DA-9801 Promotes Neurite Outgrowth via ERK1/2-CREB Pathway in PC12 Cells

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In the present study, we examined the mechanisms underlying the effect of DA-9801 on neurite outgrowth. We found that DA-9801 elicits its effects via the mitogen-activated protein kinase (MEK) extracellular signal-regulated kinase (ERK)/1-2/CAMP response element-binding protein (CREB) pathway. DA-9801, an extract from a mixture of Dioscorea japonica and Dioscorea nipponica, was reported to promote neurite outgrowth in PC12 cells. The effects of DA-9801 on cell viability and expression of neuronal markers were evaluated in PC12 cells. To investigate DA-9801 action, specific inhibitors targeting the ERK signaling cascade were used. No cytotoxicity was observed in PC12 cells at DA-9801 concentrations of less than 30 μg/mL. In the presence of nerve growth factor (NGF, 2 ng/mL), DA-9801 promoted neurite outgrowth and increased the relative mRNA levels of neurofilament-L (NF-L), a marker of neuronal differentiation. The Raf-1 inhibitor GW5074 and MEK inhibitor PD98059 significantly attenuated DA-9801-induced neurite outgrowth. Additionally, the MEK1 and MEK2 inhibitor SL327 significantly attenuated the increase in the percentage of neurite-bearing PC12 cells induced by DA-9801 treatment. Conversely, the selective p38 mitogen-activated protein kinase inhibitor SB203580 did not attenuate the DA-9801 treatment-induced increase in the percentage of neurite-bearing PC12 cells. DA-9801 enhanced the phosphorylation of ERK1/2 and CREB in PC12 cells incubated with and without NGF. Pretreatment with PD98059 blocked the DA-9801-induced phosphorylation of ERK1/2 and CREB. In conclusion, DA-9801 induces neurite outgrowth by affecting the ERK1/2-CREB signaling pathway. Insights into the mechanism underlying this effect of DA-9801 may suggest novel potential strategies for the treatment of peripheral neuropathy.

Key words DA-9801; diabetic peripheral neuropathy; neurite outgrowth; extracellular signal-regulated kinase 1/2; cAMP response element-binding protein (CREB)

Peripheral neuropathy is a common disease in diabetes patients. According to recent estimates, more than half of all diabetes patients develop diverse neuropathic symptoms.1,2 Diabetic peripheral neuropathy (DPN) is one of the most debilitating complications of Type 1 and Type 2 diabetes, and its histopathology is characterized by neuronal small fiber degeneration, demyelination, and atrophy.3–5 Hyperglycemia, a metabolic disruption known to increase oxidative stress in the peripheral nerves, is an important factor in the pathogenesis of DPN.6 In patients with diabetes, as well as those with local nerve compression that needs to be treated to prevent or repair permanent nerve damage, treatment of the underlying cause might result in partial or full pain relief.7

Nerve growth factor (NGF) itself has been considered as an option for the treatment of DPN. NGF restores the nociceptive threshold for thermal noxious stimuli,8 normalizes myelinated nerve fiber morphology,9 and reduces neurogenic vasodilatation10 in streptozotocin (STZ)-induced diabetic rats. Unfortunately, a recently concluded phase III trial failed to demonstrate the efficacy of NGF administration.11 The reasons for this failure include its limited delivery to the nervous system and an unwanted apoptotic effect elicited through the interaction with the p75NTR receptor.11–13 Additionally, the effectiveness of NGF application as a therapeutic intervention to treat brain injury is limited by the numerous unwanted side effects observed in both animals and patients, reflecting the diversity of cell types that react to NGF, both centrally and peripherally.14–16 To avoid the side effects, development of orally active small molecules that potentiate the expression and activity of NGF is warranted.17–19

