; the supernatant liquid was filtered through a syringe polyvinylidene difluoride membrane filter .45 μm and immediately analyzed by HPLC according to the methodology described by Nedden et al.[23] The chromatography was performed on a Zorbax Bonus RP column (3.5 μm, 3 × 100 mm; Santa Clara, CA) at 25°C and detection at 254 nm. Adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate (ATP) purchased from Sigma (#A4659, #A2754, #A6419, respectively) were used as standards. The amount of total protein in supernatants was determined by the Bradford assay,[24] and the calibration was performed with bovine serum albumin (Sigma, #A9511).

Quantification of Lactate

Endovascular MCAO was induced as described previously. Lactate content in brain extracts was determined 30 minutes after MCAO using the kit from Sigma-Aldrich (# MAK064). Briefly, immediately after extraction, 1 g of rat brain was homogenized in 4 mL of buffer. After centrifugation at 15,000 rpm for 10 minutes, the solution was filtrated through a membrane filter Ultra-15 (Sigma, #Z706345-8 EA) to remove proteins (molecular weight cut-off - 10 kDa). Two mL of the filtrate were mixed immediately with the reagents and analyzed on Multiskan GO microplate reader (Thermo Scientific, Waltham, MA) at 570 nm, as described in the manufacturer's protocol.

Creatine Kinase Activity Assay

The creatine kinase (CK) enzymatic assay was performed in 100 mM glycine buffer pH 9.1 at 25°C and in 50 mM TRIS-HCl buffer pH 7.4 at 25°C in the forward direction, that is, the direction of CrGEt or creatine phosphorylation, according to the procedure originally developed by Tanzer and Gilvarg.[25] The investigated compounds were dissolved in glycine buffer or in TRIS-HCl buffer right before the measurements and mixed with the other components. The typical assay mixture contained .14 mM NADH (Sigma, #43420), 1.0 mM phosphoenolpyruvate (Fluka, St. Louis, MO; #79415), .6 mM ATP (Sigma, A6419), and 1.1 mM magnesium acetate (Sigma, #M5661), whereas the substrate concentration was varied from 14 to 200 mM. The concentrations for the coupling enzymes were 144, 235, and 14 units/mL for pyruvate kinase (Sigma, P9136), lactate dehydrogenase (Sigma, L1254), and CK (Sigma, C3755), respectively. In control experiments, an impact of the CrGEt impurities on the rate of CrGEt enzyme-catalyzed phosphorylation was estimated.

Stability of CrGEt in Artificial Gastric Juice, Human Blood Serum, in the Presence of Trypsin

Stability of CrGEt in artificial gastric juice, human blood serum, and in the presence of trypsin was estimated by HPLC. The concentration of CrGEt in gastric juice and human blood serum varied from 2 to 5 mg/mL. The chromatographic analysis was performed on a Zorbax Eclipse C18 column (3.5 μm, 3 × 100 mm) at 30°C, with a mobile phase of .015 M sodium 1-octanesulfonate/0.03 M sodium diphosphate (pH = 3)-acetonitrile (91:9, v/v). The flow rate was .5 mL/minute; the detection was

at 205 nm. The products of CrGEt hydrolysis were identified by standard samples of creatine (Merck, #841470), creatylglycine (CJSC "Vertex," St. Petersburg, Russia; #1.09.13), glycine (PRS Panreac, Barcelona, Spain; #1413401209), and creatinine (Merck, Darmstadt, Germany; #105206).

The artificial gastric juice was prepared in accordance with USP 37 (ed. 32) to contain 2 g/L NaCl (Sigma, #P6887), 3.2 g/L pepsine (Merck, #EC 3.4.23.1), pH was adjusted to 1.40 ± .05 by HCl.

Human serum was obtained from blood of healthy donors and fibrinogen, and other clotting factors were removed by standard methods.

The trypsin (Sigma, #078K7020) was initially dissolved in 1 mN HCl and, then, was mixed with 46 mM TRIS-HCI buffer (pH 8.1, 11.5 mM $CaCl_2$). Solution of CrGEt in the same buffer was added to the trypsin solution to make the CrGEt/trypsin mass ratio equal 1/20.

