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Potentiation of nerve growth factor-induced elongation of neurites by gelsemiol and 9-hydroxysemperoside aglucone in PC12D cells

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Abstract

In PC12D cells, nerve growth factor (NGF) increased the proportion of neurite-bearing cells and made neurites longer. A methanol extract of *Verbena littoralis* H. B. K. collected in Paraguay only slightly potentiated the proportion of PC12D cells with neurites but markedly increased the length of neurites in the presence of NGF (2 ng mL^{-1}). The methanol extract was partitioned between ethyl acetate and water followed by further extraction of water fraction with n-butanol. The potentiating activity of NGF-action was observed in the ethyl acetate and n-butanol fractions. The n-butanol fraction was separated by silica gel chromatography, monitoring the NGF-potentiating activity to give gelsemiol and 9-hydroxysemperoside aglucone (9-OHSA). Neither compound (30–300 μM) exhibited neurite-inducing activity alone. Gelsemiol (100–300 μM) markedly enhanced an increase in the proportion of neurite-bearing cells and an extension of the neurite length in the presence of NGF (2 ng mL^{-1}). Interestingly, in the presence of NGF (2 ng mL^{-1}), 9-OHSA (100–300 μM) enhanced the elongation of neurites without affecting the increase in the proportion of cells with neurites. These results suggested that gelsemiol and 9-OHSA were major active components of *V. littoralis* in the NGF-potentiating action. It was possible that the mechanism of neurite elongation by NGF was different from that of the increase in the proportion of neurite-bearing cells, and that 9-OHSA selectively affected the neurite elongation mechanism.

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Introduction

Nerve growth factor (NGF) has held the promise of therapeutic efficacy in the treatment of neurodegenerative diseases including Alzheimer's disease and cerebrovascular dementia (Brinton & Yamazaki 1998). However, NGF can be used for medical treatment only when injected directly into the brain. This is because it is a polypeptide of large molecular weight, does not cross the blood–brain barrier and is easily metabolized by peptidases when administered peripherally. A useful strategy of addressing this drug delivery problem would be to administer drugs that either enhanced the action of NGF or increased the expression of NGF in the appropriate cell population (Aisen & Davis 1997).

Numerous natural products have been used as pharmacological tools for pharmacological, physiological and biochemical studies (Ohizumi 1997). We have found that several natural products such as goniodomin A (Matsunaga et al 1999), purealin (Takito et al 1986) and xestoquinone (Sakamoto et al 1993) modulate actomyosin ATPase activity. In the course of our search for bioactive natural substances from medicinal plants, we have devoted our attention to the occurrence

