Effect of Semax Peptide on Survival of Cultured Rat Pheochromocytoma Cells during Oxidative Stress

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We studied the effects of Semax (antiinsulin peptide with neuroprotective effect) on the survival of cultured rat pheochromocytoma cell after oxidative stress induced by short-term incubation with hydrogen peroxide. Studies with fluorescent dyes propidium iodide and Hoechst 33258 showed that cell incubation with hydrogen peroxide led to the formation of damaged cells with characteristic signs of necrosis. Semax dose-dependently reduced the number of cells damaged by oxidative stress. The efficiency of Semax depended on the time of its addition to the culture medium. The results suggest that the neuroprotective effect of Semax in ischemic stroke can be due to its capacity to protect neurons from damage caused by oxidative stress.

Key Words: *brain ischemia; oxidative stress; necrosis; Semax; cell culture; rat pheochromocytoma PC12*

Neuroprotective therapy is a very important measure at early stages of treatment of ischemic stroke. It is aimed at suppression of pathological processes caused by brain ischemia and reperfusion during the period of most active formation of infarction area. At present, the armory of traditional neuroprotectors, such as magnesium sulfate, glycine, cerebrolysine, is supplemented with Semax, one of the most perspective stroke control drugs [3]. This drug is created on the basis of Semax oligopeptide, a synthetic analog of adrenocortical hormone Met-Glu-His-Phe-Pro-Gly-Pro fragment 4-7 [1].

Clinical studies demonstrated safety and high efficiency of Semax in the treatment of carotid ischemic stroke [3,4]. Neuroprotective effect of Semax was confirmed by laboratory findings. Semax significantly reduced mortality and improved the neurological parameters in rats with experimental ischemic stroke [3,6].

The molecular mechanisms of neuroprotective effect of Semax are not quite clear. It has been demonstrated [14] that this peptide stimulates the expression of neurotrophic factor genes in glial cell culture. The fact that Semax improves neuronal survival in primary culture containing no glial cells [2] and some other reports [1,3,7,9] indicate that the effects of this peptide are mediated by different mechanisms.

For evaluation of these mechanisms, we carried out experiments on rat pheochromocytoma PC12 cell culture widely used as a neuron model. In contrast to cerebrolysine and nerve growth factor (NGF), Semax did not induce differentiation of PC12 cell and did not prevent apoptosis caused by serum deprivation [8]. These results suggest that the neuroprotective effect of Semax is not determined by direct modulation of apoptosis processes activated by cerebral ischemia and is not mediated by NGF receptors.

We investigated the effect of Semax on survival of PC12 cells under conditions of oxidative stress (OS). In our model OS was induced by short-term incubation of cells with H_2O_2, which is intracellularly transformed into extremely toxic intermediate product $OH^{\bullet}$. DNA damage induced by $OH^{\bullet}$ is known to stimulate

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processes eventually leading to cell death: hyperactivation of the nuclear enzyme poly(ADP-ribose)-polymerase, depletion of ATP pool, and dysfunction of ionic channels [10]. In ischemic stroke these processes are particularly active in ischemic areas of the brain during the first hours after impairment of cerebral circulation [11,12].

MATERIALS AND METHODS

Experiments were carried out on undifferentiated rat pheochromocytoma cells PC12. Slides coated with poly-L-lysine (0.1 mg/ml, Fluka) were put into 24-well culture plates (Corning & Costar). Cell suspension (1 ml, 30×10^3 cells/ml) in DMEM supplemented with 15% fetal calf serum (BioloT), 2 mM L-glutamine, and 80 μg/ml gentamicin sulfate was incubated for 15-18 h at 37°C in atmosphere with 5% CO_2.

Oxidative stress was induced by short-term (30 min) incubation of cells with 1 mM H_2O_2 in serum-free medium, after which the cells were cultured in a medium containing 15% serum. Semax was added in aqueous solutions. After 4- or 24-h incubation the cells were stained with fluorescent dyes propidium iodide (PI, 1 μg/ml) and Hoechst 33258 (5 μg/ml) (both from Sigma) as described previously [8]. The preparations were analyzed under an Axioscop 2 plus microscope (Zeiss) with a fluorescent attachment. In order to detect damaged cells, the intensity of fluorescence of nuclei stained with different dyes and the size and shape of the nuclei were evaluated visually. Based on these signs, damaged cells with characteristic signs of necrosis (IP^+ cells) and apoptosis ($Hoechst^+$ cells) were detected. In order to evaluate the percentage of damaged cells, several fields in each slide were analyzed, at least 600 cells.