Patch-Clamp Recordings

Hippocampal slices were prepared according to the procedure described by Vorobjev.[26] Pyramidal neurons were mechanically isolated from the CA-1 region of the slice by vibrodissociation.[26] Measurements were recorded in the configuration of "whole cell" with an EPC-7 amplifier (Welwyn Garden City, Hertfordshire, England). Transmembrane currents were induced by the L-aspartate applique (antagonism of N-methyl-D-aspartate (NMDA) receptors). Extracellular solution contained 143 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES (pH adjusted to 7.4 using HCl). Pipette solution contained 100 mM CsF, 40 mM CsCl, 5 mM NaCl, .5 mM $CaCl_2$, 5 mM EGTA, and 10 mM HEPES (pH adjusted to 7.2 using CsOH). The experiment and response recordings were controlled by a personal computer. The experiments were conducted at the potential of −80 mV in the absence of magnesium ions.

Animal Groups

Quantification of Ischemic Brain Damage and Evaluation of Neurologic Deficits

Drug administrations: (1) i.v. bolus 30 minutes before MCAO and (2) infusive (15 minutes) starting from the last 5 minutes of MCAO to the first 5 minutes of postischemic reperfusion. Animal groups: (1) control (saline), n = 10; (2) sham, n = 5; (3) CrGEt, 280 mg/kg, n = 12; (4) CrGEt, 140 mg/kg, n = 10; (5) CrGEt, 70 mg/kg, n = 10; and (6) CrGEt, 50 mg/kg, n = 7.

Quantification of Ischemic Brain Damage and Evaluation of Neurologic Deficits

Drug administrations: intragastric, 2 times/day for a 10-day period. Animal groups: (1) control (saline), n = 10; (2) sham, n = 10; (3) CrGEt, 50 mg/kg, n = 9; and (4) CrGEt, 70 mg/kg, n = 9.

Quantification of MDA Content and SOD Activity in Brain Tissues

Drug administrations: i.v. bolus, 30 minutes before MCAO. Animal groups: (1) sham, n = 9; (2) intact, n = 9; (3) control, (saline), n = 9; (4) CrGEt, 50 mg/kg, n = 9; and (5) CrGEt, 70 mg/kg, n = 9.

Quantification of Adenosine Phosphates and Lactate in Brain Tissues

Drug administrations: i.v. bolus, 30 minutes before MCAO. Animal groups: (1) intact, n = 9; (2) sham, n = 9; (3) control (saline), n = 9; (4) CrGEt, 280 mg/kg, n = 9; and (5) phosphocreatine, 255 mg/kg, n = 9.

Immunocytochemical Assessment. Drug administrations

First injection was infusive (10 minutes) starting from the last 5 minutes of MCAO to the first 5 minutes of postischemic reperfusion, second to fourth injections were bolus (1 minute), 15 hours, 40 hours, 54 hours, after MCAO. Animal groups: (1) sham, n = 3; (2) control (saline), n = 4; (3) CrGEt, 70 mg/kg, n = 3; and (4) CrGEt, 50 mg/kg, n = 3.

Statistical Analysis

Statistical analysis was performed using the software package SPSS-13 (IBM Corporation, Armonk, NY). The experimental values were expressed as means ± standard deviations. The statistical significance of the differences was estimated using the nonparametric Mann–Whitney test for independent samples.[27]

Results

Effects of CrGEt on Ischemic Brain Damage and Neurologic Status of Rats

Statistical analysis of ischemic brain damage and neurologic outcomes of the animal groups are summarized in Table 1. The single i.v. injections of CrGEt solution (280 mg/kg, 140 mg/kg, 70 mg/kg, and 50 mg/kg) 30 minutes prior MCAO reduced necrotic areas by 65%, 58%, 47%, and 35%, respectively ($P < .05$). The same dose-dependent activity of CrGEt was observed on its injections during MCAO/reperfusion. The reductions in the ischemic injury constituted 55% for the 280 mg/kg dose, 49% for 140 mg/kg, 47% for 70 mg/kg, and 33% for 50 mg/kg.

