PHARMACOLOGY AND TOXICOLOGY

Neuroprotective Effect of GK-2, a Dipeptide Mimetic of Nerve Growth Factor, during Experimental Focal Ischemia in Middle Cerebral Artery Basin

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 05, pp. 518-521, May, 2011
Original article submitted March 29, 2010

Magnetic resonance tomography, staining with triphenyltetrazolium chloride, and tests for evaluation of functional disturbances "cylinder" and "limb stimulation" showed that daily intraperitoneal injection of dipeptide mimetic of nerve growth factor GK-2 (1 mg/kg) for 6 days to rats with experimental focal ischemia provoked by unilateral intravascular occlusion of a branch of the middle cerebral artery significantly improved neurological deficit and decreased the infarction area.

Key Words: *nerve growth factor mimetic; GK2; neuroprotection; cerebral ischemia; magnetic resonance tomography*

Acute disturbances in cerebral circulation remain an important medical and social problem due to great contribution of these abnormalities to the structure of population morbidity and mortality. In Russia, mortality caused by cerebrovascular disorders ranks second after mortality caused by cardiovascular diseases [5]. The neuroprotective drugs tested until now in the multicenter clinical trials are not sufficiently effective. Thus, the search for new drugs for the treatment of pathologies related to the acute cerebrovascular disturbances is an important problem of experimental and clinical neurology. Several studies reported that nerve growth factor (NGF) can be employed to treat such pathological states and their consequences [7,13]. However, there are strong limitations for the pharmacological use of NGF related to low permeability of the blood-brain barrier (BBB) for this large molecule [15]. Evidently, the development of low-molecular-weight mimetics of NGF based on its peptide sequences and capable of crossing BBB seems to be promising. An original dipeptide mimetic of NGF bis-(N-succinyl-glutamyl-lysine) hexamethylenediamine (GK-2) was synthesized at V. V. Zakusov Institute of Pharmacology under the guidance of Prof. T. A. Gudasheva meets these requirements. Moreover, *in vitro* experiments with this agent demonstrated neuroprotective potencies of GK-2 [3].

This work was designed to study *in vivo* neuroprotective properties of GK-2 on the models of experimental focal cerebral ischemia provoked by unilateral intravascular occlusion of a branch of the middle cerebral artery. This method to induce ischemia is widely used in the studies of the neuroprotective preparations [9,10] since it produces focal ischemic injury of stan-

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0007-4888/11/1515-0584

dard volume and locality corresponding to clinical variants of ischemic lesion in the brain.

MATERIALS AND METHODS

The study was carried out on random-bred male rats weighing 250-290 g. The animals were maintained in a vivarium with a 12-hour day-night cycle on unrestricted food and water. All experimental procedures were carried out in accordance to European Economic Community Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

After behavioral testing, all the rats were randomized into three groups. Group 1 rats consisted of sham-operated (SO) animals (n=6); group 2 comprised rats with ischemic infarction (n=12); and group 3 rats comprised animals with infarction treated with GK-2 (n=12).

Ischemia was induced by transient unilateral intravascular occlusion of the middle cerebral artery with a silicone-covered nylon thread under intraperitoneal anesthesia (chloral hydrate, 300 mg/kg). After 60-min occlusion, the thread was removed to restore circulation in the basin of the middle cerebral artery. During and after surgery, temperature of the animals was maintained at 37.0±0.5°C with an infrared lamp. SO-rats were subjected to the same manipulations except for artery occlusion.

After ischemia modeling, group 3 rats were intraperitoneally injected with GK-2 (1 mg/kg body weight). The rats of other (control) groups were injected with the same volume of saline.

The baseline behavioral testing was performed 1 day before surgery. Neurological deficit was assessed after surgery in the daily limb stimulation test and by the cylinder test on day 7 after surgery.

The cylinder test assessed asymmetry of the use of forelimbs during spontaneous exploration of the cylinder wall [11].

To assess recovery of the sensorimotor status of rat limbs, a modified version of the limb stimulation test was employed [8]. This test recorded the response of hindlimbs and forelimbs to tactile and proprioceptive stimulation; it included 7 trials for the left and right sides of the body. Disturbances in limb functions were scored as follows: the rats that completely executed the task scored 2 points; the rats that performed the task with a delay of more than 2 sec or did it incompletely scored 1 point; and the rats that did not respond to limb stimulation scored 0 point.