The extracts of plants from the Dioscorea genus were reported to exhibit hypoglycemic,19 immunostimulatory,20 and anti-inflammatory effects,21 as well as anti-tumor22 and anti-osteoporotic activity.23 Dioscorea japonica Thunberg. (DJ) has been utilized in the treatment of hyperglycemia in Korea.24,25 Treatment with the extract of Dioscorea rhizome was shown to increase endogenous NGF levels in the salivary gland and sciatic nerve in mice.18 DA-9801 is an extract obtained from a mixture of DJ and Dioscorea nipponica Makino (DN). While the rhizome extracts showed a significant effect on neurite outgrowth and Trk-A phosphorylation in neurons, it remains to be determined whether DA-9801I possesses these neurotrophic properties.18,25

NGF is known to interact with two receptor proteins, TrkA and p75NTR.26,27 NGF signaling through TrkA elicits many of the classical neurotrophic actions ascribed to NGF.28 Binding of NGF stimulates the autophosphorylation of TrkA and induces a signal transduction cascade involving phosphoinositide 3-kinase (PI3K), extracellular-signal-regulated kinase (ERK), and Akt, leading to survival, regeneration, and

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determination of neurons. In PC12 cells, proteins of the Raf family mediate NGF signaling through phosphorylation (and thereby activation) of the dual-specificity mitogen-activated protein (MAP) kinase kinase (MEK1). MEK1 activation leads to the phosphorylation of two members of the MAP kinase family, ERK1/2. ERK1/2 are phosphorylated on threonine 202 and tyrosine 204 residues by MEK1, leading to activation and translocation of ERK1/2 into the nucleus. Additional transcription factors contribute to the regulation of c-fos transcription in response to NGF signaling.

In the present study, our aim was to examine the effects of DA-9801 on the promotion of neurite outgrowth in PC12 cells and elucidate the underlying mechanisms. Since several signal transduction molecules have been implicated in NGF-mediated induction of neurite outgrowth, we evaluated the effects of specific inhibitors of the ERK1/2 cellular signaling pathway on the enhancement of this particular effect of NGF by DA-9801. Our results indicate that DA-9801 potentiates the neuritogenesis-stimulating action of NGF through the ERK1/2-CREB signaling pathway.

MATERIALS AND METHODS

Materials RPNI-1640, fetal bovine serum (FBS), horse serum (HS), and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from GIBCO (Grand Island, NY, U.S.A.). Bioclot poly-d-lysine-coated 6-, 24-, and 96-well microplates and 100-mm dishes were purchased from Becton Dickinson (Bedford, MA, U.S.A.). NGF was purchased from Enzo Life Sciences (Farmingdale, NY, U.S.A.). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). GW5074 and PD98059 were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). DA-9801 was graciously provided by Dong-A Pharmaceutical Company (Yong-in, Korea). DA-9801 was dissolved in sterile water to obtain a 50 mg/mL stock solution. Anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibody was purchased from Bethyl Laboratories (Montgomery, TX, U.S.A.). Anti-p42/44, anti-phospho-p42/44, anti-CREB, and anti-phospho-CREB antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Extra pure grade methanol and ethanol was purchased from Duksan (Ansanhi, Kyonggi-do, Korea). Ultrapure Tris and EDTA disodium dihydrate was purchased from Duchefa (Haarlem, the Netherlands). GW5074, PD98059, SL327 and SB203580 were from cayman (Yong-in, Korea). DA-9801 was dissolved in sterile water at a concentration of 10 µg/mL. RPMI-1640 medium containing reduced serum levels (2% HS and 1% FBS) was purchased from Sigma. All inhibitors were dissolved in dimethyl sulfoxide (DMSO) for 72 h. GW5074, PD98059, SL327 and SB203580 also added. All inhibitors were dissolved in dimethyl sulfoxide (DMSO) with the final concentration of DMSO not exceeding 0.1%. The cell images were captured using Motic Images Plus 2.0 software (Motic Instruments Inc., Richmond, Canada). Cells with outgrowths longer than the cell body diameter were scored positive for neurites and their number was expressed as a percentage of the total cell number.

