Akt enhances nerve growth factor-induced axon growth via activating the Nrf2/ARE pathway

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Abstract. Spinal cord injury (SCI) leads to the loss of structure and function of axons. However, injured axons cannot grow or regenerate spontaneously following injury. Generally, only when treated with neurotrophins, such as nerve growth factor (NGF), will the neurons sprout new axons. Akt is one of the central kinases of neurocytes. PC12 cells are a frequently used cell model for neural differentiation and development studies. The nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway is a main mechanism in prevention from oxidative stress, which may damage the nervous system. The present study employed this cell model to investigate whether Akt could induce axon growth in PC12 cells on the basis of NGF treatments. The results showed that Akt overexpression significantly increased cell proliferation and decreased cell apoptosis. Additionally, Akt overexpression activated Nrf2/ARE pathways. In conclusion, the experiments indicated that Akt overexpression contributed to axon regeneration induced by NGF in PC12 cells through activating the Nrf2/ARE pathway.

Introduction

Spinal cord injury (SCI) leads to the consequent death of neurocytes, thus causing the dysfunction of signal transmissions between neurons and axons, which could in turn induce apoptosis of neurons. As reported, axons of the injured neurons in the adult mammalian central nervous system (CNS) can seldom spontaneously regenerate (1). This phenomenon has caused confusion for the SCI treatment. There are relevant studies that have restored the regenerative ability of the injured axons (2-11). For example, various neurotrophin treatments, including nerve growth factor (NGF) and brain-derived neurotrophic factor, have been effectively proved to promote neurons branching and sprouting (12,13), and to promote axon regeneration (14,15). However, the mechanism underlying neurotrophin treatments has not been fully investigated or established.

PC12 cells originate from a rat pheochromocytoma tumor cell line, which is sensitive to NGF treatment by differentiating into neuron-like cells (16,17). Therefore, PC12 cells have been employed as a promising, unique and frequently used cell model for neural development and protection studies (17,18). Akt is a type of neurocyte protein kinase that is associated with stress response to growth factors (19), which also has a role in tumor growth (20). The nuclear factor E2-related factor 2 (Nrf2) is a type of transcription factor, which could initiate antioxidant response element (ARE) transcription. The Nrf2 gene products include a scope of antioxidative factors participating in antioxidant function, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO-1) and γ-glutamylcysteine synthetase (γ-GCS) (21-24). The present study aimed to further understand whether Akt could activate Nrf2/ARE antioxidant systems to decrease apoptosis of neurocytes and contribute to axon regeneration.

The NGF-differentiated PC12 cells were used to investigate the effect of Akt on axon growth. Changes in axon regrowth produced by silence and overexpression of Akt were examined and the function of antioxidant enzyme activities in the presence of NGF in PC12 cells was identified. Understanding the mechanisms between Akt and the Nrf2/ARE pathway in PC12 cells is important for the development of new methods to prevent or treat neurodegenerative diseases, such as SCI-caused axon degeneration.

Materials and methods

Cell culture and differentiation. PC12 cells were purchased from Riken Cell Bank (Tsukuba, Ibaraki, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) horse serum and 5% (v/v) fetal bovine serum (FBS) (all from Hyclone, Logan, UT, USA). The dishes had

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Abbreviations: SCI, spinal cord injury; NGF, nerve growth factor; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; CNS, central nervous system; HO-1, heme oxygenase-1; NQO-1, NAD(P)H:quinone oxidoreductase-1; γ-GCS, γ-glutamylcysteine synthetase

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been previously coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated at 37˚C in a humidified 5% CO_2 atmosphere. PC12 cells were differentiated with 100 ng/ml NGF (Invitrogen, Carlsbad, CA, USA) for ≤72 h.

Recombinant adenovirus construction and transfection. Recombinant adenovirus vectors were purchased from Genomeditech Biotechnologies (Shanghai, China). Briefly, the genes encoding Akt were amplified and identified, followed by conjugation with shuttle vector pAdTrack-CMV. The pAdTrack-CMV and adenoviral gene expression vector pAdEasy-1 were co-transfected into HEK293 cells in non-serum DMEM medium to produce recombinant adenovirus using Lipofectamine 2000 (Invitrogen). The recombinant adenoviruses were harvested, amplified, concentrated and purified, and the titers were measured prior to use. Cells treated with empty carrier LacZ instead of recombinant adenovirus were used as negative control.

