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Levetiracetam protects hippocampal neurons in culture against hypoxia-induced injury

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Abstract: Many experimental studies indicate that some antiepileptic drugs possess neuroprotective properties in varied models of neuronal injury. Levetiracetam is a second-generation antiepileptic drug with a novel mechanism of action. In the present study, we evaluated the putative neuroprotective effect of levetiracetam on primary hippocampal cultures at seven day *in vitro.* Cell death was induced by incubation of neural cultures in hypoxic conditions over 24 hours. Neuronal injury was assessed by morphometric investigation of death/total ratio of neurons in light microscopy using Trypan blue staining and by evaluation of lactate dehydrogenase (LDH) release in the culture medium. Our results indicate that pre-conditioning of hippocampal cultures with high concentrations of levetiracetam (100 μM and 300 μM) protects neurons against hypoxia-induced death. Two-fold higher number of neurons remained viable as compared to control cultures without drug. Lack of neuroprotective action of the drug on hippocampal neural cultures was observed, when a low concentration (10 μM) of levetiracetam was used. *(Folia Histochemica et Cytobiologica 2011, Vol. 49, No. 1, 148–152)*

Key words: levetiracetam, neuroprotection, hippocampal culture, hypoxia

Introduction

Disturbances to the function of the central nervous system, occurring both acutely and chronically, activate the cascade of complex biochemical processes, which lead to degeneration and finally to the death of neurons [1]. A classical example from clinical practice could be an ischemic stroke, triggered by an occlusion of cerebral artery. A deep energetic deficit severely disturbs the functions of cellular and mitochondrial enzymes and imbalances the ion system. Degeneration of the neurons is probably triggered by excessive Ca^{2+} influx, additionally amplified by Ca^{2+} efflux from the endoplasmic reticulum [2]. Increased intracellular concentration of Ca^{2+} results in rapid efflux of glutamate to the synapse, and glutamate-mediated neurotoxicity [3, 4]. Sustained elevation in Ca^{2+} is assumed to set in motion various pathological processes, which could degenerate neurons by activating proteases, lipases, endonucleases, pro-apoptotic genes and nitric oxide synthases and by promoting the formation of free oxygen radicals [5, 6].

There are known to be two pathways of neural death: necrosis and apoptosis. Recent research indicates that these processes coincide and can be termed ‘aponecrosis’ [7, 8]. Recent decades have seen many scientific reports about the putative neuroprotective effects of various drugs and chemical compounds. Neuroprotectants stop or delay various stages of the complex mechanism of neuronal death. Neuronal cultures are often used as an *in vitro* model to assess the effects of various chemical compounds in the field of neuroprotection [9]. Hippocampal or cortical neurons are usually preferred because these neurons are selectively vulnerable to the effects of hypoxia and ischemia,

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both *in vivo* and *in vitro* [10]. Because of their complex, multidirectional mechanism of action, some traditional, and some new, antiepileptic drugs have been suggested as promising neuroprotectants, and have been studied in neuronal cultures, in which varied cell death-inducible factors have been used [11, 12].

Levetiracetam (LEV), the a-ethyl analogue of the nootropic piracetam, is a relatively new antiepileptic drug, with a unique chemical structure and mechanism of action. LEV has a very favorable pharmacokinetic profile: excellent bioavailability, linear kinetics, minimal plasma protein binding, and quick achievement of steady state concentrations [13]. LEV is widely used for protection against partial seizures, and is also effective in the treatment of primary generalized seizures [14].

The mechanism of action of LEV is distinct from first-generation and other second-generation AEDs. LEV appears not to work by the three classic routes of other AEDs: sodium channel modulation, low-volt -age-activated (T-type) calcium channel modulation, and gamma-aminobutyric acid (GABA) facilitation. LEV's mode of action is not fully elucidated, but it has been found to target high-voltage, N-type calcium channels [15] as well as the synaptic vesicle protein 2A (SV2A). The drug binds reversibly and specifically to this plasma membrane protein receptor site [16, 17]. The functional role of SV2A is not well understood, but it seems to be a positive modulator of synaptic transmission that may act by preparing vesicles for fusion [18]. We decided to undertake our study because the scientific reports discussing the putative neuroprotective action of LEV are both scarce and controversial, especially as regards neuronal cultures.

Material and methods

Culture of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague–Dawley rats following Brewer et al. [19] and Brewer and Price [20]. Dissected hippocampi were purchased commercially and delivered in B27/Hibernate E from Brain Bits (http://www.BrainBitsLLC.com). Tissues were incubated with papain (Worthington) in Hibernate E medium (BrainBits) at 30°C for 20 minutes, followed by mechanical trituration with a fire-polished Pasteur pipette. The mixture was transferred into B27/Hibernate E medium and the cells were centrifuged at 200 × g for one minute. The supernatant was quickly aspirated, and the cells were re-suspended 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 a hemacytometer.

