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Induction of Neurite Outgrowth by (-)-(7*R*, 8*S*)-Dihydrodehydrodiconiferyl Alcohol from PC12 Cells

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A lignan derivative, (-)-(7*R*, 8*S*)-dihydrodehydrodiconiferyl alcohol (DHDA), was isolated from *Kalopanax septemlobus* L. and was observed to have neuritogenic activity. DHDA at 50 μM caused a marked induction of neurite outgrowth and an enhancement of nerve growth factor (NGF)-mediated neurite outgrowth from PC12 cells. However, it did not exhibit any neurotrophic action. At 50 μM, DHDA enhanced NGF-induced neurite-bearing activity. This activity was partially blocked by the mitogen-activated protein kinase (MAPK) inhibitor PD98059 and by GF109203X, a protein kinase C (PKC) inhibitor. These results suggest that DHDA can induce neurite outgrowth and enhance NGF-induced neurite outgrowth from PC12 cells by amplifying up-stream steps such as MAPK and PKC.

Key words: (-)-(7*R*, 8*S*)-Dihydrodehydrodiconiferyl alcohol, *Kalopanax septemlobus*, Neurite outgrowth, PC12 cells

INTRODUCTION

Nerve growth factors (NGF) and other neurotrophic factors stimulate neurite outgrowth in neuronal cells and play an important role in their survival and maintenance in the central nervous system (Patrick *et al.*, 1996). Therefore, it is proposed that NGF and NGF-like agents may be used to treat the neurodegeneration seen in such illnesses as Alzheimer's and Parkinson's diseases (Connor and Dragunow, 1998). Although the NGF molecule is too large to cross the blood-brain barrier, NGF-like low molecular weight compounds can be considered as candidates for treating neurodegenerative diseases.

Several compounds have been found to enhance NGF action in the neurite outgrowth from PC12 cells; simvastatin (Sato-Suzuki and Murota, 1996), AIT-082 (Middlemiss *et al.*, 1995), SR57746A (Pradines *et al.*, 1995), Aroclor 1254 (Angus and Contreras, 1995), nerfilin (Hirao *et al.*, 1995), and nardosinone (Li *et al.*, 1999). However, these compounds have not been evaluated for clinical applications yet.

An inducer of neurite outgrowth of PC12 cells was isolated from bioactive components of the stem bark of *Kalopanax septemlobus* by activity-guided fractionation. It was identified as a lignan derivative, (-)-(7*R*, 8*S*)-dihydrodehydrodiconiferyl alcohol (DHDA) (Fig. 1). The stem bark of *K. septemlobus* L. (Araliaceae) has been used in traditional medicine to treat neuralgia, rheumatic arthritis, lumbago, furuncle, diarrhea, and scabies (Namba, 1980). The major components of *K. septemlobus* are saponins, polyacetylenes, phenylpropanoid glycosides, lignans, and simple phenolic glycosides (Li *et al.*, 2002).

In the presence of NGF, rat pheochromocytoma PC12 cell lines differentiated and extended neurite outgrowth. Therefore, these cells have been used as a model system for sympathetic neuron-like cells (Gysbers and Rathbone, 1992).

In this study, we investigated the partial neuritogenic mechanism of DHDA and its effects on neurite outgrowth from PC12 cells.

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MATERIALS AND METHODS

Materials

RPMI 1640, donor horse serum (HS), and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, U.S.A.). 2.5S NGF, poly-L-lysine, antibiotics, PD98059, and GF109203X were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade. The stem bark of *K. septemlobus* was collected from Jinbu, Kangwon province, Korea and a voucher specimen was deposited at the herbarium of the College of Pharmacy, Chungbuk National University (Cheongju, Korea).

Separation and identification of DHDA

Air-dried stem bark of *K. septemlobus* (5 kg) was extracted with MeOH. The dried MeOH extract (930 g) was then partitioned in turn with CH_2Cl_2, EtOAc, BuOH, and water. The CH_2Cl_2 extract (81 g) was separated on a 9230-400 MESH silica gel column (Fischer Scientific) and Sephadex LH-20 (25-100 μm, Pharmacia) with MeOH to obtain five subfractions (KP221-KP225). DHDA (15 mg, t_R=20 min) was isolated from the third subfraction KP223 by a preparative HPLC system (Waters 515 pumps, Waters, Milford, MA, U.S.A.) equipped with photodiode array detector (Waters 2996, Waters) and YMC ODS-H-80 column (20 x 150 mm i.d., YMC Co., Wilmington, NC, U.S.A.) eluted with CH_3CN-H_2O (23:77, v/v) (Hanawa *et al.*, 1997). Optical rotation was measured with a JASCO DIP-370 spectrometer and CD spectra were determined with a JASCO J-715 spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AMX-500 MHz NMR spectrometer. EI-MS was measured on a Hewlett Packard 598A mass spectrometer.

