

Survival of cultured hippocampal neurons upon hypoxia: neuroprotective effect of gabapentin

Przeżycie hodowli komórek nerwowych hipokampa w warunkach niedotlenienia: neuroprotekcynny efekt gabapentyny

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ABSTRACT

Introduction: Gabapentin (GBP) is a novel analogue of GABA used widely in the treatment of epileptic partial seizures and neuropathic pain. GBP blocks Ca²⁺ channels in neural cell membrane and diminishes excitation of neurons. Such mechanism of action of this drug can predict GBP as a potential neuroprotectant. **Aim of the study:** To investigate the putative protective effect of GBP against hypoxia-induced neurotoxicity in primary culture of rat hippocampal neurons. **Material and methods:** An experiment was performed on dissociated hippocampal cultures at seven day *in vitro*. Cell death was induced by incubation of neural cultures in hypoxic conditions over 24 hours. The cultures (except control) were treated with 30 μ M, 100 μ M and 300 μ M concentrations of GBP to cause a neuroprotective effect. Neuronal injury was assessed by morphometric investigation of death/viable neurons in light microscopy using Trypan blue staining. **Results and conclusions:** None of the used concentrations of GBP exerted *per se* a toxic effect on cultured neural cells. Death of one third of neurons was observed in non-treated cultures upon hypoxia. GBP was found to inhibit hypoxia-induced neuronal damage in a dose-dependent manner: in cultures treated with high concentrations of the drug (100 μ M and 300 μ M), about two-fold higher number of neurons remained viable when compared to non-treated cultures. The results suggest that GBP has promising neuroprotective properties *in vitro* and prevents hypoxia-induced cell damage in primarily cultured hippocampal neurons. **Key words:** gabapentin, neuroprotection, hippocampal culture, neurons

STRESZCZENIE

Wprowadzenie: Gabapentyna (GBP) jest chemicznym analogiem GABA stosowanym w leczeniu padaczki z napadami częściowymi oraz w terapii bólu neuropatycznego. GBP blokuje funkcję kanałów Ca²⁺ w błonie komórkowej neuronu i ogranicza napływ Ca²⁺ do wnętrza komórki nerwowej, czego efektem jest zmniejszenie jej pobudliwości. Taki mechanizm działania GBP sugeruje, że antyepileptyk może wykazywać działanie ochronne na komórki nerwowe poddane czynnikom wywołującym neurodegenerację. **Cel pracy:** Celem pracy była ocena potencjalnych właściwości neuroprotekcynnych GBP w warunkach pierwotnej hodowli komórek nerwowych hipokampa poddanej stresowi oksydacyjnemu. **Materiał i metodyka:** Doświadczenie przeprowadzono na 7-dniowej rozproszony hodowli komórek nerwowych hipokampa. Uszkodzenie neuronów wywołano 24-godziną inkubacją hodowli w warunkach hipoksji. Do medium hodowli (z wyjątkiem hodowli kontrolnych) dodano roztwór GBP w stężeniach 30 μ M, 100 μ M i 300 μ M celem wywołania potencjalnego efektu neuroochronnego. Uszkodzenie komórek nerwowych w poszczególnych hodowlach oceniano w mikroskopie świetlnym po zabarwieniu neuronów błękitem trypanu. **Wyniki i wnioski:** Żadne z zastosowanych w doświadczeniu stężeń GBP nie wywołało *per se* uszkodzenia neuronów. W hodowlach pozbawionych leku stres oksydacyjny spowodował śmierć 30% neuronów. Wykazano dobre, proporcjonalne do stężenia właściwości neuroochronne leku. W hodowlach zawierających wysokie stężenia GBP (100 μ M i 300 μ M) przeżyła prawie 2-krotnie większa ilość neuronów w porównaniu do hodowli kontrolnych bez leku. Uzyskane wyniki wskazują, że GBP w warunkach *in vitro* wykazuje efekt neuroochronny i zapobiega śmierci komórek nerwowych hipokampa w hodowli poddanej stresowi oksydacyjnemu. **Słowa kluczowe:** gabapentyna, neuroprotekcja, hodowle neuronów hipokampa, neurony

INTRODUCTION

Scientific data from recent years indicates that many different mechanisms are involved in the neurodegenerative processes. There are known to be two pathways of neural death: necrosis and apoptosis. These two processes com-

monly occur simultaneously and are referred to as apoptosis [1]. Cell death molecular mechanism is often initiated by hypoxia, especially in the immature brain. Results of numerous investigations have demonstrated the leading role of glutamate in hypoxic damage to neurons [2,3]. Oversti-

mulation of glutamate receptors due to excessive exposure to the neurotransmitter glutamate has been implicated as the most important factor contributing to neuronal injury and has been referred to as excitotoxicity [3,4]. The result of increased glutamate activity on the postsynaptic membranes is rapid inflow of Ca^{2+} into the neurons, followed by activation of Ca^{2+} -dependent proteases, lipases, endonucleases, pro-apoptotic genes and by promoting the formation of free oxygen radicals, which lead to death of nerve cells [5]. Therefore, pharmacological antagonizing excitotoxicity by inhibition of glutamate receptors and Ca^{2+} channel function in neurons is a particularly attractive target for neuroprotection. Some of the antiepileptic drugs due to their multidirectional mechanism of action including glutamate receptors blockade, Ca^{2+} channel inhibition and GABAergic system potentiation, have been suggested as promising neuroprotectants.

