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Cellular levels of TrkB and MAPK in the neuroprotective role of BDNF for embryonic rat cortical neurons against hypoxia in vitro

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Abstract

Intrauterine asphyxia often results in neonatal loss or mental retardation. Brain-derived neurotrophic factor (BDNF) has been shown to be a protective agent against hypoxic damage to neurons. To understand the signaling mechanism underling the neuroprotective function of BDNF and to find therapeutic interventions for intrauterine asphyxia, we utilized an immunofluorescent technique to measure the intracellular levels of tyrosine kinase B (TrkB), phosphorylated TrkB, and the mitogen-activated protein kinase (MAPK) in the rat embryonic cortical neurons cultured in hypoxic conditions with and without BDNF pretreatment. The results showed that the fluorescent intensity of TrkB and phosphorylated TrkB in the cytoplasm and the fluorescent intensity of MARK in both cytoplasma and nucleus of the neurons were significantly increased in the presence of BDNF. The results indicate that the neuroprotective function of BDNF against hypoxia-induced neurotoxicity requires the participation of TrkB and is transduced via the Ras–MAPK signaling pathway.
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Keywords: Embryonic cortical neuron; BDNF; TrkB; Ras–MAPK

1. Introduction

Prenatal asphyxia is one of the main factors leading to cerebral injuries and neurodevelopmental impairments and can be fatal in severe cases. Extensive research has been carried out to discover therapeutic means that reduce asphyxia-related neonatal mortality and morbidity.

Endogenous brain-derived neurotrophic factor (BDNF) has been shown to be a protective agent against hypoxic-ischemic damage to the brain (Walton et al., 1999; Schabitz et al., 1997). Our previous study showed that the death of the embryonic rat neurons was delayed in the presence of BDNF when challenged by hypoxia. To understand the signaling mechanism underlying the neuroprotective function of BDNF, we attempted, in this study, to address whether tyrosine kinase B (TrkB), the BDNF receptor, was phosphorylated and whether the level of TrkB was elevated upon a BDNF treatment. We also tried to address whether the Ras–mitogen-activated protein kinase (MAPK) signaling pathway was activated by the BDNF treatment. We cultured fetal rat cortical neurons in vitro under hypoxic conditions to mimic an intrauterine hypoxia and observed an increase of the intracellular levels of TrkB, phosphorylated TrkB, and MAPK in neurons treated with BDNF.

2. Materials and methods

2.1. Culture and identification of embryonic cortical neurons

Primary cortical cultures were prepared from the SD fetal rats of 16–18 gestational days according to the procedure modified from that described previously (Li et al., 2001; Zhou et al., 2002). Briefly, the cortex from fetal rats was digested for 15 min by trypsin (0.05%, Sigma) and type-II collagenase (Sigma). The digestion was stopped by addition of calf serum and then allowed to pass through a 100-eye sieve. The cell suspension was centrifuged at 1000 rpm for 5 min and washed three times with a basic culture medium. The cells were planted into a neurobasal medium

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supplemented with B27 supplement, 20% fetal bovine serum, and 1% Glutamine (Gibco), 100 U ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, pH 7.2–7.4. The final cell density was adjusted to 1.5×10^6 to $1.8 \times 10^6\ ml^{-1}$. Twenty-four hours after plating, the cells were treated with 2.5 $\mu g\ ml^{-1}$ of cytosine arabinoside (Sigma) to inhibit proliferation of the non-neuronal cells. The growth medium was changed twice per week. The neuronal cells were identified by the anti-tubulinβ-III monoclonal antibody (Neomarkes). Cortical neurons of the 5- to 7-day culture in vitro (DIV5–7) were selected as study subjects.

2.2. *Hypoxic culture of cerebral cortical neurons and group assignment*

The cultured cerebral cortical neurons were randomized into four groups. Group A, the baseline control group, was cultured in normal condition without pretreatment of BDNF. Group B, the experimental group, was treated with 100 ng ml^{-1} of BDNF (Sigma) before hypoxia-culture and was sub-grouped into Groups B1 and B2 according to the duration of BDNF treatment. Group B1 received BDNF 24 h before hypoxia-culture and Group B2 received BDNF just before hypoxia-culture. Group C, the control group, underwent hypoxia-culture without pretreatment with BDNF. The hypoxia-culture chamber was filled with 99.99% nitrogen gas.