In addition, the neurologic deficit observed in the control animals 48 hours after MCAO (12.2 ± 1.9 points on Garcia scale) was markedly minimized in the CrGEt-treated animals (16.6 ± 1.1 points for 280 mg/kg and

Table 1. *Effect of single intravenous CrGEt injections on cerebral ischemic damage and neurologic status of rats*

Animal groups	Necrotic area as percentage of hemisphere area, %		Neurological status on Garcia scale		
	CrGEt injection 30 min before MCAO	CrGEt injection during MCAO	24 h before MCAO	48 h after MCAO	72 h after MCAO
Control, n = 10	38.2 ± 4.1	38.0 ± 3.0	18.0 ± .0	12.2 ± 1.9	15.2 ± 1.2
Sham, n = 5	.1 ± .2	.2 ± 0.2	18.0 ± .0	18.0 ± 1.0	18.0 ± .0
CrGEt, 280 mg/kg, n = 12	13.3 ± 3.1*	17.2 ± 2.4*	18.0 ± .0	16.6 ± 1.1*	17.5 ± .5*
CrGEt, 140 mg/kg, n = 10	16.2 ± 3.3*	19.5 ± 2.1*	18.0 ± .0	15.4 ± .8*	17.1 ± .5*
CrGEt, 70 mg/kg, n = 10	20.2 ± 1.9*	20.2 ± 1.9*	18.0 ± .0	14.7 ± 1.5*	16.8 ± 1.2*
CrGEt, 50 mg/kg, n = 7	25.0 ± 3.1*	25.3 ± 2.8*	18.0 ± .0	14.7 ± 1.9*	16.5 ± 1.3*
Phosphocreatine, 255 mg/kg, n = 7	18.0 ± 2.4*†	21.1 ± 3.0*†	18.0 ± .0	14.9 ± 1.5*	16.3 ± .8*

*$P < .05$ versus control.
†$P < .03$ versus CrGEt, 280 mg/kg.

15.4 ± .8 points for 140 mg/kg). Seventy-two hours after MCAO, the neurologic status of rats, treated with CrGEt in the 280 and 140 mg/kg doses, was restored closely to the status of sham animals. Similar, but less prominent, effects were observed on the CrGEt injections in the smaller doses. The neuroprotective effects of CrGEt at the doses 280 mg/kg (.84 mmole/kg) and 140 mg/kg (.42 mmole/kg) were more pronounced than at the higher dose of phosphocreatine (255 mg/kg, 1 mmole/kg).

Next, neuroprotective activity of CrGEt was examined on its course intragastric administrations (Table 2). In comparison with the control group, the ischemic injury in the CrGEt-treated rats was reduced by 36% (30 mg/kg) and 47% (50 mg/kg). In comparison with the control group, the ischemic injury in the CrGEt-treated rats was reduced by 36% (30 mg/kg) and 52% (50 mg/kg), respectively.

The saline-treated rats showed prominent neurologic deficits 48 hours after reperfusion, 12.3 ± .1 pts. In contrast, a course administration of the CrGEt led to a substantial improvement of the neurologic status of rats, namely 18% and 26% at doses of 30 and 50 mg, respectively ($P < .05$).

To confirm the neuroprotective activity of the CrGEt, immunocytochemical assessment was performed immediately after ischemia/reperfusion (I/R; Fig 2).

As shown in Figure 2, the perivascular edema and the loss of neurons were observed in the brain tissues of the saline-treated rats, and an obviously smaller loss of hippocampal neurons occurred in the brains of the CrGEt-treated rats, particularly in those with dose of 70 mg/kg. These results confirmed the neuroprotective activity of the CrGEt during I/R.

