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Codonopsis pilosula (Franch) Nannf total alkaloids potentiate neurite outgrowth induced by nerve growth factor in PC12 cells

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KEY WORDS *Codonopsis*; alkaloids; mitogen-activated protein kinases; nerve growth factors; neurites

ABSTRACT

AIM: To explore the effect of *Codonopsis pilosula* (Franch) Nannf total alkaloids (DSA) on differentiation induced by nerve growth factor (NGF) in PC12 cells. **METHODS:** After culturing PC12 cells with DSA in the presence or absence of NGF, neurite outgrowth in PC12 cells and correlated protein kinases were assayed. **RESULTS:** DSA alone did not exhibit neurotogenic activity, but caused a significant enhancement of NGF (2 µg/L)-induced neurite outgrowth in PC12 cells, and increased the phosphorylation of mitogen-activated protein kinase (MAPK). Furthermore, this enhancing effect was completely blocked by a specific MAPK kinase inhibitor, PD98059. **CONCLUSION:** DSA enhanced the NGF-induced neurite outgrowth in PC12 cells by amplifying an up-stream step of the MAPK-dependent signaling pathway.

INTRODUCTION

Nerve growth factor (NGF) is essential for the differentiation and maintenance of peripheral sympathetic neurons and certain nociceptive neurons. Various neurons of the central nervous system may also respond to NGF^[1]. The effects of NGF are mediated through interaction with two specific receptors, the high-affinity tyrosine kinase receptor TrkA and the low-affinity receptor p75^{NGFR}^[2]. NGF is a target-derived growth factor and in the whole organism, target tissues innervated by the sympathetic neurons and small sensory neurons are the major sites of production. It reported that NGF might be useful for the treatment of many peripheral nervous system diseases such as amy-

trophic lateral sclerosis (ALS) and diabetic neuropathy^[3]. However, current clinical trials with recombinant protein growth factors have caused a number of problems such as delivery, short half-lives, poor penetration through the blood brain barrier. New compounds with neurotrophic activity that have potential for treating such nervous diseases need to be developed.

The pharmacology and clinical application of traditional Chinese medicine (TCM) has been well documented for centuries in China. Some Chinese herbs are effective in promoting good health and vitality, and in curing various diseases. *Codonopsis pilosula* (Franch) Nannf (Dangshen) has an important effect on antiaging, and curing various diseases^[4]. *Codonopsis pilosula* (Franch) Nannf total alkaloids (DSA), abstracted with 20 % alcohol, had been demonstrated that they were helpful to cure the animal model of remembering dysfunction and prevent the decrease of acetylcholine level and choline acetyltransferase activity^[5].

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The most widely used bioassays to determine activities of trophic agents involve either assessing neurite outgrowth from tissue explants, or measuring cell survival and neurite outgrowth in cell cultures^[6]. The aim of our study is to explore the effect of DSA, on the neuronal differentiation induced by NGF in PC12 cells, which might provide not only as a useful compound for studying the mechanism of NGF neurotrophic action, as well in development of drugs for the injury or degenerating diseases in nervous system.

MATERIALS AND METHODS

Cell culture Chemicals and materials were purchased from Sino-America Bio-company (SABC, China) unless noted. PC12 cells, a rat pheochromocytoma cell line that responds to NGF by expressing neurite-like processes, were purchased from the American Type Culture Collection (Manassas, VA, USA). PC12 cells are cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose formulation) supplemented with 5 % fetal bovine serum (FBS, Gibco) and 10 % horse serum (HS, Gibco). Medium was replaced every 2-3 d, cells were maintained at 37 °C in a humidified 5 % CO₂ incubator. For the experiment of regeneration of neurites in PC12 cells, cells were grown in 24-well plate precoated with poly-*L*-Lysine. After serum-starvation for 12 h in serum free DMEM, cells were treated with different dose of DSA and/or NGF in tested medium (DMEM supplemented with 1 % FBS, 2 % HS) at 37 °C for indicated time. A specific inhibitor of MAPKK, PD98059 (Calbiochem, 513000) was prepared as a 50 μmol/L solution in dimethyl sulfoxide (Me₂SO) and diluted directly into the medium. The concentration of Me₂SO never exceeded 0.5 % (v/v), which had no effect on the cell viability. Cells were preincubated for 15 min in the presence or absence of PD98059 before addition of the DSA and/or NGF.

