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Panaxynol protects cortical neurons from ischemia-like injury by up-regulation of HIF-1α expression and inhibition of apoptotic cascade

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ABSTRACT

Apoptosis is one of the major characteristics of delayed neuronal degeneration in neuronal injury following cerebral ischemia. Hypoxia-induced apoptosis may be co-regulated by HIF-1α as well as many other factors. In recent years, numerous studies concerning panaxynol (PNN) have been reported. However, whether PNN can show anti-hypoxia properties is still unknown. In this study, the protective effects of PNN on OGD-induced neuronal apoptosis and potential mechanisms were investigated. Pretreatment of the cells with PNN for 24 h following exposure to OGD resulted in a significant elevation of cell survival determined by MTT assay, LDH assay, Hoechst staining and flow cytometric assessment. In addition to enhancing the expression of HIF-1α, PNN also normalized the caspase-3 expression/activation and increased the Bcl-2/Bax ratio. In our study, the increased level of HIF-1α with decreased cellular apoptosis suggested an important role for HIF-1α in hypoxic neurons. These results indicated that the neuroprotective effects of PNN on hypoxic neurons were at least partly due to up-regulation of HIF-1α and raised the possibility that PNN might reduce neurodegenerative disorders and ischemic brain diseases.

1. Introduction

The ischemic damage and ischemia/reperfusion injury may proceed through necrosis or by apoptosis, depending upon the severity and duration of the insult [1]. Hypoxia-induced apoptosis is co-regulated by HIF-1α as well as many other factors [2]. HIF-1α activates the expression of multiple genes, including anti-apoptotic proteins like Epo, VEGF, BCL-xL and pro-apoptotic proteins such as Nip 3 and Nix [3–6]. The increased level of HIF-1α with decreased cellular apoptosis suggests an important role for HIF-1α in developing rat brain [7]. On the contrary, prolonged hypoxia causes HIF-1α binding with p53 which facilitates apoptotic cell death via typical mitochondrial apoptotic cascades [8]. Although HIF-1α is essential for adaptation to low oxygen levels, the roles of HIF-1α in regulating apoptosis in CNS injury remain contradictory. A multitude of studies currently show that the function of HIF-1α in regulating apoptosis may depend on the hypoxic models, species, cell types or insult that induces neuronal death [9]. Therefore, to find selective promising target from various signaling cascades and cell type-specific hypoxia models for drug discovery and development is very challenging.

Panaxynol (PNN; syn. falcarinol) and related aliphatic C17 acetylenes are among highly bioactive compounds that occur in many medicinal plants (e.g. *Panax ginseng*) and food plants (e.g. carrots, celery and knob celery). They are likely to be responsible for at least a part of the beneficial of intake related plants [10]. In recent years, numerous studies concerning PNN and its analog panaxydol (PND) as well as their neuroprotective effects and other bioactivities have been reported by our group [10–14]. For example, PND could protect primary cultured Schwann cells (SCs) against hypoxia-induced injury [12]. PNN and PND could protect neurons against sodium nitroprusside (SNP)-induced apoptosis via regulating the apoptotic related genes [13] and both could alleviate neuron injury caused by Aβ25–35 [11]. In addition, the ability to enhance cerebral blood flow of PND and PNN [15] and the inhibition to nitrite production by inhibiting inducible nitric oxide synthase (iNOS) [16] also demonstrate their neuroprotective effects. However, whether PNN can show anti-hypoxia properties is still unknown. Since oxygen availability is crucial for cellular viability and HIF-1α is the major oxygen homeostasis regulator functioning as a widely operative transcriptional control system responding to physiological levels of cellular hypoxia [17], we hypothesized that PNN might have a potential effect on the HIF-1α activity. In the present study, we investigated the neuroprotective effects of PNN on primary

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cultured cortical neurons challenged directly with hypoxia, and the mechanism involved using the common model of oxygen/glucose deprivation (OGD) and by comparing HIF-1α expression and cellular apoptosis.

2. Materials and methods

2.1. Reagents

PNN was isolated and purified as described previously [10,14] from the roots of *P. notoginseng* and its purity checked by GC was over 98%. Its DMSO stock solution (100 mM) was stored at −20 °C. Neurobasal medium, B27 supplement, glucose-free Dulbecco's minimum essential medium (DMEM) and Hanks' balanced salts (HBSS) were purchased from Gibco (Grand Island, NY). Poly-L-lysine, Hoechst 33342 and cytosine-β-D-arabinofuranoside were from Sigma–Aldrich (St. Louis, MO). Anti-HIF-1α, Anti-caspase-3, Anti-Bcl-2, anti-Bax and anti-β-actin antibodies were from Cell Signaling Technology (Danvers, MA). BCA protein assay kit, ECL kit and stripping buffer were from Pierce (Rockford, IL).