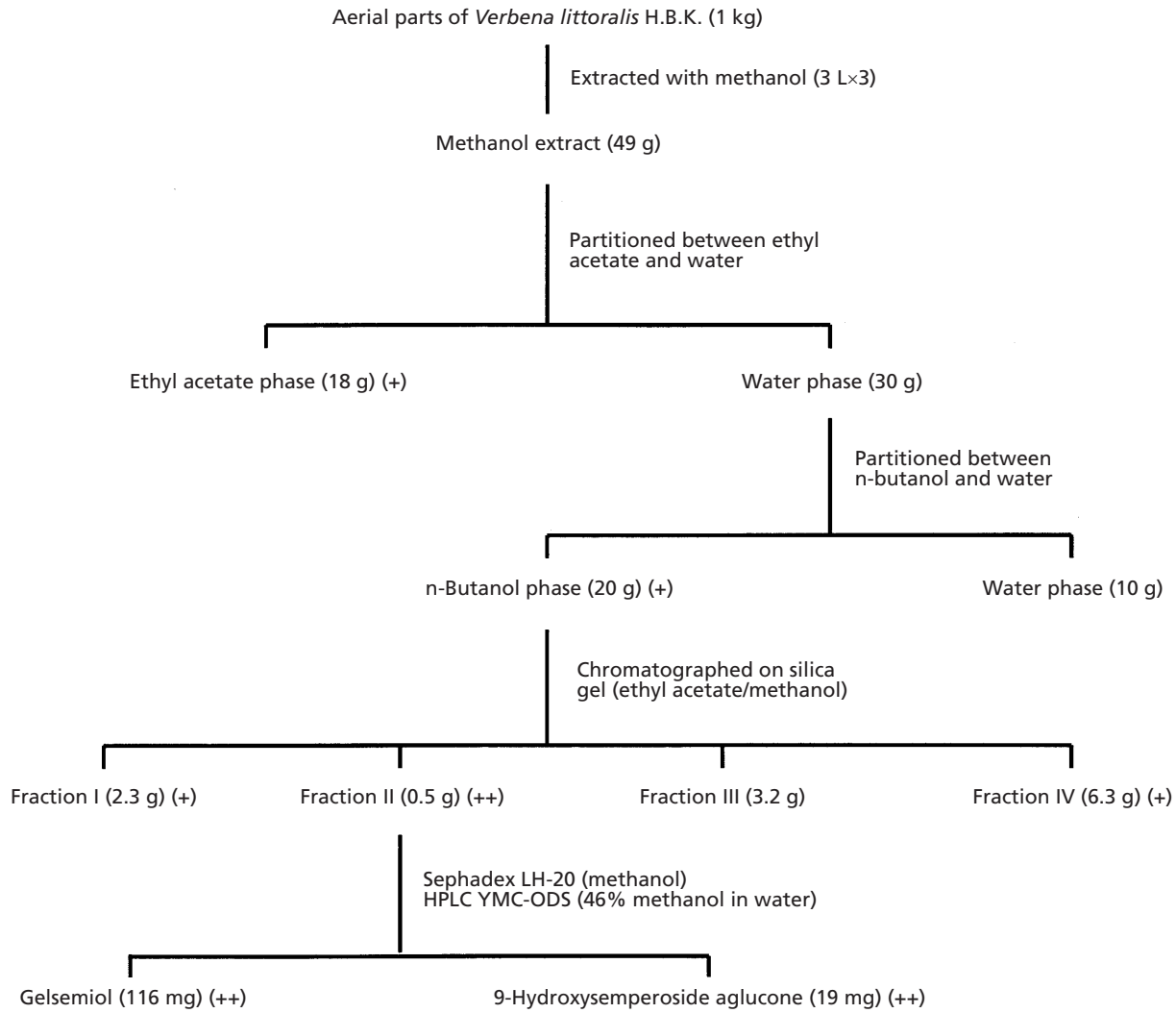

Figure 1 Isolation procedure used to obtain gelsemiol and 9-hydroxysemperoside aglucone (9-OHSA). Each fraction was added at a concentration of 30 μg mL^{-1}. +, enhancement.

of natural products, but not peptides, that possess neurotrophic activity (Obara et al 1998) or NGF-potentiating activity. These compounds are very useful for basic and clinical studies on neurodegenerative diseases (Brinton & Yamazaki 1998). It has been reported that several natural products and synthetic compounds possess NGF-potentiating activity (Middlemiss et al 1995; Ito et al 1999). Recently, we found that nardosinone from *Nardostachys chinensis* (Li et al 1999) and picrosides from Picrorhizae Rhizoma potently enhanced the population and the neurite length of neurite-bearing PC12D cells in the presence of NGF, and that scabronines stimulated the secretion of NGF from 1321N1 human astrocytoma cells (Obara et al 1999). More recently a crude methanol extract of *Verbena littoralis* H.B.K. has been shown to potentiate the NGF-action in PC12D cells. The aerial parts of *V. littoralis* have been used widely in folk medicine as an antidiarrhoeic, and for typhoid fever and tonsillitis in South America (Umana & Castro 1990). However, only a few studies concerning the constituents of this plant have been conducted (Umana & Castro 1990). In this paper, we present the first report on the successful isolation of gelsemiol and 9-hydroxysemperoside aglucone (9-OHSA) from *V. littoralis* and their biological activity as enhancers of NGF-action.

