Note
Potential of Nerve Growth Factor-Induced Neurite Outgrowth in PC12 Cells by a Coptidis Rhizoma Extract and Protoberberine Alkaloids

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A methanol extract of Coptidis Rhizoma effectively enhanced the outgrowth of neurite in PC12 cells induced by nerve growth factor (NGF). Following solvent partition and preparative HPLC, berberine was isolated as the major active compound. Berberine enhanced the proportion of neurite-bearing cells in a dose-dependent manner without cytotoxicity. Its structural relatives, palmatine and coptisine, showed a slightly weaker NGF-enhancing effect than berberine. These three alkaloids inhibited acetylcholinesterase activity at a level comparable to that of physostigmine, but this inhibition was not responsible for the potentiation of NGF-induced neurite outgrowth. It is demonstrated for the first time that protoberberine alkaloids potentiated the NGF-induced differentiation of neural cells.

Key words: neurite outgrowth; PC12 cell; nerve growth factor; protoberberine alkaloid; Coptidis Rhizoma

Neurotrophic factors play an essential role in the development of the nervous system. Among them, nerve growth factor (NGF) is known to function in the survival and maintenance of cholinergic neurons in the central nervous system. Dementia such as Alzheimer’s disease is one of the most common neural disorders of an aged population and is considered to be caused by neural cell death. Therefore, these factors have been investigated for use in the treatment of neurodegenerative disorders.1) However, such polypeptides may not be applicable for medical treatment, since they are easily metabolized by proteases and cannot be transported through the blood-brain barrier when administered peripherally.

There have been some reports presented on the search for novel low-molecular-weight neurotrophic substances from plants.2–7) In many cases, these compounds were found to stimulate the outgrowth of neurites in undifferentiated neural cells, including rat pheochromocytoma PC12 cells,5) in the absence or presence of NGF. It is very important to search for a variety of compounds that may contribute to the development of medical drugs for treating various neurodegenerative diseases, including dementia. In this paper, we describe the potentiation effect of a Coptidis Rhizoma extract on the neural differentiation in PC12 cells.

Rhizomes of Coptis japonica Makino (Coptidis Rhizoma, “oren” in Japanese) and bark of Phellodendron amurense Ruprecht (Phellodendri Cortex, “obaku” in Japanese) were purchased from Tochimoto Tenkaido Co. (Osaka, Japan). 2.5S NGF, berberine chloride, physostigmine sulfate (eserine hemisulfate), and substrates for the cholinesterase assay were obtained from Sigma (NY, U.S.A.). Palmatine chloride and coptisine chloride were products from Matsuura Yakugyo Co. (Nagoya, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. All other chemicals used were of analytical grade from commercial sources. Rat PC12 cells were obtained from RIKEN Cell Bank (Saitama, Japan) and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, NY, U.S.A.) supplemented with 10% fetal bovine serum (Filtron, Brooklyn, Australia) and 10% horse serum (Gibco BRL) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The cells (3.5 × 10^4 cells/well) were seeded in a 12-well collagen-coated plate (Iwaki, Chiba, Japan) and cultured overnight. After NGF (10 ng/ml) and various concentrations of each sample had been added to the medium, the cells were cultured for a further 48 h. All samples were dissolved in DMSO and added at a final concentration of less than 0.1% DMSO. In another experiment for determining the activity of authentic compounds, PC12 cells were differentiated in a serum-free medium. Precultured cells were washed once with DMEM and then cultured in fresh serum-free DMEM containing an N-2 supplement (Gibco BRL). After 2 h, NGF (10 ng/ml) and each test compound were added to
Fig. 1. NGF-Potentiating Effect of an Extract and Its Partitioned Fractions Obtained from Coptidis Rhizoma.

The methanol extract and its partitioned fractions from Coptidis Rhizoma were dissolved in DMSO at concentrations of 100 and 50 μg/ml, respectively. After their addition at the indicated concentrations together with 10 ng/ml of NGF, PC12 cells were induced to differentiate for 48 h in a serum-containing medium and examined for their rates of neurite formation. For comparison, the cells were treated with NGF alone at concentrations of 10 and 100 ng/ml. *Significantly different from the NGF (10 ng/ml)-treated group (p < 0.05).

Fig. 2. NGF-Potentiating Effect of the Active Compound Isolated from the Coptidis Rhizoma Extract.

PC12 cells were treated with the active compound (Fr. 6) at the indicated concentrations described in Fig. 1, and then examined for their rates of neurite formation. The cells were also treated with NGF alone at concentrations of 10 and 100 ng/ml. *Significantly different from the NGF (10 ng/ml)-treated group (p < 0.05).