Preparation of small interference RNA (siRNA) and transfection. The siRNA was synthesized by GenePharma Co., Ltd. (Shanghai, China). Briefly, the medium had been changed to non-serum medium 30 min before transfection. siRNA (5 μl; Akt siRNA sc-108059; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added into 245 μl of non-serum DMEM (solution A). Lipofectamine 2000 (10 μl; Invitrogen) was also diluted in 245 μl of non-serum DMEM (solution B) and incubated for 5 min at room temperature. Subsequently, solution B was gently added into solution A, mixed and incubated for 20 min at room temperature. The mixtures were equally distributed into the 6-well cultured cells at drop speed with successive agitation, followed by incubation at 37˚C, prior to conducting further analysis. Cells treated with siRNA instead of Akt siRNA were used as the negative control.

Neurite outgrowth measurement of PC12 cells. The length of axons that extended from cell bodies was measured by Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). Subsequently, the number of neurites extending from cells was also calculated by counting the number and percentage to determine differentiation efficiency.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability evaluation. Cell viability was evaluated by the MTT assay. Briefly, PC12 cells were cultured in 96-well plates with a density of $2x10^4$ cells/well. Subsequently, cells were incubated with 20 μl MTT solution (5 mg/ml) in fresh medium (10% FBS) for 4 h in a 37˚C incubator. Following this, the mixtures were centrifuged at 12,890 x g for 15 min and the supernatant was carefully discarded using a vacuum pump, and formazan crystals were dissolved in dimethylsulfoxide (0.1% final concentration; Sigma-Aldrich). The absorbance of samples was measured at 490 nm using the EnVision® Multilabel Reader (Perkin-Elmer, Waltham, MA, USA).

Hoechst assay and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) experiment for apoptosis evaluation. For the Hoechst assay, cells were seeded at a density of $1x10^4$/well in 96-well plates, followed by the addition of 200 μl fresh medium and incubation at 37˚C in 5% CO_2. When cells grew to a confluence of 80%, apoptosis was detected via the Hoechst Staining kit (Beyotime, Beijing, China) according to the manufacturer's instructions. For the TUNEL experiment, cells were firstly fixed with 4% paraformaldehyde for 10 min at room temperature. Subsequently, cells were washed with phosphate-buffered saline (PBS) twice and permeabilized by 0.1% Triton X-100 under ice-cold incubation for 10 min. Following washing with PBS again, cell apoptosis was evaluated by a TUNEL Apoptosis Detection kit (Merck Millipore, Billerica, MA, USA) following the manufacturer's instructions. Cells were observed under a fluorescent microscope (Olympus, Tokyo, Japan). The positive cells were counted in randomly selected fields.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of targeted PC12 cells was isolated using the TRIzol reagent (Life Technologies, Rockville, MD, USA). Reverse transcription was conducted using 1 μg of total RNA from each sample via the oligo(dT) primer, using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). RT-qPCR analysis, including Akt, HO-1, NQO-1 and γ-GCS, was performed using the SYBR-Green PCR kit (Takara, Shiga, Japan) on a Bio-Rad CFX96 Real-Time PCR Detection system. β-actin served as the reference gene and data were further analyzed using the ΔCt method.

Western blot analysis. Proteins were harvested using radioimmunoprecipitation assay buffer supplemented with protease inhibitor phenylmethanesulfonylfluoride (both from Sigma-Aldrich). A total of 20 μg proteins were fractionated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be transferred onto a nitrocellulose (NC) membrane (Amersham, Little Chalfont, UK). Subsequently, the NC membrane was incubated in blocking buffer consisting of 4% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline to block non-specific binding for 1 h. Subsequently, the membrane was incubated with primary antibodies (rabbit antibodies including Akt (sc-8312; 1:500), HO-1 (sc-10789; 1:500), NQO-1 (sc-16464; 1:600) and γ-GCS (sc-22755; 1:500), all from Santa Cruz Biotechnology, Inc.) diluted in blocking buffer overnight at 4˚C. On the following day, the NC membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (sc-2004; diluted at 1:1,000; Santa Cruz Biotechnology, Inc.) for 1 h. The protein signal was visualized using the Amersham ECL™ Plus Western Blotting Detection kit (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis. All the data are presented as mean ± standard deviation. Comparisons between the two groups and among multiple groups were performed by Student's t-test and one-way analysis of variance, respectively. $P<0.05$ was considered to indicate a statistically significant difference. All the statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA).