Cell proliferation and cytotoxic assays Using the cell proliferation (MTT) kit (Jiancheng, Nanjing, China), the effect of DSA on cell proliferation was monitored by measuring the reduction of the tetrazolium salt. Briefly, PC12 cells were preincubated overnight in 24-well plates. The cells were then treated with different concentration of DSA (0-100 mg/L) for 2 or 4 d. MTT assays were performed by incubating the DSA-treated PC12 cell with MTT labeling solution. After 4 h of incubation, the cell lysates and the purple formazan crystals were solubilized for detection at 570

nm. At least three independent experiments were performed for each study.

The cytotoxicity of DSA was determined by the cytotoxicity detection kit (Jiancheng, Nanjing, China) that was based on the detection of lactate dehydrogenase (LDH) released from dead cells as a result of cytotoxicity. Cell-free culture supernatants from DSA-treated PC12 cells were collected and then transferred to 96-well plates. Substrate mixture containing tetrazolium salt was added, and then incubated for 30 min. The formazan dye formation was quantitated by measuring the absorbance at 440 nm. At least three independent experiments were performed for each study.

Cell counts and neurite analyses After treatment, cell counts were performed in random microscopic field and all counts were performed at least five times. With the software of Image Pro Plus, neurite outgrowth was evaluated based both on the number of neurites per cell and on the relative lengths of neuritis. A minimum of 100 clumps per culture were scored per dish and examined by strip counting.

Western blot analysis of mitogen-activated protein kinases Immunoblotting was performed as described previously^[7,8]. Cell extracts were fractionated by SDS-PAGE with 10 % polyacrylamide gel. Proteins were electroblotted onto a polyvinylidene difluoride (PVDF, Novex) membrane. MAPK were immunassayed with the monoclonal antibodies (Gene, Cal-444944) against MAP kinases. The antibodies used have been shown to bind both p42/44 MAPK^[9]. For determination of the tyrosine phosphorylation of MAPK, PVDF membranes were generally incubated in a 5 % solution of nonfat dry milk in Tris-HCl buffer 10 mmol/L (pH 7.6) saline with 0.1 % Tween-20 (TBS-T) and washed once in TBS-T. The blots were probed with the phospho-p42/44 MAPK antibody (Gene, Cal-444945) at a dilution of 1:300 in a blocking buffer (5 % nonfat dry milk in TBS-T) at room temperature for 1 h. Membranes were incubated with horseradish peroxidase-conjugated antibodies against mouse immunoglobulin G. Peroxidase activity was detected using the enhanced chemiluminescence's light-based detection system. Western blotting results were quantified by the analysis of X-ray films using the software of Labworks.

Statistical analysis The data were analyzed by Student's *t* test. All results were expressed as mean±SD of triplicate determination. Each experiment was repeated 3 times.

RESULTS

Effect of DSA on proliferation of PC12 cells

PC12 cells were collected and plated with 2×10^4 cells/cm² in normal medium. After the cells attached, NGF 50 μ g/L and different concentrations of DSA in the presence or absence of NGF 2 μ g/L were separately added to PC12 cells cultured in test medium (DMEM supplemented with 1 % fetal bovine serum and 2 % horse serum). Treatment of PC12 cells with DSA at high concentration (>3 mg/L) decreased the proliferating rate compared with the control cells treated with NGF 2 μ g/L alone (Fig 1). More than 15 % decrease in cell proliferation was observed when PC12 cells were treated with DSA 30 mg/L+NGF 2 μ g/L for 4 d (Fig 1).