The significance of differences was evaluated using Student's *t* test.

For evaluation of the stability of Semax, 10 μCi tritium-labeled peptide was added into the medium during culturing and aliquots (100 μl) were collected for the analysis of radioactive products after different periods. Parallel samples were pooled, pH was adjusted to 2 with hydrochloric acid, and peptides were extracted with acetonitrile. After removal of the solvent under vacuum the samples were analyzed by high performance liquid chromatography (Beckman) on a Kromasil column (4×140 mm, 5 μm) in the following system: A) 0.082% trifluoroacetic acid and 0.018% heptafluorobutyric acid in water; and B) 80% acetonitrile in eluent A. Radioactivity of fractions corresponding to Semax and its degradation products was measured on a liquid scintillation counter.

RESULTS

Incubation of PC12 cells with 1 mM H_2O_2 for 30 min yielded 30% PI^+ cells and only 3% $Hoechst^+$ cells in the culture as soon as after 4 h. No damaged cells were detected either immediately or 1 h after incubation with H_2O_2, and the number of these cells increased just negligibly 4-24 h after OS. Since damaged cells with typical signs of necrosis predominated in the culture, in further experiments we evaluated cell survival by staining with PI alone.

In order to confirm the necrotic type of injuries in OS, we studied the effects of apoptosis and necrosis suppressors on cell survival (Fig. 1, *a*). Substances

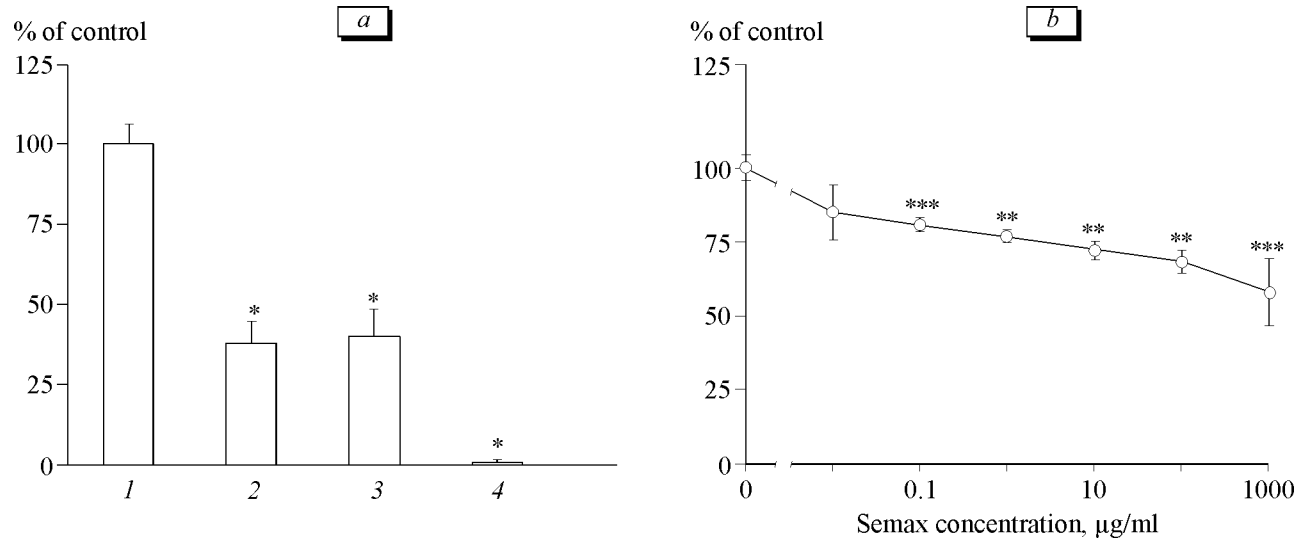

Fig. 1. Effects of cytoprotectors (*a*) and Semax (*b*) on the survival of rat pheochromocytoma PC12 cell culture after oxidative stress. *1*) control (no cytoprotectors); *2*) 3-aminobenzamide (1 mM); *3*) nerve growth factor (100 ng/ml); *4*) culturing without H_2O_2. Here and in Fig. 2.: ordinates: percentage of damaged cells stained with propidium iodide. *$p<0.001$, **$p<0.02$, ***$p<0.05$ compared to the control.