Results

NGF promotes neural differentiation of PC12 cells. The study first confirmed the function of NGF to induce neural differentiation of PC12 cells. NGF-treated cells had a significant

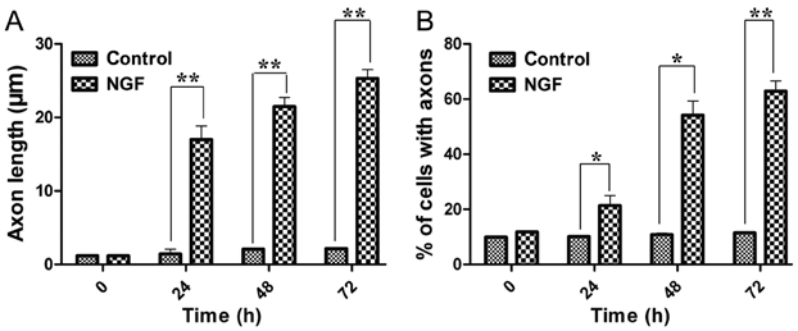

Figure 1. Effects of NGF on axon growth. (A) Average axon length was calculated 0, 24, 48 and 72 h post-NGF treatment. (B) The ratio of axon-possessing versus total cells was calculated. Each column represents mean ± standard deviation. n=3/group. *P<0.05 and **P<0.01 vs. control. NGF, nerve growth factor; control, untreated cells.