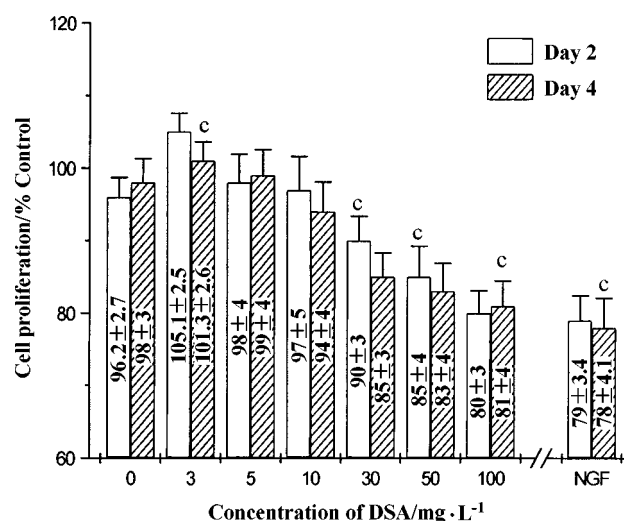


Fig 1. Effect of DSA on the cell proliferation of PC12 cells in the presence of NGF 2 μ g/L. NGF 50 μ g/L was used as a positive control in this experiment. The mean absorbance obtained using NGF (2 μ g/L) as control was designated 100 %. $n=6$. Mean \pm SD. ^c $P<0.01$ vs control.

Since the cell proliferation MTT assays measured the metabolic activities of the cells, the reduction in the cell number could either be a consequence of cell death or the reduction in the cell proliferation. The cytotoxicity test was then performed to examine whether treatment of PC12 cells with DSA could induce cell death. Increasing concentrations of DSA (0-100 mg/L) were added to PC12 cells and the release of LDH was monitored after overnight incubation. It was observed that DSA did not induce any cytotoxic effect (Tab 1).

DSA enhanced the neuronal differentiation of PC12 cells The addition of NGF alone to PC12 cells resulted in major change in their appearance at 48 h, suggesting of differentiation into a neuronal phenotype

Tab 1. Cytotoxic effect of DSA on PC12 cells. $n=6$. Mean \pm SD. ^b $P<0.05$ vs control.

Treatment	Average absorbance
DSA 0 mg/L	0.198 \pm 0.023
3	0.214 \pm 0.014 ^b
5	0.22 \pm 0.03
10	0.235 \pm 0.022
30	0.23 \pm 0.04 ^b
50	0.229 \pm 0.024 ^b
100	0.245 \pm 0.013
Control	
Total cell lysate	2.74 \pm 0.08
NGF 50 μ g/L	0.224 \pm 0.023 ^b

as described previously^[9]. NGF caused a dose-dependent increase in cells with neurites longer than one or more cell body diameters (Fig 2B). With NGF 2 μ g/L, very few cells extended neurites longer than one cell body diameter (Fig 2A). Although DSA alone had no effect on morphology, DSA added concurrently with NGF 2 μ g/L had a dramatic effect on the appearance of PC12 cells, including compaction of cell bodies and extension of neurites (Fig 2C). The presence of DSA also strongly increased the neuronal differentiation number of PC12 cells responding to NGF, as well as the magnitude of their response (Fig 3).

In the presence of DSA (30 mg/L), the number of PC12 cells responding to NGF 2 μ g/L with neurites greater than one cell body diameter or three cell body diameters were increased from 7 % \pm 3 % to 52 % \pm 8 % or from 1.3 % \pm 0.7 % to 18 % \pm 6 % respectively compared with control. But, when DSA was increased to 100 mg/L, no significant difference in effect was observed (Fig 4).

DSA enhanced the neuronal differentiation of PC12 cells induced by NGF based on the pathway of MAPK The activated forms of MAPK were observed on Western blot of lysates from NGF-stimulated PC12 cells (Fig 5A). The activated states of the kinases were confirmed by immunostaining with phosphotyrosine specific antibodies. When PD98059 was added to cultures, the phosphorylated forms of both M_r 42 000 and 44 000 MAPK (Fig 5B) were decreased distinctly. Furthermore, the number of neurites per cell and the length of neurites were dramatically decreased (Fig 2D). When PC12 cells were treated with DSA in the presence of NGF (2 μ g/L), both M_r 42 000 and 44 000