2.3. *Immunofluorescent detection of intracellular levels of full-length TrkB (TrkB794), phosphotyrosine, and MAPK*

The DIV5–7 cortical neuronal cells, which had been cultured in hypoxia for 3–5 h were fixed with 4% polyformalin for 15 min, permeablized with 0.1% Triton X-100/PBS for 5 min, treated with 3% H_2O_2 for 15 min to block endogenous peroxidase, and incubated with 1% bovine serum albumin (BSA) for 15 min to reduce non-specific binding. Then, anti-TrkB (794, Santa Cruze) at a working dilution of 1:75 and anti-p-Tyr (PY99, Santa Cruze) and anti-MAPK (Sigma) at a working dilution of 1:100 were added to each group, respectively. The cells were incubated at 4 °C overnight. After addition of the respective secondary fluorescent antibodies (Dako) at a working dilution of 1:100, cells were incubated at 37 °C for 1 h. The laser scanning confocal microscope was used to examine the cell.

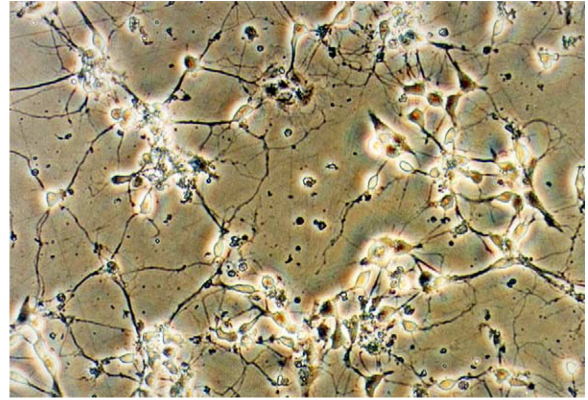

Fig. 1. Cerebral cortical neurons of a rat embryo on day 7 of ex vivo culture. LSAB 200×. On day 7, the neurons were growing well. Each neuron was surrounded by a clear pale halo. Neuronal processes interconnected with each other to form a relatively dense reticulation.

2.4. *Statistical analysis*

Data were processed with the variance analysis, and the probability of 0.05 or less was considered statistically significant.

3. Results

3.1. *Primary culture of cortical neurons*

3.1.1. *Morphological observation of cultured neurons*

Under the inverted phase contrast microscope, some neurons were seen attached to the wall of the culture flask

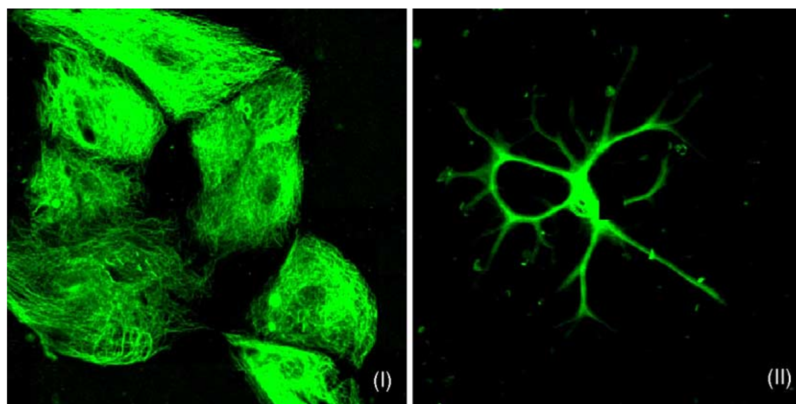

Fig. 2. Identification of neurons with the laser scanning confocal microscope. LACS 600×. Immunofluorescent staining of neuronal cells was visualized by a laser scanning confocal microscope. (I) Neurons were stained with anti-tubulin β-III monoclonal antibodies. (II) Astrocytes were stained with anti-GFAP polyclonal antibodies.

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