Materials and Methods

Materials

V. littoralis was obtained from Seiwa Pharmaceuticals, Ltd (Ibaragi, Japan). 7S NGF and poly-L-lysine were purchased from Sigma Chemical Co. (St Louis, MO). Dulbecco's Modified Eagle's medium (high glucose; DMEM) was from Gibco RBL (Grand Island, NY). Fetal calf serum (FCS) and horse serum were from Cell Culture Laboratory (Cleveland, OH) and ICN Biochemical, Inc. (Costa Mesa, CA), respectively. Glutaraldehyde was purchased from Wako Pure Chemical (Tokyo, Japan). All other reagents or drugs were of analytical grade.

Preparation of extracts

Figure 1 shows the separation procedure used to obtain gelsemiol and 9-OHSA. The aerial parts (1 kg) of *V. littoralis* were extracted with methanol at room temperature. The crude methanol extract (49 g) was partitioned between ethyl acetate and water and the water fraction was extracted with n-butanol. The butanol soluble materials (20 g) were chromatographed over silica gel (ethyl acetate/methanol), monitoring the potentiation of the NGF-action in PC12D cells. An active fraction II (0.52 g) was repeatedly chromatographed on Sephadex LH-20 (methanol) and reversed-phase semi-preparative HPLC on YMC-ODS (46% methanol in water) to give two active compounds. These compounds were elucidated to be gelsemiol and 9-OHSA (Figure 2) on the basis of 1D and 2D NMR data and EIMS fragmentation (Jensen et al 1987).

Cell culture

PC12D cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in DMEM supplemented with 5% FCS, 10% horse serum and 2 mM glutamine (Li et al 1999).

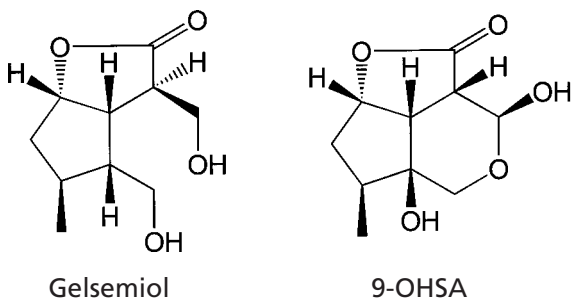

Figure 2 The chemical structures of gelsemiol and 9-hydroxysemperoside aglucone (9-OHSA).

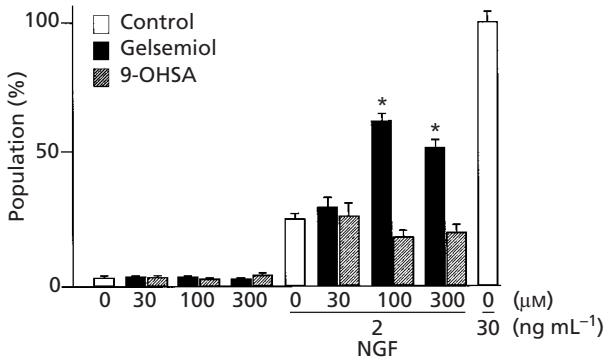

Figure 3 Effects of gelsemiol and 9-hydroxysemperoside aglucone (9-OHSA) on the proportion of neurite-bearing PC12D cells in the presence or absence of NGF. The proportion of neurite-bearing cells was expressed as a percentage against the maximum response to NGF (30 ng mL^{-1}, 100%) in the absence of gelsemiol and 9-OHSA. Values are mean ± s.e. from four experiments. $^*P < 0.01$ compared with the control (2 ng mL^{-1} NGF) in the absence of gelsemiol and 9-OHSA.

