PHARMACOLOGY AND TOXICOLOGY

Effects of Selective Anxiolytic Afobazole on Active Caspase-3

T. A. Antipova, D. S. Sapozhnikova, M. Yu. Stepanichev, M. V. Onufriev, N. V. Gulyaeva, and S. B. Seredenin

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We studied the effects of afobazole on apoptosis through active caspase-3. Afobazole in a final concentration of 10^{-8} M inhibits hyperactivation of effector apoptotic caspase-3 in HT-22 cell culture under conditions of glutamate toxicity.

Key Words: *afobazole; apoptosis; caspase-3; neuroprotection*

Biochemical and morphological signs of apoptosis (chromatin condensation, DNA fragmentation, cell shrinkage) are observed in different types of neurodegenerative diseases (transient cerebral ischemia, intracerebral hemorrhage, and local thermal brain damage) and can be found in postmortem material from patients with schizophrenia, Alzheimer disease, Parkinson disease, Huntington disease, multiple sclerosis, and in experimental animal with modeled Alzheimer disease [2]. Caspase activation is a principal stage of apoptosis cascade [10].

Caspases play a key role in apoptosis by cleaving specific proteins and causing cell death [13]. Caspase-3 is of special interest; its activation is observed in the zone of ischemic penumbra [9,12,13,14].

Original psychoactive agent afobazole appeared as a result of longstanding fundamental investigations performed at V. V. Zakusov Institute of Pharmacology. Neuroprotective effects of afobazole were demonstrated in various models of neural pathologies *in vitro* and *in vivo* [1,5]. The protective effect of afobazole observed 1, 16, 20 and 24 h after administration *in vitro* of glutamic acid [3] agrees with *in vivo* findings on neuroprotective activity of the agent after its administration within 3 and 6 h after focal ischemia induced by occlusion of the middle cerebral artery [4].

Published data suggest that caspase-3 in rats is activated 12-48 h after ischemia [14]. During modeling of glutamate toxicity in cell culture, morphological changes in cell organelles, Goldgi apparatus, endoplasmic reticulum, mitochondria, and DNA, were observed in a similar time interval [6,8]. Addition of caspase inhibitors to cell culture HT-22 significantly reduced cell death [11]. For evaluation of the mechanisms underlying the neuroprotective properties of afobazole, we studied its effects on caspase-3 activation in glutamate toxicity model.

MATERIALS AND METHODS

Experiments were performed on cell culture HT-22 (immortalized mouse hippocampal cells) [11]. The cells were incubated in DMEM containing 5% fetal bovine serum. Glutamate toxicity was induced by adding glutamic acid in a final concentration 5 mM to the culture medium [11]. The cells were incubated with glutamic acid for 18 h at 37°C and 5% CO_2. Afobazole was administered immediately after replacement of cultural medium containing glutamic acid with normal one. Incubation with afobazole in a final concentration

Laboratory of Pharmacology of Neuroprotection, V. V. Zakusov Institute of Pharmacology, Russian Academy of Medical Sciences; Laboratory of Functional Biochemistry of Nervous System, Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Russia. ***Address for correspondence:*** niipharm@mail.ru. T. A. Antipova

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10^{-8} M lasted 24 h under the same conditions. Thereafter, the cells were fixed with 4% paraformaldehyde in 0.01 M phosphate buffered saline for 30 min at room temperature.

For evaluation of changes in cells induced by glutamic acid, some cells were stained with specific nuclear fluorescent dye Hoechst 33258. This method allows evaluation of the percent of cells with damaged membrane and destructed organelles (necrotic cells) and cells with intact membrane, chromatin condensation, and nucleus fragmentation (pyknosis, apoptotic cells). To this end, the cells were incubated with water solution of Hoechst 33258 (Serva) with a final concentration of 5 μg/ml for 30 min at room temperature. After incubation, the cells were washed in phosphate buffered saline and examined under a fluorescent microscope at ×200 magnification and 365 nm wavelength.

Number of cells with signs of apoptosis was calculated in five 500×500 μ random fields of view on each slide (24×24 mm). Cells with intensively stained nuclei and with condensed or fragmented nuclei were considered apoptotic. Slightly stained cells with normal nucleus morphology were considered to be alive. Arithmetic mean for 5 fields of view was calculated, and the mean value for 3 replications was used for statistical analysis.

