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Potentiation of Nerve Growth Factor-Mediated Nerve Fiber Production in Organ Cultures of Chicken Embryonic Ganglia by Ginseng Saponins: Structure–Activity Relationship

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Potentiation of the nerve growth factor (NGF)-mediated nerve fiber production in organ cultures of chicken embryonic dorsal root ganglia (DRG) and lumbar sympathetic ganglia (SymG) by saponins isolated from *Panax ginseng* C. A. MAYER and *Panax japonicum* C. A. MAYER and related compounds was studied in order to elucidate the structure–activity relationship. Panax saponins and related compounds so far tested did not promote nerve fiber production, but some 20(*S*)-protopanaxadiol glycoside having glucose units in their two sugar moieties potentiated the effect of NGF. Removal of glucose or introduction of a hydroxy group into the side chain of ginsenoside Rd reduced the activity. Little difference was observed in the potentiation of the NGF effect by the saponins in organ cultures of DRG and SymG.

Keywords——*Panax ginseng*; saponin; nerve growth factor; tissue culture

In the previous paper,[1] ginsenoside Rb_1 (GRb_1) was reported to potentiate the nerve growth factor (NGF)-mediated nerve fiber production in organ cultures of chicken embryonic dorsal root ganglia (DRG) and lumber sympathetic ganglia (SymG), but it did not promote nerve fiber production. We therefore observed the effect of ginseng saponins on the action of NGF. Marked potentiation of the NGF effect was observed with GRb_1 and GRd, 20(*S*)-protopanaxadiol glycosides having glucose units in their two sugar side chains. The present studies were performed to elucidate the structure–activity relationship between 20(*S*)-protopanaxadiol glycosides with various oligosaccharide moieties and the potentiation of the NGF-mediated nerve fiber production in organ cultures of chicken embryonic DRG and SymG. As materials for the investigation, purified ginseng saponins with established structures were used.[2-5] We also studied the NGF potentiation by prosapogenins and sapogenins of ginsenosides and related compounds.

Materials and Methods

The samples were dissolved in physiological saline, dimethyl sulfoxide (DMSO) or ethanol (EtOH), and diluted to 30 μM (final concentration) with Dulbecco's modified Eagle's medium (DMEM); the final DMSO and EtOH concentrations were less than 0.03%. NGF was isolated from adult male mouse submandibular glands as the 2.5 S

subunit according to the procedure of Bocchini and Angeletti.[6] The purity was checked by poly acrylamide gel electrophoresis. Protein concentrations were determined by the method of Lowry *et al.*[7]

Spinal DRG were dissected from 8- to 9-day-old chick embryos under a dissecting microscope, and lumber SymG, from 12-day-old chick embryos. NGF activity was determined by biological assay following the modified procedure of Fenton.[8] Three ganglia were placed on each well of a polystyrene tissue culture plate (Falcon 3072, Becton Dickinson Labware) in culture medium consisting of 25 μl of DMEM with 165 U/ml thrombin. Then 25 μl of DMEM containing NGF at various concentrations and a test substance, and finally 25 μl of rooster plasma, were added. After incubation at 37 °C in 95% air and 5% CO_2 for 24 h, the nerve fiber production from each ganglion was observed by phase contrast microscope. Intensity scores (index 0—8) were assigned to each ganglion according to the procedure of Saito *et al.*[1] Optimal nerve fiber production (dence halo of fibers, maximal fiber length) was designated as index 4 and corresponded to one biological unit (BU) of NGF.[8] The optimal concentration of NGF for index 4 was calculated from the equation of the regression line, when the correlation coefficient (r) was more than 0.9. Then the ratio of the optimal concentration of NGF to that of NGF with test substance was obtained.

Results

Effect of 2.5 S NGF

Figure 1 shows a gradually increase of nerve fiber production induced by 2.5 S NGF at various concentrations from 0.1 ng/ml to 26.0 ng/ml after 24 h in organ cultures of chick embryonic DRG. The optimal response (index 4) was obtained with 2.4 ng/ml (0.8—3.2 ng/ml) of NGF. The dose–response relationship for NGF was shown in Fig. 2.

