Diosgenin from Dioscorea nipponica Ameliorates Diabetic Neuropathy by Inducing Nerve Growth Factor

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Diabetic neuropathy is characterized by axonal degeneration, demyelination, and atrophy in association with failed axonal regeneration, remyelination, and synaptogenesis. Recent reports suggest that reduced levels of nerve growth factor (NGF) may play a significant role in the pathogenesis of diabetic polyneuropathy. In this study, we investigated the regulation of NGF by steroid diosgenin (DG) in a diabetic neuropathy rodent model. We found that DG, the primary spirostane-type steroid in several Dioscorea species, increased NGF levels in the sciatic nerve of diabetic rats. Additionally, DG increased neurite outgrowth in PC12 cells and enhanced nerve conduction velocities in the diabetic neuropathy mouse model. DG-treated diabetic mice showed reduced disorganization of the myelin sheath and increased area of myelinated axons by electron microscope studies and exhibited improvement in the damaged axons. Our data further suggest that DG increased the nerve conduction velocity through induction of NGF. Thus, our findings indicate that DG, a major sapogenin obtained from Dioscorea nipponica, reverses functional and ultrastructural changes and induces neural regeneration in a diabetic neuropathy model.

Key words diabetic neuropathy; diosgenin; nerve conductivity; nerve growth factor; neurite outgrowth

The increasing prevalence of diabetes heightens concerns over diabetic complications such as neuropathy. Diabetic neuropathy is one of the most debilitating complications of type 1 and type 2 diabetes.1) The histopathology of the condition is characterized by axonal degeneration, demyelination, and atrophy in association with failed axonal regeneration, remyelination, and impaired synaptogenesis.2) Previous reports have suggested that reduced availability of nerve growth factor (NGF) plays a significant role in the pathogenesis of diabetic polyneuropathy.3) Retrograde axonal transport of NGF is impaired in animals with diabetes mellitus (DM),4) and its transcription in neuronal target tissues is reduced.5) NGF itself has been considered an option for the treatment of diabetic peripheral neuropathy. Although systemic administration of exogenous recombinant human NGF was found to prevent neuropathy in a phase II clinical trial,6) unfortunately efficacy of NGF was not demonstrated in a completed phase III trial due to its limited delivery to the nervous system and adverse effects after subcutaneous injection.7) An alternative therapeutic approach is the use of small molecules to induce or enhance neurotropic factor production. Saito and colleagues reported that the orally active small molecule MCC-257, a sialic acid derivative, elevated NGF levels in the sciatic nerve and improved nerve conduction velocity in a diabetic rodent model.8) Also, retinoic acid induced or enhanced NGF levels in a diabetic rodent model.9) Hence, increased NGF concentrations in nerve terminals.

Our study focused on diosgenin (DG), a major steroid sapogenin isolated from Dioscoreaceae. DG is the primary spirostane-type sapogenin found in several plants, including fenugreek, Costus speciosus, and the Dioscorea species.10) DG has been shown to be useful in maintaining healthy blood cholesterol levels,11) and is a precursor for several hormones such as dehydroepiandrosterone.12)

The present study examined the neuroprotective effect of DG on diabetic peripheral neuropathy through increasing NGF levels in a diabetic rodent model.

MATERIALS AND METHODS

Isolation of Diosgenin from Dioscorea nipponica Rhi-zomes of Dioscorea nipponica (DN) MARINO (Dioscore-aceae), referred to as Cheonsangyong in China and as Buchema in Korea, were obtained from the Kyungdong Herb Market in Seoul, Korea. They were identified by Dr. C. S. Yook, Department of Pharmacy, Kyung Hee University. Dried DN rhizomes (1 kg) were extracted 3 times using 85% methanol under ultrasonic apparatus for each 2 h and evaporated to dryness. Such an extraction process was repeated twice. The residue (147 g) was then partitioned between water-saturated n-butanol and water, and the n-butanol layer was separated, which was repeated 5 times, and evaporated to dryness. The yield was 3.4%. The residue (147 g) was then partitioned between water-saturated n-butanol and water, and the n-butanol layer was separated, which was repeated 5 times, and evaporated to dryness. The yield was 3.4%. The residue was subsequently taken up into 2.5 N HCl for 4 h at 94 °C, extracted using CHCl3, filtered, and evaporated to dryness. Finally, the residue was crystallized using 95% ethanol at 4 °C, and compound 1 was isolated by recrystallization using acetone at 4 °C.13) Based on the 1H-NMR, 13C-NMR, and FAB-mass spectral data, the structure of compound 1, a white amorphous powder, was identified using reference data as 3β,25R-spirostan-5-en-3-ol.14)