The brain of all experimental rats was examined by magnetic resonance tomography (MRT) on day 7 after surgery. All MRT examinations were performed on a BioSpec 70/30 (Bruker) apparatus with magnetic induction 7 T and a gradient system of 105 mT/m. The total scan time was 4 min 48 sec and section width was 0.5 mm. Before MRT, the rats were anesthetized with chloral hydrate and placed in a setup with stereotaxic system and thermal stabilization.

The size of infarction zone was assessed by T2-weighted MRT-images or by morphometry of the cerebral sections stained with triphenyltetrazolium chloride. To analyze the images, an ImageJ 1.38x (NIH) software was used [4,6].

The data on infarction size and rat behavior were analyzed using a double-blind method. Normalcy of distribution of the sign in the sample was examined using Shapiro–Wilk W-test. The behavioral and infarction size data were processed statistically using

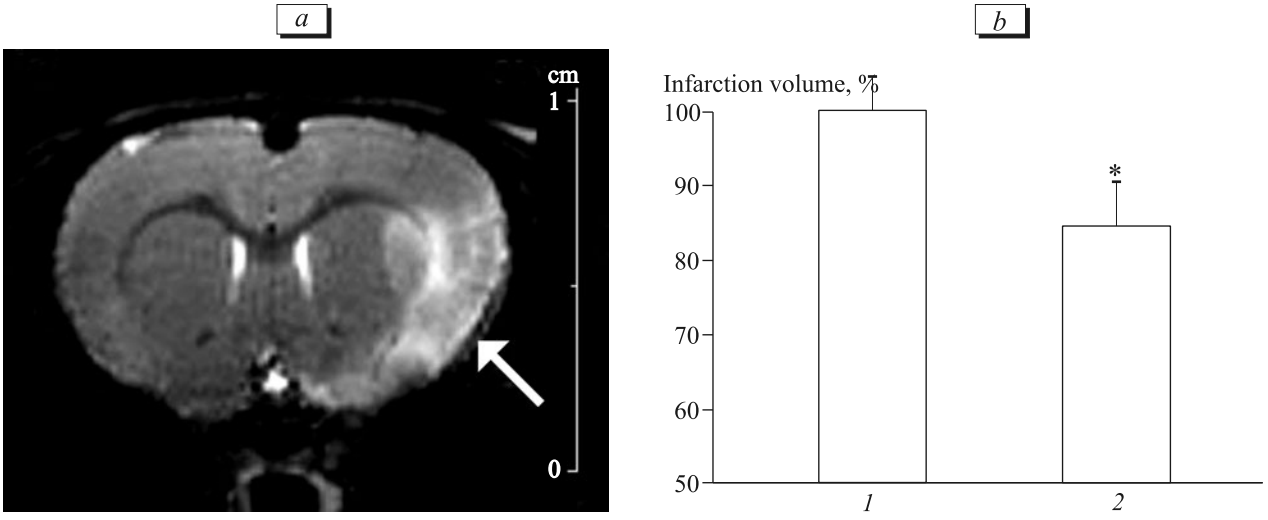

Fig. 1. Effect of GK-2 on the volume of cerebral infarct caused by transient occlusion of the middle cerebral artery (day 7 after surgery). *a*) MRT-image of the developed ischemic focus (arrow indicates injury area); *b*) infarct volume in control group 2 (ischemia, *1*) and in experimental group 3 (ischemia+GK-2, *2*). *p<0.05 in comparison with control group 2 (ischemia, Mann–Whitney U-test).

Mann–Whitney *U*-test and Student *t*-test, respectively. The data were presented as mean±error of the mean.

RESULTS

In MRT-study of ischemic rat brain, the injured region was detected by enhanced signal (hyperintensity) on T2-weighted images compared to the intact white matter due to cytotoxic edema. The most damaged structures were cortex and the adjacent subcortical part of striatum (Fig. 1, a).

The size of ischemic focus in the rats receiving saline was 298.5±13.9 mm^3. In rats treated with GK-2, the size of ischemic focus significantly decreased to 251.9±18.7 mm^3 (p<0.05, Fig. 1, *b*). GK-2 decreased the ischemic focus by on average 16%.

Quantitative data attesting to a decrease of the ischemic volume under the action of GK-2 completely corresponded to the data on sensorimotor disturbances in the limbs of these rats revealed by the behavioral testing. The limb stimulation test showed that treatment with GK-2 significantly moderated the neurological deficit in rats with ischemia. Before ischemia modeling, the rats scored 14 points in this test, but after intravascular occlusion this index dropped to 3.0±0.4 and then insignificantly changed to day 7 after ischemia (Fig. 2). SO-animals scored the same points as the intact rats. Starting from day 4 after surgery, the rats receiving GK-2 demonstrated rapid sensorimotor recovery of the limbs in contrast to group 2 rats receiving saline. On day 7 after surgery, the sensorimotor index in group 3 rats was higher by 4 points in comparison with group 2 rats (Fig. 2).