Cell culture

PC12 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated HS, 5% heat-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin as a normal medium at 37°C in 5% CO_2/humidified air (Greene and Tischler, 1976). The cells (ca. 1×10^5 cells/cm^2) were treated with DHDA and were then incubated for 96 h for experiments on neurite extension.

Assay for neurite outgrowth

The morphology of PC12 cells was observed according to the method of Sano and Kitajima (1998). PC12 cells were dissociated by incubation with 1 mM EGTA in phosphate-buffered saline for 1 h and were seeded in 24-well culture plates (ca. 2×10^4 cells/well) coated with poly-L-lysine. After 24 h, the cells were transferred to a test medium containing 2% HS and 1% FBS that was appropriate for neurite-bearing cells. Outgrowth of neurite from PC12 cells was monitored under a phase-contrast microscope. Processes with lengths equivalent to one or more cell body diameters were counted as neuritis. A minimum of 100 cells was examined for each data point. The concentration of 2.5S NGF was used for 2 or 30 ng/mL. The data was analyzed by one-way ANOVA followed by Tukey's test for significance.

RESULTS AND DISCUSSION

A bioactive component-induced neurite outgrowth was isolated from *K. septemlobus* and identified as a lignan derivative, (-)-(7*R*,8*S*)-dihydrodehydrodiconiferyl alcohol (DHDA) (Fig. 1): colorless oil; $[\alpha]_D^{25}$ -5.4° (*c* 0.21, MeOH); CD (MeOH, c 1.4×10^{-4}): $[\theta]_{295}$ -2030, $[\theta]_{265}$ +145, $[\theta]_{245}$ -7150, $[\theta]_{225}$ +4500; EI-MS *m/z*: 360 $[M]^+$; ^{1}H-NMR and ^{13}C-NMR spectra and physical constants were identical with the previous report (Hanawa *et al.*, 1997).

The effects of DHDA on neurite outgrowth from PC12 cells were examined in the absence or presence of NGF. DHDA at 50-100 μM induced neurite outgrowth of PC12

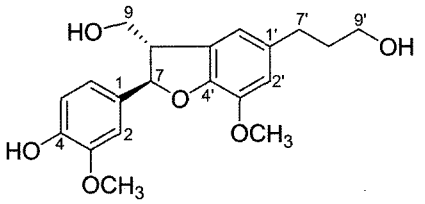

Fig. 1. Chemical structure of (-)-(7*R*,8*S*)-dihydrodehydrodiconiferyl alcohol (DHDA)

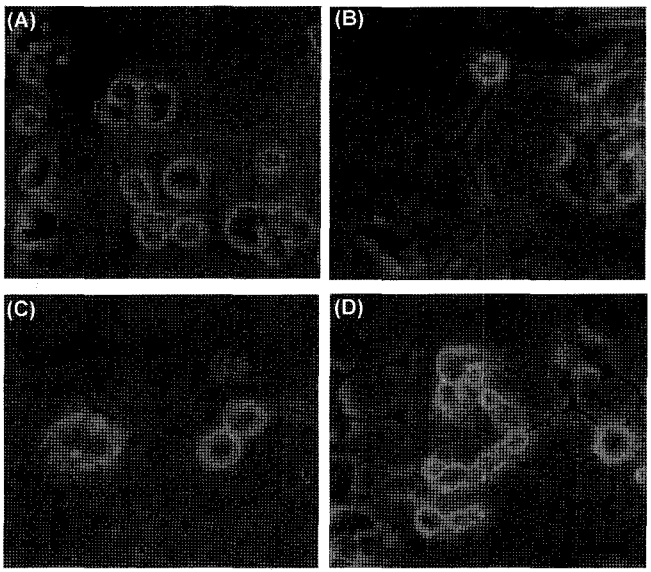

Fig. 2. Effects of DHDA and nerve growth factor (NGF) on the morphology of PC12 cells. The cells were treated for 96 h without (A, C) or with (B, D) DHDA (50 μM) in the absence (A, B) or presence (C, D) of NGF (2 ng/mL) respectively. Scale bar = 50 μm.