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, 9% O_2. Half of the medium was replaced with NbActiv4 medium (BrainBits) every three days. Under these conditions, cell cultures comprise more than 95% neurons [19, 21]. The experiment was performed after seven days in culture.

Drug preparation

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

Hypoxia in culture

Before starting exposure of the cultured neurons to hypoxia, the medium was replaced with fresh NbActiv4 medium with 10 μM, 100 μM or 300 μM levetiracetam. Control cultures were grown without levetiracetam. 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 a hypoxia experiment. Hypoxia was maintained by flushing the incubator with 20% CO_2 which resulted 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 Trypan blue positive cells (death cells), were counted via a light microscope under magnification × 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.

Evaluation of cell death by LDH assay

Neural injury was evaluated additionally by measuring lactate dehydrogenase (LDH) activity released in the media 24 hours after hypoxia cessation using the colorimetric assay (Roche). The experiment was performed as per the manufacturer's instructions. The intensity

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of red color formed in the assay and measured at a wavelength of 490 nm was proportional to LDH activity and to the number of damaged cells. Data were normalized to the activity of LDH released from control culture media (100%) and expressed as a percentage of the control. The experiment was performed three times with three wells per condition each time.

Data analysis

Data after normalization as a percentage of control ± ± SEM were 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 *post hoc* Tukey test, with the significant differences marked in the following way: *p < 0.01 and **p < 0.001 (vs. control cultures), #p < 0.001 (vs. 20% CO_2 treated cultures). Data were expressed as means ± SD. A level of p < 0.05 was considered statistically significant.

Results

Morphometric findings

The percentage of dead nerve cells after seven days in dissociated culture in normoxic conditions was about 6%, and it did not differ statistically among individual plates of cultures with different concentrations of LEV. 24-hour oxidative stress caused death of over a quarter of the population of neurons in control cultures without LEV and in the culture with a low, 10 μM, concentration of the antiepileptic drug (p < 0.001). In cultures with a high concentration of LEV, (100 μM and 300 μM), the percentage of dead cells was 16% and 13%, respectively (Figure 1).

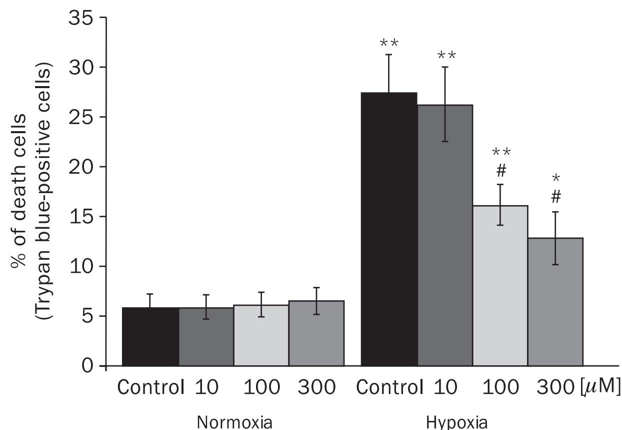

Figure 1. The effect of levetiracetam (10 μM, 100 μM and 300 μM) on hypoxia-induced neuronal injury. Cultures were treated with levetiracetam for 26 h and Trypan blue staining was applied. Each bar represents a mean percentage ± SD of death neurons. *p < 0.01; **p < 0.001 vs. control cells in normoxic conditions; #p < 0.001 vs. control cells in hypoxic condition, respectively

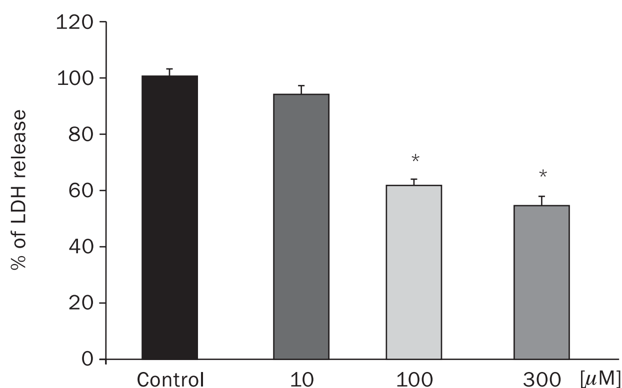

Figure 2. The effect of levetiracetam (10 μM, 100 μM and 300 μM) on LDH activity 24 h after cessation of hypoxia-induced injury. Each bar represents a mean percentage ± SD of LDH activity in culture medium when compared to the LDH activity in control culture without LEV. *p < 0.001 vs. control

Measurement of lactate dehydrogenase (LDH) activity

In biochemical assessment of LDH efflux from disintegrating neurons to the culture media, we found statistically significant 40% and 46% decreases of LDH concentration in cultures concerning 100 μM and 300 μM of LEV, respectively, compared to a control culture without the drug (p < 0.001). The activity of LDH in dishes concerning 10 μM concentration of LEV was comparable to the control (Figure 2).

