In Vitro Study of Neuroprotective Properties of GK-2, a New Original Nerve Growth Factor Mimetic

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New nerve growth factor (NGF) mimetic GK-2, a substituted dimeric dipeptide, in a concentration of up to 10^{-9} M produced a protective effect on the culture of immortalized mouse hippocampal neurons (line HT-22) after addition of H_2O_2 and glutamate. GK-2 in a concentration of 10^{-8} M protected rat PC-12 pheochromocytoma cells from the neurotoxin MPTP. The neuroprotective effect of this peptide on the model of oxidative stress was also observed in the primary culture of embryonic rat hippocampal neurons.

Key Words: *neuroprotection; GK-2; oxidative stress; glutamate toxicity; MPTP*

Over the past decades, numerous studies have shown that neurotrophins NGF and BDNF are involved in the pathogenesis of neurodegenerative diseases [5,13]. The neuroprotective effect of NGF and BDNF was confirmed by pharmacological researches. The use of NGF and BDNF in pharmacotherapy is limited because of difficulties in crossing the blood-brain barrier, influence of endogenous peptidases, and other problems associated with systemic treatment with these drugs [2].

Recent studies at the V. V. Zakusov Institute of Pharmacology were directed towards the construction of a small molecule, which possesses the properties of NGF. A low-molecular-weight mimetic of the 4th loop of NGF (GK-2) was synthesized [1]. GK-2 is a substituted dimeric dipeptide, bis-(N-succinyl-glutamyl-lysin) hexamethylenediamine.

This work was designed to study the *in vitro* neuroprotective properties of GK-2 on the models of oxidative stress (OS), glutamate toxicity, and MPTP-induced neuronal damage.

MATERIALS AND METHODS

Experiments were performed on the culture of immortalized mouse hippocampal neurons (line HT-22), primary culture of rat hippocampal neurons (HN), and dopamine-positive PC-12 cells of rat pheochromocytoma (tumor of the adrenal cortex).

Primary culture of HN was obtained from 18-day-old embryos of MR (Maudsley reactive) rats. Dissection of the brain and isolation of midbrain neurons were performed in Hank's medium (BioloT) [6]. The isolated HN were put in centrifuge tubes with DMEM/F-12 medium (ICN), resuspended, and centrifuged twice at 1500 rpm for 10 min. The cells were repeatedly resuspended, counted, and inoculated in 48-well culture plates (density 350,000-400,000 cells per well) with the same medium. Experiments were conducted on the 6th day after cell inoculation. HT-22 cells were inoculated (3500 cells per well) in 96-well plates with DMEM medium (ICN) containing 2 mM glutamine (ICN) and 5% FBS (ICN). Incubation was performed until the formation of monolayer. The cells were cultured at 37°C and 5% CO_2.

OS was induced by H_2O_2 in a final concentration of 1.5 mM [7]. HT-22 cells were incubated with H_2O_2 at 37°C and 5% CO_2 for 30 min. The culture medium with H_2O_2 was replaced for normal medium. Cell viability was estimated after 4 h.

For modeling MPTP-induced damage in culture of PC12 cells, neurotoxin MPTP (Sigma) in a final concentration of 1 mM was added to the cell medium containing 1% FBS (24 h after passage of cells) [12]. The peptide was put to the cell medium simultaneously or 24 h before addition of MPTP.

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Glutamate toxicity was induced by adding glutamate (final concentration 5 mM) to the culture medium. After 24-h incubation at 37°C and 5% CO_2, the medium was replaced with normal medium. Cell viability was estimated after 24 h [9,14]. The peptide was added 24 h before damage or immediately after washing out glutamic acid.

Cell viability was evaluated in the test with bromide 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, Sigma) [7]. Optical density was measured on a Multiscan EX spectrophotometer (Thermo).

For evaluation of the neuroprotective effect of GK-2, the peptide was added 24 h before or immediately after the exposure to adverse factors. NGF (Sigma) in a final concentration of 100 ng/ml was used as the positive control.

The significance of differences between the treatment and control groups was estimated by Student's *t* test.

RESULTS

Addition of GK-2 after treatment with H_2O_2 prevented death of immortalized mouse HT-22 hippocampal neurons (Table 1). NGF has a similar effect, which is probably related to modulation of antioxidant enzyme activity in cells [15].

Addition of GK-2 (final concentration up to 10^{-9} M) 24 h before treatment with H_2O_2 produced a neuroprotective effect on HT-22 cells (Table 1). The neuroprotective effect is also observed in experiments with addition of NGF 24 h before OS, which results from an increase in the synthesis of neuroprotective heat-shock proteins [4,10].