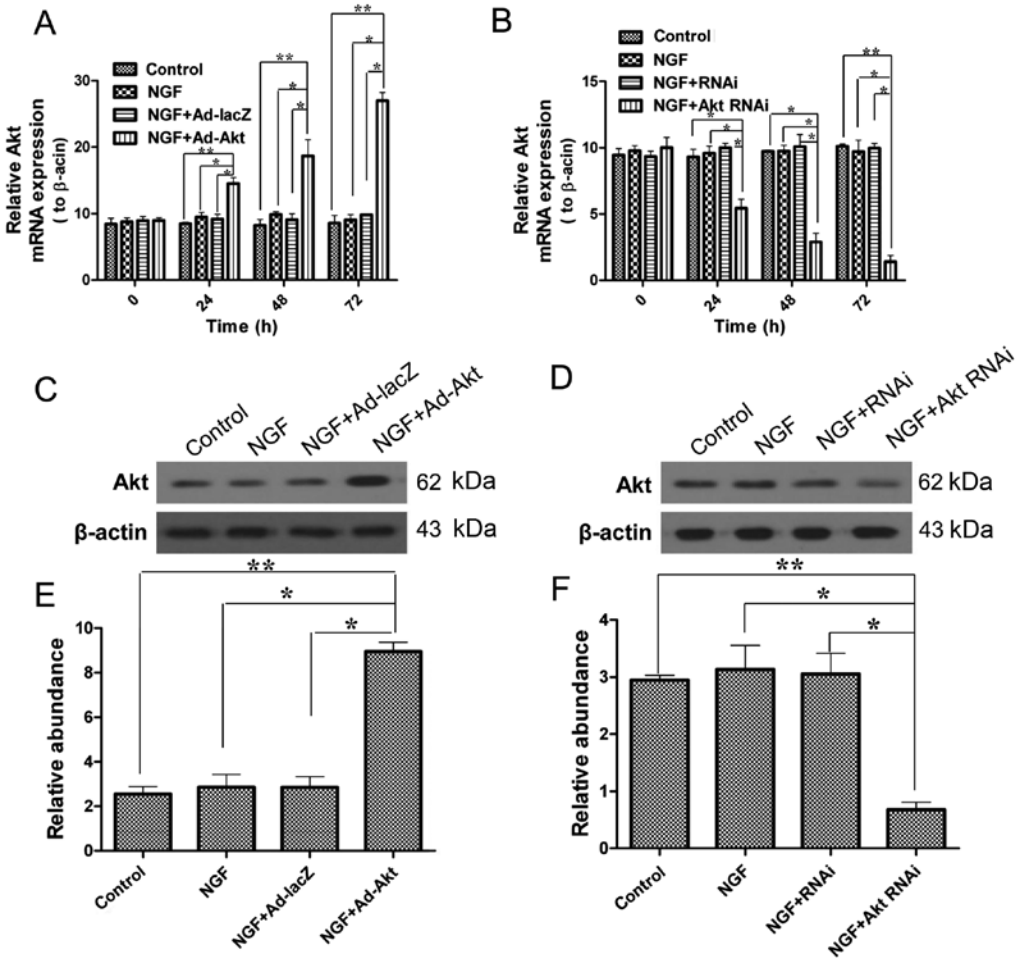

Figure 2. Overexpression and silencing of Akt at the mRNA and protein level. Reverse transcription-quantitative polymerase chain reaction for Akt (A) over-expression and (B) silencing. Western blotting for Akt (C) overexpression and (D) silencing. (E and F) Quantitative analysis of the western blot results. Each column represents mean ± standard deviation. n=3/group. *P<0.05 and **P<0.01 vs. control. NGF, nerve growth factor; control, untreated cells.

increase in the neurites length and also in the number of neurite-possessing cells in a time-dependent manner (Fig. 1). After 72 h induction, the average axon length was 21.4-fold longer than previously and the percentage of axon-attached neurocytes increased to 62.8±3.8%. These results demonstrated that NGF could induce neural differentiation of PC12 cells.

Akt is overexpressed or silenced in PC12 cells. Subsequently, in order to conduct the further experiments under defined conditions, the effects of adenovirus transfection and siRNA on PC12 cells were confirmed. Fig. 2 shows that Akt mRNA and protein expression levels were significantly upregulated by adenovirus transfection, and by contrast, siRNA knocked down Akt expression.

Akt promotes the proliferation of PC12 cells. Cell viability is important for neural repair and regeneration, and therefore, PC12 cells were subjected to MTT assays. The results showed

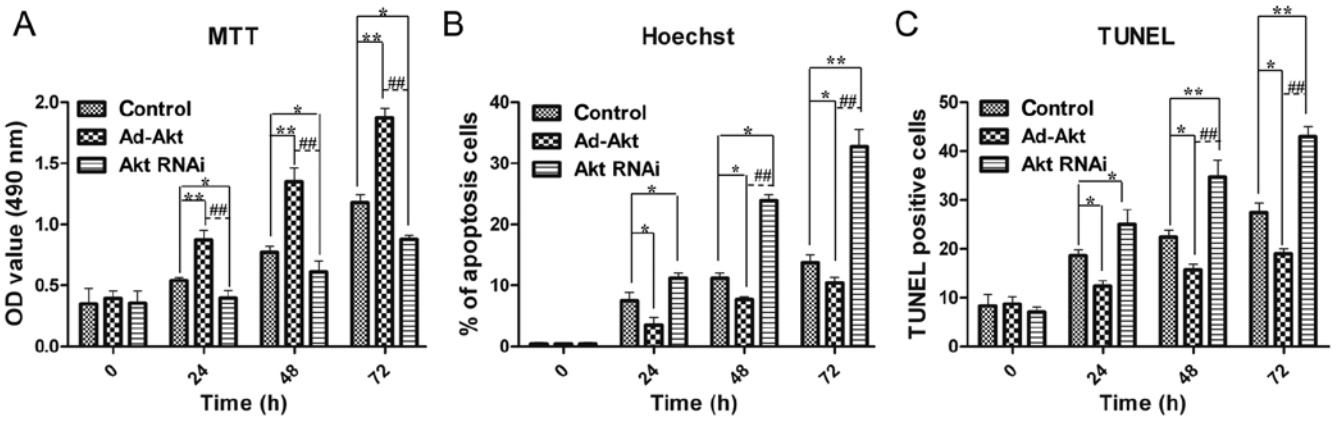

Figure 3. Effects of Akt on proliferation and apoptosis. (A) MTT assay for cell proliferation. OD value was measured at γ=490 nm. (B) Hoechst staining for cell apoptosis. The ratio of apoptotic versus total cells was calculated. (C) TUNEL assay for cell apoptosis. The number of apoptosis-positive cells was counted. Each column represents mean ± standard deviation. n=3/group. *P<0.05 and **P<0.01 vs. control; $^{\#\#}$P<0.01 Ad Akt vs. Akt RNAi. Control, untreated cells; OD, optical density; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

that cell proliferation was significantly improved in the existence of Akt overexpression, and by contrast, cell growth was inhibited by Akt silencing (Fig. 3A).