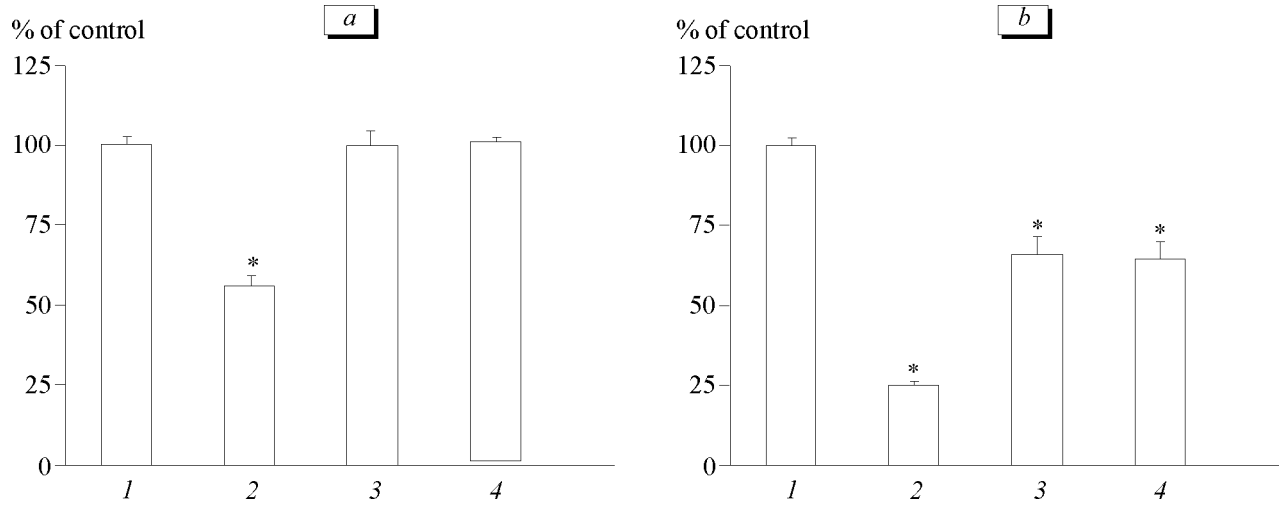

Fig. 2. Cytoprotective efficiency of Semax (1 mg/ml) added to culture medium at different terms before and after oxidative stress. *a*) cells stained with propidium iodide 4 h after oxidative stress. Time of Semax addition: *2*) immediately after stress; *3*) 6 h before stress; *4*) 24 h before stress. Here and in Fig. 2, *b*: *1*) control (no Semax). *b*) cells stained with propidium iodide 24 h after oxidative stress. Time of Semax addition: *2*) immediately after stress; *3*) 4 h after stress; *4*) 20 h after stress.

with antinecrotic activity (3-aminobenzamide and NGF) decreased the number of PI^+ cells, while proapoptotic protease inhibitor did not change the percentage of these cells.

Hence, cell damage caused by OS in PC12 culture is characterized by the following morphological and biochemical signs of necrosis: plasma membrane damage, shrinking of the nucleus, and activation of poly(ADP-ribose)-polymerase. Membrane damage seems to result from a cascade of reactions initiated by OS and taking at least 1 h.

Semax decreased the number of cells damaged by OS in a dose-dependent manner (Fig. 1, *b*). A significant ($p<0.05$) decrease in the number of damaged cells was observed starting from peptide concentration of 1 μg/ml.