HPLC Analysis of Diosgenin A quantitative analysis of the DG in DN was performed using an analytical HPLC unit (HP 1100 system, Agilent Technologies, Santa Clara, CA, 2011 Pharmaceutical Society of Japan
U.S.A.) and a reverse-phase C18 column Prontosil (Bischoff Chromatography, Leonberg, Germany) HPLC column (150×4 mm, Eurobond C18, 5 μm). The solvent system composed of water and acetonitrile was used in the present study and prepared. The linear gradient was 0—10 min, 70% and 10—40 min, 70—100% acetonitrile (v/v), with a constant flow rate of 1.0 ml/min at 40 °C. For detection, a UV–Vis detector was used at 208 nm.

**Cell Culture** The level of secreted NGF was determined using an enzyme-linked immunosorbent assay (ELISA) development kit (R&D System, Minneapolis, MN, U.S.A.) in DG-conditioned media from C6 glioma cells and primary astrocytes from the cortex of 1-d-old neonatal Sprague-Dawley rats. Cells (2×10^5 cells/dish) were seeded onto 100-mm dishes. After 24 h, the cells were treated with 0.1, 1, 2.5, 5 and 10 μg/ml DG for 1 d. The supernatant of the harvested media was used for our assay. Cell viability was assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.

Rat PC12 pheochromocytoma cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) and grown in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum (FBS), 10% heat-inactivated horse serum, and 1% penicillin/streptomycin (PS) at 37 °C in a humidified incubator with 5% CO2 and 95% air. The cells were grown on culture dishes that were precoated with poly-D-lysine. The cells (2×10^4 cells/well) were seeded onto 6-well culture dishes that were precoated with poly-D-lysine (50 μg/ml) in sterile water overnight.

C6 glioma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) PS and maintained in a humidified incubator at 37 °C and 5% CO2. C6 cells (3×10^5 cells/well) were seeded onto 100-mm dishes. After 24 h, the C6 glioma cells were treated with DG (10 μg/ml). The medium was collected after a further 24 h of incubation, and stored at −20 °C until treatment of PC12 cells.

**Neurite Outgrowth Assay** The neurotrophic effects of DG and NGF were evaluated by microscopically monitoring their potency in inducing neurite outgrowth of PC12 cells. PC12 cells (3×10^5 cells/well) were seeded onto 6-well plates, and after 24 h the cells were treated with DG-conditioned media from the C6 glioma cells or with NGF (50 ng/ml). The DG-conditioned medium and NGF-containing medium was changed every other day. After 4 d, randomly selected fields of neurites were photographed using a camera attached to an Olympus optical inverted phase-contrast microscope (model CK-2; X100 magnification) and Optimas 6.5 program (Media cybernetics, U.S.A.). Neurite extension was evaluated with respect to a length equivalent to one diameter of the cell body by three independent observers. More than 10 cells were examined for the measurement of neurite length at each data point. The differentiation of PC12 cells was scored as follows: cells without neurite outgrowth, 0; cells bearing neurite as long as one cell diameter, 1; cells bearing neurite two times longer than their diameter, 2; and cells that had a synapse-like neurite, 4.

**Animals and Diabetes Induction** All experimental procedures were performed according to the Principles of Laboratory Animal Care (NIH Publication, # 85-23, revised in 1985) and the Animal Care and Use Guidelines of Nambu University, Korea. Seven-week-old male ICR mice and SD rats (Jung-Ang Lab Animal, Seoul, Korea) were housed individually, with a 12/12-h light/dark cycle and food and water were available ad libitum.