Akt inhibits the apoptosis of PC12 cells. As Akt could promote PC12 cells proliferation, Akt was assumed to be able to decrease apoptosis for cell accumulation. The Hoechst and TUNEL experiments were performed to verify this hypothesis. The results proved that Akt overexpression reduced the apoptosis rate to 10.4±1.0%, while Akt silencing contributed to cell death (32.7±2.8%) (Fig. 3B). TUNEL results exhibited similar results as the above descriptions (Fig. 3C). These results confirmed the role of Akt in alleviating apoptosis of PC12 cells, which contributed to cell number increase collaborating with cell viability promotion.

Overexpression of Akt enhances axon growth induced by NGF. As NGF was proved to promote neural differentiation, the present study aimed to discover a synergetic effector to enhance this function. Overexpression of Akt significantly improved the average axon length (33.4±1.6 μm) compared with the NGF-treated (26.9±1.7 μm) and control groups (2.14±0.14 μm) (Fig. 4A). Furthermore, the number of differentiated cells was also increased by 18.1 and 86.2% compared with the NGF-treated and control groups, respectively (Fig. 4B).

Silencing of Akt diminishes axon growth induced by NGF. To further identify the function of Akt in axon growth, its expression was knocked out via siRNA. Fig. 4A and B showed that 24 h after siRNA transfection, there was a 1.5-fold decrease of the average length in the siRNA-treated group, while 48 h later there was a 1.6-fold decline, compared with the NGF-treated group. Additionally, the differentiated cells were shown to have an average 21% decrease (from 24 to 48 h after siRNA transfection). As a result, knockdown of Akt expression attenuated NGF-induced axons outgrowth.

Akt cannot influence axon growth without NGF. The above results confirmed the effects of Akt on the proliferation, apoptosis and axon sprouting of PC12 cells. However, all the aforementioned results were acquired in the presence of NGF, and therefore cannot distinguish whether, how and to what extent Akt alone had participated in these functions. Therefore, the present experiment was conducted to monitor neural differentiation without NGF. The results proved that Akt alone could not promote or attenuate neurite growth, so therefore, Akt did not influence axon growth in the absence of NGF (Fig. 4C and D). This phenomenon confirmed the necessary role of NGF to foster the growth of axons, and Akt could reinforce this effect induced by NGF.

NGF cannot alter the expression of HO-1, NQO-1 and γ-GCS. As aforementioned, NGF is a necessity for axon extending. Therefore, whether NGF-induced axon growth is associated with the Nrf2/ARE signaling pathway was examined. NGF-treated and non-NGF-treated cells were collected to evaluate the changes of HO-1, NQO-1 and γ-GCS expression in mRNA and protein levels. The mRNA expression was not significantly changed in the presence and absence of NGF, which was also similar with the protein expression, and NGF was not closely associated with Nrf2/ARE signaling (Fig. 5).

Akt increases the expression of HO-1, NQO-1 and γ-GCS during axon growth. Subsequently, whether Akt has a role in the Nrf2/ARE signaling pathway was investigated. The results showed that Akt overexpression upregulated HO-1, NQO-1 and γ-GCS expression to 4.3-, 3.2- and 3.8-fold in the mRNA level compared with the control group, respectively (Fig. 6A-C); the western blot results also showed a significant increase in the protein level. By contrast, the knockdown of Akt led to a significant decrease of HO-1, NQO-1 and γ-GCS expression at the mRNA and protein levels (Fig. 6D-G). These results confirmed that Akt participates in Nrf2/ARE signaling.

