Psychotropic Drug Tenoten Activates Mitogen-Activated MAP/ERK Kinase Regulatory Cascade Controlling the Neuroprotective Effects

O. I. Epstein, O. V. Vorobieva*, L. N. Grinkevich*, O. A. Kharchenko*, and M. B. Shtark**

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For evaluation of the molecular mechanisms underlying the effects of tenoten, a preparation based on ultralow-dose antibodies to S100 proteins and intended for the therapy of anxious and depressive disorders, we studied its influence on mitogen-activated ERK kinase cascade in *Helix lucorum* subpharyngeal ganglion complex. Western blot analysis showed that incubation of the ganglion preparation with tenoten resulted in significant activation of mitogen-activated ERK kinases, which was reduced by PD98059 (blocker of upstream protein kinase). This attests to a specific effect of the drug on mitogen-activated ERK kinase cascade. It is hypothesized that the therapeutic efficiency of tenoten is realed to activation of mitogen-activated ERK kinase cascade.

Key Words: *mitogen-activated ERK protein kinases; antidepressants; tenoten; central nervous system; Helix lucorum*

The number of diseases associated with depressions is increasing [3,10]. Drugs for their treatment are created, but the molecular architecture of depressions and the mechanisms underlying the effects of antidepressant drug are still the key problems of psychopharmacology.

MAP-ERK kinase cascade (KC) is a possible target for some antidepressants [4]. The role of adaptive intracerebral phenomena realized through intracellular signal systems: primarily KC, regulating total neuron tolerance, neuroregeneration, and, which is particularly important, plasticity mechanisms in the nervous system is intensively investigated [8,13]. KC regulates neuron survival and regeneration of neuronal processes and effectively modifies structural and functional plasticity [7]. KC activation is observed during the formation of some types of conditioned defense behavior in higher vertebrates and in invertebrates [1,9,10].

MAP kinases ERK, p38, and JNK play an extremely important role in adaptive processes in the brain. Depending on the degree of activation of each component, the neuron decides, whether it survives or dies via stress-induced apoptosis, increases the efficiency of synaptic contacts or forms new ones. The ERK1/2 kinase cascade is responsible for cell growth and survival, while stress-induced p38 and JNK kinases are proapoptotic [8]. Dysfunction of KC is the cause of many mental and neurological diseases, including severe depressions associated with suicide [3,4,10]. KC modulate genome-dependent processes in cells through phosphorylation of some DNA-binding transcription factors, including TCF and CREB [2]. KC are activated by bioactive substances (growth factors, hormones, neurotransmitters).

Materia Medica Holding, Moscow; *I. P. Pavlov Institute of Physiology, Russian Academy of Sciences, St. Petersburg; **Institute of Molecular Biology and Biophysics, Siberian Division of Russian Academy of Medical Sciences, Novosibirsk. ***Address for correspondence***: mark@soramn.ru. Shtark M.B.

Tenoten is a new drug recommended for the treatment of patients with anxiety and depressive symptoms and various cognitive disorders. This drug is created on the basis of ultralow-dose antibodies to multifunctional S100 neurospecific proteins. S100 proteins are Ca^{2+}-binding proteins involved in regulatory intracellular Ca^{2+}-dependent processes [15]. Moreover, S100B and S100A1 are characterized by neurotrophic activity. In the nervous system, S100 proteins participate in the regulation of differentiation of neuronal cells, protection of neurons from destruction, and in learning and memory mechanisms [5-7,14,15]. High level of antibodies to this antigen is diagnosed in Alzheimer's disease, schizophrenia, and psychoses. Extensive studies of the mechanisms and therapeutic effects of low and ultralow doses of antibodies to S100 proteins persuasively demonstrated their involvement in the formation of the so-called "bipathic" phenomenon arresting or modulating the inhibitory effects of high doses of the same antibodies [5].

For better understanding of the molecular mechanisms of the effect of tenoten, we studied its influence on KC.

MATERIALS AND METHODS

The study was carried out on a simple nervous system, subpharyngeal ganglion complex of *Helix lucorum* mollusk. Due to long-term survival of the mollusk CNS *in vitro*, processes of associative and nonassociative behavioral forms can be modeled on this object. The expression of MAP/ERK kinases in *Helix* CNS is comparable to that in higher vertebrates and is activated during training [17].

Before isolation of the CNS, the animals of similar weight were anesthetized with magnesium, after which the CNS was incubated for 20 h in saline for cold-blooded organisms with tenoten (affinity purified antibodies to brain-specific S100 protein: a mixture of C12, C30, and C200 dilutions) and proproten (antibodies to S100 brain-specific protein: C1000 dilution). Potentiated water containing no antibodies was added in control samples instead of the test preparations. PD98059 (20 μM), a blocker of upstream MEK kinase was used for blockade of MAP/ERK kinase.