Another portion of cells was used for active caspase-3 evaluation. To this end, the cells were washed with buffer 0.1 M Tris-HCl, 150 mM NaCl, 0.1% Triton X-100 (TBS-T) 3 times 10 min each. Thereafter the cells were additionally incubated in the same buffer, but with 0.3% Triton X-100 for 5 min. After blockade of nonspecific binding with 1% normal goat serum in TBS-T, the cells were incubated with primary polyclonal antibodies against caspase-3 [Asp175] (rabbit IgG, Cell Signaling Technology) in dilution 1:50. Incubation with antibodies was performed in a humidified chamber at 4°C overnight. Then cells were washed with TBS-T (3×10 min), incubated with FITC-conjugated secondary antibodies (Sigma) diluted 1:80 for 3 h at room temperature, washed with 0.1 M TBS (3×10 min), and the slides were imbedded in special mounting Fluoromount G (EMS). Expression of caspase-3 was examined under a fluorescent microscope at 488 nm.

RESULTS

Cell staining with nuclear dye Hoechst after glutamate administration revealed typical morphological changes indicative of apoptotic neurons [8,11]: nucleus pyknosis and chromatin fragmentation. Thus, glutamic acid produced lesions corresponding to morphological signs of apoptosis.

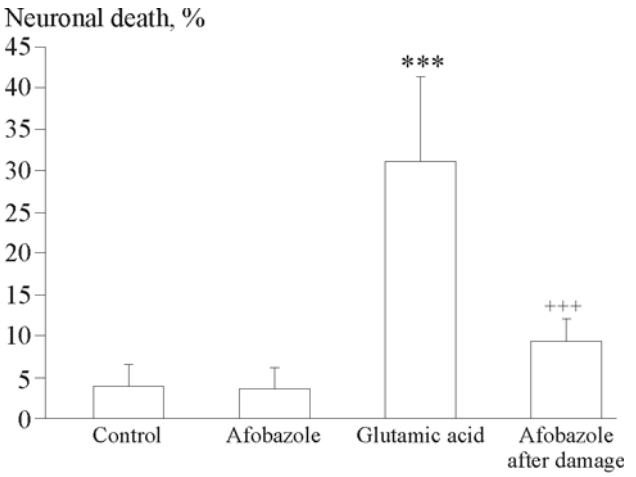

Fig. 1. Protective effect of afobazole (10^{-8} M) on the model of glutamate toxicity in cultured HT-22 cells. $p<0.001$ compared to: ***control, +++glutamic acid.

Incubation of cells with afobazole after application of glutamic acid significantly reduced the number of cells with pyknotic nuclei. Afobazole application in the absence of glutamic acid did not affect nucleus morphology, and cells exposed to afobazole did not significantly differ from the control (Fig. 1). The percent of neurons with damaged nuclei in the control and after glutamate application was 3.97 and 34.5%, respectively ($p<0.001$). Afobazole application in a final concentration of 10^{-8} M after damaging exposure to glutamate toxicity significantly decreased the number of neurons with pyknotic nuclei to 7% (Fig. 1).

Cell labeling with antibodies against active caspase-3 demonstrated significant activation of this enzyme in cells exposed to toxic glutamate influence. These findings agree this published data on the presence of caspase-3 in the zone of ischemia [13]. Afobazole application in a final concentration of 10^{-8} M prevents caspase-3 hyperactivation (Fig. 2). It should be noted, that active caspase-3 was found in some cells in cultures not exposed to glutamate; in this case, afobazole application had no significant effect on the expression of active caspase-3 in HT-22. These findings appear to be important, since the presence of active form of this enzyme under normal conditions agrees the concept on possible non-apoptotic role of caspase-3 in mammalian CNS. Caspase-3 activity underlies proteolysis essential for long-term neuroplasticity. Inhibition of caspase-3 impairs active avoidance learning in rats and results in cell death through necrosis [7]. Thus, limiting hyperactivation of caspase-3, but not inhibition of enzyme activity observed in this study of afobazole in glutamate toxicity model suggests that this effect is an element of neuroprotective activity of the agent which supports the possibility of its use in anti-ischemic therapy.

