Promotion of Neuronal Differentiation of PC12h Cells by Natural Lignans and Iridoids

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We studied the effect of (+)- and (–)-syringaresinol, (+)-syringaresinol glucosides, syringin, aucubin and catalpol on neurite outgrowth of a cultured cell line of paraneuron, PC12h cells. Of these compounds, (+)-syringaresinol diglucoside and partly glucosidase-hydrolyzed aucubin were found to be the most potent in promotion of the neurite outgrowth and stimulated responses to a high concentration of KCl and to carbachol in the cells, as observed by increase of the concentration of cytosolic free calcium. It is suggested that some of these herb-derived compounds can induce neuronal differentiation in PC12h cells.

Keywords lignan; iridoid; neurite outgrowth; calcium channel; PC12 cell

The most important feature in neuronal cell differentiation is outgrowth of neurites, and regulation of this outgrowth is considered to be one of the most basic mechanisms in development and regeneration of the nerve tissues. Search for materials inducing neurite outgrowth in pre-differentiated cells or para-neurons, therefore, is expected to lead to elucidation of the basic mechanism of neuronal differentiation and also to the development of anti-dementia drugs, because senile dementia like Alzheimer's disease is characterized by progressive neuronal degeneration in specific regions of the brain.¹⁾

Nerve growth factor (NGF)²⁾ and fibroblast growth factors (acidic and basic FGF),³⁾ all of which are demonstrated to be effective in prolonging the survival of brain neurons,⁴⁾ have been found to elicit notable induction of neurite outgrowth in a rat pheochromocytoma cell line, PC12 cell. The cell is also responsive to other various neuroactive substances such as cAMP,^{3a)} glycolipids (gangliosides),⁵⁾ cell adhesive substances (laminin, NCAM, etc.).^{3a,6)} manganese,⁷⁾ staurosporine,⁸⁾ adenosine,⁹⁾ triap,¹⁰⁾ specific peptides (pituitary adenylate cyclase-activating polypeptides, etc.),¹¹⁾ FK506¹²⁾ and lipophilic components of oriental restorative crude drugs¹³⁾ with consequence of extending neurites and differentiation.

Many crude drugs including those prepared from

Acanthopanax senticosus HARMS and *Rehmannia glutinosa* LIBOSCHITZ are also used in Kampo medicine as a tonic drug or 補腎, 補氣, and several lignans or iridoids or their glucosides have been isolated from those crude drugs as possible active agents.

We report in this communication the effects of syringaresinol and its glucosides, catalpol and aucubin on neurite outgrowth, response to carbachol and the voltage-dependent calcium channels of PC12h cells.

MATERIALS AND METHODS

Chemicals Compound I (Chart 1) (white amorphous powder, $[\alpha]_D^{25} + 31.0^\circ$ ($c=0.20$, CHCl_3)) was obtained by enzymic hydrolysis of compound III (mp 189°C , $[\alpha]_D^{25} - 13.6^\circ$ ($c=0.13$, MeOH), which had been isolated from roots of *Scutellaria baicalensis* GEORGI. Compound II (white amorphous powder, $[\alpha]_D^{23} - 16.5^\circ$ ($c=0.18$, CHCl_3)) was isolated from leaves of *Scutellaria scandens* BUCH.-HAM. ex D. DON. Compound IV (mp $268\text{--}269^\circ\text{C}$, $[\alpha]_D^{25} - 4.3^\circ$ ($c=0.21$, pyridine)) and compound V (mp $189\text{--}190^\circ\text{C}$) were isolated from *Acanthopanax senticosus* HARMS and generously offered us by Yakuhan Pharmaceutical Co., Ltd., Hokkaido, Japan. Compounds VI and VII were purchased from Wako Pure Chemicals Co., Ltd.,

Chart 1

Japan and Carl Roth KG, Germany, respectively. All of the compounds tested, except for I and II, were dissolved in distilled water to make stock solutions 500-fold as high in concentration as in test media. Compounds I and II were concentratedly dissolved in 50% dimethyl sulfoxide (DMSO) solution. DMSO was less than 0.1% in culture medium and had no influence on the tests in this work. Compounds VI and VII were partly hydrolyzed before supplementary experiments by β -glucosidase in water at pH 5 at initial concentrations of 0.05 and 0.5 mg/ml in an incubator (37 °C) for about 10 h. The reaction was terminated by addition of ethanol and the mixtures were centrifuged to separate the supernatant, which was evaporated under vacuum to obtain solutions free of ethanol. They were then diluted with distilled water up to their starting volume, 0.5 ml, and used as stock solutions. Indo-1-AM was purchased from Dojin Chemical Co., Japan and dissolved in dimethyl sulfoxide as stock solution.