Effects of CrGEt on Energy Metabolism in Brain Tissues

Effects of the CrGEt on concentrations of high energy phosphates and lactate content in ischemic brains were explored (Table 3). A 280 mg/kg injection of CrGEt resulted in a preserve of adenosine phosphates, mainly ATP, and led to the 1.4-fold increase in the brain lactate. A similar single injection of the phosphocreatine before ischemia did not contribute to the maintenance of high levels of phosphates and ATP in particular (Table 3).

To explore the possible mechanism underlying the ability of CrGEt to normalize the energy metabolism in the ischemic rat brains, interactions of CrGEt with CK, the key enzyme involved in the ATP metabolism in brain tissues, was investigated. The enzymatic assay was performed in 50 mM TRIS at pH 7.4 and 100 mM glycine buffers at pH 9.1. The CrGEt was substantially stable in

Table 2. *Effect of the CrGEt course intragastric administrations on cerebral ischemic injury and neurological status of rats before MCAO*

Animal groups	Necrotic area as percentage of hemisphere area, %	Total number of points on Garcia scale		
		24 h before MCAO	48 h after MCAO	72 h after MCAO
Control. n = 10	43.4 ± 3.2	18.0 ± .0	12.3 ± .1	13.1 ± .4
Sham, n = 10	3.9 ± 2.1	18.0 ± .0	18.0 ± .0	18.0 ± .0
CrGEt, 30 mg/kg, n = 9	27.7 ± 2.4*	18.0 ± .0	14.5 ± 1.3*	17.0 ± .5*
CrGEt, 50 mg/kg, n = 9	22.8 ± 2.3*	18.0 ± .0	15.5 ± .8*	17.6 ± 1.1*

†P < .03 versus CrGEt, 280 mg/kg.
*P < .05 versus control.

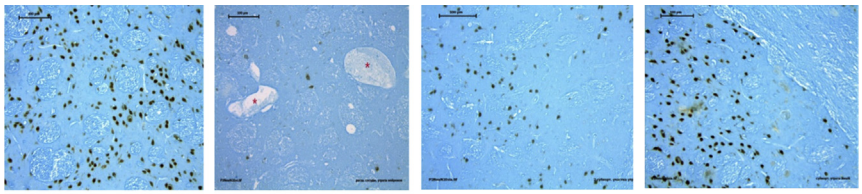

Figure 2. *Effect of creatylglycine ethyl ester fumarate (CrGEt) on neuronal injury in the ischemic cerebral cortex of rats (NeuN immunocytochemistry and astra blue counterstaining). Representative images.*

the solution under investigation, the enzyme–substrate interaction, and the impurity content remained almost constant during the assay measurements. The contribution of the CrGEt impurities on the rate of the CrGEt phosphorylation was also estimated and found to be insignificant, less than 3%, and was accounted in the K_m calculation. To verify the accuracy of the assay procedure, a creatine–CK assay was conducted under the same conditions. The K_m value obtained for creatine (10.0 ± .5 mM) was in a good agreement with the published data.[28]

In Figure 3, the rate of phosphorylation of CrGEt by CK was measured as a function of concentration CrGEt. The reaction followed the Michaelis–Menten kinetics. The found value of K_m was 135 ± 13 mM (n = 2) in TRIS-HCl buffer at pH 7.4 and 123 ± 11 mM (n = 4) in glycine buffer at pH 9.1 (Fig 3, A,B).

Effects of CrGEt on SOD Activity and MDA Content in Brain

To further explore the mechanism of neuroprotective activity of CrGEt, effect of the compound on the levels of the brain antioxidant SOD and MDA, marker of lipid peroxidation processes, were estimated (Fig 4).

In ischemic brains of the control animals, the SOD activity was decreased on 33% (from 47.75 to 31.91 KU/g protein), compared with the sham-operated rats. Pretreatment with CrGEt in the 70 mg/kg and 50 mg/kg doses enhanced the SOD activity on 69% (from 31.91 to 53.86 U/g protein) and on 41% (from 31.91 to 44.94 KU/g protein), respectively.