Single addition of Semax (1 mg/ml) to the culture medium 6 and 24 h before OS did not protect the cells (Fig. 2, *a*). By contrast, the peptide added to the medium 4 and even 20 h after incubation with H_2O_2 considerably decreased the number of damaged cells (Fig. 2, *b*). Addition of the peptide into the medium immediately after incubation with H_2O_2 was most effective. These results suggest that OS leads to rapid death of cells in PC cultures, while some cells are partially damaged (detected by PI) and remain viable for a long time. These cells can recover under the effect of Semax 4 and 20 h after OS. Hence, Semax not only prevents damage to

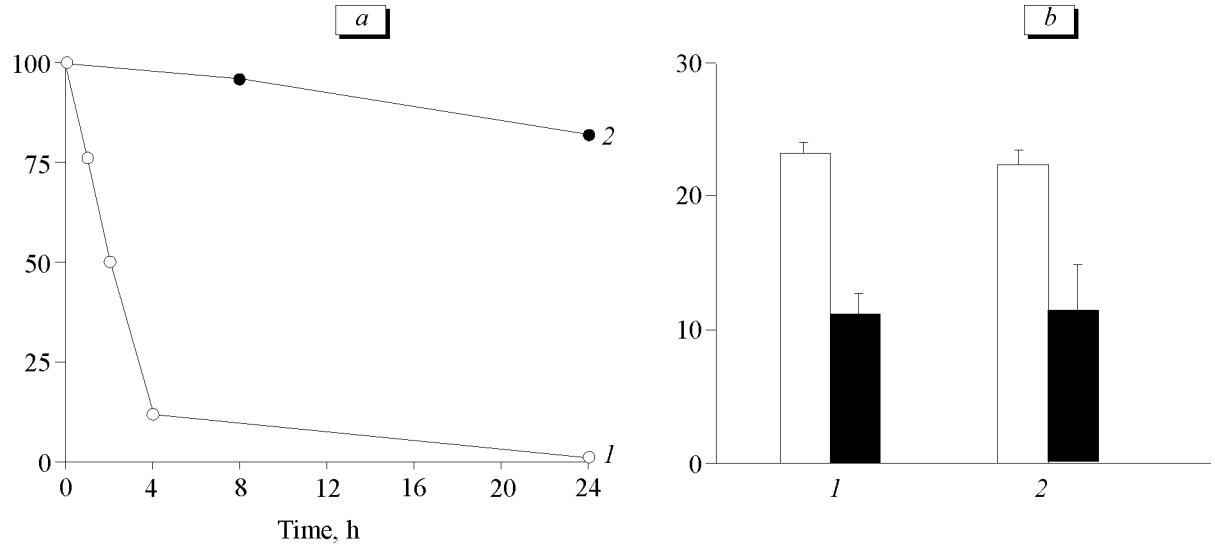

Fig. 3. Comparison of Semax stability (*a*) and cytoprotective activity (*b*) during cell culturing in medium with serum (*1*) and in serum-free medium (*2*). *b*) control (light bars); Semax (dark bars), 0.1 mg/ml. Ordinates: *a*) Semax concentration, μM; *b*) percentage of cells stained with propidium iodide.

plasma membrane, but also activates processes leading to restoration of cell membrane in damaged cells.

Previous experiments revealed rapid proteolytic degradation of Semax in the blood [13] and in primary neuronal and glial cultures [5]. In our model stability of Semax can also be an important factor determining its efficiency.

Chromatography showed rapid degradation of Semax in PC12 culture under standard conditions (Fig. 3, *a*): its half-life period was about 2 h. The main products of peptide degradation were detected in the amino acid and dipeptide fraction (data not presented). Incubation of Semax in a serum-free medium for 24 h practically did not decrease the content of whole peptide. However, the protective effect of the peptide was virtually the same in serum-free and serum-containing medium (Fig. 3, *b*). These results indicate that degradation of Semax during culturing is caused by the presence of high peptidase activity in the serum used for cell culturing. At the same time, active concentration of the peptide does not decrease during the period when the peptide can prevent cell death.

Our results suggest that the neuroprotective effect of Semax *in vivo* can be explained by its capacity to protect neurons from OS by direct interaction with damaged cells. Further studies should be aimed at evaluation of the pharmacological effects of Semax in diseases involving OS and massive DNA damage.

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