Discussion

The spine consists of 26 hollow vertebras filled with vast spinal cord containing abundant neurocytes. The axons extending from the neurocytes are responsible for the signal

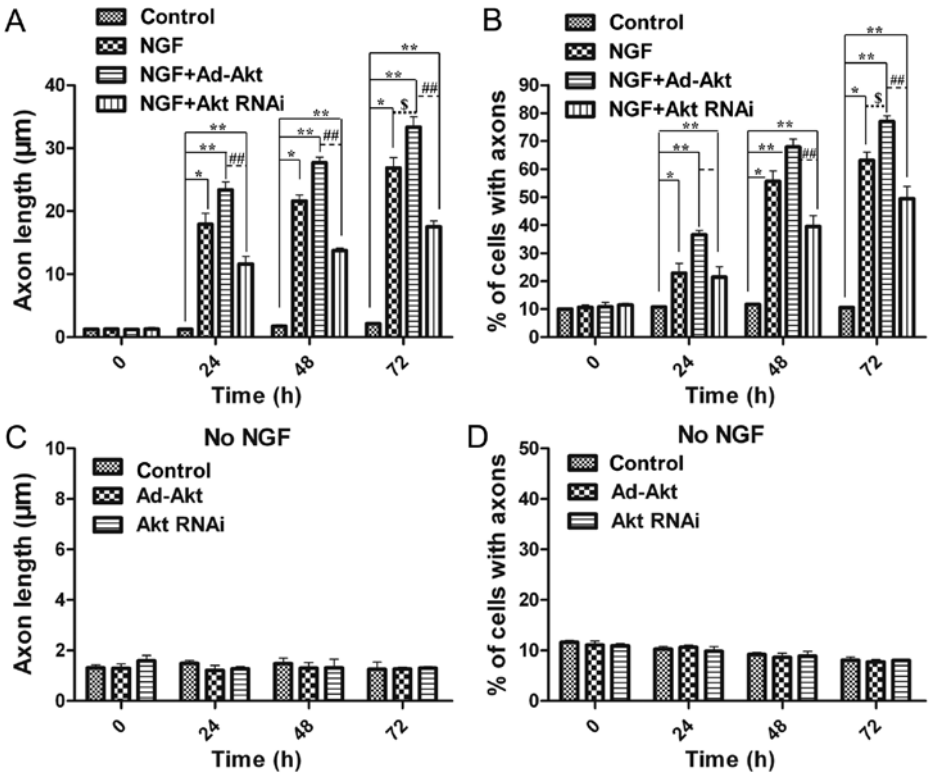

Figure 4. Effects of Akt on axon growth. (A) Average axon length following Akt overexpression and silencing. (B) Data are expressed as a percentage of control. (C and D) Length and percentage of axon following Akt overexpression and silencing without NGF treatment. Each column represents mean ± standard deviation. n=3 group. *P<0.05 and **P<0.01 vs. control; $^{\#\#}$P<0.01 Ad Akt vs. Akt RNAi; $^{\$}$P<0.05 Ad Akt vs. NGF alone. Control, untreated cells; NGF, nerve growth factor.