2.2. Cell culture

Animal handling and surgery were performed in accordance with the Standard outlined in Ministry of Health of China (1998) for the care and use Laboratory Animals. Cultures of pure cortical neurons were obtained from E18 embryos of Sprague–Dawley as previously reported [13]. Briefly, cortical tissue was dissected and maintained in MEM Eagle's HBSS medium at 4 °C during dissection. After dissociation with trypsin (1 mg/ml) and DNAse (10 μg/ml) and centrifugation, the cells were resuspended in neurobasal (NB) medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 2% B-27 (Invitrogen), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Neurons were plated onto 96-well or 12-well plates pre-coated with poly-L-lysine (25 μg/ml, Sigma, St. Louis, MO) at a density of 0.3×10^5 cells/well to a 96-well plate or 5×10^5 cells/well to a 12-well plate. Cultures were kept at 37 °C in a humidified incubator in 5% CO_2 atmosphere. Cytosine-β-D-arabinofuranoside was added (5 μM final concentration) 3 days after plating, and the cultures were incubated for 24 h to halt the growth of non-neuronal cells. The medium was changed twice weekly and experiments were performed on 8 days in vitro (DIV). Purity of neuronal cultures was identified by immunocytochemistry using anti-β-tubulin antibody as before.

2.3. OGD treatment

To mimic cerebral ischemia/reperfusion in vitro, OGD was performed as described [18]. Conditioned medium was taken and stored in an incubator at 37 °C. The cells were washed and incubated in the pre-gassed glucose-free DMEM. The plate was transferred to an anaerobic chamber flushed with a mixture of 5% CO_2 and 95% N_2 at 37 °C for 60 min incubation. Then, the OGD treatment was stopped by replacing the glucose-free DMEM with the saved conditioned media and the cells were allowed to reoxygenate under normal conditions for 0–24 h prior to following experiments. Control group was exposed to oxygenated DMEM containing 5.5 mM glucose in normal conditions during identical period as the OGD cultures. PNN (1–20 μM) was added to the medium which was incubated for 24 h before OGD treatment if needed.

2.4. MTT reduction assay

Cell viability was assessed by phase-contrast microscopy with Trypan Blue exclusion assay and quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. At the end of reoxygenation after OGD, MTT was added at a final concentration of 0.5 mg/ml. The medium was discarded after the incubation for 4 h at 37 °C and the insoluble dark blue formazan crystals were dissolved in 100 ml of DMSO. Absorbance was subsequently measured at 570 nm with a reference wavelength of 630 nm using a microtiter plate reader (Quant, Bio-Tek Instruments Inc.). The sample solution was freshly prepared, in which DMSO was lower than 0.1%. No effect on cell survival was observed in the vehicle group.

2.5. LDH release assay

Lactate dehydrogenase (LDH) in the culture medium was detected with Cytotoxicity Detection Kit according to manufacturer's procedure. Briefly, 50 μl of supernatant from each well was transferred to a 96-well plate and 50 μl of reconstituted substrate mix was added to each well and the plates were kept for 30 min in dark at room temperature. Stop solution was added and then red color developed. The intensity was measured at 490 nm with an ELX 800UV microtiter plate reader (Bio-Tek Instruments Inc.). The results were expressed as a percentage of LDH release *vs*. a control obtained in untreated sister culture. Experiments were performed in triplicate, and repeated with at least three separate batches of cultures.

2.6. Hoescht 33342 staining

Cortical neurons were seeded on poly-L-lysine-coated glass coverslips (20 mm) at a density of 5×10^5 cells/dish. On DIV 8 cells were exposed to OGD and at the end of reoxygenation cells were loaded with 1 μM Hoescht 33342 for 5 min at 37 °C in Krebs–Henseleit solution. Cells were then washed twice with Krebs–Henseleit solution (NaCl 120, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, $NaHCO_3$ 27, KH_2PO_4 1.0, and glucose 5.5 in millimolar concentrations; pH 7.4 ± 0.05) and the fluorescence was observed by using an Olympus BX60 microscope with the appropriate fluorescence filters (excitation wavelength of 350 nm and emission wavelength of 450 nm). For cell counts, 5 random fields (about 60 cells each field) were observed per coverslip. Results were expressed as percentage of Hoescht-positive nuclei (condensed or fragmented) relative to the total number of nuclei counted per coverslip for each experimental condition.

2.7. Flow cytometric assessment of apoptosis

Apoptosis in primary cortical neurons subjected to various treatments was determined using an Annexin V-FITC (fluorescein isothiocyanate) staining kit from Becton Dickinson Company (San Jose, CA, USA). Propidium iodide (PI) was used to differentiate apoptotic cells with membrane integrity ($Annexin^+/PI^-$) from necrotic cells that had lost membrane integrity ($Annexin^+/PI^+$). The assay was performed as described [19]. Following OGD and drug treatments, cells were harvested using trypsin-EDTA, washed once in ice-cold PBS, and resuspended in 1 ml of Annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 1×10^5 cells were stained with 5 μl of Annexin V fluorescein isothiocyanate and 5 μg/ml PI in 100 μl of Annexin V binding buffer at 4 °C. After 20 min, 400 μl of binding buffer was added to each tube and samples were analyzed using a tri-laser FACS Calibur flow cytometer.

2.8. Western blot analysis

After treatment, cortical neurons were washed with PBS and lysed with a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 4 μg/ml leupeptin,