PC12h Cells PC12h cells, a subclone of PC12 cell, established by Hatanaka were cultured with Dulbecco's modified minimum essential medium (DMEM) supplemented with 5% (v/v) horse serum and 5% (v/v) precolostrum newborn calf serum in 50 ml plastic culture flasks for growth under 5% CO₂/95% air (humidified) at 37 °C. In experiments determining neurite outgrowth, cells were plated in 35 mm culture dishes coated with collagen at the density of 2×10^4 in 2 ml of medium per dish. The next day the culture medium was renewed with the mixture of DMEM (high glucose) and Ham's F12 medium (1:1) supplemented with transferrin, insulin and progesterone as described,¹⁴⁾ containing test compounds as specified in the Results, to observe daily morphological change. In experiments monitoring cytoplasmic free calcium concentration, cells were plated in 35 mm culture dishes with a cover glass-attached window (10 mm in diameter) in the bottom of each at the cell density of 4×10^4 in 2 ml of medium. Following 3 or 4 d of treatment with test compounds, cells were loaded with Indo-1-AM (1 μ M) dissolved in HEPES-buffered Krebs medium by incubation for 30 min at 37 °C. After incubation in fresh medium without the dye for 15 min, cells were stimulated with carbachol or a high concentration of KCl (final amount of 0.1 and 40 mM in medium, respectively) as described in the Results. The change in fluorescence intensity (ratio of emissions at 485 and 405 nm with excitation at 355 nm) at intervals of 20 s was converted to time curve (up to 3 min)

of absolute calcium concentration in each cell on the basis of a standard curve for various calcium concentrations below 500 nM, using an argon laser-equipped fluorocytometer, ACAS 570 (Meridian Instruments Co., U.S.A.). Maximal values after the addition of stimulators ("induced" values) or vehicle ("not induced" values) were adopted as intracellular calcium concentration for comparison.

Evaluation of Neurite Outgrowth Outgrowth of the processes (neurites) from cells cultured with various concentrations of test compounds or vehicle alone (control) was evaluated by measuring their total area per cell with an image processor (XL-500, Olympus) attached to a phase-contrast microscope and computer (PC-9801RA, NEC) using application software (Image Command 4198, RATOC System Engineering Co., Ltd.). Ten to 15 selected cells in more than 5 fields of two culture dishes were subjected to analysis for each treatment or control every day.¹³⁾ Average value (less than 20 μ m²/cell) of neurites for a group in one day before treatment was subtracted from the value of each cell on culture days 2 and 3 for evaluation and expression in the Results.

Statistic Analysis Significance of the difference between mean values of control and treatment was statistically determined by Student's *t*-test and ANOVA Student's *t*-test in assays of two and more groups, respectively.

RESULTS AND DISCUSSION

Figure 1 shows profiles of the neurite outgrowth of PC12h cells cultured with 0.1–10 μ g/ml of glucosides of (+)-syringaresinol (compound III or IV) in comparison with controls (vehicle only). The processes of cells observed after treatment with the glucosides were considered as an expression of neuronal differentiation of PC12h cells, since the long processes possessed terminal growth cones and cells could be immunocytochemically stained by the method utilizing anti-neuron-specific enolase antibody¹⁵⁾ on day 3 of treatment with compounds. Interesting features of these data are the significant outgrowth of the neurite exceeding the controls in all of the doses on culture day 2 and/or 3 in both compounds, and rather prominent outgrowth in 0.1 μ g/ml of compound IV, in contrast to the lowest outgrowth in the same concentration of compound III. However, the morphological appearance of the cells cultured with compounds III and IV at

Fig. 1. Neurite Outgrowth of PC12h Cells Induced by Syringaresinol Glucosides

Cells were cultured with vehicle (control ●) or with 0.1 (Δ), 1 ($\square$) or 10 μ g/ml ($\circ$) of compound III or IV for 3 d. Average area of neurites per cell (ordinate) for each group was calculated daily using an image processor connected to a phase-contrast microscope for 10–15 selected cells in more than 5 fields of two culture dishes. S. E. of each value is shown by vertical bar. * $p < 0.01$ against control value on each culture day.

Fig. 2. Morphological Change of PC12h Cells after Treatment with Syringaresinol Glucosides

A, control; B, compound III; C, compound IV. Cells were treated with the glucosides (0.1 $\mu\text{g/ml}$) as indicated for 4 d as described in Materials and Methods and observed by a phase-contrast microscope.