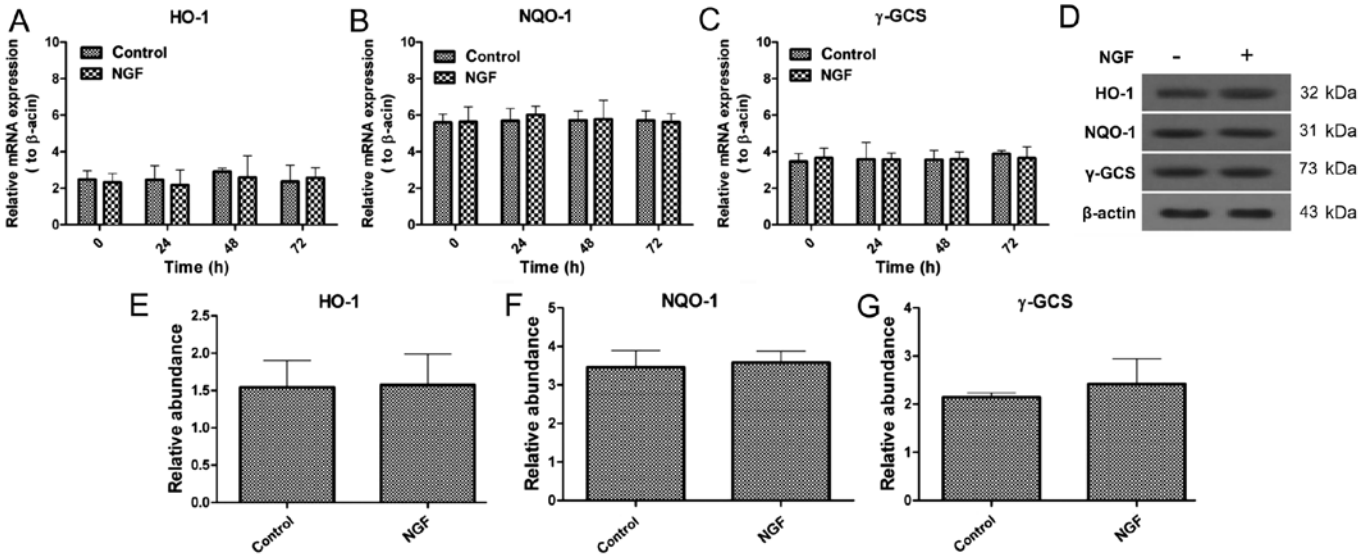

Figure 5. Effects of NGF on HO-1, NQO-1 and γ-GCS expression. The relative expression was normalized to β-actin. (A-C) Reverse transcription-quantitative polymerase chain reaction for HO-1, NQO-1 and γ-GCS mRNA expression. (D) Western blotting for HO-1, NQO-1 and γ-GCS protein expression. (E-G) Quantitative analysis of the western blot results. Each column represents mean ± standard deviation. n=3/group. NGF, nerve growth factor; HO-1, heme oxygenase-1; NQO-1, NAD(P)H:quinone oxidoreductase-1; γ-GCS, γ-glutamylcysteine synthetase; control, without NGF treatment.

transmission from and to the brain (25,26). SCI is the most serious complication resulting from spinal injury, leading to severe dysfunction and disorder below the injured segment, such as paralysis and quadriplegia. The loss of the function and intercurrent sequelae are mainly due to the death and apoptosis of neurocytes surrounding the lesion sites (27), cutting the signal transmission and losing control of body parts, and the axons could rarely spontaneously regenerate. Therefore, it is important to find techniques for efficient axon regeneration.

Numerous studies have proposed certain methods to promote axon regeneration in animal models. The methods include transplantation of neural stem cell grafts (28);