Gabapentin [1-(aminomethyl)cyclohexane acetic acid] (GBP) is a recently introduced structural analog of GABA used in the treatment of partial epilepsy and neuropathic pain [6,7]. Although originally designed as a GABA mimetic, GBP has no significant activity on GABA receptors [8]. GBP blocks $\alpha_2\delta$ - Ca^{2+} channels [9-12] and modulates mitochondrial ATP-sensitive K^{+} channels [13, 14]. Therefore, the choice of GBP for our experiment was not incidental. GBP has several mechanisms of action that may contribute to its neuroprotective activity including antagonistic effects on Ca^{2+} channels, which play important role in the excitotoxic neuronal damage. Hippocampal neurons are selectively vulnerable to the effect of excitotoxic damage because of high density of glutamate receptors on their cell membranes. Therefore, dissociated hippocampal cultures have been used extensively to study neuroprotective effects of drugs and cell death pathways under hypoxic/excitotoxic conditions [15]. Scientific reports discussing neuroprotective properties of GBP in hypoxia-injured neuronal cultures are very scarce. This fact has inclined us to undertake the current experiment. The aim of the study was to estimate putative protective effects of GBP on cultured hippocampal neurons in the experimental model of hypoxic stress.

MATERIALS AND METHODS

Culture of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague–Dawley rats as described previously [16]. Dissected hippocampi were purchased commercially and delivered in B27/Hibernate E from Brain Bits (BrainBits, USA) Tissues were incubated with papain (Worthington) in Hibernate E medium (BrainBits, USA) at 30°C for 20 min, followed by mechanically trituration with a fire-polished Pasteur pipette. The mixture was transferred into B27/Hibernate E medium and the cells were centrifuged at $200\times g$ for 1 min. The supernatant was quickly aspirated and the cells were resuspended in 1 mL of B27/Neurobasal medium (Invitrogen) with 0.5 mM Glutamax and 25 μM glutamate. Once in suspension, the number of viable cells was determined by trypan blue exclusion using hemacyto-

meter. Next, cells were plated on 24-well plates coated with poly-D-lysine (Becton Dickinson) at a density of 32×10^3 cells/ 2 cm^2 . Cultures were grown in a humidified incubator at 37°C , 5% CO_2 . Half of the medium was replaced with NbActiv4 medium (BrainBits, USA) every 3 days. Under these conditions cell cultures comprise more than 95% neurons. The experiment was performed after 7 days in culture.

Drug preparation

Gabapentin was supplied from Sigma-Aldrich and dissolved in NbActiv4 medium (BrainBits, USA) at a concentration of 1mM as a stock solution. The solution was further diluted with the same medium to obtain desired concentrations.

Hypoxia in culture

Before starting the exposure of the cultured neurons to hypoxia, the medium was replaced with fresh NbActiv4 medium with 30 μM , 100 μM , 300 μM GBP or NbActiv4 medium without drug (control). Cultures were then transferred into an incubator at normoxic conditions for two hours. Next, cultures (except control) were replaced into an incubator set up for hypoxia experiment. Hypoxia was maintained by flushing the incubator with 20% CO_2 resulting in a reduction of the oxygen concentration in the incubator atmosphere to less than 1%. Cultures were exposed to hypoxia or normoxia (control) for 24 hours.

Morphometric study of neuronal cell survival

After cessation of hypoxia, neuronal cultures were stained with 0.4% Trypan blue (Sigma-Aldrich). Unstained cells were regarded as viable and stained cells were regarded as dead. Total cell number and number of cells excluding the dye (death cells), were counted in light microscope under magnification $\times 200$ by an independent, blinded investigator. For each condition, five non-overlapping fields of three different wells (i.e. 15 fields per condition) were analyzed.

Data analysis

After normalization as a percentage of control \pm SD the data was analyzed using Statistica software. One-way analysis of variance (ANOVA) was used to determine overall significance. Differences between control and experimental groups were assessed with *posthoc* Tukey test, with the significant differences marked in the following way: * $p < 0.01$, ** $p < 0.001$ (compared to the control cultures upon normoxia); # $p < 0.01$ and ## $p < 0.001$ (compared to the control cultures upon hypoxia). Data was expressed as means \pm SD. Differences were considered statistically significant at $p < 0.05$.