Effects of DMSO and EtOH Used as Solvents of Test Substances

Figure 2 shows the effects of DMSO at various concentrations on the NGF action in organ cultures of chicken embryonic DRG. DMSO at a concentration of less than 0.03% had no influence on the dose–response relationship of NGF. DMSO at a concentration of 0.3% moved the dose–response plot in parallel to the right, and the ratio was 0.7. NGF with DMSO at a concentration of 3% did not promote nerve fiber production. EtOH at a concentration of less than 0.03% also had no influence on the dose–response relationship of NGF. EtOH at concentrations of 0.3 and 3% moved the plot in parallel to the right, and the ratios were 0.8 and 0.7, respectively. No difference in the effects of DMSO and EtOH was observed between organ cultures of DRG and SymG. Thus, DMSO and EtOH were used as solvents at final concentrations of less than 0.03% in the culture medium.

Effects of Saponins and Related Compounds

Saponins and related compounds tested did not promote nerve fiber production at concentrations between 3 and 100 μM in organ cultures of chicken embryonic DRG. The ratios of the optimal concentration of NGF to that of NGF with saponins in organ cultures of chicken embryonic DRG, and the structures of the ginseng saponins, are shown in Table I. GRb_1, GRd, GF_2 and GRa_1 potentiated the effect of NGF. These saponins are 20(S)-protophanaxadiol glycosides having glucose units in their two sugar side chains. Thus, the effect of the numbers of glucose units attached to 20(S)-protopanaxadiol in the above saponins on the potentiation of NGF action in the organ cultures of both DRG and SymG was studied using the prosapogenins and sapogenins. The results are shown in Table II. Marked potentiation of the NGF effect was observed with GRb_1 and GRd. Removal of glucose from the sugar moieties and hydroxylation at the side chain of GRd at position 17 reduced the activity. No marked difference was observed between the potentiations of the NGF effect in DRG and SymG.

Discussion

In view of the reported potentiation of the effect of NGF in organ cultures of chicken embryonic DGR by GRb_1,[1] the promoting activities of saponins isolated from *Panax ginseng*