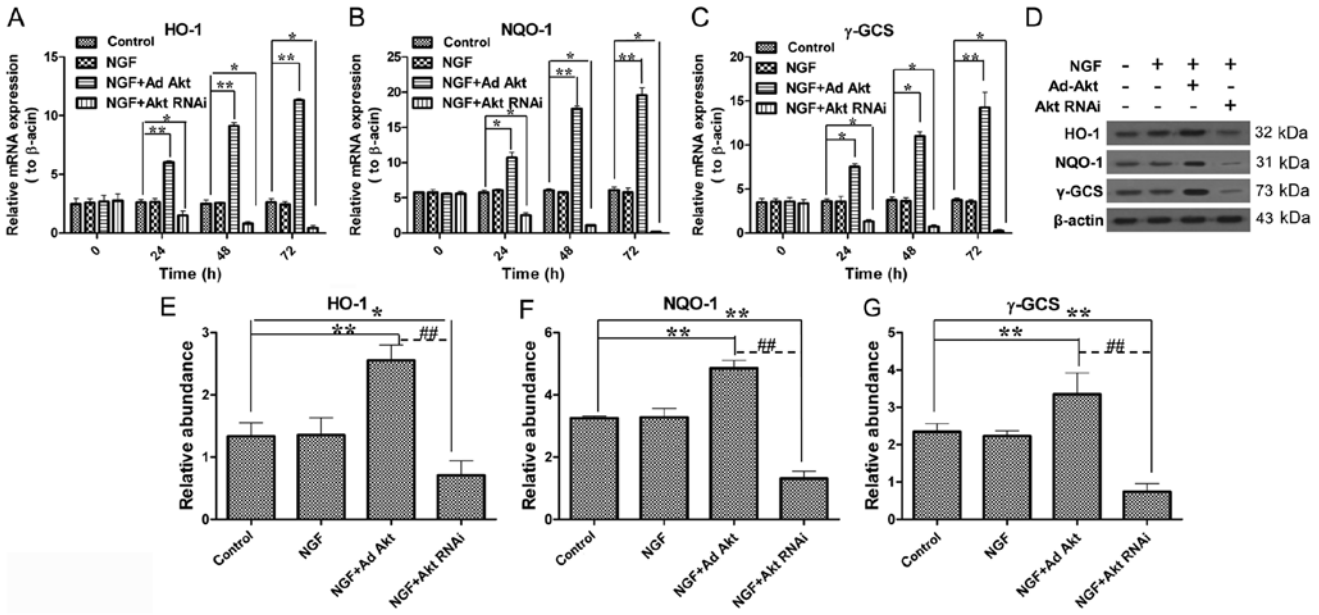

Figure 6. Effects of Akt on HO-1, NQO-1 and γ-GCS expression. (A-C) mRNA expression of HO-1, NQO-1 and γ-GCS. (D) Protein expression of HO-1, NQO-1 and γ-GCS. (E-G) Quantitative analysis of the western blot results. Each column represents mean ± standard deviation. n=3/group. *P<0.05 and **P<0.01 vs. control; $^{\#\#}$P<0.01 Ad Akt vs. Akt RNAi. HO-1, heme oxygenase-1; NQO-1, NAD(P)H:quinone oxidoreductase-1; γ-GCS, γ-glutamylcysteine synthetase; control, untreated cells; NGF, nerve growth factor.

injection of human induced pluripotent stem cells into the lesion sites (29); inhibition of myelin-associated glycoprotein (MAG) to promote neurites sprouting from transplanted neurons (30,31); avoidance of myelinated tracts (4,32); removal of myelin (33); and the application of antibodies to inhibit myelin inhibitors (2,34). Another means to induce axon regeneration has relied on neurotrophin treatments. For example, different neurotrophin treatments have not only increased neuron regeneration in adult CNS, but also stimulated axonal growth and sprouting following injury (12,13,35,36). All the above methods merely focus on promoting axon regeneration, however, none of the associated signaling mechanisms have been further excavated or investigated.

In the present study, the effects of NGF to promote neural differentiation in PC12 cells were first confirmed. On the basis of NGF, the role of Akt in promoting proliferation and inhibiting apoptosis of PC12 cells was subsequently investigated. Akt was overexpressed and silenced via adenovirus and siRNA transfection. The results showed that increased Akt expression could promote axon growth, contrary to the growth inhibition by Akt silence. Of note, the promoting or inhibitory effects had a precondition that PC12 cells must be treated with NGF. Therefore, NGF is a determinant of axon growth. The Nrf2/ARE signaling pathway is an antioxidative system for neuroprotection. HO-1, NQO-1 and γ-GCS are three molecules that have important roles in this antioxidant system. Whether this known neuroprotection is associated with the Akt effects requires elucidating. As expected, Akt overexpression upregulated HO-1, NQO-1 and γ-GCS expression. By contrast, Akt knockdown had a negative effect on HO-1, NQO-1 and γ-GCS expression. Therefore, Akt has a positive correlation with Nrf2/ARE signaling. To distinguish the role of NGF in Nrf2/ARE, an experiment was conducted to evaluate HO-1, NQO-1 and γ-GCS levels with or without NGF. The results indicated that NGF could not affect Nrf2/ARE signaling.

In the present study, Akt not only promoted the proliferation, but also inhibited the apoptosis of PC12 cells. These all contributed to neuroprotection, such as in SCI treatment, as neurocyte death and reduction are the main reason for SCI complication (37). Additionally, the promotive effect toward axon growth contributed to branching and sprouting of neurocytes to carry signals effectively. However, the present study is limited of animal experiments to verify Akt effects for SCI recovery *in vivo*, which requires further analysis. In the present study, a synergistic effect was discovered between Akt and NGF. Therefore, only Akt could activate the Nrf2/ARE signaling pathway and its downstream genes. NGF was responsible for neural differentiation, Akt alone had no influence; Akt was able to boost neural differentiation induced by NGF, which was likely to be involved in the Nrf2/ARE pathway. The Nrf2/ARE signaling pathway is a main protective mechanism versus oxidative damage. The upregulation of HO-1, NQO-1 and γ-GCS by Akt was coordinated with axon growth induced by NGF; they all contribute synergistically and systematically to neuroprotection and functional recovery of neurocytes.

In conclusion, an association between Akt and a potential of the Nrf2/Akt signaling pathway to enhance NGF-induced axon growth was reported, which contributes to the treatment of neural degenerative diseases, such as SCI and its subsequent complications.

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