Discussion

Numerous reports have suggested that conventional, as well as recently introduced, antiepileptic drugs have some neuroprotective activity in the experimental models of hypoxic/ischemic brain injury [11, 22, 23]. The neuroprotective activity of certain antiepileptic drugs seems to be connected to their complex mechanisms of action. The commonest actions were shown on ion channels, GABA-ergic and glutamatergic metabolism, receptors or secondary messengers [24]. Those biochemical mechanisms are connected tightly with necrosis/apoptosis pathways in the neurons. Modulation of these processes by mostly antiepileptic drugs results in inhibition or limitation of them, otherwise neuroprotection.

The trials of a new generation of antiepileptic drugs seem to be of great interest from a practical point of view. Newer antiepileptic drugs, when compared to

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conventional drugs, have equal or greater efficacy, but fewer toxic effects and better tolerability [25].

The results of our study indicate that none of the three concentrations of LEV used in the experiment exerted *per se* a toxic effect on neuronal cultures. There were no statistically significant differences between cultures in morphometric and LDH activity assessment in normoxic conditions.

LEV, when used in higher concentrations, has a beneficial neuroprotective effect on hippocampal neurons in culture after hypoxia-induced injury. In cultures with a concentration of LEV of 100 μM and 300 μM, an increased survival of nerve cells in the morphometric evaluation was noted, of 47% and 59%, respectively. Comparable neuroprotective effects of the drug have been confirmed by assessing LDH activity in the culture medium.

Our results indicate that higher concentrations of LEV protect at least half of hippocampal neurons in culture, when compared to non-treated cultures.

Published reports looking at the neuroprotective actions of LEV are scarce, controversial and have been performed mostly as experimental *in vitro* studies. Hanon and Klitgaard [26] demonstrated the neuroprotective properties of LEV in the experimental model of ischemic stroke triggered by occlusion of medial cerebral artery. When a high dose of LEV was applied to the rats *i.p*. before artery occlusion, the insult area was about 33% smaller. Promising neuroprotective effects of LEV have also been observed in murine models of closed head injury and subarachnoid hemorrhage [27]. In experimental models of kainic acid-induced neurotoxicity, the results were controversial. Marini et al. found a neuroprotective effect of LEV in hippocampal neuronal cultures [28], while Kitano et al. observed a similarly beneficial effect only when the n-propyl analogue of LEV, nefiracetam, was used rather than LEV itself [29]. The neuroprotective properties of LEV have also been observed in animal models of an electrically-induced epileptic state. This effect could be seen by the positive influence of the drug on the efficiency of energetic processes of neurons in mitochondria [30]. Custer et al. found a reduction of excitatory synaptic potential responses in hippocampal neuron cultures from mice in which SV2A had been deleted by gene targeting [18].

Published data evaluating the neuroprotective effect of LEV *in vitro* are inconclusive. There has been found to be no protective effect of LEV on neurons in hypoglycemia and hypoxia-induced damage of organotypic hippocampal culture. But some other antiepileptic drugs did have a protective effect [31]. We have not found in the literature any studies concerning the potential neuroprotective effects of LEV in dissociated cultures of hippocampal neurons. Studies in neuron cultures have focused on efforts to explain the mechanisms of LEV action which have a putative neuroprotective effect. Some studies have confirmed that LEV inhibits ryanodine and IP3 receptors in hippocampal culture [32]. These receptors are responsible for excessive accumulation of Ca^{2+} in the cytoplasma of neurons. This is an important stage in the process of cell death [32]. Another experiment found that LEV modulates AMPA receptors in cortical neuronal cultures and leads to impairment of postsynaptic potential in these cells [33]. Angehagen et al. reported that LEV inhibits the caffeine-induced and Ca^{2+}-dependent epileptiform discharges in hippocampal neuronal culture [34]. This drug also inhibits high potential-dependent calcium channels in conditions in vitro [35]. No effect of LEV on Na^{+} and T-type Ca^{2+} currents has been found [36]. Cardile et al. found that LEV enhances brain-derived neurotrophic factor expression in astrocytes culture [37], which is the next neuroprotective mechanism of action of this drug.

With respect to action mechanisms, LEV is distinct from traditional antiepileptic drugs such as Na^{+} channel blockers, GABA enhancers and T-type Ca^{2+} channel blockers. Previous data have demonstrated that mechanisms of LEV action have a near connection with the excitotoxic pathway of neuronal death, which is triggered by hypoxia. The results of our study confirmed also this neuroprotective effect of LEV.

Conclusions

This experimental study demonstrates that a second-generation antiepileptic, levetiracetam, can prevent hypoxia-inducible neuronal injury in cultured hippocampal neurons. The drug exerts neuroprotective effects only when used in higher concentrations in the culture medium: 100 μM and 300 μM. Such concentrations of LEV caused that two-fold higher number of neurons remained viable as compared to control cultures without drug.

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