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Effect of panaxydol on hypoxia-induced cell death and expression and secretion of neurotrophic factors (NTFs) in hypoxic primary cultured Schwann cells

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ABSTRACT

It has been shown that panaxydol (PND) can mimic the neurotrophic effect of nerve growth factor (NGF) normally secreted by Schwann cells (SC) and protect neurons against injury. To evaluate the effect of PND on hypoxia-induced SC death and expression and secretion of neurotrophic factors (NGF and brain derived neurotrophic factor (BDNF)), hypoxic SCs were cultured in vitro and then treated with PND (0–20 μM). The MTT (3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay, immunocytochemistry, ELISA and RT-PCR were employed to examine the effects. We found that hypoxia resulted in a significant decrease in SCs viability (MTT: 64 ± 4.7% of control group) and nearly a 3.3-fold increase of intracellular level of active caspase-3. PND (5–20 μM) treatment significantly rescued the SCs from hypoxia-induced injury (85 ± 8.2%; 92 ± 8.6%; 87 ± 7.3%) and reduced caspase-3 activity with the maximal effect occurred at 10 μM ($P < 0.01$), reducing to about 1.6-fold of control level. Furthermore, PND treatment also enhanced NGF and BDNF mRNA levels in hypoxic SCs and promoted protein expression and secretion. BDNF mRNA in hypoxic SCs was restored to about 90% of normal level and NGF mRNA was elevated to 1.4-fold of control after 10 μM PND treatment. These observations showed that PND protects primary cultured SCs against hypoxia-induced injury and enhances NTF-associated activities.

1. Introduction

Schwann cells (SC) are the myelin-forming cells of the peripheral nervous system and play a key role in peripheral nerve regeneration. However, SC can be injured by various pathologies, and SC pathology usually leads to regeneration failure of the peripheral nerve. Trauma, for example, can cause peripheral nerve injury, resulting in Wallerian degeneration and SC apoptosis. Traumatic injury can also damage nearby blood vessels, resulting in ischemia and SC exposure to hypoxia. Thus, SC in the lesion site would undergo ischemia–reperfusion injury due to oxidative stress, intracellular calcium overload, and generation of reactive oxygen species (ROS) [1,2]. In diabetic neuropathy, hypoxia, hyperglycemia and increased oxidative stress contribute both directly and indirectly to SCs dysfunction [3,4], including impaired paranodal barrier function, damaged myelin structure, reduced antioxidative capacity and decreased neurotrophic support for axons [5–7].

The use of artificial nerves is nowadays considered as an effective strategy for the repair of large nerve defect. This, however, requires a large amount of viable SCs. Despite the fact that SCs express NGF and BDNF in vitro [8], the biologi-

Abbreviations: DEPC, diethyl pyrocarbonate; DMSO, dimethyl sulfoxide; PND, panaxydol; SCs, Schwann cells.

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cal activities of these cells seeded in grafts decays with time. Regeneration of nerve fibers in the middle of a conduit is usually poorer than that at both ends after graft transplantation [9,10], and this may be associated with SCs exposure to local hypoxia induced by ischemia. Thus, it is very important to find out how to effectively protect impaired SCs and to enhance their biological activities in order to promote peripheral nerve growth and regeneration.

Recently, more than 10 naturally occurring polyacetylenes were isolated from white ginseng and *Panax quinquefolium*, some of which exhibit anti-inflammatory properties [11], anti-platelet action [12], inhibition of lipoxygenase [13], cytotoxic activity against leukemia cells [14], inhibition of 15-hydroxyprostaglandin dehydrogenase [15], and inhibition of platelet aggregation, release reaction, and thromboxane formation [16]. It is well known that PND ((3*R*)-9,10-epoxy-1-ene-4,6-diyn-3-ol) is a highly bioactive polyacetylene compound that occurs in many food plants (e.g. carrots, celery, and konb celery) and in many medicinal plants (e.g. *Panax ginseng*) [17]. Previous studies have shown that PND can mimic the neurotrophic effect of NGF and forskolin, promote neurite outgrowth in PC12 cells dose dependently and protect cultured cortical neurons against injuries induced by sodium nitroprusside [18,19]. Furthermore, we have previously shown that PND is able to promote the growth of primary SCs and stabilize their mitochondrial membrane. Thus, we hypothesize that PND protects SCs against hypoxic injury.