Diabetes mellitus (DM) was induced by an intraperitoneal (i.p.) injection of alloxan (Sigma, St. Louis, MO, U.S.A.) in 0.89% sterile saline into mice (150 mg/kg) or rats (120 mg/kg) that had been food deprived for 18 h. A strip-operated blood glucose sensor (OneTouch Ultra Inverness Medical Ltd., MA, U.S.A.) was used to measure the fasting blood glucose in samples obtained from a tail prick. Blood glucose levels were estimated 3 d later to confirm that the animals were exhibiting DM. Mice or rats with blood glucose levels ≥300 mg/dl at 1 week after the alloxan injection were used in the study.

**Sciatic Nerve Conduction Velocity (NCV) Measurements** For NCV measurements, the diabetic mice were divided into three groups: NGF, DM, and DG groups. Animals in the DM group were treated orally once a day with 0.5 ml vehicle (n=14); the DG group were treated orally once a day with 10 mg/kg DG (n=14), and the NGF group received a subcutaneous injection of 1 mg/kg NGF (R&D Systems, Minneapolis, MN, U.S.A.) for 2 months after the induction of diabetes (n=7). The non-diabetic mice (normal) received the same volume/weight of the vehicle (ethanol: dimethyl sulfoxide (DMSO) (1:3)) (n=14). Preparations of DG were dissolved in ethanol: DMSO (1:3) daily immediately prior to treatment.

NCV tests were performed at 1 or 2 months after the induction of diabetes. At 1 and 2 months, mice in the normal, DM, and DG groups (n=7/group at each time point) were anesthetized with isofluorane, and their sciatic nerves were isolated. At 2 months, mice in the NGF group (n=7/group) were anesthetized with isofluorane, and their sciatic nerves were isolated. Each isolated nerve was placed immediately in saline in a petri dish and connected to a stimulator and sensor probes with aeration. The distance between the sensor and stimulator probes was 15.0 mm. A digital storage oscilloscope (Tektronix 2211, Madell Function Generator, CA, U.S.A.) was used to record the conduction times (3–5 V stimulation and 5-ms duration). The NCV was calculated based on the distance between the electrodes and the time.

**NGF Assay and DG Quantification in Sciatic Nerves** For DG level or NGF ratio profiling, the sciatic nerves of rats in the DG10 with DM group (DG 10 mg/kg; n=30) and DG10 without DM group (DG 10 mg/kg; n=30) were isolated at 1, 6, 12, 24, and 48 h after DG treatment (n=6/group at each time point), and DG or NGF concentrations were measured using HPLC analysis as described above or a NGF ELISA kit purchased from R&D Systems. Briefly, tissue samples were homogenized in NGF lysis buffer (Tris–HCl 100 mm, bovine serum albumin (BSA) 2%, NaCl 1 m, EDTA–2Na2H2O 4 mm, Triton X-100 2%, sodium azide 0.1%, pH 7.0, and phenylmethylsulfonylfluoride 17 μg/ml), centrifuged at 4 °C and 10000×g, and the supernatant was subjected to ELISA in 96-well plates according to the manufacturer’s instructions. The amount of NGF/β in the samples was calculated using a standard curve. The NGF concentration ratios were determined by dividing the NGF levels of DM or DG groups by the mean NGF level of the normal group.

**Sciatic Nerve Morphology** Sciatic nerve morphology was examined using electron microscopy 2 months after the...
induction of diabetes. Mice in the normal, DM, DG, and NGF groups were anesthetized with isofluorane, and their sciatic nerves were isolated. Each isolated nerve was immersed in a fixative (2.5% glutaraldehyde in phosphate buffered saline (PBS)) and incubated at 4 °C for at least 12 h. Each specimen was then immersed in 2% osmium tetroxide in 0.1M phosphate buffer for 2 h at 4 °C, dehydrated through a graded series of ethanol solutions up to 100% ethanol, and embedded in Epon 812. Following polymerization, ultrathin sections (50—60 nm thick) were cut and stained with uranyl acetate and lead citrate. The nerve morphology was examined using transmission electron microscopy (TEM; H7100, Hitachi, Ibaraki, Japan).

The data were analyzed using SigmaPlot software (Systat Software, Inc., San Jose, CA, U.S.A.). All data were expressed as the mean±standard error (S.E.M.). One-way analyses of variance (ANOVA) were used to compare differences between treatments and followed up using Dunnett’s multiple comparison post-hoc test. Values of $p\leq0.05$ were considered to be statistically significant.

