Nerve Regeneration Potential of Protocatechuic Acid in RSC96 Schwann Cells by Induction of Cellular Proliferation and Migration through IGF-IR-PI3K-Akt Signaling

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Abstract

Peripheral nerve injuries, caused by accidental trauma, acute compression or surgery, often result in temporary or life-long neuronal dysfunctions and inflict great economic or social burdens on the patients. Nerve cell proliferation is an essential process to restore injured nerves of adults. Schwann cells play a crucial role in endogenous repair of peripheral nerves due to their ability to proliferate, migrate and provide trophic support to axons via expression of various neurotrophic factors, such as the nerve growth factor (NGF), especially after nerve injury. Protocatechuic acid (PCA) is a dihydroxybenzoic acid, a type of phenolic acid, isolated from the kernels of Alpinia oxyphylla Miq (AOF), a traditional Chinese herbal medicine the fruits of which are widely used as a tonic, aphrodisiac, anti-salivation and anti-diarrheatic. This study investigated the molecular mechanisms by which PCA induces Schwann cell proliferation by activating IGF-IR-PI3K-Akt pathway. Treatment with PCA induces phosphorylation of the insulin-like growth factor-I (IGF-I)-mediated phosphatidylinositol 3 kinase/serine - threonine kinase (PI3K/Akt) pathway, and activates expression of cell nuclear antigen (PCNA) in a dose-dependent manner. Cell cycle analysis after 18 h of treatment showed that proliferation of the RSC96 cells was enhanced by PCA treatment. The PCA induced proliferation was accompanied by modulation in the expressions of cell cycle proteins cyclin D1, cyclin E and cyclin A. Knockdown of PI3K using small interfering RNA (siRNA) and inhibition of IGF-IR resulted in the reduction in cell survival proteins. The results collectively showed that PCA treatment promoted cell proliferation and cell survival via IGF-I signaling.
Key Words: IGF-I, IGF-I signaling, peripheral nerve regeneration, protocatechuic acid (PCA), RSC96 Schwann cells

Introduction

Nerve cells are the primary functional units of the nervous system. Neurons do not undergo cell division but glial cells are generated into neurons when needed. Glial cells are present in both the central and the peripheral nervous systems (PNS). Schwann cells are the major glial cell type of the PNS and have an important role in nerve development and regeneration. Schwann cells can differentiate into the myelin sheath and proliferate and migrate to the distal end of injured nerve areas (3, 10, 22). Schwann cell migration is essential for axonal elongation and remyelination of injured nerves (2, 39). In peripheral nerve injuries, Schwann cells are activated to synthesize the essential neurotrophic factors, adhesion molecules, cytokines and growth-promoting surface molecules (19, 37). But the action mechanisms that regulate Schwann cell proliferation remain unclear.

Insulin-like growth factor-I (IGF-I) is a well-known biochemical marker of the survival mechanism in mammalian cells. IGF-I is secreted in response to growth hormone to stimulate tissue growth (25). Muscle regeneration and hypertrophy are known to be mediated by the activation, proliferation and differentiation of muscle satellite cells and appear to be modulated by the mitotic and myogenic activities of locally produced IGF-I, which functions in an autocrine/paracrine mode (30). Early up-regulation of IGF-I in Goldfish retinal ganglion cells (RGCs) after optic nerve injury leads to enhanced survival and axonal regeneration in adult goldfish retinas via phosphatidylinositol 3 kinase/threonine - threonine kinase (PI3K/Akt) signaling (24).

Targeting Schwann cells with herbal medicines that potentially promote neuron regrowth may be a possible therapeutic approach to treat injured nerves. Alpinate Oxyphyllae Fructus (Alpinia oxyphylla Miq) (AOF) is one of most important traditional Chinese medicines, which is used for treating diarrhea, polyuria, ulceration, dementia, antitumor and gastralgia according to the Chinese Pharmacopoeia (5, 18, 35). Several studies found that AOF might be a neuroprotective agent both in water and ethanol extracts (1, 23, 35, 36, 42). Protocatechuic acid (PCA) is a simple phenolic compound isolated from the kernels of AOF. This study investigated the molecular mechanisms by which PCA-treated Schwann cell survival may potentially contribute to neuron regeneration. The roles of MAPK (ERK1/2, JNK and p38) pathways for PCA-induced matrix-degrading proteolytic enzyme (PAs and MMP2/9) production in RSC96 Schwann cells were investigated. This study, therefore, applied an in vitro system to investigate the effects of AOF on Schwann cell migration and neuron regeneration.