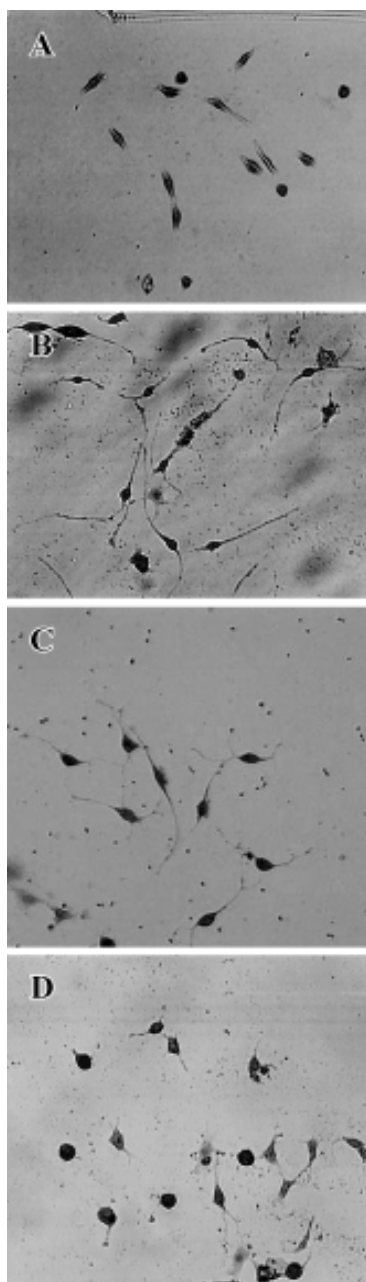


Fig 2. Effect of DSA and/or NGF on the morphology of PC12 cells ($\times 100$). A: control (NGF 2 $\mu\text{g/L}$); B: NGF 50 $\mu\text{g/L}$; C: NGF 2 $\mu\text{g/L}$ +DSA 30 mg/L ; D: NGF 2 $\mu\text{g/L}$ +DSA 30 mg/L +PD98059 20 $\mu\text{mol/L}$.

MAPK were activated for at least 1 h, when preincubation with PD98059, the phosphorylation of mitogen-activated protein kinase was inhibited. These data indicated that pretreatment of PC12 cell with PD98059 efficiently inhibited the phosphorylation of mitogen-activated protein kinases.

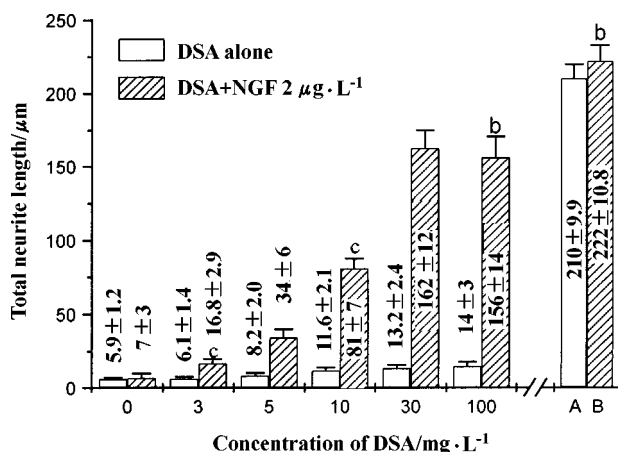


Fig 3. Effect of DSA on PC12 neuritogenesis in the presence of NGF 2 $\mu\text{g/L}$. $n=3$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control. A: DSA 30 mg/L +NGF 50 $\mu\text{g/L}$; B: NGF 50 $\mu\text{g/L}$.

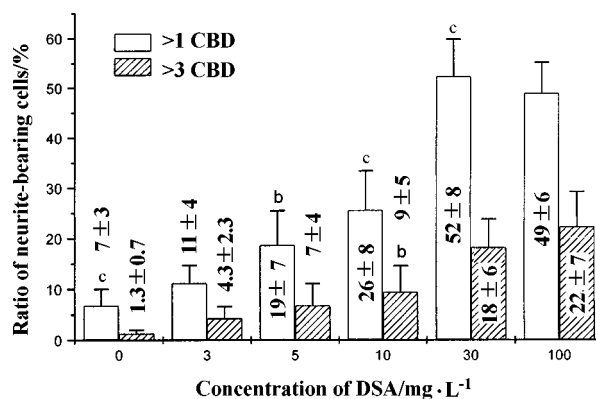


Fig 4. DSA increases the ration of neurite-bearing cells in PC12 cells in the presence of NGF 2 $\mu\text{g/L}$. $n=3$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control.