RESULTS

The percentage of dead nerve cells after seven days in dissociated culture in normoxic conditions was about 7%, and it did not differ statistically among individual plates of cultures with different concentrations of GBP. 24-hour oxidative stress caused death of one third of the population of neurons in control cultures without drug and in the culture with a low, 30 μM , concentration of the antiepileptic drug ($p < 0.001$ vs. control upon normoxia). In cultures with a high concentrations of GBP, the percentage of dead cells was significantly

lesser and it amounted 18% and 21% for 100 μM and 300 μM concentrations of GBP, respectively (Figure 1).

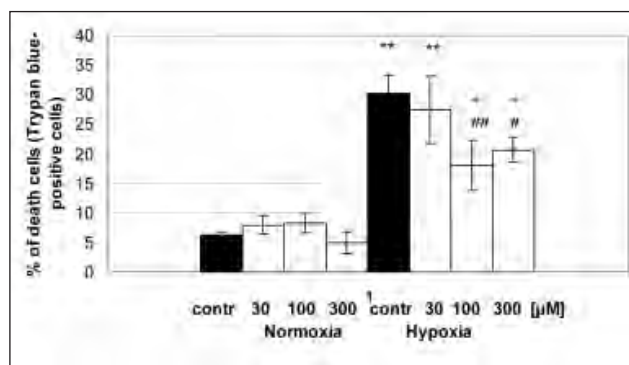


Figure 1. The effect of gabapentin (30 μM , 100 μM and 300 μM) on hypoxia-induced neuronal injury. Cultures were treated with gabapentin for 26 h and Trypan blue staining was applied. Each bar represents an average percentage \pm SD of death neurons. * $p < 0.01$; ** $p < 0.001$ (compared to the control cultures upon normoxia); # $p < 0.01$; ## $p < 0.001$ (compared to the control cultures upon hypoxia).

DISCUSSION

It has been postulated that the abnormal release of excitatory amino triggers the cellular events leading to neuronal death after hypoxia [3,17]. Calcium signaling pathways play a vital role in the survival of neurons. Increased intracellular concentrations of Ca^{2+} result in rapid efflux of glutamate to the synapse and glutamate-mediated neurotoxicity [18]. The pharmacological modulation of Ca^{2+} -dependent glutamate release has been frequently studied in varied models of experimental and clinical neuroprotection. Numerous reports have suggested that conventional as well as a novel antiepileptic drugs have some neuroprotective activity in the experimental models of brain injury [19,20]. Inhibition of postsynaptic voltage-dependent Ca^{2+} currents has been reported for some of antiepileptic drugs, including GBP [21,22]. The results of our study indicate that none of the three used concentrations of GBP exerted a toxic effect *per se* on cultured neurons. After 24-hour incubation of hippo-

campal cultures in hypoxic condition death of 30% of non-treated neurons was observed. In cultures containing high GBP concentrations: 100 μM and 300 μM , two-fold higher number of nerve cells remained viable as compared to control cultures without drug.

Neuroprotective properties of GBP were described in a few experimental studies. Comi et al. [23] determined neuroprotective effect of GBP in the model of ischemic injury in the immature brain of the rat. Hippocampal neurodegeneration in streptozotocine-induced diabetic rats has been significantly attenuated by GBP treatment [24]. Protective effect of GBP has been evaluated *in vitro* by Kim et al. [25] in organotypic hippocampal slice cultures. Pre-treatment with GBP reduced the degree of neuronal damage induced by NMDA exposure in cultured hippocampal slices, especially in CA1 sector. Results of another experimental study performed on cultured hippocampal slices demonstrated a beneficial neuroprotective effect of GBP via inhibition of $\text{K}^{(+)}$ -evoked glutamate release [26].

The most important mechanism of GBP neuroprotective effects is blocking the influx of calcium into neurons via the $\alpha 2\delta$ -2 subunits of voltage-dependent Ca^{2+} channels, thus GBP prevents intracellular calcium accumulation in neurons [27]. The hippocampus has been shown to have high density of the $\alpha 2\delta$ -2 subunits. Via this mechanism, GBP may reduce synaptic release of excitatory neurotransmitters and post-synaptic neuronal excitation [28].

CONCLUSIONS

24-hours incubation of dissociated hippocampal cultures in hypoxic conditions caused death of about 30% neurons. In cultures treated with high concentrations of GBP (100 μM and 300 μM) almost two-fold higher number of neurons remained viable. The drug at low 30 μM concentration was ineffective as a neuroprotectant. In summary, the present study demonstrates that GBP induces significant neuroprotection in the experimental model of brain hypoxia. This effect was correlated with concentration of the drug in culture medium.

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