The MDA content in brain tissues of sham-operated animals was 4.51 μM/g. Ischemia significantly elevated MDA concentration by 44% (from 4.51 to 6.49 μM/g). However, pretreatment with CrGEt in the doses of 70 mg/kg and 50 mg/kg remarkably leads to a significant reduction of the MDA content in brain tissue, namely by 48% (from 6.49 to 3.39 μM/g) and 46% (from 6.49 to 3.5 μM/g), respectively, compared with the control group of rats.

CrGEt in Modulation of NMDA Receptors

Modulation of NMDA receptors by different compounds is widely explored for potential treatment of neurodegenerative diseases and ischemia.[29] In this regard, the CrGEt-to-NMDA receptor interactions were studied in this work (Figs 5 and 6).

In the absence of glycine, CrGEt caused a dose-dependent increase in the stationary component of the response to 100 μM L-aspartate (Fig 5). Maximum potentiating effect was observed at the CrGEt concentrations of 300 μM. The maximum response of CrGEt was 75 ± 5% of the response in the presence of saturating concentrations of glycine or D-serine (10 μM). Activity of CrGEt (EC_{50} = 19 ± 2 μM) was 25 times lower than glycine or D-serine (EC_{50} = .8 ± .1 μM). Thus, action of CrGEt was analogous to the action of glycine, however, with the lower efficacy and affinity.

The specificity of the CrGEt binding to the glycine site of the NMDA receptors was studied next. In the presence of both 10 μM glycine and 300 μM CrGEt, the response of NMDA receptors was reduced by 15 ± 5%, compared with the response in the presence of glycine only. Thus, modulation of NMDA receptors by glycine and CrGEt was nonadditive. Moreover, the reduction in the intensity of the response can be explained by the competition between glycine (a full agonist) and CrGEt (a partial agonist).

Table 3. *Effect of CrGEt on concentrations of adenosine phosphates and lactate in ischemic tissues*

Groups	AMP nmole/mg protein	ADP nmole/mg protein	ATP nmole/mg of protein	L-Lactate nmole/mg protein
Control, n = 9	.21 ± .01*	.57 ± .05*	1.06 ± .10*	690.8 ± 42.1*
Sham (saline), n = 9	.13 ± .01†	.35 ± .03†	2.40 ± .06†	-
Intact animals, n = 9	.14 ± .01†	.33 ± .04†	2.51 ± .05†	430.4 ± 38.0
CrGEt, 280 mg/kg, n = 8	.23 ± .01*	.40 ± .03*†	1.87 ± .10*†	490.8 ± 37.1†
Phosphocreatine, 255 mg/kg, n = 8	.22 ± .02*	.52 ± .06*	1.16 ± .04*	671.1 ± 59.4*

*$P < .5$ versus intact animals.
†$P < .05$ versus control.

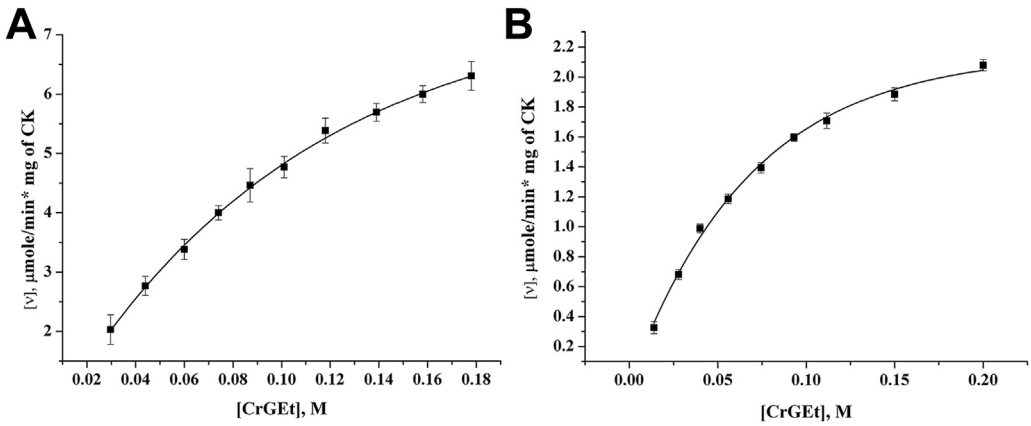

Figure 3. *Michaelis–Menten plot of the creatylglycine ethyl ester fumarate (CrGEt) and creatine kinase (CK) interactions in (A) 50 mM TRIS buffer pH 7.4 and (B) in 100 mM glycine buffer pH 9.1. Data are presented as mean ± standard deviation (n = 4, n = 2).*