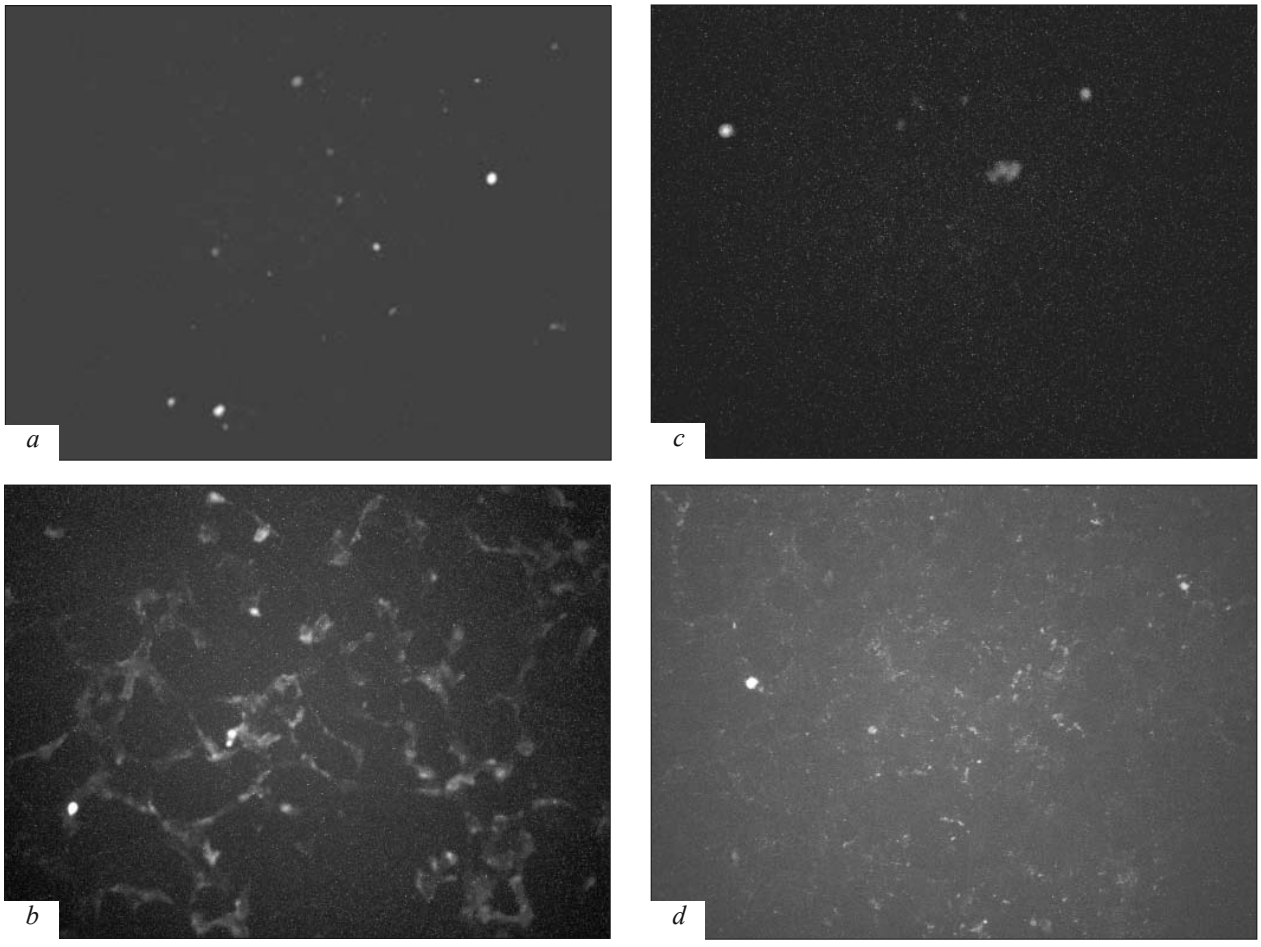

Fig. 2. Detection of caspase-3 activity in neuron culture HT-22 on glutamate toxicity model using specific antibodies against active caspase-3, ×200. *a*) intact cells; *b*) cells, incubated with glutamic acid (5 mM), *c*) cells incubated with afobazole (10^{-8} M) after damage caused by exposure to glutamic acid, *d*) cells incubated with afobazole (10^{-8} M).

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