The content and/or activation of MAP kinases were evaluated by Western blot hybridization. Since MAP/ERK kinase is a highly conservative "product" (it was cloned in many animal species, from mollusks to higher vertebrates and humans; according to gene data banks, its conservatism by protein is estimated as 95%), commercial antibodies were used. The content of MAP/ERK kinases was evaluated using antibodies to total forms of this kinase, while activation was studied using antibodies to threonine- and tyrosine-phosphorylated kinase forms. Phosphorylation of MAP-ERK kinases by upstream MEK kinase by these amino acids leads to its activation.

Mitogen-activated kinases were extracted from the CNS nerve cells using buffer I: 10 mM Tris-HCl (pH 7.6), 1% Triton X-100, 1 mM dithiotreitol, 50 mM NaCl, 0.2 mM phenylmethylsulfonylfluoride, leupeptine, aprotinine, pepstatin (Sigma, 10 μg/ml each), 0.1 mM Na_3VO_4, and 50 mM NaF. The supernatant was collected after centrifugation (20 min at 15,000*g* and 0°C). Protein concentration in the extracts was measured spectrophotometrically after Bradford, after which the extracts of nerve cells containing the target proteins were separated by electrophoresis in 10% PAAG (Laemmli system) in Tris-glycine buffer (pH 8.3) with 0.1% sodium dodecylsulfate. Protein samples were preliminary boiled with buffer for samples (50 mM Tris, pH 6.7; 6% sodium dodecyl sulfate; 20% glycerol, 8% 2-mercaptoethanol; 0.25% bromophenol blue) in 1:1 ratio for 5 min. Novex color proteins served as molecular weight markers. Separated proteins were transferred onto nitrocellulose filters by semidry blotting (1.5 mA/cm^2 membrane, duration of transfer 1.5 h). The quality of protein transfer onto membrane was verified by Ponceaus staining.

After procedures reducing nonspecific adsorption (incubation with 3% milk), nitrocellulose filters were successively incubated with first and second (conjugated with horseradish peroxidase) antibodies (according to recommendations of Amersham Pharmacia Biotech: Protocol for ECL Handling). First antibodies to ERK and p-ERK (Cell Signaling) were used in 1:1000 dilution, second antibodies (ECL system; Amersham) in dilutions 1:1500-1:2500.

Incubation with the first antibodies was carried out overnight at 4°C. Visualization and quantitative analysis of bound antibodies was carried out by the chemiluminescent method (ECL system; Amersham). The membranes were exposed with X-ray films at ambient temperature for 1-20 min (depending on fluorescence intensity), after which they were washed in glycine buffer (pH 2.8) 2×20 min at 55°C, incubated with milk, and used for detection of total MAP-ERK kinase forms. X-Ray films were scanned.

Quantitative analysis was carried out using Gel Pro Anal software. The data were statistically processed using ANOVA method.

RESULTS

Incubation of CNS with tenoten significantly increases expression of phosphorylated (activated)

MAP/ERK kinases, while expression of MAP/ERK kinases (content of total forms) did not change (Fig. 1, *a*). Quantitative processing and statistical analysis of the data of 6 independent experimental series confirmed these differences ($F(3.30)=4.3$; $p<0.01$; Fig. 1, *b*). The content of phospho-ERK kinases in animal CNS increased significantly under the effect of tenoten in comparison with the effect of potentiated water and proproten (Post-hoc Scheffe's test: $p<0.015$ and 0.014, respectively).

The tenoten-stimulated increase of activation was reduced by PD98059, an upstream MEK protein kinase blocker ($p<0.014$, Student's *t* test), which attests to specific effect of the drug on KC.

Proproten used for the treatment of the abstinent syndrome caused no KC activation in the studied time intervals, which indicated specific functioning of the intracellular signaling systems under the effects of these two drugs.

Hence, tenoten and proproten differ by the intensity of their anxiplytic effects; in the mapped nervous system of *Helix* mollusk they activate neurons of the motor and regulatory pools differing by their functions. This conclusion correlates with previous findings demonstrating the relationship between the effects of potentiated antibodies to S100 and their arbitrary dose (dilution). Since S100 protein (antibodies to this protein are the basis of tenoten) promotes Ca^{2+} release from intracellular depot through interaction with ryanodine receptor, Ca^{2+}-dependent activation of MAP-ERK kinases can be a mechanism of the effect of tenoten [14], which should be taken into consideration in analysis of the mechanisms of therapeutic effects of this drug and, which is more important, in studies aimed at further improvement of this class of drugs (derivatives of antibodies to S100 proteins).