To further support the hypothesis, we probed the ability of 7-chlorokynurenic acid, the competitive antagonist of this site, to prevent binding and action of CrGEt. Figure 6 demonstrates that 7-chlorokynurenic acid inhibited the NMDA receptor response in the presence of CrGEt. The inhibitory action of both .3 and 3 μM 7-chlorokynurenic acid was significantly smaller against 300 μM (21 ± 5% and 55 ± 8% inhibition for .3 and 3 μM 7-chlorokynurenic acid, respectively) than against 30 μM of CrGEt (42 ± 6% and 89 ± 7% inhibition for .3 and 3 μM 7-chlorokynurenic acid, respectively).

Taken together, the previously mentioned strongly suggested that CrGEt acts as a partial agonist of the glycine site of the NMDA receptors.

Stability of CrGEt

The stability of CrGEt was tested in artificial gastric juice, human blood serum, in the presence of trypsin (Fig 7). The change in the CrGEt concentration was assessed by HPLC.

The CrGEt decomposition halftime was about 18 hours and 35 hours in human blood serum and in the presence of trypsin, respectively (Fig 7). CrGEt was highly stable in the artificial gastric juice, after 24 hours incubation at 37.0 ± .5°C, the concentration of the CrGEt decreased only on 15%. Metabolites of CrGEt under the studied conditions were creatine, creatinine, glycine, and creatylglycine.

Discussion

Our recent studies revealed the neuroprotective properties of creatine amides at the high dose (280 mg/kg for CrGEt)[7-8]; the current work experimentally confirmed that the CrGEt can manifest its activity at the significantly lower doses. The i.v. and intragastric administrations of CrGEt before ischemia as well as at the early stages of MCAO resulted in a dose-dependent (30-280 mg/kg) decrease in the area of ischemic brain damage and in higher neuronal survival. The study also showed that CrGEt not only acts as neuroprotectant but

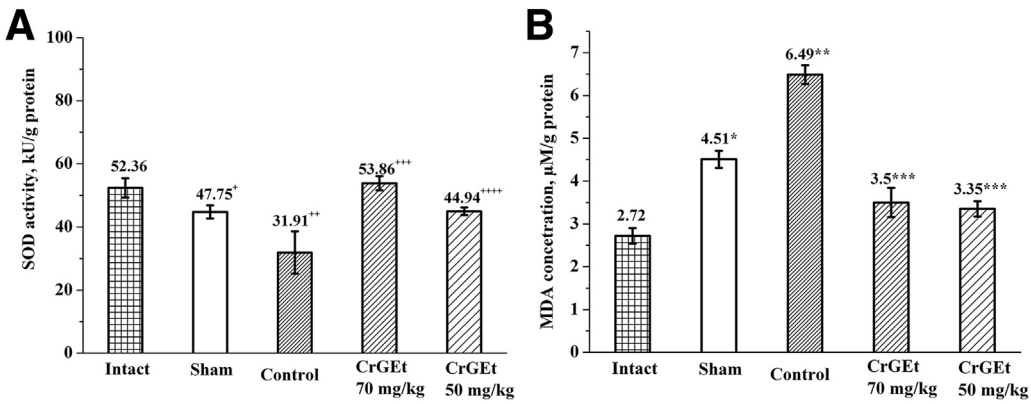

Figure 4. *Effect of creatylglycine ethyl ester fumarate (CrGEt) on (A) superoxide dismutase (SOD) activity and on (B) malondialdehyde (MDA) content in the brain tissues. Data are expressed as the mean ± SD (n = 9).* *P < .01 *versus intact animals;* **P < .01 *versus sham-operated animals;* ***P < .001 *versus control (saline/MCAO) group.* $^{+}$P < .01 *versus intact animals;* $^{++}$P < .01 *versus sham-operated animals;* $^{+++}$P < .01 *versus control (saline/MCAO) group;* $^{++++}$P < .06 *versus control (saline/MCAO) group.*