TABLE I. Effect of Natural Lignans and Iridoids on Neurite Outgrowth of PC12h Cells

Compd.	Concn. $\mu\text{g/ml}$	Neurite image on culture day 3 (% control $\pm$ S.E.)
I	0.1	111 $\pm$ 8.5
	1	138 $\pm$ 32
II	0.1	176 $\pm$ 31*
	1	148 $\pm$ 15*
III	0.1	164 $\pm$ 28
	1	222 $\pm$ 45*
IV	0.1	188 $\pm$ 21*
	1	179 $\pm$ 25*
V	0.1	138 $\pm$ 20*
	1	139 $\pm$ 14*
VI	0.1	104 $\pm$ 4.5 (219 $\pm$ 58*)
	1	104 $\pm$ 5.6 (245 $\pm$ 41*)
VII	0.1	159 $\pm$ 6.8* (131 $\pm$ 7.9*)
	1	202 $\pm$ 9.6* (136 $\pm$ 5.6*)

Cells were cultured with or without (control) 0.1 or 1 $\mu\text{g/ml}$ of each compound indicated for 3 d. Neurite image calculated as described in the legend to Fig. 1 is expressed as % control $\pm$ S.E. Values shown in parentheses for compounds VI and VII represent those of their partial hydrolysate mixtures at nominal concentrations indicated of the respective compounds. Details of the method are described in Materials and Methods. * $p < 0.05$ against control. Refer to Fig. 1 for ordinary range of absolute values of neurite image of control cells on day 3 of culture.

0.1 $\mu\text{g/ml}$ for 4 d was very similar in a microscopic survey (Fig. 2). The effect of compound III on the neurite outgrowth was comparatively small in the short term at 0.1 $\mu\text{g/ml}$, but apparently durable and progressed for several days.

Table I shows the effect of lignans (compounds I and II), iridoids (compounds VI and VII) and syringin (compound V) on the PC12h neurite outgrowth in comparison with that of compounds III and IV (based on the data shown in Fig. 1) at concentration levels of 0.1 and 1 $\mu\text{g/ml}$. Compound V is comparable in structure to one half of the symmetric molecule of compound IV. Compounds II, III, IV, V and VII were all considered to be effective in the stimulation of neurite outgrowth. Compound IV was apparently more active than any other of these compounds at 0.1 $\mu\text{g/ml}$, although compounds III

and VII were more effective than compound IV at the higher concentration. Configuration of syringaresinol (compounds I and II) seems to be important to the neurite outgrowth promoting action. Nevertheless, glucosides of (+)-syringaresinol, which has been shown to be little effective even at 1 $\mu\text{g/ml}$, have significant effect on the neurite outgrowth. Sugar group(s) of the glucosides possibly modulate the structure of the compounds so that they adapt to the reactive site (probably in the plasma membrane) or fix these molecules to the membrane of the cells. Compound IV, but not (–)-syringaresinol-di- O - β -D-glucoside, was reported to have a considerably inhibitory effect on the activity of cAMP phosphodiesterase *in vitro*, its aglycone being less active.¹⁶⁾ The effect of compounds II and IV was apparently reduced at 1 $\mu\text{g/ml}$ as compared to that at 0.1 $\mu\text{g/ml}$. They were apparently partially depressive on the cells at higher concentrations (*cf.* Fig. 1), for the cells showed a partial rounding of their shape on day 3 of culture in 1 $\mu\text{g/ml}$ of both compounds.

Aucubin (compound VI) itself had little effect on neurite outgrowth, but partly hydrolyzed aucubin (hydrolysate mixture prepared by reaction with β -glucosidase at pH 5 and 37 $^{\circ}\text{C}$ for about 10 h) was very effective in the promotion of neurite outgrowth. The hydrolysate produced a progressively colored substance(s) with time of reaction and even after termination of the reaction and during storage in a refrigerator. After developing the hydrolysate of aucubin on silica gel TLC plate with the solvent mixture, CHCl_3 :MeOH: H_2O =25:8:1, on the day of the addition to test medium, several colored bands (R_f values: 0.42 (main band), 0.34, 0.03 and 0 (original spot)) other than that (very minor) corresponding to aucubin (R_f value: 0.13) were detected by spraying with concentrated sulfuric acid followed by heating of the plate. Compound VII was rather more effective on the neurite outgrowth than its partial hydrolysate which showed a colored main band on the TLC plate at R_f : 0.05 accompanied by minor bands at R_f : 0.35 and 0.31 and very minor bands at R_f : 0.13 (catalpol), 0.03 and 0 by the same method as above.

