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Cytokine IL-10, activators of PI3-kinase, agonists of α-2 adrenoreceptor and antioxidants prevent ischemia-induced cell death in rat hippocampal cultures

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ABSTRACT

In the present work we compared the protective effect of anti-inflammatory cytokine IL-10 with the action of a PI3-kinase selective activator 740 Y-P, selective agonists of alpha-2 adrenoreceptor, guanfacine and UK-14,304, and compounds having antioxidant effect: recombinant human peroxiredoxin 6 and B27, in hippocampal cell culture during OGD (ischemia-like conditions). It has been shown that the response of cells to OGD in the control includes two phases. The first phase was accompanied by an increase in the frequency of spontaneous synchronous Ca^{2+}-oscillations (SSCO) in neurons and Ca^{2+}-pulse in astrocytes. Spontaneous Ca^{2+} events in astrocytes during ischemia in control experiments disappeared. The second phase started after a few minutes of OGD and looked like a sharp/avalanche, global synchronic (within 20 s) increase in $[Ca^{2+}]_i$ in many cells. Within 1 h after OGD, a mass death of cells, primarily astrocytes, was observed. To study the protective action of the compounds, cells were incubated in the presence of the neuroprotective agents for 10–40 min or 24 h before ischemia. All the neuroprotective agents delayed a global $[Ca^{2+}]_i$ increase during OGD or completely inhibited this process and increased cell survival.

1. Introduction

A stroke of the brain is a very transient process. Upon termination of blood vessels in the brain already after 15 s there is a change in the bioelectric neuronal activity. Moreover, after 4–5 min, irreversible neuronal damage occurs in the area of stroke [8]. Ca^{2+} ions play a key role in neuronal death. $[Ca^{2+}]_i$ increase, the secretion of glutamate, activating AMPA, kainite and NMDA receptors lead to depolarization, which again activates influx of Ca^{2+} through potential-dependent Ca^{2+}-channels [11].

Previously it was shown that anti-inflammatory cytokine IL-10 protects neurons from hyperexcitability and death during hypoxia/ischemia *in vitro* and *in vivo* [37] [32]. In experiments with neurons in culture, IL-10 suppressed the effect of hyperexcitability during reoxygenation after brief hypoxia inhibiting IP3-dependent release of Ca^{2+} from endoplasmic reticulum (ER) [34]. IL-10 protective effect is abolished by inhibitors of PI3-kinase [33]. In control experiments, inhibitors of PI3-kinase provoked a global $[Ca^{2+}]_i$ increase in individual neurons and their subsequent death, even after short-term hypoxia [33]. These experiments indicated that neuroprotective effects of IL-10 during ischemia are mainly determined by the activation of PI3-kinase-dependent signaling pathways of cell survival [29].). Thus, activation of PI3-kinase signaling pathway, leading to activation of PKB - and PKG-dependent phosphorylation of protein targets and anti-inflammatory gene expression [15], can be a strategy to protect neurons from death during an ischemic stroke. It is known that the protective PI3-kinase pathway, stimulated by IL-10, is activated by other receptors, in particular receptors coupled with G_i proteins [Lopez-Ilasaca et al., 1997]. Thus, it is possible to predict the neuroprotective effect of agonists of this receptor group.

It is known that an ischemic stroke is accompanied by inflammation and generation of reactive oxygen species [5,16]. Therefore,

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the use of antioxidants as neuroprotective agents often also has a positive effect [2,18]; [22].

In the present work, with the aim to strengthen the protection of brain cells from death during ischemia, we compared the effect of anti-inflammatory cytokine IL-10, agonists alpha-2-adrenergic receptor, activators of the PI3-kinase signaling pathway, and antioxidant enzymes on the impulse activity of neurons and astrocytes, on the global Ca^{2+} increase and cell death during ischemia in cell culture of rat hippocampus. The cytokine IL-10, an activator of PI3-kinase 740 Y–P, agonists of alpha-2 adrenoreceptor, guanfacine and UK-14,304, antioxidant enzyme - peroxiredoxin 6, and B27 have been used as neuroprotective agents in the experiments.

2. Material and methods

2.1. Hippocampal cell isolation

Rat hippocampus was excised with clippers, put in a test-tube and incubated for 2 min. Supernatant was removed with pipette, and 2 ml trypsin (0.1% in Ca^{2+}- and Mg^{2+}-free Hanks' solution) was added to the pellet to cover the whole tissue. The preparation was being incubated for 15 min at 37 °C with constant shaking at 600 rpm. Then, trypsin was inactivated by equal volume of cold embryo serum, and the preparation was centrifuged at 300 g for 5 min. To remove trypsin, the cells were centrifuged twice in DMEM (Dulbecco's Modified Eagle's Medium). Then, the cells were resuspended in this medium with the addition of glutamine (0,5 mM), FBS (10%) and gentamycin (20 μg/ml). 200 μl of the suspension was put in a glass ring with the internal diameter of 6 mm standing on a round coverslip, 25 mm in diameter (VWR International), covered by poly-L-lysine (one hippocampus for five glasses). The preparations were put in a CO_2-incubator at 37 °C for 5 h for cell attachment. After cell attachment, the glass rings were removed. On the third day the medium in the dishes was replaced with a fresh portion of medium, and incubation in CO_2 atmosphere was continued for 24 h. After that the medium was replaced with fresh culture medium. Then, the culture medium (2/3 of the volume) was replaced every 3 days. To keep the ratio of astrocyte/neuron, we used a cytosine-arabinoside which inhibits proliferation process.