RNA Isolation and Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). For real-time qPCR, 0.1 µg of total RNA was reverse transcribed using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed in triplicate using the MyiQ Single-Color Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.). PCR amplification was performed using the Bio-Rad CFX Connect Real-Time System (Bio-Rad, Hercules, CA, U.S.A.) for 40 cycles. Thermal cycling conditions included pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 s, annealing at 57°C for 15 s, and extension at 72°C for 20 s. The primer sequences used were as follows: GAPDH, forward (5'-GGA TGC AGG GAT GAT GTT C-3') and reverse (5'-GGA TGC AGG GAT GAT GTT C-3').
neurofilament-L (NF-L), forward (5′-AGA CAT CAG CGC CAT GCA-3′) and reverse (5′-TTC GTG CTT CGC AGC TCA T-3′). Relative expression was normalized to GAPDH levels.

**Western Blot Analysis** Cells were homogenized by sonication at 0°C in homogenization buffer (25 mM Tris, 1.25 mM EDTA, and 0.1% Triton X-100, pH 7.5 (Amresco, Solon, OH, U.S.A.)) containing Protease Inhibitor Cocktail and PhosStop (Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged at 2000 × g at 4°C for 10 min, and the supernatant was collected. Proteins in the lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Bio-Science, NJ, U.S.A.). The membrane was blocked for 1 h by using Tris-buffered saline containing 5% bovine serum albumin (BSA) (Bioworld, Dublin, OH, U.S.A.) and 0.1% Tween 20 (Amresco). Immunoblots were performed with anti-phospho-ERK1/2 (Thr 202/Tyr204; 1:1000 dilution), anti-ERK1/2 (1:1000), GAPDH (1:1000), anti-phospho-CREB (Ser 133), and anti-CREB antibodies (Cell Signaling Technology, Beverly, MA, U.S.A.) overnight at 4°C in the same buffer. Membranes were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) (Cell Signaling Technology) for 2 h, and proteins were detected using the Enhanced Luminol Chemiluminescence system (GE Healthcare, Bio-Science, NJ, U.S.A.).

**Statistical Analysis** All data are expressed as means±S.E.M. Statistical differences between groups were analyzed by one-way ANOVA with subsequent Tukey’s tests. In all cases, p<0.05 was considered statistically significant.

**RESULTS**

**Effect of DA-9801 on PC12 Cell Viability** The viability of PC12 cells exposed to DA-9801 for 72 h was evaluated using CCK-8. DA-9801 showed a negative effect on cell viability at concentrations of 100 µg/mL and above. However, DA-9801 at concentrations of 30 µg/mL and below increased PC12 cell viability in a dose-dependent manner in the presence or absence of NGF in the media (Fig. 1D). Therefore, the maximum concentration tested in subsequent experiments was 30 µg/mL. We also conducted the cell viability assessment following 0-, 24-, and 48-h incubation with DA-9801. DA-9801 showed no immediate (0 h) toxicity. It reduced cell viability at concentrations of 500 and 200 µg/mL with 24-h and 48-h incubation, respectively (Figs. 1A–C).

**Effect of DA-9801 on Neurite Outgrowth** The effect of DA-9801 on neurite outgrowth was investigated in PC12 cells. After 72 h of exposure to DA-9801, PC12 cells showed a dose-dependent increase in the percentage of neurite-bearing cells.
cells in media containing 2 ng/mL of NGF (Figs. 2A, B). Conversely, treatment with DA-9801 alone did not induce neurite outgrowth in PC12 cells. NGF (2 ng/mL) plus DA-9801 (30 µg/mL) increased neurite outgrowth in PC12 cells 1.5-fold, compared to that observed with NGF (2 ng/mL) treatment alone (p < 0.05). NGF (2 ng/mL) plus DA-9801 (30 µg/mL) induced neurite sprouting to an extent equivalent to that achieved with 50 ng/mL NGF, suggesting that DA-9801 potentiates NGF-induced neurite outgrowth (Fig. 2B).

Effect of DA-9801 on NF-L Expression in PC12 Cells
The effect of DA-9801 on the mRNA expression of NF-L, a marker of neuronal differentiation, was investigated in PC12 cells. Compared to the control treatment, the incubation with 2 and 50 ng/mL NGF for 72 h resulted in 1.2- and 7.5-fold increases in the relative mRNA levels of NF-L, respectively (Fig. 3). To determine if the effect of DA-9801 is synergistic with that of NGF, NF-L gene expression was analyzed after 72 h of exposure to NGF plus DA-9801. Compared to the incubation with NGF (2 ng/mL) alone, incubation with NGF (2 ng/mL) plus DA-9801 (30 µg/mL) increased the expression of NF-L mRNA (p < 0.05; Fig. 3).