Analysis of asymmetrical use of the forelimbs in the cylinder test showed that on day 7 after surgery, the use of the affected limb in group 2 rats decreased by 60% and simultaneous use of both limbs by 67% in comparison with SO-rats. This effect was less pronounced in rats treated with GK-2 for 6 days after ischemia: the use of affected limb decreased significantly by only 27% (p<0.05) and simultaneous use of both limbs decreased by 34% in comparison with SO-rats (Fig. 3).

Thus, this study attests to a significant effect of GK-2 on cerebral plastic processes and on recovery of the sensorimotor functions after experimental ischemia. GK-2 is a dipeptide mimetic of NGF, an analog of a fragment of loop IV of this protein. The data obtained well correlate with those on the neuroprotective effect of NGF previously shown in various ischemia models [7,13]. The therapeutic effect of NGF on the ischemic injury to the brain is related to active involvement of this factor in the axonal growth, synaptic plasticity; it can induce differentiation of growth of neurons [2,12]. Moreover, NGF moderates the inflammatory response

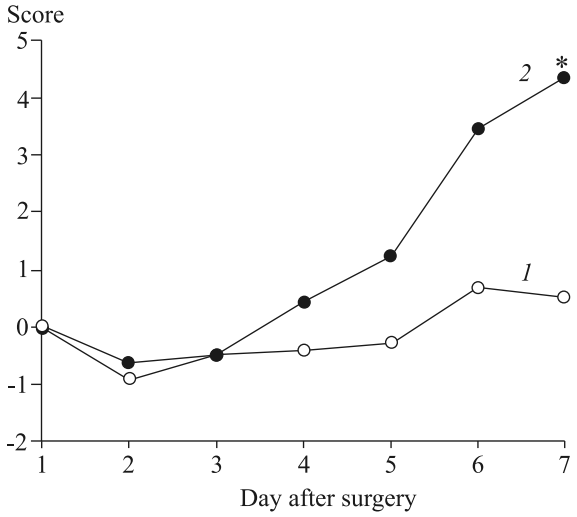

Fig. 2. Effect of course intraperitoneal injections of GK-2 (1 mg/kg) on neurological deficit scored in the limb-stimulation test over 7 days after occlusion of the middle cerebral artery. *1*) group 2 (ischemia); *2*) group 3 (ischemia+GK-2). *p<0.05 in comparison with control group 2.

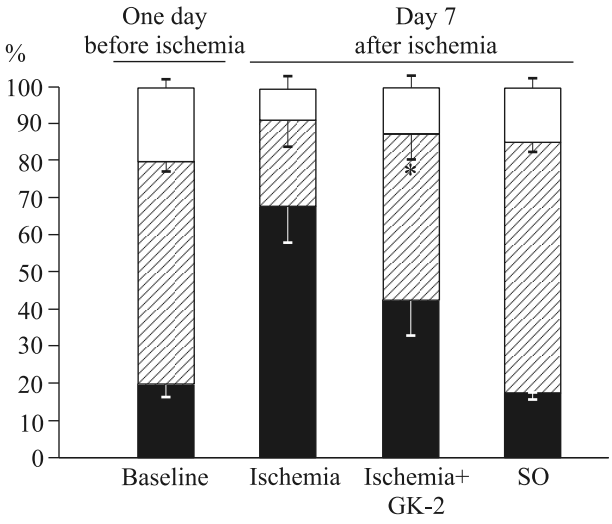

Fig. 3. Effect of course intraperitoneal injections of GK-2 (1 mg/kg) on asymmetrical use of forelimbs in the cylinder test on day 7 after occlusion of middle cerebral artery. Open sector: the use of the contralateral limb; closed sector: the use of ipsilateral limb, dashed sector: the use of both limbs simultaneously. *p<0.05 in comparison with control group 2 (ischemia without GK-2).

after cerebral ischemia [14]. During the development of ischemic injury, high level of trophic factors ensures amelioration of neurological deficit even during the morphological deficiency that provoked it [1]. The therapeutic effect of peptide analogs to NGF during the postischemic period results mostly from moderation of neurological deficit and not from morphological reduction of the focal damage.

Our data corroborate the *in vitro* neuroprotective properties of GK-2 and attest to a great potential of

further development of this agent as an anti-ischemic drug.

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