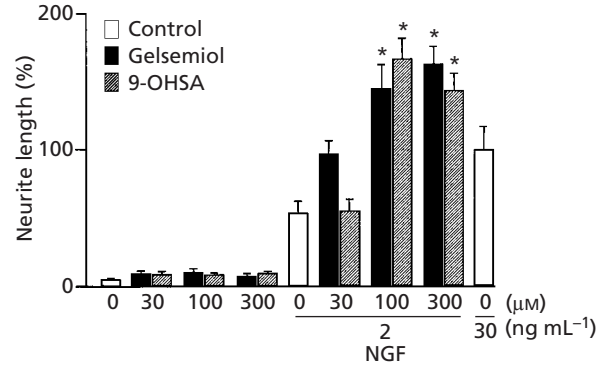

Figure 4 Effects of gelsemiol and 9-hydroxysemperoside aglucone (9-OHSA) on the neurite length of PC12D cells cultured in the presence or absence of NGF. Neurite length was expressed as a percentage against the maximum response to NGF (30 ng mL^{-1}, 100%) in the absence of gelsemiol and 9-OHSA. Values are mean ± s.e. from four experiments. $^*P < 0.01$ compared with the control (2 ng mL^{-1} NGF) in the absence of gelsemiol and 9-OHSA.

Measuring the proportion of neurite-bearing cells and neurite length

The morphology of PC12D cells was observed as reported by Li et al (1999). Cells were dissociated by incubation with 1 mM EGTA (*O*, *O*′-bis(2-aminoethyl) ethyleneglycol-*N*,*N*,*N*′,*N*′,-tetraacetic acid) in phosphate-buffered saline (PBS) for 20 min and were seeded in 24-well culture plates (2×10^4 cells/well) coated with

poly-L-lysine. After 24 h, the medium was changed to an appropriate test medium containing 1% FCS and 2% horse serum. After 48 h, each culture was fixed with 2% glutaraldehyde in PBS and stored in PBS solution. The outgrowth of neurites from PC12D cells was monitored under a phase-contrast microscope. Processes with lengths equivalent to one or more diameters of a cell body were scored as neurites. A minimum of 100 cells was examined for each data point.

Microscopy

Fixed cultures in 24-well culture plates prepared as described above were examined under an Olympus IMT-2 microscope. Phase-contrast photomicrographs of representative fields were obtained (magnification $\times 400$).

Statistical analysis

Values were mean $\pm$ s.e. A statistically significant difference was determined by the Student's t-test. $P < 0.05$ was considered significant.

Results and Discussion

The effects of gelsemiol and 9-OHSA on PC12D cells were examined in the absence or presence of NGF. NGF (30 ng mL^{-1}) increased the proportion of PC12D cells with neurites and increased neurite length (Figure 3). Gelsemiol (30–300 μM) or 9-OHSA (30–300 μM) did not induce neurite outgrowth from PC12D cells in the absence of NGF (Figure 3). Gelsemiol (100–300 μM) markedly potentiated the NGF (2 ng mL^{-1})-induced increase in the proportion of neurite-bearing cells (Figure 3) but 9-OHSA had no effect on this (Figure 3). As shown in Figure 4, gelsemiol or 9-OHSA alone did not produce elongation of the neurites of PC12D cells in the absence of NGF. However, both the compounds (100–300 μM) markedly enhanced the length of neurites in the presence of NGF (2 ng mL^{-1}) (Figure 4). In the microscopic observation, when cultured in the absence of NGF for 48 h, very few PC12D cells had neurites greater than one cell diameter (Figure 5A). Neither gelsemiol (100 μM) nor 9-OHSA (100 μM) caused a morphological change in the absence of NGF (Figure 5B