Materials and Methods

Cell Culture and Treatments

RSC96 Schwann cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamate, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose in humidified atmosphere of 5% CO2 and 95% air. Cells were cultured in serum-free medium for 4 h and treated with PCA at different concentrations. Cells were then harvested after 16-24 h of incubation for further analysis.

Flow Cytometer

Cells were suspended in phosphate buffered saline (PBS, pH 7.4) and fixed with 70% ethanol at -20°C for 12-16 h. Cells were washed with PBS after removing ethanol and stained for 30 min with 0.005% propidium iodide (PI). Using a FACS Calibur cytometer (BD Biosciences, San Jose, CA, USA), cellular PI content was measured and data were analyzed using Mod fit LT software.

Migration Assay

For migration assay, Boyden chamber and polyvinyl-pyrrolidone-free polycarbonate membranes with 8 μm pores (Neuro Probes, Inc., Gaithersburg, MD, USA) were used. DMEM medium supplemented with 10% FBS was used to fill in the bottom wells of the chamber, which was subsequently covered with a membrane sheet. In the top chamber, serum-free medium was added in the wells. After being incubated for overnight, the membrane was stained with Giemsa stain (Sigma). By using a counting grid fitted in the eyepiece of a phase contrast microscope, cells were counted after migration.

Western Blotting

Cultured RSC96 cells were scraped and washed using a lysis buffer and centrifuged at 13,000 rpm for 30 min at 4°C and the supernatant was collected. Proteins were then separated in 12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Non
specific protein binding was prevented by the use of a blocking buffer (5% milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20) and blotted with specific antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in the blocking buffer at 4°C overnight. Western blot stripping buffer (Pierce Biotechnology, Inc., Rockford, IL, USA) was used for repeated blotting.

Inhibitor and Small Interfering RNA (siRNA) Application

For chemical inhibition assay, RSC96 Schwann cells were treated with 5, 10 or 15 μM AG1024, a IGF-1R inhibitor (Promega, Madison, WI, USA). For siRNA studies, double-stranded siRNA sequences targeting PI3K mRNAs were obtained from Dharmacon, (Lafayette, CO, USA). RSC96 cells were cultured in 100-mm plates in DMEM without FBS and transfected with double-stranded siRNA using the DharmaFECT Duo Transfection Reagent (Dharmacon) according to the manufacturer’s instructions. A non-specific duplex (Dharmacon) was used as a control. Using Western blot, down-regulation of the PI3K protein level was detected.

Statistical Analysis

Statistical differences were assessed using one way ANOVA. P < 0.05 was considered statistically significant. Each experiment was duplicated at least three times. Data are expressed as the mean ± SEM.

Results

PCA Enhances the Survival Mechanism of RSC 96 Cells

PCA treatment effectively enhanced the molecular evens of the survival mechanism in RSC 96 cells. PCA
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treatment significantly induced the activation of PI3K, and Akt proteins increased significantly in various treatment doses (Fig. 1). The increases in the expression levels of survival proteins were correlated with increases in the expression of IGF-I and p-IGF-IR, indicating a role of IGF-I signaling in the molecular events of enhanced cell survival (Fig. 1A). The level of p-PI3K and Akt were increased considerably (Fig. 1B).

**PCA Suppresses Pro-Apoptotic Proteins**

PCA treatment also modulated the B-cell lymphoma 2 (Bcl-2) family of regulatory proteins that regulate cell death (apoptosis). Bad, a pro-apoptotic
protein, was down-regulated, and the pro-survival proteins p-Bad and Bcl-xL were up-regulated in RSC 96 Schwann cells on PCA treatment (Fig. 1C).

PCA Promotes Proliferation of RSC 96 Cells

Treatment with 1, 1.5 or 2 mM PCA enhanced the expression of proliferating cell nuclear antigen (PCNA) in RSC 96 Schwann cells. While there was a dose-dependent increase in PCNA expression, G1 to S-phase cell cycle transition also increased in a time-dependent manner (Fig. 2A). Analysis on the cell cycle proteins showed an increase in the expression of the cell cycle regulators cyclins D1, E and A (Fig. 2A). Cells in the S-phase increased from 16% before treatment to 17.7%, 19.9% and 25.9% after 6, 12 and 18 h of 1 mM PCA treatment, respectively (Fig. 2B). All the tested concentrations of PCA enhanced the expression of the cyclin family proteins and showed a dose-independent effect.

PCA Enhances Cell Survival and Migration by IGF-IR-Mediated Modulation of PI3K

PCA induced the survival proteins in the RSC96 cells and the induced proteins were suppressed when
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pretreated with an IGF-IR inhibitor (Fig. 3A). IGF-IR also inhibited the PCA-enhanced migration of RSC96 Schwann cells. The migration effect of PCA on Schwann cells was also suppressed when treated with IGF-IR (Fig. 3B). Therefore, PCA enhances Schwann cell proliferation and migration by complementation of the IGF-I survival pathway.