RESULTS

A quantitative analysis of the DG in DN was performed using an analytical HPLC (data not shown). The DG content was increased from 0.38 to 16.7 mg/ml by acid hydrolysis of the DN BuOH fraction extract. The increase in DG content may be the result of the hydrolysis of the sugar moiety of
diosgenyl glycoside to aglycone, DG.

The data of effects of diosgenin on NGF secretion in C6- and primary astrocyte-conditioned media was examined (Fig. 1). NGF level in the various concentration of DG (0.1, 1, 2.5, 5, 10 μg/ml) did not increase in C6 glioma cells. The effect of DG on neurite outgrowth in undifferentiated PC12 cells was examined. The PC12 cells treated with DG-conditioned media showed neurite outgrowth. NGF (50 ng/ml), which was used as the positive control, also triggered the differentiation of PC12 cells (Fig. 2). The effect of DG (10 μg/ml) was 70% that of NGF (50 ng/ml). Both DG and NGF showed a significant neuritogenic effect compared with the control group.

Conductivity of the sciatic nerve was measured to determine whether DG was effective in preventing diabetic neuropathy in mice. One month after the induction of diabetes, the NCV in the DM group was not reduced compared with that of the normal group, whereas at 2 months after the induction of diabetes, the NCV of the DM group was significantly decreased by 37% compared with that of the normal animals (p<0.001). Treatment with DG for 2 months significantly increased NCV by 33% compared with that of the DM group (p<0.001), and the NCVs in the DG group were similar to those in the NGF or normal groups. These data indicate that DG improved NCV following damage in diabetic mice with peripheral neuropathy (Fig. 3).

To investigate the time dependent correlation between DG and NGF, we examined the 48-h profile of DG or NGF concentrations after DG treatment in sciatic nerves of rats. The DG concentrations of normal or DM groups increased 12 h after DG treatment; however, the NGF concentration of these groups increased 24 h after DG treatment. The NGF concentration ratios of normal groups were increased by 333% at 24 h after DG treatment compared with those of the no-treatment rats. The NGF concentration ratios of DM groups were decreased by 39% at 1 h after DG treatment, but at 24 h after DG treatment, the NGF concentration ratios of DM groups were increased by 140% compared with those of the no-treatment rats (Fig. 4). These data indicated that oral treatment with DG might induce NGF secretion.

The ultrastructural changes of sciatic nerves were evaluated by electron microscopy. We found that the sciatic nerve of the diabetic mice exhibited loss of the myelin sheath and axon and an increased endoneural space resulting from edema after 2 months of diabetic induction. However, the myelin sheath and axon showed recovery in the DG- or NGF-treated groups (Fig. 5). These data support the proposal that DG induces nerve regeneration or prevents damage to the myelin or axons in diabetic neuropathy animals.

Fig. 3. Sciatic Nerve Conductivity in Diabetic Mice Treated with Vehicle (DM), 10 mg/kg DG (DG), or 1 mg/kg NGF (NGF), and in Non-diabetic Mice (Nor)

Data are presented as the mean±S.E.M. for each group (n=7/group at 1 or 2 month). ***p<0.001 compared with the DM group.

Fig. 4. DG Level and NGF Ratio Profiles in the Sciatic Nerves of Normal or Diabetic Rats over 48 h Following Treatment with 10 mg/kg DG (DG10)

For DG profiles, data are presented as the mean±S.E.M. for each group (n=6/group at each time points). For NGF profiles, data are presented as the NGF concentration ratio mean±S.E.M. for each group (n=6/group at each time points). The NGF concentration ratios were calculated by dividing the NGF levels of the DG treatment group by the NGF level means of the no-treatment group.
DISCUSSION

In a previous study, we showed that the steroidal saponin spicatoside A, isolated from Liriope platyphylla, had neurotrophic effects and also stimulated NGF induction and activated the tyrosine kinase signaling pathway. Spicatoside A is 25S-ruscogenin 1-O-\{\beta-D-glucopyranosyl-(1→2)\}-[\beta-D-xylopyranosyl-(1→3)]-\beta-D-fucopyranoside}. Because DG has a similar chemical structure to ruscogenin, the active chemical moiety of spicatoside A, we hypothesized that DG might also have neurotrophic activity.