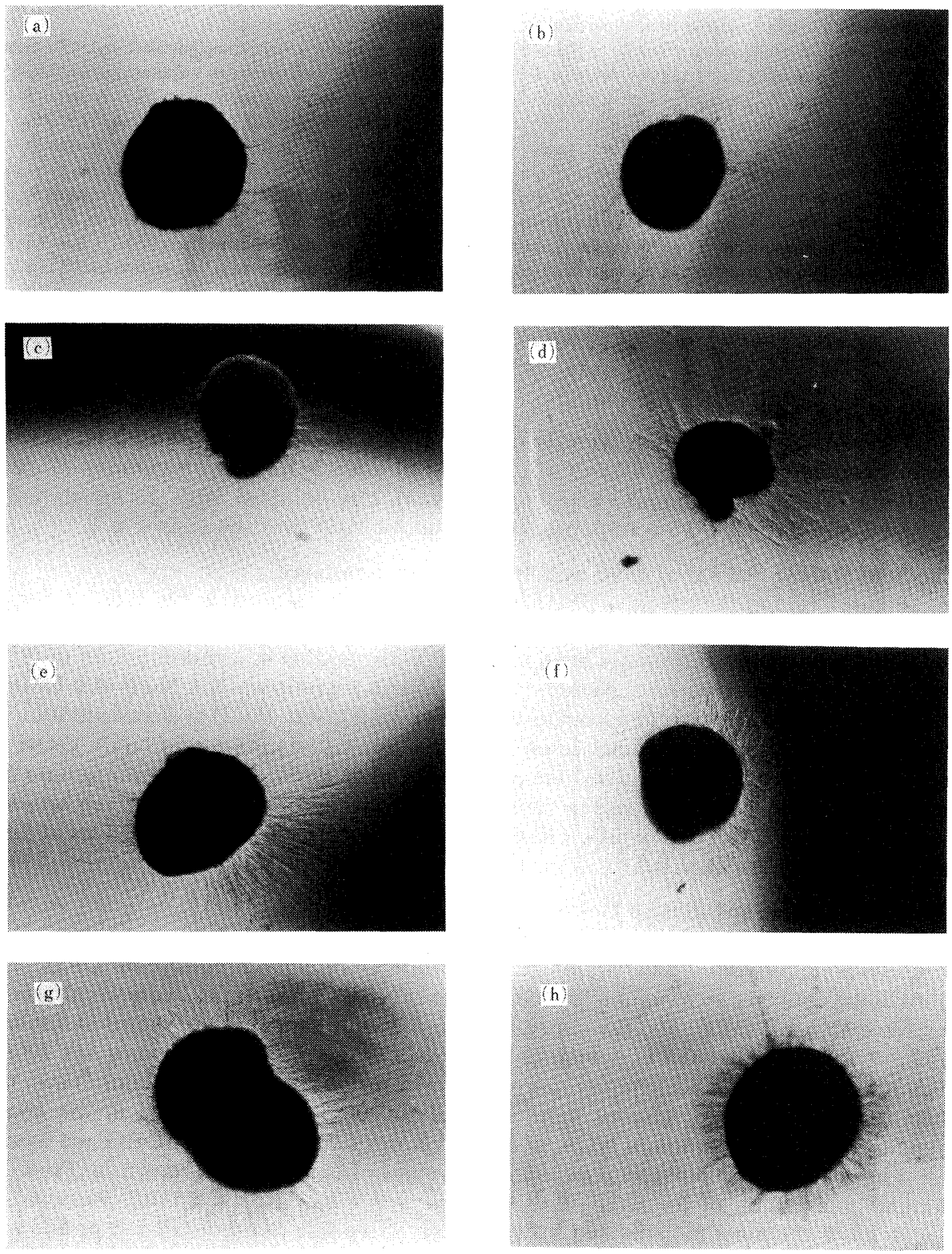


Fig. 1. Effect of NGF on Nerve Fiber Production in Organ Cultures of Chicken Embryonic Dorsal Root Ganglia

(a), negative response (score 0 or 8), no NGF in culture medium; (b), score 1, NGF 0.2 ng/ml; (c), score 2, NGF 0.4 ng/ml; (d), score 3, NGF 0.8 ng/ml; (e), score, 4, NGF 1.6 ng/ml; (f), score 5, NGF 3.3 ng/ml; (g), score 6, NGF 6.5 ng/ml; (h), score 7, NGF 13 ng/ml.

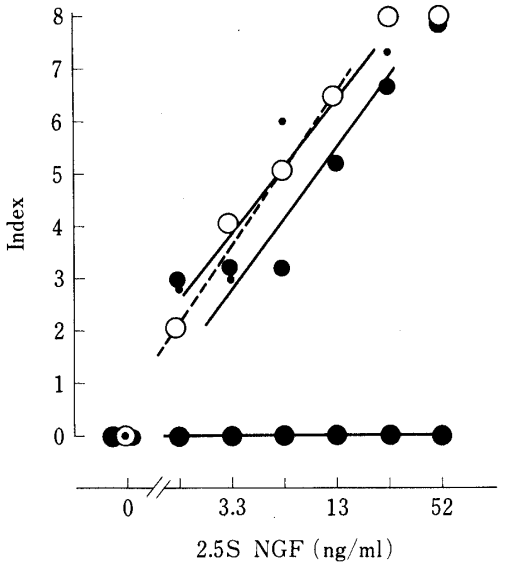
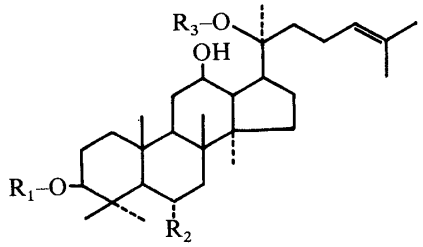


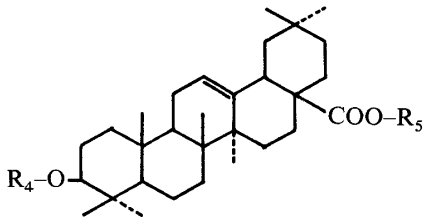
Fig. 2. Effect of DMSO on the NGF-Mediated Nerve Fiber Production in Organ Cultures of Chicken Embryonic Dorsal Root Ganglia

---○---, control; —•—, 0.03% DMSO; —●—, 0.3% DMSO; —⬤—, 3% DMSO.