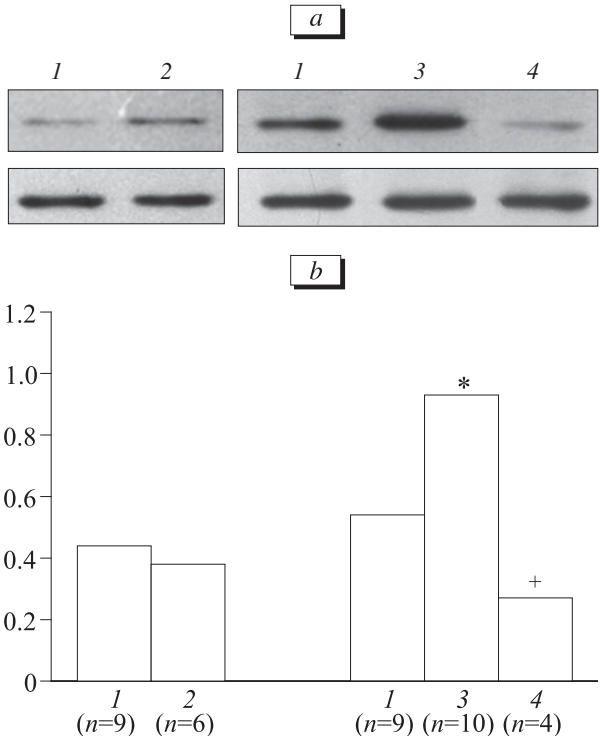

Fig. 1. Effects of tenoten and proproten created on the basis of ultrahigh dilutions of antibodies to S100 brain-specific protein, on KC activation in CNS. *a*) Western blot analysis of phosphorylated (activated) and total forms of MAP/ERK kinases; *b*) content of phosphorylated MAP/ERK kinases in comparison with total forms. Abscissa: content of phosphorylated MAP/ERK kinases in comparison with total forms. *1*) potentiated water; *2*) proproten; *3*) tenoten; *4*) tenoten+PD98059. $p<0.05$ compared to: $^{*}1$, $^{+}3$.

REFERENCES

1. L. N. Grinkevich, P. D. Lisachev, K. A. Baranova, and O. A. Kharchenko, *Ros. Fiziol. Zh.*, **92**, No. 5, 536-545 (2006).
2. S. Davis, P. Vanhoutte, C. Pages, *et al.*, *J. Neurosci.*, **20**, No. 12, 4563-4572 (2000).
3. Y. Dwivedi, H. S. Rizavi, R. C. Roberts, *et al.*, *J. Neurochem.*, **77**, No. 3, 916-928 (2001).
4. H. Einat, P. Yuan, T. D. Gould, *et al.*, *J. Neurosci.*, **23**, No. 19, 7311-7316 (2003).
5. O. I. Epstein, A. Beregovoi, N. S. Sorokina, *et al.*, *Front. Biosci.*, No. 8, a79-a84 (2003).
6. O. I. Epstein, I. F. Pavlov, and M. B. Shtark, *Evid. Based Complement. Alternat. Med.*, **3**, No. 4, 541-545 (2006).
7. O. I. Epstein, T. A. Zapara, O. G. Simonova, *et al.*, *Front. Biosci.*, No. 9, 809-815 (2004).
8. D. R. Kaplan and F. D. Miller, *Curr. Opin. Neurobiol.*, **10**, No. 3, 381-391 (2000).
9. J. M. Kornhauser and M. E. Greenberg, *Neuron*, **18**, No. 6, 839-842 (1997).
10. S. V. Kyosseva, *Int. Rev. Neurobiol.*, **59**, 201-220 (2004).
11. K. C. Martin, D. Michael, J. C. Rose, *et al.*, *Neuron*, **18**, No. 6, 899-912 (1997).
12. X. Tao, S. Finkbeiner, D. B. Arnold, *et al.*, *Ibid.*, **20**, No. 4, 709-726 (1998).
13. G. M. Thomas and R. L. Huganir, *Nat. Rev. Neurosci.*, **5**, No. 3, 173-183 (2004).
14. S. Treves, E. Scuatri, M. Robert, *et al.*, *Biochemistry*, **36**, No. 38, 11498-11503 (1997).
15. D. B. Zimmer, E. H. Cornwall, A. Landar, and W. Song, *Brain Res. Bull.*, **34**, No. 4, 417-429 (1995).