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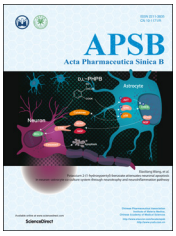

ORIGINAL ARTICLE

Potassium 2-(1-hydroxypentyl)-benzoate attenuates neuronal apoptosis in neuron–astrocyte co-culture system through neurotrophy and neuroinflammation pathway

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Abstract Potassium 2-(1-hydroxypentyl)-benzoate (D,L-PHPB), a new drug candidate for ischemic stroke at the phase II clinic trial, has been shown to protect neurons by inhibiting oxidative injury and reducing neuron apoptosis in previous studies. But the mechanisms of D,L-PHPB remain to be studied. In this study, a neuron–astrocytes co-culture system was used to elucidate the roles of astrocytes in neuroprotection of D,L-PHPB under oxygen-glucose deprivation/reoxygenation (OGD/R) condition. Our data showed that D,L-PHPB reduced neuronal apoptosis in mono-culture system and this effect was enhanced in neuron–astrocyte co-culture system under the OGD/R condition. Meanwhile, D,L-PHPB obviously increased the levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), which were mainly secreted from astrocytes, in the co-culture system after OGD/R. The PI3K/AKT and ERK signaling pathways as well as the p-TRKA/B receptors were involved in the process. In addition, the levels of TNF-α and IL-1β secreted from astrocytes after OGD/R were markedly reduced after D,L-PHPB treatment, which was mainly due to the suppression of phosphorylated p38. In conclusion, the present study demonstrates that the neuroprotective effects of D,L-PHPB were improved by astrocytes, mainly mediated by increasing the release of BDNF/NGF and attenuating inflammatory cytokines.

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1. Introduction

Brain ischemic stroke is a leading cause of death and disability. Most of researches have focused on neurons injury after brain ischemia, because neurons are far more susceptible to ischemic injury than their neighboring astrocytes[1]. Astrocytes, as the most abundant cell type in brain, distribute in all regions of the central nervous system (CNS). Aastrocytes had been regarded as the passive partners of neurons in the CNS. However, this view was challenged by more and more evidences that astrocytes may play the important roles in the CNS[2,3]. Astrocytes may provide the metabolic and trophic support to neurons, participate in the synaptic function and neuroplasticity, and maintain the extracellular balance of ions, fluid, and transmitters. In addition, astrocytes respond to a variety of injury of CNS, such as cerebral ischemia[4,5], neurodegenerative disease[6] and infection.

Potassium 2-(1-hydroxypentyl)-benzoate (D,L-PHPB) is a novel drug candidate for the treatment of cerebral ischemia. Previous study[7] showed that D,L-PHPB improved the neurobehavioral deficits and reduced infarct volume in the cerebral ischemic animal model. It might protect neurons against H_2O_2-induced apoptosis by modulating the protein kinase C (PKC) signaling pathway[8]. Recently, D,L-PHPB was shown to improve learning and memory deficits, reduce oxidative stress and glia activation in the cerebral area of hypo-perfused rats[9] and attenuate amyloid and τ pathologies in a mouse model of Alzheimer's disease[10]. These results suggested that the neuroprotective roles of D,L-PHPB in cerebral ischemia might be related to astrocytic functions, but the details are still unclear.

Most studies of neuroprotective agents were carried out separately on neurons or astrocytes. However, in brain both cells are interacted closely at physiological and pathological condition. Therefore, to understand the function of astrocytes under the cerebral ischemia and the effects of astrocytes on neuroprotective agent are very important. In the present study, we investigated the protective effects of D,L-PHPB on neuronal injury *in vitro* after oxygen-glucose deprivation/reoxygenation (OGD/R) using a neuron–astrocyte co-culture system. It was found that the neuronal protective effects of D,L-PHPB were different in presence and absence of astrocytes. The relevant mechanisms were studied.