TABLE I. Potentiation by Ginseng Saponins of the NGF-Mediated Nerve Fiber Production in Organ Cultures of Chicken Embryonic Dorsal Root Ganglia



20(*S*)-Protopanaxadiol glycosides

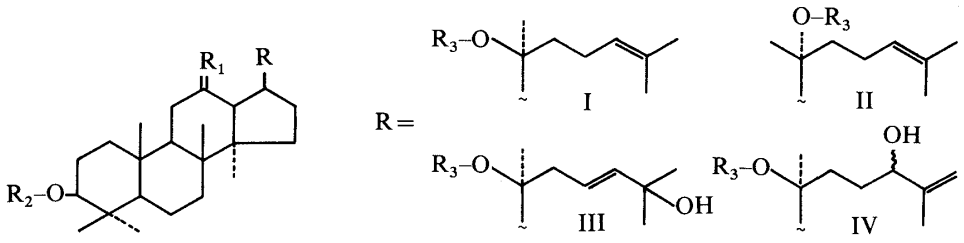


Oleanolic acid glycosides

Saponins tested (30 μM)	R_1	R_2	R_3	Ratio
20(*S*)-Protopanaxadiol glycosides				
Ginsenoside-Ra_1	glc-^{2}glc	H	xyl-^{4}ara(p)-^{6}glc	3.8
Ginsenoside-Rb_1	glc-^{2}glc	H	glc-^{6}glc	6.2
Ginsenoside-Rb_2	glc-^{2}glc	H	ara(p)-^{6}glc	1.8
Ginsenoside-Rb_3	glc-^{2}glc	H	xyl-^{6}glc	1.0
Ginsenoside-Rc	glc-^{2}glc	H	ara(f)-^{6}glc	1.3
Ginsenoside-Rd	glc-^{2}glc	H	glc	5.8
Ginsenoside-F_2	glc	H	glc	4.3
20(*S*)-Ginsenoside-Rg_3	glc-^{2}glc	H	H	1.3
20(*R*)-Ginsenoside-Rg_3	glc-^{2}glc	H	H	1.5
Chikusetsusaponin III	xyl$\diagdown^{4}$glc, glc$\diagup^{2}$	H	H	2.2
20(*S*)-Protopanaxatriol glycosides				
Ginsenoside-Re	H	rha-^{2}glc-O	glc	1.2
Ginsenoside-Rf	H	glc-^{2}glc-O	H	1.5
Ginsenoside-Rg_1	H	glc-O	glc	2.5
20(*S*)-Ginsenoside-Rg_2	H	rha-^{2}glc-O	H	1.5
20(*R*)-Ginsenoside-Rg_2	H	rha-^{2}glc-O	H	1.5
20(*S*)-Ginsenoside-Rh_1	H	glc-O	H	2.6
20(*R*)-Ginsenoside-Rh_1	H	glc-O	H	1.4

	R_4	R_5	Ratio
Oleanolic acid glycosides			
Ginsenoside-Ro	glc-^{2}glcUA	glc	1.9
Chikusetsusaponin IV	ara(f)-^{3}glcUA	glc	1.8

TABLE II. Potentiation by 20(*S*)-Protopanaxadiol Glycosides with Glucose Units in Their Oligosaccharide Moieties of the NGF-Mediated Nerve Fiber Production in Organ Cultures of Chicken Embryonic Dorsal Root Ganglia and Sympathetic Ganglia



Saponins and related compounds tested (30 μM)	R	R_1	R_2	R_3	Ratio DRG	Ratio SymG
20(*S*)-Protopanaxadiol	I		H	H	1.1	0.9
Compound K	I		H	glc	2.2	1.6
12-Keto-compound K	I	=O	H	glc	1.8	
Damarenediol-I-3-*O*-β-glucoside	II		glc	H	2.4	2.3
20(*S*)-Ginsenoside-Rg_3	I		glc-^{2}glc	H	1.3	
20(*R*)-Ginsenoside-Rg_3	II		glc-^{2}glc	H	1.5	
Ginsenoside-F_2	I		glc	glc	4.3	3.2
Ginsenoside-Rd	I		glc-^{2}glc	glc	5.8	6.3
Ginsenoside-Rb_1	I		glc-^{2}glc	glc-^{6}glc	6.2	6.4
Ginsenoside-$M_{6\text{-}a}$	III		glc-^{2}glc	glc	4.6	4.3
Ginsenoside-$M_{6\text{-}bc}$	IV		glc-^{2}glc	glc	1.8	2.2

