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Neuroprotective effects of the β-carboline abecarnil studied in cultured cortical neurons and organotypic retinal cultures

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Abstract

Presently there is no neuroprotective pharmacological treatment of proven clinical safety and efficacy available. The purpose of this study was to investigate whether the β-carboline, abecarnil (Abe), which has already passed clinical phase III trials in patients with anxiety disorders, is neuroprotective in in vitro models of cerebral ischemia or excitotoxicity. Abe (100 nM) protected cultured cortical neurons when applied 20 min before or 20 min after combined oxygen glucose deprivation (OGD). Furthermore, cultured cortical neurons were protected from NMDA excitotoxicity when Abe (100 nM) was administered 20 min before or concurrent with 100 μM NMDA. In contrast, in adult rat organotypic retinal cultures, Abe failed to protect retinal ganglion cells (RGCs) against glutamate (Glu) excitotoxicity. Thus, although our data demonstrate that Abe is a potential neuroprotectant in cultured neurons, the lack of effect in an organotypical model of Glu toxicity indicates that further study is required before Abe might be considered for human neuroprotection trials.

Keywords: Abecarnil; Cortical neuron; Excitotoxicity; Neuroprotection; Organotypic retinal culture; Oxygen glucose deprivation

1. Introduction

Cerebral ischemia triggers a cascade of detrimental cellular pathways. Early events such as rapid depletion of intracellular ATP and accumulation of extracellular excitotoxic transmitters occur in the excitotoxic phase. Subsequently, intra- and extracellular ion homeostasis are irreversibly affected by complex interactions of different transmitter systems. In addition to progressively increased intracellular Na^+ and Ca^{2+} concentrations, increased levels of extracellular K^+ are observed, which together with the burst release of Glu from intravesicular stores might propagate deleterious depolarization waves (Dirnagl et al., 1999). In addition to Glu, gamma-aminobutyric-acid (GABA) neurotransmission modulates ischemia-induced neuronal cell death (Schwartz-Bloom and Sah, 2001). One of the major effects of GABA after ischemia is an increase in intracellular Cl^-. GABA thereby reduces Cl^- gradients found in GABAergic interneurons within hippocampal area CA1 (Inglefield and Schwartz-Bloom, 1998).

Besides cerebral ischemia, excitotoxic mechanisms are also center stage in current models of glaucoma pathophysiology. Glaucoma represents a progressive neurodegenerative disease involving neuronal death of retinal ganglion cell (RGC)

Abbreviations: Abe, abecarnil; BSS, basic salt solution; CNS, central nervous system; div, days in vitro; GABA, gamma-aminobutyric acid; GCL, ganglion cell layer; Glu, glutamate; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartate; OGD, oxygen glucose deprivation; PBS, phosphate buffered saline; RGC, retinal ganglion cell.

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axons, soma, and dendrites (Osborne et al., 2004). Local ischemia/hypoxia, possibly due to impaired blood flow autoregulation, has been implicated as one major factor contributing to the progression of glaucoma. Furthermore, excessive stimulation of Glu receptors, especially of the *N*-methyl-D-aspartate (NMDA) subtype, has also been proposed to contribute to neuronal death of RGCs in glaucoma (Lipton, 2003). A number of NMDA receptor antagonists were tested in preclinical trials to prevent loss of RGCs (Osborne et al., 2004). As yet, none of the tested compounds were introduced into clinical practice due to failure of effectiveness or unexpected side effects.

Although a central role for glutamatergic mechanisms of cell death is generally acknowledged for cerebral ischemia, glaucoma, and other acute and chronic neurodegenerative disorders, attempts to treat these disorders with anti-glutamatergic drugs has led to disappointing clinical results in numerous trials (Muir and Lees, 2003; Areosa et al., 2004). One important reason for these negative clinical results has been the failure to be able to treat with sufficiently high dosages of anti-excitotoxic agents, which may have serious side effects. Thus, the identification of clinically safe anti-glutamatergic neuroprotectants remains a high priority. In a number of models of experimental cerebral ischemia benzodiazepines and benzodiazepine agonists modulating $GABA_A$ receptor transmission protected populations of hippocampal neurons (Schwartz et al., 1995; Schwartz-Bloom et al., 2000; Hall et al., 1997). In an oxygen glucose deprivation model (OGD), diazepam led to recovery of the K^+ Cl^- cotransporter 2, indicating one possible mechanism of benzodiazepine mediated neuroprotection (Galeffi et al., 2004).

The β-carboline, abecarnil (Abe), acts as a partial benzodiazepine agonist and is approximately 100 times more potent than diazepam (Stephens et al., 1990). Abe directly modulates the $GABA_A$ receptor (Pribilla et al., 1993). Similar to diazepam, Abe potentiates GABA-induced chloride currents. Recent electrophysiological evidence (Brückner et al., unpublished observations) suggested that Abe, in addition to its $GABA_A$-receptor modulatory effect, also acts in a direct anti-glutamatergic fashion. Furthermore, a number of models of experimental stroke have shown a neuroprotective effect of the β-carboline stobadine (Stolč et al., 1997). Abe has been developed and intensively tested in several phase III clinical studies for treatment of anxiety disorders, among other indications (Rickels et al., 2000; Small and Bystritsky, 1997). In these trials, Abe was found to have a highly favorable clinical safety profile.