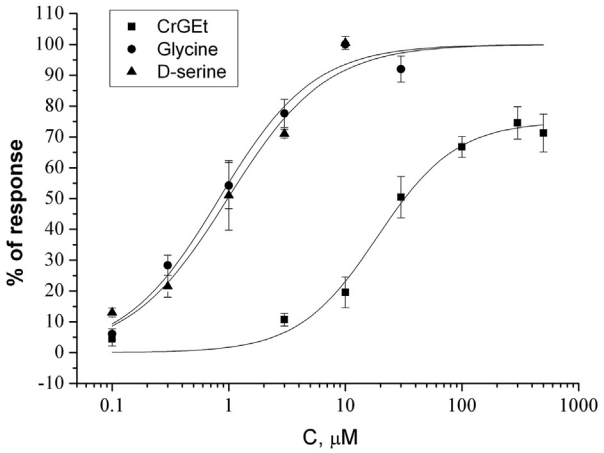

Figure 5. *Creatylglycine ethyl ester fumarate (CrGEt), glycine, and D-serine dose-response curves of NMDA receptors of pyramidal neurons in hippocampal zone CA1. The data were recorded in the presence of 100 μM L-aspartate. Data are presented as mean ± standard deviation (n = 6).*

also mediates functional recovery after the stroke. The CrGEt administration led to dose-dependent (30-280 mg/kg) reduction of the neurologic deficits and restoration of the cognitive functions in the animals after ischemia, as assessed by the sensorimotor tests.

The neuroprotective effect of CrGEt results from its involvement in the key processes of the I/R pathogenesis. Acute cerebral ischemia is firstly characterized by energy metabolism failure with a sharp drop in ATP and accumulation of lactate.[30-34] Here, we showed that the neuroprotective activity of CrGEt was defined by its ability to normalize energy metabolism under ischemic conditions. Even a single i.v. injection of CrGEt 30 minutes before ischemia promoted the preservation of the ATP amount in brains 30 minutes after bilateral occlusion of the common carotid arteries and the reduction in lactate levels in 30 minutes after MCAO.

The stability of CrGEt in serum (rate of the hydrolysis—less than 9% over 60 minutes) shown in our studies and similar results by Garbati et al[35] allows us to assume that the observed change in the ATP production was largely defined by the biologic action of CrGEt rather than by the activity of its metabolites. For instance, it seems that a part of the energetic benefits of CrGEt may be related to its interactions with CK (K_m = 135 ± 13 mM at pH 7.4).

In contrast to our studies, Garbati et al[35] failed to observe activity of 2 mM CrGEt in the CK assay. One potential explanation of this controversy is that creatine amides are characterized by the K_m values greater than 100 mM (unpublished data) and, thus, the catalytic reaction can be detected in vitro only at the substrate concentrations significantly higher than 2 mM. However, such high values of K_m also suggest that CrGEt may be involved in other biochemical interactions supporting the ATP preservation.

Oxidative stress leading to the reactive oxygen species production is also considered as a key mechanism underlying ischemic brain injury.[36] In the normal cells, reactive oxygen species can be neutralized by the antioxidant systems; however, in the ischemic tissues, this system is greatly suppressed. In this regard, it was particularly interesting that treatment with CrGEt inhibited the I/R-related oxidative stress in rat brains as evidenced by increased levels of SOD, brain antioxidant, and reduced levels of brain MDA amount, an index of lipid peroxidation.