In the present study, we investigated the protective effect of PND against hypoxia-induced injury to SCs as well as its effect on the expression and secretion of NTFs.

2. Materials and methods

2.1. Reagents

PND was isolated and purified as described previously [20] from the roots of *Panax notoginseng*. Briefly, PND was isolated by using silica gel column chromatography and thin layer chromatography (TLC) and, its chemical structure was identified by spectroscopic techniques including IR, (1)HNMR, (13)CNMR, DEPT, HMQC and HMBC. The purity checked by GC was over 98%. PND was dissolved in DMSO (100 mM) and stored at −20 °C until use.

2.2. Primary cultures of Schwann cells

SCs cultures were obtained utilizing the method of Brockes et al. [21], with minor modifications. Briefly, sciatic nerves and brachial plexus from 2 to 3-day-old Sprague–Dawley rats were harvested and digested with 0.12% collagenase and 0.25% trypsin (Sigma, St. Louis, MO, USA) at 37 °C for 10–13 min. Twenty-four hours after planting, cells were treated with cytosine arabinoside (5 μg/ml; Sigma) for 3 days to eliminate proliferating fibroblasts. Following this treatment, the culture medium was replaced with fresh medium supplemented with forskolin (4 μM; Sigma) and basic fibroblast growth factor (bFGF; 20 ng/ml; R&D Systems, Minneapolis, MN, USA) to allow for Schwann cell proliferation. After 8 days, SCs were detached with 0.25% EDTA–trypsin (Sigma) and passaged. The culture medium was changed three times per week. Cells were treated with PND after the second passage.

2.3. Hypoxia

Hypoxic conditions were induced according to the method of Kitano et al. [22], with minor modifications. Briefly, the incubation chamber was filled with a mixture of 5% CO_2/92% N_2 and monitored by an oxygen analyzer (Shanghai Instrument Co., Shanghai, China) with an oxygen sensitive metal electrode. The chamber was sealed and placed at 37 °C in a humidified incubator for 15 min when the concentration of oxygen was lower than 1%. Subsequent to the hypoxic treatment, the medium was replaced with fresh medium containing different concentrations of PND (5, 10, 20 μM) and cells were then cultured for 24 h under normoxic conditions. Three replicates were used in each experiment.

2.4. Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde and probed with the following polyclonal antibodies: rabbit anti-S-100 (1:200, DAKO, Glostrup, Denmark) or rabbit anti-active caspase-3 (Stressgen Bioreagents, Victoria, BC, Canada). Cells were then incubated with peroxidase-conjugated goat anti-rabbit IgM or IgG (1:200) for immunocytochemistry, or with FITC-conjugated goat anti-rabbit IgG (Cappel/Organon Teknika Corp., Aurora, OH, USA) for immunofluorescence. The results of immunostaining were examined with a Leica DM2500 microscope using a DDC 2/3 camera and analyzed with RS IMAGE Pro™ Version 4.5 (Roper Scientific, Trenton, NJ, USA).

2.5. MTT assay

The MTT (3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay was used to study the effect of PND on SC survival. Cells in a 96-well plate were treated with PND for 24 h. Ten microliters of MTT stock solution (10 mg/ml) (Sigma) was then added to the remaining medium and the cultures were incubated for another 4 h at 37 °C. The medium was discarded after the incubation and the insoluble dark blue formazan was dissolved in 100 μl of DMSO and quantified at 570 nm with a reference wavelength of 630 nm using a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA). Survival of the positive control group was defined as 100% and that of treated groups was expressed as percentage of positive control group.

2.6. Reverse transcriptase–polymerase chain reaction (RT–PCR)

Twenty-four hours following PND treatment, the total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed in a final volume of 20 μl, containing 1 μg of total RNA, 50 pM oligodT, 10 μM of each deoxyribonucleoside triphosphate (dNTP), 20 units RNasin and 10 units AMV Reverse Transcriptase (Promega),