The neurite formation of PC12 cells was examined under a phase-contrast microscope, and processes with a length more than the cell diameter were scored as neurites. The percentage of neurite-bearing cells to the total number of cells counted was examined for each culture well. Each value is expressed as the mean ± SD from triplicate wells for each treatment. Statistical analyses were carried out by Fisher’s protected least significant difference test after an arcsine transformation of all percentage values.

Methanol extracts of various Chinese medicinal plants were examined for their activities to potentiate the NGF-induced neurite outgrowth of PC12 cells in the serum-containing medium. The active component in the Coptidis Rhizoma extract was isolated by extracting Coptidis Rhizoma (500 g) with MeOH (1 liter × 3) at room temperature, suspending the combined extract (45.0 g) in water (500 ml), and partitioning with hexane, EtOAc, and water-saturated n-BuOH (300 ml each × 3). A part (500 mg) of the most active aqueous fraction (22.9 g) was subjected to preparative HPLC separation with a Jaigel-GS310 column (20 mm × 50 cm) and isocratic elution by H2O–MeCN–AcOH–Et3N (75:25:0.3:0.8). The active fraction (Fr. 6, 50 mg) was further purified by recrystallization with EtOH to give a brownish-yellow crystal (103 mg) [mp 150–153°C (decomp.)]. An authentic compound. The compound afforded a yellow crystal [mp 200–203°C (decomp.)] which was identified as berberine chloride by direct comparison (mixed mp, TLC, IR, EI-MS, and 1H-NMR) with an authentic compound.

During the course of our search for promising medicinal plants, the methanol extracts of Coptidis Rhizoma and Phellodendri Cortex, each at a concentration of 100 μg/ml, markedly potentiated the neurite outgrowth in PC12 cells induced by 10 ng/ml of NGF. The Coptidis Rhizoma extract showed more effective dose-dependence than the Phellodendri Cortex extract, and there has been no previous report of such an effect of these two Chinese medicinal plants. The active component was then isolated from the Coptidis Rhizoma extract. When treated with partitioned fractions at a concentration of 50 μg/ml, the aqueous fraction showed the highest proportion of neurite-bearing cells and, moreover, this value was demonstrated to be higher than that induced by a high concentration (100 ng/ml) of NGF (Fig. 1). Preparative HPLC of the aqueous fraction gave six peaks, the sixth (Fr. 6) being demonstrated to have potent NGF-enhancing activity at a concentration of 20 μg/ml. Figure 2 shows its dose-dependent potentiation of the neurite outgrowth induced by 10 ng/ml of NGF. This active fraction significantly potentiated the NGF action at a concentration of 5 μg/ml, and its treatment at 20 μg/ml produced a higher proportion of differentiated cells than that by a single addition of 100 ng/ml of NGF. The treated cells showed not only an increased rate of neurite formation, but also an extension of the bipolar processes when compared with the cells treated with 10 ng/ml of NGF alone. This fraction proved to be highly homogeneous by
that these alkaloids in the same concentration range had no neurotrophic activity in the absence of NGF.

These alkaloids were determined for their inhibitory activities toward acetylcholinesterase in the rat brain and toward butyrylcholinesterase in the rat plasma by using acetylthiocholine iodide and butyrylthiocholine iodide, respectively, as their specific substrates.9) The three alkaloids showed potent inhibition of the acetylcholinesterase activity, but not of the butyrylcholinesterase activity, their potency being in the order berberine > palmatine > coptisine (data not shown). This inhibition was comparable to that of physostigmine, the most potent inhibitor toward both types of cholinesterase. However, physostigmine, as well as carbamylcholine, a stable cholinergic agonist, had no effect on neurite outgrowth in PC12 cells. These results suggest that the NGF-potentiating activity of berberine was not mediated through the inhibition of acetylcholinesterase and/or the accumulation of acetylcholine. In addition, berberine showed no cytotoxic effect on the growth of PC12 cells in the concentration range of 3.1–25 μg/ml judged by the WST-1 method for the cytotoxicity assay.

Coptidis Rhizoma as well as Phellodendri Cortex have been widely used as traditional medicines for treating diarrhea and gastrointestinal disorders. Berberine and its structural relatives, the major alkaloids in these plants, have been reported to have various biological activities,10,11) including antimicrobial, antimalarial and antifungicidal activities. Furthermore, these alkaloids exhibit a wide variety of pharmacological effects via their inhibition of enzymes and/or their interaction with proteins.9,12,13) Our findings demonstrate for the first time that berberine and its relatives had a potent effect to enhance the NGF-induced differentiation in PC12 cells. Although the mechanism for enhancing the NGF action remains to be elucidated, these alkaloids might be useful for developing pharmacological agents which have the function to restore and maintain neural cells in the central nervous system.

References