PI3K Is an Essential Mediator of IGF-IR-Survival Mechanism Induced by PCA

The role of PI3K on PCA-induced cellular proliferation was examined by siRNA-mediated inhibition of PI3K expression. Knockdown of PI3K significantly inhibited expression levels of the survival proteins pAkt and Bcl-2 (Fig. 4A). Inhibition of PI3K also suppressed the migration of RSC96 Schwann cells indicating a major role played by PI3K in PCA-induced Schwann cell migration (Fig. 4B).

Discussion

Schwann cells are the major glial cells of the PNS, and play a key role in the survival, function and
regeneration of neurons. Schwann cells assist the organization of the neural extracellular matrix and provide structural and trophic support to axonal regrowth (15, 22). Various immortal Schwann cell lines have been established and widely used to study the regulatory mechanisms of Schwann cells that promote nerve growth and regeneration (32). RSC 96 Schwann cells have been previously used to investigate the nerve regenerative effects of various herbal medicines (6, 9, 27). AOF is an efficient traditional Chinese medicine with neuroprotective potentials (1, 23, 35, 36, 42). PCA, purified from AOF, was found to possess effective neuroprotective potential and, therefore, can be considered as the active principle behind the neuroprotective effects of AOF.

Neurotrophic factors are a family of growth factors that support and influence the growth and regenerative capacity of neurons, such as IGFs neurons (13, 14, 20, 21, 33). Recent studies have demonstrated that IGF-I signaling plays a crucial role in nerve cell proliferation and survival (24, 25). The PI3K/Akt signaling activated by IGF-I mediated through IGF-IR is a well-established cascade of cell proliferation and survival pathway. IGF-I up-regulation directly induces neurite outgrowth via an PI3K/Akt-dependent mechanism (4, 16, 17, 26, 28, 31).

PI3K are important mediators of IGF-I signaling in rescuing Schwann cells from apoptosis and progression from G1 to S-phase of the cell cycle (8, 17). Inhibition of PI3K by using inhibitors blocked the antiapoptotic and protective effects of IGF-I, indicating the essential role of PI3K in Schwann cell survival (26, 28). Activation of IGF-I and thereby induction of the phosphorylation of the PI3K/Akt pathway and promotion of the expression of anti-apoptotic proteins, PCNA and cyclins could be an ideal phenomenon to enhance Schwann cell proliferation and nerve regeneration. Based on these facts, our results demonstrated that PCA stimulated Schwann cell proliferation and survival through the PI3K/Akt system mediated by IGF-I. The migration of RSC96 Schwann cells along the growth direction is an important phenomenon that is essential for nerve regeneration and it helps in repairing damaged peripheral nerves (11). Our results further showed that IGF-II/Akt/PI3K may play an important role in the PCA-induced Schwann cell migration.

Cell proliferation is regulated by several check points in the cell cycle, such as the first gap phase (G1) to the synthesis phase (S) transitions. Cyclin A plays an important role in DNA replication in the S phase and in the initiation of mitosis (M phase) (12, 34, 40). Our data showed that PCA induced cell cycle progression at the G1 to S phase transition (Fig. 3B). PCA promoted DNA replication and growth of RSC96 cells by up-regulating the sequential expression of cyclin A, thereby elevated cell proliferation. Previous studies on the role of IGF-I in cultured fibroblasts and mammary epithelial cells have revealed that IGF-I enhances cell cycle progression by facilitating G1 or the G0/G1 transition (7, 29, 38). Our observations are in agreement with previous studies in that the cell cycle is not only regulated directly by cyclins but is also mediated by IGF-I. Collectively, we suggest that cell cycle alterations may be critical determinants of the increased proliferation potency of the PCA extract. IGF in certain cells, such as hematopoietic cells, functions as an inhibitor of cell death (41). Activation of the PI3K/Akt pathway by PCA promotes cell survival. Akt activation leads to the phosphorylation of Bad and connects to a proximal survival signal with the Bcl-2 family to protect cells from apoptosis.

In conclusion, our study provides evidences for the potential mechanism by which PCA extract promotes neuron regeneration. PCA enhances proliferation and survival of Schwann cells potentially by up-regulation of IGF-I and activation of the PI3K/Akt signaling. However to support this conclusion, further studies such as cell proliferation assays should be performed after utilizing the IGF-1R inhibitor and/or siRNA-induced knockdown of PI3K. The PCA also modulates cell cycle and up-regulates the anti-apoptotic proteins. Further analyses are needed to determine the in vivo effects of PCA to promote cell survival and proliferation.

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References