In the present study we used different models of ischemic and excitotoxic neuronal cell damage to test the hypothesis that Abe acts as a neuroprotectant. To mimic ischemic conditions in vitro, primary cortical neurons were exposed to combined OGD or an excess of NMDA. In organotypic retinal cultures with maintained cellular composition, a model of Glu excitotoxicity was established (Rzeczinski et al., 2006). In both models we tested whether Abe protects neurons against stimuli which are relevant in various CNS disorders (stroke, glaucoma, etc.).

2. Materials and methods

2.1. Preparation of primary cortical neurons

Primary neuronal cultures of cerebral cortex were obtained from embryos (E17) of Wistar rats (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany). Cultures were prepared according to Brewer (1995) with small modifications described before (Ruscher et al., 2002). Cultures were kept at 36.5 °C and 5% CO_2 and fed beginning from 4 days in vitro (div) with cultivating medium (starter medium without Glu) by replacing half of the medium twice a week. The cultures were used for experiments after 8 div containing <10% astroglial cells.

2.2. Oxygen glucose deprivation (OGD)

Culture medium was washed out with phosphate buffered saline (PBS), and OGD was induced with deoxygenated aglycemic solution (pH 7.4, Na^+ 143.8 mM, K^+ 5.5 mM, Ca^{2+} 1.8 mM, Mg^{2+} 1.8 mM, Cl^- 125.3 mM, HCO_3^- 26.2 mM, PO_4^{3-} 1.0 mM, SO_4^{2-} 0.8 mM) in an anoxic atmosphere. Hypoxia was generated in a dedicated humidified, gas tight incubator (Concept 400, Ruskinn Technologies Ltd., UK), flushed with gas of the following composition: 5% CO_2, 85% N_2 and 10% H_2. During OGD (120 min) oxygen tensions in the media were below 1 mmHg (Polarographic Probe, Licox, GSM, Germany). In control experiments, medium was replaced by basic salt solution (BSS: pH 7.4, Na^+ 143.8 mM, K^+ 5.5 mM, Ca^{2+} 1.8 mM, Mg^{2+} 1.8 mM, Cl^- 125.3 mM, HCO_3^- 26.2 mM, PO_4^{3-}1.0 mM, SO_4^{2-} 0.8 mM, glucose 20 mM) after washing with PBS, and cells were incubated in a normoxic atmosphere containing 5% CO_2. Immediately after OGD solutions were replaced by 75% fresh NBM supplemented with B27 and 25% stored medium from untreated neurons.

2.3. Drug administration in cultured cortical neurons

Matured cortical neurons were treated with 100 μM of NMDA (Sigma, Taufkirchen, Germany) for different intervals. Stock solutions were prepared in Neurobasal medium supplemented with B27 and used as a 1:100 dilution in the experiments.

Prior treatment with Abe (kindly provided by Dr. Mann Pharma, Berlin, Germany) dimethyl sulfoxide (DMSO, final concentration 0.1%) was added to the culture medium. An Abe stock solution (final concentration 1 mM) was prepared in DMSO. Different dilutions were used to treat cells with 100 nM, 1 μM or 10 μM Abe. Final concentration of DMSO in the culture medium was 0.2%. For controls, only DMSO was added to the culture medium. Higher concentrations of Abe were toxic and resulted in precipitation of the drug in the culture medium (data not shown). Diazepam (Ratiopharm, Ulm, Germany) was purchased as a liquid and was diluted in 100% ethanol. Final concentration of diazepam in the culture medium was 1 μM. For pretreatment, Abe and diazepam were added to the culture medium for 20 min prior to OGD and washed out immediately before OGD. For concurrent treatment, both substances were present in the incubation buffer during OGD and BSS for 120 min and washed out immediately after treatment. For posttreatment, Abe and diazepam were applied for 24 h beginning 20 min after OGD and BSS, respectively.

2.4. Lactate dehydrogenase (LDH) assay

At the indicated time points cell injury was assessed by measurement of lactate dehydrogenase (LDH) activity in the supernatant medium as described previously (Bruer et al., 1997). LDH activities are presented in a normalized fashion. LDH values from OGD treated cells were set to 100%, BSS treated cells were set to 0%. All other LDH activities are presented as a ratio of these conditions.

2.5. Dissection and roller cultivation of the adult rat retina

Organotypic roller cultures of rat retinas were prepared as described previously (Rzeczinski et al., 2006). Briefly, for each experiment the eyes of four male Wistar rats (220–240 g) were enucleated under sterile conditions. After punching a hole at the cornea–sclera junction with a scalpel, a circular