In the present study, we found that DG-conditioned media affected the induction of PC12 neurite outgrowth. However, NGF level in the concentration of 0.1—10 μg/ml of DG did not increase in C6 glioma cells. We presume that this perhaps is the difference of NGF secretion mechanism between animal and C6 glioma cells. The neurotogenic effect of DG may be due to NGF mimetic property in PC12 cells. Although NGF plays an important role in the induction of PC12 neurite outgrowth, it is known that other NFs such as neurotrophic factor-3 (NT-3) and interleukin-6 (IL-6) also trigger the differentiation of PC12 cells. Therefore, it is possible that DG might stimulate the secretion and/or synthesis of other NFs as well as NGF.

Decreased conduction velocities are correlated with decreased myelinated fiber density and changes in axonal caliber. Nerve conduction velocity in diabetic rodents has been the preclinical ‘gold standard’ for evaluating the therapeutic potential of drugs for diabetic neuropathy. In the present study, the NCV was not significantly decreased in diabetic mice at 1 month after diabetes induction, but was significantly decreased at 2 months after induction, indicating that the onset of diabetic neuropathy required more than 1 month under hyperglycemia. Another study reported that the NCV was significantly reduced in diabetic rats at 6 weeks after diabetes induction. Morphological examination of myelinated fibers by electron microscopy indicated the presence of neural damage such as axon demyelination and loss in the sciatic nerve of diabetic mice. We showed that DG prevented or suppressed the demyelination and axon loss in the sciatic nerve. Thus, the decrease in NCV associated with diabetic neuropathy may be reversed by treatment with DG.

Neurotrophins such as NGF are critical for the development and physiology of the peripheral nervous system. A previous study showed that the concentration of NGF in the sciatic nerves of streptozotocin (STZ)-induced diabetic rats decreased 3 weeks after STZ injection. Furthermore, replacement therapy with exogenous NGF in diabetic rats normalizes key molecular and functional aspects of the neuropathy. However, subcutaneous administration of NGF failed to improve diabetic neuropathy in patients. The reasons for this failure were its limited delivery to the nervous system and severe pain after subcutaneous injection, which made it necessary to find an orally effective small molecule to induce NGF secretion.

We investigated the time-dependent NGF profile after DG treatment. DG was distributed in sciatic nerves of normal and diabetic mice when a single dose of DG 10 mg/kg was given orally. DG concentration in the sciatic nerves of normal or diabetic rats was maximized at 12 h after DG treatment. In comparison, NGF levels reached a maximum at 24 h. We therefore think that it may take 12 h to induce a maximum level of NGF in sciatic nerves after DG distribution. In our study, DG concentrations showed the different in
normal rat and DM rat and standard error at 24 h treatment of DG in normal rat was too big. We presume that the difference of DG concentration between normal and diabetic rats may be due to pharmacokinetic alteration of DG under diabetes mellitus status. And, presumably, because individual variations of some rats may be too high, standard error at 24 h treatment of DG in normal rat is too big.

Examination of the sciatic nerves in diabetic mice by electron microscopy showed nerve fiber loss and the presence of pathological features, including thin myelin, dystrophic axons, and a cluster of small regenerating axons. However, the myelin sheaths and the axons of the DG groups were not different from those of normal groups. The relationship between these morphological changes and the NGF concentration profile data suggests that the preventive efficacy of DG treatment in diabetic neuropathy might be related to NGF secretion. DG is a primary spirostane-type saponin that potentiates the action of NGF, it can be administered orally, and it may prevent adverse effects related to injection-site pain. These natural products therefore show potential for treatment of diabetic peripheral neuropathy.

This report shows that the regulatory effect of diosgenin in diabetic neuropathy is related to the role of neurotrophins in sciatic nerves. However, it remains to be determined whether DG can activate other neurotrophic molecules in addition to NGF, such as brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and ciliary neurotrophic factor (CNTF). Thus, further studies are warranted to clarify the neuroprotective effect of DG via regulation of NGF and other neurotrophins.

Acknowledgements This study was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (A081053). We thank May Kim at Korea International School and Jinwha Hong at Kyung Hee University for technical assistance.

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