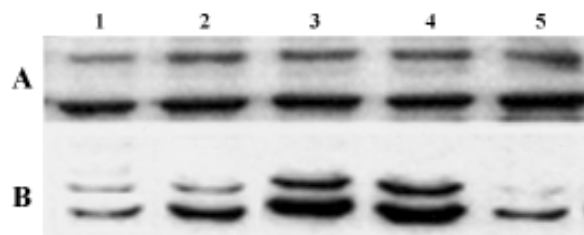


Fig 5. Biphosphorylation of mitogen-activated protein kinases in response to DSA and/or NGF. Mitogen-activated protein kinases were detected by Western blotting analysis with anti-total MAPK (A) or anti-biphosphorylated MAPK antibody (B). Proteins were transferred to PVDF membranes and immunoassayed with antibodies against MAPK (A) and against phosphorylated MAPK (B). Lane 1: NGF 2 $\mu\text{g/L}$; Lane 2: PD98059 20 $\mu\text{mol/L}$ +NGF 50 $\mu\text{g/L}$; Lane 3: NGF 50 $\mu\text{g/L}$; Lane 4: DSA 30 mg/L +NGF 2 $\mu\text{g/L}$; Lane 5: DSA 30 mg/L +PD98059 20 $\mu\text{mol/L}$ +NGF 2 $\mu\text{g/L}$.

DISCUSSION

Nervous system function is dependent on highly specific neuron connections during development. The patterning and specificity of these connections require neurite extension toward the proper targets guided by the growth cone in response to environmental signals. However, the processes that involve signal-induced morphological changes resulting in coordinated cytoskeletal remodeling in the specialized growth cone are poorly understood^[10]. In the present study, we utilize a model system, rat pheochromocytoma PC12 cells, to demonstrate the presence of neuroactive compounds in traditional Chinese medicine. DSA, with low-dose NGF, resulted in neuronal differentiation of PC12 cells, as well as inhibiting the cell proliferation, moreover, DSA unregulated the phosphorylation of MAPK in the presence of NGF (2 µg/L). All these results suggest that DSA enhancing PC12 cell differentiation might be involved in MAPK pathways. Our findings not only demonstrate that *Codonopsis pilosula* (Franch) Nannf comprises a rich resource of neuroactive compounds, but also reveals the potential signaling molecules involved in its action. This pathway involves protein kinases that are important in regulating the growth and differentiation of PC12 cells^[11,12].

NGF activates the MAPK pathway to cause neurite outgrowth from PC12 cells via phosphorylation of MAPK^[13]. Phosphorylation of both the tyrosine and threonine residues of MAPK by MAPKK is required for full enzymatic activation^[14,15]. Recently, it has been reported that MAPK-dependent and independent pathways are involved in NGF-induced formation of processes in PC12 cells^[15]. In this experiment, NGF-induced neurite outgrowth was only partially blocked by PD98059, a representative MAPKK inhibitor. This observation suggested that the MAPK-dependent signaling pathway play only a partial role in the neurotrophic effect of NGF. Since PD98059 completely inhibited the DSA-induced enhancement of the NGF-mediated outgrowth of neurites from PC12 cells, it indicated a MAPK-dependent action of DSA. Western blotting analysis was performed in order to elucidate whether the site of action DSA is up- or down-stream step in the MAPK-dependent signaling pathway. The result indicated that DSA could stimulate NGF-induced MAPK activation, but has no effect on the phosphorylated MAPK. These observations demonstrated that DSA enhanced the NGF-in-

duced neurite outgrowth in PC12 cells by amplifying an up-stream step in the NGF receptor-mediated intracellular MAPK-dependent signaling pathway.

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