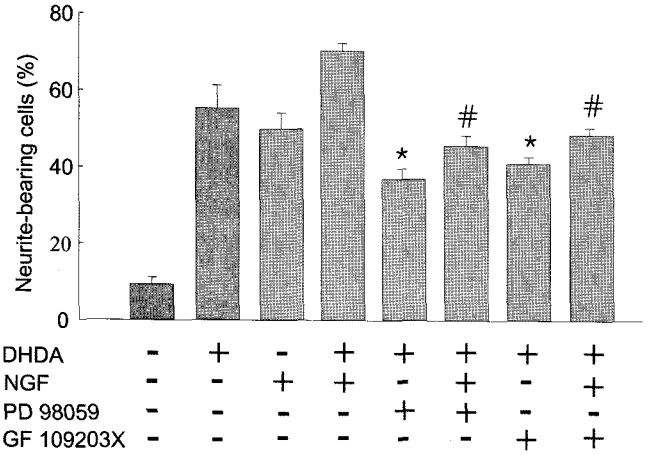

Fig. 3. Effects of PD98059 and GF109203X on DHDA-induced neurite-bearing activity by NGF. The number of neurite-bearing cells was expressed as a percentage of maximum number in response to NGF (30 ng/mL, 100%) in the absence of DHDA. The concentrations of DHDA, NGF, PD98059, and GF109203X were 50 μM, 2 ng/mL, 30 μM and 100 nM, respectively. Each point represents the means±SEM from three experiments. *, $p<0.05$ compared with DHDA alone; #, $p<0.05$ compared with corresponding DHDA plus NGF (ANOVA followed by Tukey's test).

cells. DHDA at 50 μM alone significantly induced neurite elongation from PC12 cells (Fig. 2B). In addition, DHDA-induced neurite outgrowth was enhanced by co-treatment with NGF at 2 ng/mL (Fig. 2D). Upon microscopic observation, PC12 cells exposed to DHDA (50 μM) and/or DHDA with NGF formed long neurites, which extended to neighboring cells over distances of 50 μm. However, when PC12 cells treated with DHDA were cultured in serum (HS and FBS)-free media, they started the apoptotic process earlier than untreated PC12 cells. This data suggested that DHDA was neuritogenic but not neurotrophic.

It is well known that neurite outgrowth in PC12 cells is stimulated by mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) (Leprince *et al.*, 1996; Tsuji *et al.*, 2001). DHDA alone showed significant neuritogenic activity at 50 μM and also enhanced NGF (2 ng/mL)-induced neurite outgrowth in the appropriate test medium for 24-36 h (Fig. 3). However, DHDA-induced enhancement of the NGF-neuritogenic action was partially blocked by PD98059, a representative MAPK inhibitor, and GF109203X, a PKC inhibitor (Fig. 3). These results suggested that the neuritogenic action of DHDA was partially involved in amplification of the up-stream steps of MAPK and PKC in the NGF receptor-mediated intracellular signaling pathway.

Among the various neuritogenic agents, simvastatin at 0.5-1.0 μg/mL alone extends neurite outgrowth (Sato-Suzuki and Murota, 1996). AIT-082 (100 μM), nerfilin I (0.5-0.05 μg/mL), nardosinone (100 μM), aroclor 1254 (25-50 μg/mL), and SR57746A (0.5 μM) only enhance NGF-mediated neurite outgrowth (Angus and Contreras, 1995; Hirao *et al.*, 1995; Middlemiss *et al.*, 1995; Pradines *et al.*, 1995; Li *et al.*, 1999). DHDA at similar concentration ranges of 20-50 μM exhibited neuritogenic activity. In addition, DHDA at 20-100 μM did not show any stimulatory effects on dopamine biosynthesis in PC12 cells (data not shown). According to MTT assays, DHDA at concentrations up to 150 μM did not exhibit cell cytotoxicity either.

In conclusion, naturally-derived DHDA induced neurite outgrowth and enhanced neurite outgrowth in association with NGF by mediating the MAPK and PKC signal pathways in PC12 cells. The mechanism of DHDA and its in vivo applications need to be studied further.

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