2.2. Ca^{2+} measurements

To measure the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), we used Carl Zeiss Cell Observer on the basis of an inverted motorized microscope Axiovert 200M with a high-speed monochrome CCD-camera AxioCam HSm and a high-speed light filter replacing system, Ludl MAC5000. For Fura-2 excitation and registration, we used the 21HE filter set (Carl Zeiss, Germany) with excitation filters BP340/30 and BP387/15, beam splitter FT-409 and emission filter BP510/90, objective lens Plan-Neofluar 10×/0.3, excitation light source HBO 103 W/2. Calcium responses to OGD were recorded with double wavelength fluorescent probe Fura-2. During image processing, the calcium response amplitudes of Fura-2 loaded cells expressed as the ratio of fluorescence intensities of Fura-2 upon excitation at wave lengths 340 and 380 nm were being measured. ImageJ and Origin 8 software packages were used for data processing, graph creation and statistical analysis. All values are given as mean ± SEM or as typical calcium responses of most of the cells. The data were statistically compared using the paired *t*-test and were considered significantly different at $p \leq 0.05$. To discriminate the neurons and astrocytes, short-term application of 35 mM KCl and 10 μM ATP were used.

The technique for induction of OGD in primary cultured hippocampal cells Ischemia-like conditions were obtained by omitting glucose (HBSS medium without glucose) and by displacing dissolved oxygen with nitrogen or argon in the special vacuum system. The level of oxygen in the medium was measured using a Clark electrode. Oxygen tensions reached values 30–40 mm Hg or less within 20 min after displacing in the vacuum system. Ischemia-like conditions lasting 40 min were created by means of supplying the Oxygen glucose deprivation (OGD)-medium into the chamber, which contained cultured hippocampal cells. Constant argon feed into the experimental chamber was used to prevent the contact of the OGD-medium with the atmospheric air. The effects of OGD on neurons and astrocytes were evaluated by measuring the amplitude of cell calcium response and assessment of cell viability before and after ischemia-like conditions.

2.3. Assessment of cell viability

Cell death induced by OGD exposure was assessed by propidium iodide (PI, 1 μM) before and after OGD in the same microscopic field. Since PI stains both dead astrocytes and neurons, analysis of calcium signals upon 35 mM KCl application before OGD was used to identify the type of the cell. Neurons were identified due to the quick transient calcium signal upon KCl addition. Furthermore, as an additional indicator of cell viability, we used the Ca^{2+}-signals (presence or absence of a global increase in $[Ca^{2+}]_i$ during OGD). Also we evaluated the cell damage using output of not penetrating through the membrane dye, Fura-2 (Figs. 1F, 2G and 3F). Each experiment was repeated three or more times using separate cultures. All values are given as mean ± SEM. ImageJ and Origin 8 software packages were used for data processing and graph creation. Statistical analysis was carried out with Prism 5 (GraphPad Software, La Jolla, CA). Differences between the experimental groups/treatments were tested for statistical significance by one-way or two-way analysis of variance (ANOVA) followed by the post-hoc Tukey-Kramer test. Differences with $p < 0.05$ were considered to be significant.

2.4. Reagents

The following reagents were used in experiments: an activator of PI3-kinase 740Y-P, guanfacine and UK-14,304 (Tocris Bioscience, UK), neurobasal medium, B-27 supplement, Fura 2AM, Propidium iodide (Invitrogen, USA); interleukin-10 (Chemicon, USA). Transgenic peroxiredoxin 6 was kindly provided by prof. Novoselov V. I (ICB RAS).

2.5. The choice of the optimal model

To evaluate the effect of ischemia we measured cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) and the number of dying cells in the culture. Previously it was shown that these two parameters are related to each other [19,38]. To investigate the protective properties of the compounds, we chose the model with prolonged ischemia without reperfusion. In preliminary experiments it was shown that ischemia evoked $[Ca^{2+}]_i$ increase in the control, and the time during which the cells were with increased concentration of Ca^{2+} has implications for the reaction direction in response to reperfusion. After short-term ischemia reperfusion caused a $[Ca^{2+}]_i$ decrease to baseline and complete survival of cells. With more prolonged ischemia, reperfusion caused an additional sharp increase in $[Ca^{2+}]_i$ and additional cell death. In this case, cell death was strongly increased compared to conditions without reperfusion, indicating that reperfusion is the primary cause of their death. As this model reflects primarily the impact of reperfusion injury, and the response depending on the duration of ischemia and on the presence of the protectors was characterized as all-or-nothing, the option with