TABLE 1. Effect of GK-2 on Viability of Mouse HT-22 HN after OS (*M*±*m*, *n*=12)

Group	Optical density	
	24 h before OS	immediately after addition of H_2O_2
Control	0.36±0.07	0.47±0.07
H_2O_2, 1.5 mM	0.24±0.02*	0.35±0.05*
NGF, 100 ng/ml	0.34±0.07+	0.460±0.047+
GK-2, 10^{-5} M	0.32±0.07+	0.44±0.05+
GK-2, 10^{-6} M	—	0.37±0.06
GK-2, 10^{-7} M	—	0.39±0.07
GK-2, 10^{-8} M	0.35±0.07+	0.47±0.07+
GK-2, 10^{-9} M	—	0.41±0.06+
GK-2, 10^{-10} M	—	0.38±0.06

Note. *$p \le 0.05$ compared to the control; +$p \le 0.05$ compared to H_2O_2.

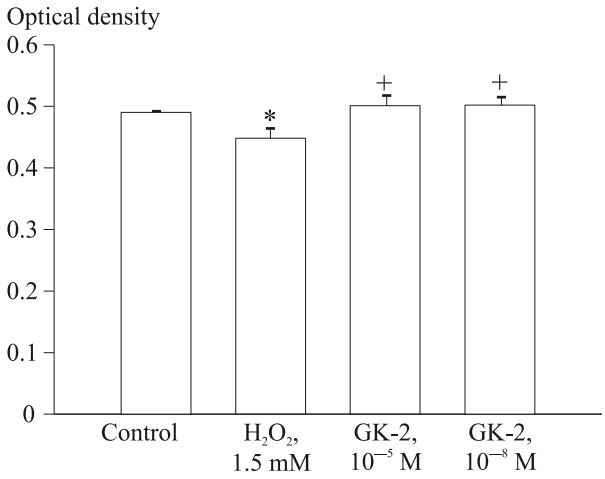

Fig. 1. Effect of GK-2 on viability of HN from 18-day-old rat embryos (*n*=12) after OS. The peptide is added 24 h before treatment with H_2O_2. *$p \le 0.05$ compared to the control; +$p \le 0.05$ compared to H_2O_2.

Experiments on the model of OS showed that GK-2 in the specified concentrations produed a neuroprotective effect not only on immortalized neurons, but also on the primary culture of embryonic HN (Fig. 1). The protective effect of GK-2 was also observed upon exposure to a variety of adverse factors.

The neurotoxin MPTP is used for modeling of the cellular stage of Parkinson's disease. This agent causes a selective damage to dopaminergic neurons due to mitochondrial dysfunction and impairment of energy metabolism in the neuron [11]. In these experiments, GK-2 was added simultaneously or 24 h before treatment with 1 mM MPTP [12]. GK-2 exhibited protective properties under these conditions (Fig. 2).

The neuroprotective effect of KG-2 was also revealed under conditions of glutamate toxicity. The peptide was added 24 h before or immediately after washing out glutamic acid. GK-2 in the test concentration had a strong protective effect on cells under both conditions of treatment with glutamic acid (Fig. 3).

We conclude that GK-2 in low concentrations produces a neuroprotective effect on the models of OS, glutamate toxicity, and MPTP-induced cell damage. Activity of GK-2 is similar to that of NGF. GK-2 is effective in a concentration of 10^{-9} M. It should be emphasized that the most potent agonist of non-peptide TrkA receptors, Gambogic amide [8], only in concentrations of up to 0.5×10^{-6} M protects the culture of T17 and SN56 cells from apoptotic death. *In vitro* study showed that L1L4 (strong peptide agonist of NGF) only in a concentration of 5×10^{-6} M exhibits NGF-like properties [3]. L1L4 is a 14-membered bicyclic peptide with a molecular weight >2000 Da. The dimeric dipeptide GK-2 has a molecular weight of 830 Da. A smaller number and variability of peptide