Furthermore, CrGEt exerts neuroprotective effects through NMDA receptor modulation acting as a partial agonist of the NMDA-receptors. Partial agonists of the NMDA receptors bind to the same site as full agonists but induce less channel activation; at high concentrations, they are considered as antagonists. Stanton et al[37] showed that compounds GLYX-13 serves as a partial agonist at the concentration .1-1 μM, whereas at concentrations 10-50 μM becomes an antagonist and, more importantly, delayed neuronal death in 2 different stroke models. Similar, Gill et al[38] explained protective effects of

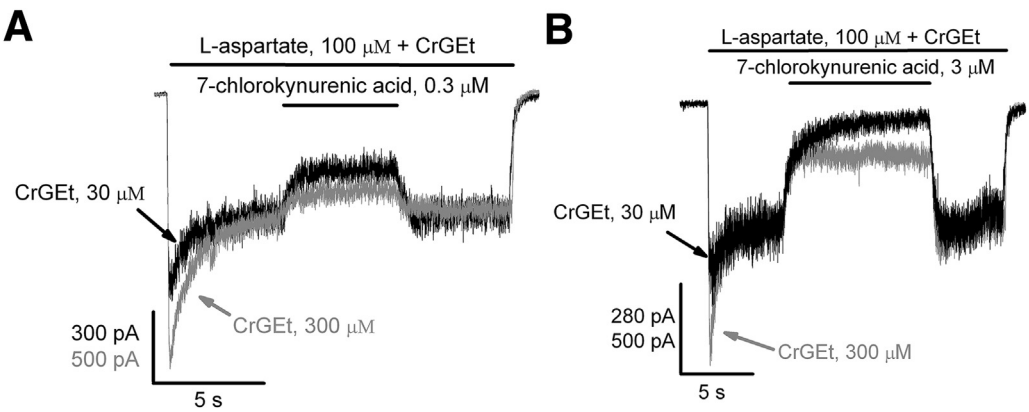

Figure 6. *Typical normalized current traces, demonstrating inhibition of responses to 100 μM L-aspartate +30 or 300 μM creatylglycine ethyl ester fumarate (CrGEt) by two different concentrations of 7-chlorokynurenic acid (.3 μM [A] and 3 μM [B]). In both cases the inhibition by 7-chlorokynurenic acid is significantly smaller in case of high (300 μM) CrGEt concentration.*

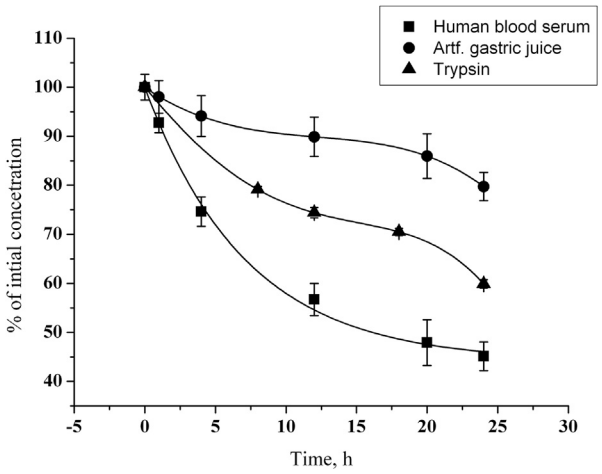

Figure 7. *Stability of creatylglycine ethyl ester fumarate (CrGEt) in human blood serum, in artificial gastric juice, in the presence of trypsin at temperature 37.0 ± .5°C. Concentration (percentage of the initial value) vs time (hours). Data are presented as mean ± standard deviation (n = 5).*

L-687,414 in a rat model of focal ischemia by the partial agonist properties of this compound. Based on these observations, we hypothesize that the CrGEt acts analogous, prevents the overactivation of NMDA receptors and, as a consequence, suppresses mechanisms leading to neuronal dysfunction and death.

Altogether, this work suggests CrGEt as a potential compound for the treatment and prevention of ischemic brain disorders. However, more research is required to reveal other pharmacologic activities and pharmacokinetics of CrGEt and to further elucidate the mechanisms of the CrGEt neuroprotective action.

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