2. Materials and methods

2.1. Animals

Neonatal pups of Wistar rats (within 24 h after birth) were obtained from Vital River Laboratories, Beijing, China. All experimental protocols in this study were approved by the Laboratories Institutional Animal Care and Use Committee of the Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China.

2.2. Materials

D,L-PHPB (purity > 98% by HPLC) was synthesized by the Department of Medical Synthetic Chemistry, Institute of Materia Medica (Beijing, China) and dissolved in phosphate buffered saline (PBS) with the current use. DMEM/F12, DMEM and Neurobasal-A media were purchased from Invitrogen Co. (Carlsbad, CA, USA). Hoechst 33342 dye was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) ELISA kits, as well as tumor necrosis factor-α (TNF-α), interleukelin-1β (IL-1β) and interleukelin-6 (IL-6) ELISA kits were purchased from BOSTER Co. (Wuhan, China). Antibodies used in Western blot analysis, including phosphorylated tyrosine kinase receptorA/B (TRKA/B), protein kinase B (AKT), extracellular regulated protein kinases (ERK), cAMP-response element binding protein (CREB) and p38 antibodies, as well as their respective total protein and β-actin antibodies, were obtained from Cell Signaling Technology Co. (Danvers, MA, USA). Other antibodies, including cleaved-caspase 3, B-cell lymphoma-2 (BCL-2), BCL-2 assaciated X protein (BAX), phosphorylated c-Jun N-terminal kinase (JNK) and total JNK, phosphatidylinositol 3-kinase (PI3K) and glial fibrillary acidic protein (GFAP) antibodies were obtained from Santa Cruz Biotechnology Co. (San Diego, CA, USA). Anti-mouse and anti-rabbit IgG were purchased from Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). All other reagents were analytical grade.

2.3. Neuron–astrocyte co-culture

Astrocytes were cultured and purified as described previously[11]. Neonatal pups of Wistar rats were decapitated and the cerebral hemispheres were immediately transferred to the cold DMEM/F12 media, then the meninges were carefully removed under a dissecting microscope. The cerebral tissue was treated with a 0.25% trypsin solution for 20 min at 37 °C. An equal volume of DMEM/F12 medium containing 10% fetal bovine serum (FBS) was added to stop the trypsin and the mixture was centrifuged at 800 × *g* for 3–5 min. The pellet was resuspended with the medium. The cultures were incubated at 37 °C in a humid 5% CO_2/95% air environment. The complete medium was changed after 24 h and half medium was changed every three days. After about 12 days, the astrocytes were treated with 0.25% trypsin solution for 3–5 min at 37 °C and were harvested. The destiny of the astrocytes was adjusted to 2×10^5 cells/mL. And they were planted on the upper chamber of transwell (0.4 μm, Corning, NY, USA).

Rat hippocampal neurons were isolated and cultured from Wistar rats postnatal within 24 h according to protocols modified from Kaech and Banke[12]. Hippocampus were isolated and incubated in trypsin (0.25%) for 20 min at 37 °C to obtain neurons. Then, neurons were planted on the lower chamber of transwell at the destiny of 4×10^5 cells/mL. Neurons were firstly cultured in DMEM with 10% FBS and 10% horse serum on poly-D-lysine (Sigma–Aldrich) coated glass coverslips. After 4 h the medium was removed and changed to Neurobasal-A medium with 2% B27 supplement (Invitrogen). Then culture medium was half-changed every 3 days. A final concentration of 10 μmol/L cytosine arabinoside (AraC, Sigma–Aldrich) was added on day *in vitro* (DIV) 2, and removed on DIV 3. At the same time, the upper chamber containing astrocytes was inserted above the lower chamber containing neurons to establish the neuron–astrocyte co-culture system. And the medium was change to Neurobasal-A with 2% B27 supplement (2%). Until DIV 7, the neuron–astrocytes were used to OGD/R treatment.

2.4. OGD/R model

OGD/R experiments were performed based on previous procedures[13] by replacing the culture medium (Neurobasal-A) with