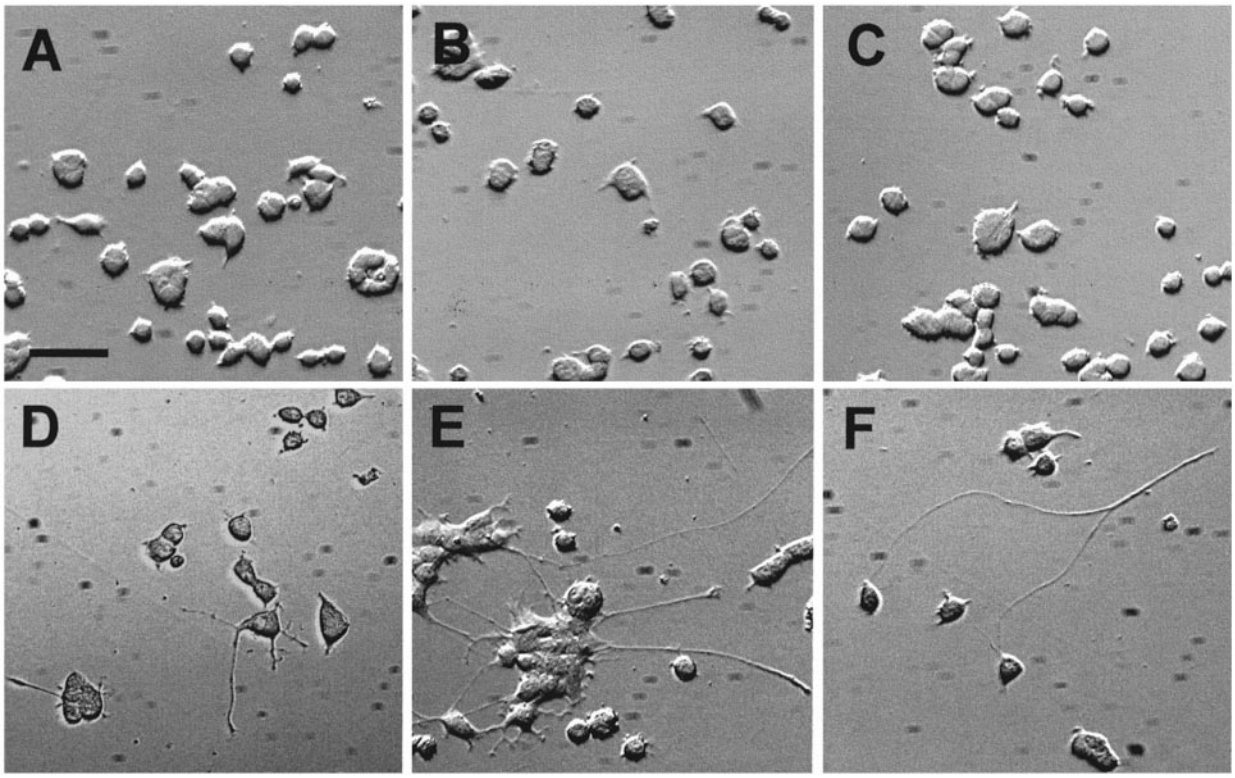

Figure 5 Effects of gelsemiol, 9-hydroxysemperoside aglucone (9-OHSA) and NGF on the morphology of PC12D cells. The cells were treated for 48 h without (A, D) or with gelsemiol (100 μM) (B, E) or 9-OHSA (100 μM) (C, F) in the absence (A, B and C) or the presence (D, E and F) of NGF (2 ng mL^{-1}). Scale bar indicates 50 μm.

and C). NGF (2 ng mL^{-1}) slightly elongated neurites from PC12D cells (Figure 5D). PC12D cells exposed to gelsemiol (100 μM) or 9-OHSA (100 μM) with NGF (2 ng mL^{-1}) formed long neurites, which extended to neighbouring cells over distances of 100 μm (Figure 5E and F).

We successfully isolated gelsemiol and 9-OHSA from *V. littoralis* as active components that enhanced the NGF-action in PC12D cells. Neurite outgrowth was not caused by either of the compounds. Gelsemiol markedly enhanced an increase in the proportion of neurite-bearing cells and an extension of the neurite length in the presence of NGF. It is interesting that in the presence of NGF, 9-OHSA markedly enhanced the elongation of the neurite length, whereas the increase in the population bearing neurites was not affected. The data may be interpreted in that gelsemiol enhanced NGF-signalling pathways resulting in an increase in the population bearing neurites and neurite elongation, but that 9-OHSA preferentially potentiated the NGF-signal transduction of neurite elongation. Gelsemiol and 9-OHSA may provide useful pharmacological tools for studying the mechanism of neurotrophic action of NGF.

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