Hydrolysates of compounds VI and VII had a positive

Fig. 3. Enhancement of High KCl- and Carbachol-Induced Increases of Cytoplasmic Calcium Concentration of PC12h Cells after Treatment with Compound IV

Cells were cultured in dishes with cover glass-attached windows in the bottom with or without (control) 0.5 μ g/ml of compound IV for 4 d and subjected to determination of cytoplasmic free calcium concentration as described in Materials and Methods. Control and compound IV-treated cells were stimulated (striped columns) to induce elevation of the calcium concentration with a high concentration of KCl (A) or carbachol (B) or were not stimulated (open columns). Each value shown is average $\pm$ S.E. of 20–40 cells of two dishes.

* $p < 0.01$ against induced control.

Fig. 4. Enhancement of High KCl and Carbachol-Induced Increases of Cytoplasmic Calcium Concentration of PC12h Cells after Treatment with Compound VI Hydrolysate

Cells were cultured with or without (control) 0.5 μ g/ml of partly hydrolyzed compound VI mixture for 3 d and subjected to determination of cytoplasmic free calcium concentration as stated in the legend to Fig. 3. Control and compound VI hydrolysate-treated cells were stimulated (striped columns) to induce elevation of the calcium concentration with a high concentration of KCl (A) or carbachol (B) or were not stimulated (open columns). Each value shown in average $\pm$ S.E. of 30–50 cells of two dishes. * $p < 0.01$ against induced control.

effect on neurite outgrowth, the former seemingly producing more active substance(s). More complicated products, *i.e.* with higher potential for forming non-active secondary products, from compound VII (at least 5 bands of reaction products in TLC compared to 4 bands from compound VI as mentioned above) could cause the lower activity of the hydrolysate of compound VII.

Figure 3 shows the effect of culture with compound IV (0.5 μ g/ml of medium) on intracellular concentration of free calcium ion after stimulation of PC12h cells with 40 mM KCl or 0.1 mM carbachol.

Control cells cultured with vehicle for only 4 d responded to both a high concentration of KCl and carbachol and increased the calcium concentration within 2 min after the addition of these stimulators, as determined by cytofluorometric analysis using Indo-1. Treatment with compound IV significantly enhanced the increase of calcium concentration in response to both stimulators. KCl increased cytoplasmic free calcium concentration of the control cells by 22% compared to 72% in the treated cells. Carbachol increased the calcium concentration by 36% in the control compared to 106% in the treated cells.

Treatment with partly hydrolyzed aucubin (0.5 μ g/ml as original compound for 3 d) also significantly enhanced the increase of calcium concentration in response to KCl (Fig. 4). KCl increased cytoplasmic free calcium concentration

of the control cells by 23% compared to 63% in the treated cells. Carbachol increased the calcium concentration of the treated cells significantly above the induced control, but the increments due to carbachol stimulation in control and treated cells differed little (43% and 47%, respectively).

The above mentioned changes in morphology and the response to a high concentration of KCl and carbachol after treatment with compound IV of PC12h cells for 2–4 d indicate that this compound is capable of inducing differentiation of para-neurons with consequent activation of the voltage-dependent calcium channels and acetylcholine receptor. The monoglucoside (compound III) and related compound (compound V) possibly have the same effect, although they are different in potency. The experimental finding that optical isomer of compound I and truncated form (compound V) of compound III are even more effective on neurite outgrowth than compound I suggests that the essential structure for the activity of those compounds is in a rather narrow region of the molecules.

Predominant effectiveness of the glucosides over their aglycone, syringaresinol, in the stimulation of neurite outgrowth suggests that the hydrophilic form can be accessible to the site of action, probably on the surface of cells, and has the advantage of avoiding decomposition

by cellular metabolic activities.

Acanthopanax senticosus HARMS is the source of the crude drug "Shigoka," which is useful in Kampo medicine as a restorative and is also evaluated in other traditional medicine as an "adaptogen" capable of augmenting nonspecific resistance to pathological influences, as is "Ninjin" or the *Panax ginseng* root.¹⁷⁾ Compounds VI and VII are contained in the *Rehmanniae* root, which has restorative and antiphlogistic activities as a crude drug. Harmonious vitality of the whole body should be basically maintained by the complete functioning of the central nervous system and peripheral nerve cells. In this respect it is very interesting that the constituents of the restorative crude drugs have the same activity on the paraneuronal cells as neurotrophic factors such as nerve growth factor.

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