and *Panax japonicum* on the NGF action were studied by the same procedure. As shown in Table I, ginseng saponins can be classified into three types: 20(*S*)-protopanaxadiol, 20(*S*)-protopanaxatriol and oleanolic acid types. Marked potentiation of the NGF effect was observed only with GRb_1, GRd and GF_2, which are 20(*S*)-protopanaxadiol glycosides having glucose units in their two sugar side chains attached to $C_{(3)}$ and $C_{(20)}$. Compounds of the 20(*S*)-protopanaxatriol and oleanolic acid types so far tested showed no influence on the NGF effect. GRb_2, GRb_3 and GRc also showed no influence, though they are 20(*S*)-protopanaxadiol glycosides having a sugar moiety consisting of two glucoses at their $C_{(3)}$ position. GRb_1 possesses two glucose units attached at $C_{(20)}$, whereas GRb_2, GRb_3 and GRc contain different sugar units in the sugar side chain. GF_2 which possesses only one glucose each at $C_{(3)}$ and $C_{(20)}$, was less effective than GRb_1. The effect of the numbers of glucose molecules attached to 20(*S*)-protopanaxadiol on the potentiation of NGF action in the organ cultures of both DRG and SymG is shown in Table II. 20(*S*)- and 20(*R*)-GRg_3, 20(*S*)- and 20(*R*)-prosapogenins of GRb and GRc, compound K, a prosapogenin derived from ginseng saponin, and damarenediol-I-3-*O*-β-glucoside, which all possess one or two glucose units in their sugar moieties, showed slight effects, whereas 20(*S*)-protopanaxadiol had no effect. Among the 20(*S*)-protopanaxatriol glycosides tested, only GRg_1, which possesses one glu-

cose attached to each of $C_{(6)}$ and $C_{(20)}$, showed a slight effect in organ cultures of DRG and SymG (ratios; 2.5 and 2.7 respectively).

Though DMEM contains 5.6 mM glucose, which is equivalent to a final concentration of 1.9 mM in the culture medium tested, we observed the effect of 30 μM 20(*S*)-protopanaxadiol with 30, 60, 90 or 120 μM glucose on the action of NGF, and we found no positive effect. Glucose at a concentration of less than 0.1 M in the culture medium neither promoted the nerve fiber production nor influenced the effect of NGF. 20(*S*)-Protopanaxatriol and oleanolic acid were also tested with the same manner and gave the same result as 20(*S*)-protopanaxadiol.

These results indicate that glucose rather than other sugars, and the location and number of glucose units, have a considerable effect on the NGF-mediated nerve fiber production. Exceptionally, GRa_1 with –Glc–Ara(f)–Xyl at the $C_{(20)}$ position and two glucose units at the $C_{(3)}$ position also showed potentiation of the NGF effect in organ cultures of chicken embryonic DRG.

GRd, GM6-a and GM6-bc possess the same sugar moieties, but a hydroxyl group is present at $C_{(25)}$ in GM6-a, and at $C_{(24)}$ in GM6-bc. The observed ratios were 5.8, 4.8 and 1.8 in DRG and 6.3, 4.3 and 2.2 in SymG, respectively. These results indicate that hydroxylation at the side chain attached to $C_{(17)}$ of sapongenin also influences the potentiation of the NGF effect.

NGF, GRb_1 and NGF in combination with GRb_1 did not alter the level of cAMP in chicken embryonic DRG and SymG in culture. Though NGF increased the incorporation of both ^{3}H-leucine and ^{3}H-uridine into both DRG and SymG in culture, GRb_1 neither altered the incorporation nor potentiated the action of NGF. Colchicine and vinblastine antagonized the NGF effect on nerve fiber production in organ cultures of both ganglia. GRb_1 protected the NGF effect from colchicine inhibition, but potentiated the inhibitory effect of vinblastine on the NGF action.[9] Therefore, it is possible that GRb_1 alters the membrane properties, influencing the uptake of colchicine and vinblastine into the ganglionic neurons, or it may have an intraneuronal effect on tubulin assembly. Although the precise mechanism is still unknown, the results of the present experiments indicate that 20(*S*)-protopanaxadiol glycosides having two glucosyl moieties are effective in promoting nerve fiber production induced by NGF.

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