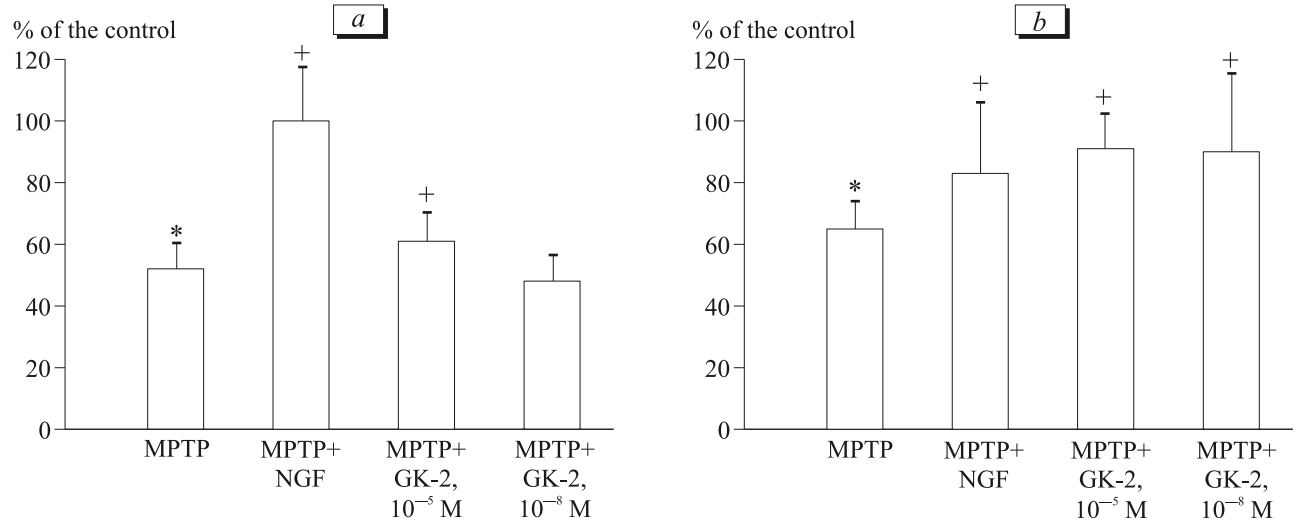

Fig. 2. Effect of GK-2 on PC12 cell viability during MPTP-induced damage. (*a*) Addition of GK-2 simultaneously with MPTP; (*b*) addition of GK-2 24 h before treatment with MPTP. *$p \le 0.05$ compared to the control (100%); $^{+}p \le 0.05$ compared to MPTP.

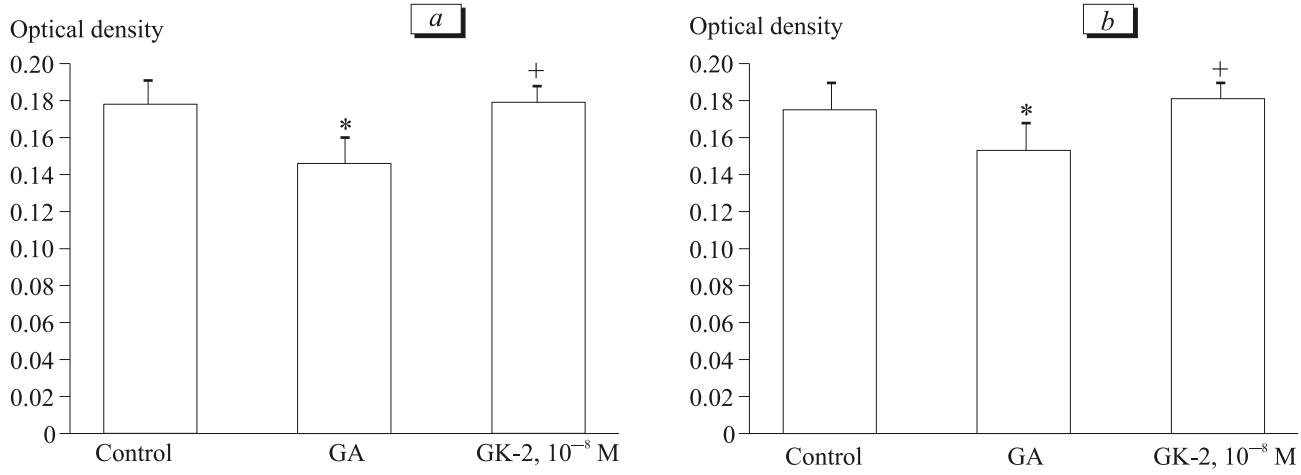

Fig. 3. Effect of GK-2 on the viability of mouse HT-22 HN under conditions of glutamate toxicity. (*a*) Addition of the peptide 24 h before treatment with glutamic acid (GA); (*b*) addition of the peptide immediately after washing out GA. *$p \le 0.05$ compared to the control; $^{+}p \le 0.05$ compared to glutamic acid.

bonds in GK-2 determine higher resistance of this agent to peptidases. The ability of GK-2 to cross the blood-brain barrier is associated with its relatively low molecular weight.

The properties of GK-2 should be evaluated in *in vitro* and *in vivo* experiments.

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