Effect of DA-9801 on the Raf-1/MEK/ERK1/2 Signaling Pathway in PC12 Cells
To identify the signaling mechanism by which DA-9801 induces neurite outgrowth, we focused on the Raf-1/MEK/ERK1/2 signaling cascade, one of the key signaling pathways associated with the control of neurite outgrowth. To further determine whether DA-9801-mediated neurite outgrowth is mediated by the Raf-1/MEK signaling pathway, GW5074 (a Raf-1 kinase inhibitor) and PD98059 (an MEK inhibitor), compounds known to inhibit the ERK1/2 pathway, were used. Compared to the treatment with NGF plus DA-9801 alone, GW5074 and PD98059 significantly at-
The MEK inhibitor, PD98059, potentiated NGF-induced activation of ERK1/2 (Fig. 5B). NGF, or both. Treatment of PC12 cells for 15 min with 30 µg/mL DA-9801 resulted in a 1.7-fold increase in the levels of Ser^{33} phosphorylated CREB protein (p < 0.05). Levels of total CREB and GAPDH proteins were unaffected (Fig. 6A). Treatment of PC12 cells with 2 ng/mL of NGF induced a 1.9-fold increase in the levels of Ser^{33} phosphorylated CREB at 5 min. The levels of Ser^{33} phosphorylated CREB remained elevated at 15 min (p < 0.05). Levels of total CREB and GAPDH proteins were unaffected (Fig. 6B). The effect of co-treatment with DA-9801 and NGF on the activation of CREB was investigated in PC12 cells. PC12 cells were treated with DA-9801, NGF, and a combination of both. DA-9801 and NGF stimulated the activation of CREB, respectively. NGF (2 ng/mL) plus DA-9801 (30 µg/mL) increased the phosphorylation of CREB in PC12 cells compared to the treatment with NGF (2 ng/mL) alone (p < 0.05; Fig. 6C).

PC12 cells were then pretreated with the MEK inhibitor, PD98059 (10 µM, 30 min), and stimulated with DA-9801 (30 µg/mL, 60 min) plus NGF (2 ng/mL, 15 min). The DA-9801-treated cells without PD98059 induced an increase in the levels of Ser^{33} phosphorylated CREB protein (p < 0.05). On the other hand, pretreatment with PD98059 blocked Ser^{33} phosphorylated CREB protein (p < 0.05). Similarly, PD98059 blocked the NGF (2 ng/mL) plus DA9801-induced the phosphorylation of CREB (p < 0.05). The protein level of CREB and GAPDH was unchanged from the control level after treatment with DA-9801 and NGF (Fig. 6D).

**DISCUSSION**

Treatment of neuropathic pain remains a challenge because a significant proportion of patients do not attain adequate pain relief with currently available therapy. The effectiveness of a number of drugs, including antidepressants (norepinephrine and serotonin reuptake inhibitors), calcium channel α2-δ ligands, opioid analgesics, and topical lidocaine, was consistently demonstrated in randomized controlled clinical trials and meta-analyses. While these agents are commonly administered for management of neuropathic pain, they are associated with a high incidence of side-effects.

DA-9801 is a herbal extract from *Dioscorea* species used in the prevention or treatment of DPN. Treatment with DA-9801 has been shown to improve sensory nerve conductivity velocity (SNCV), motor nerve conductivity velocity (MNCV), and thermal hyperalgesia in Type 2 db/db mice. In contrast to peptide neurotrophic factors, DA-9801 can be administered systemically and was demonstrated to improve nerve conduction velocity and facilitate recovery from neuronal degeneration. In the present study, DA-9801 elicited no cytotoxic effect in PC12 cells in the presence or absence of NGF at concentrations below 30 µg/mL (Fig. 1). PC12 cell viability was significantly reduced at 100 µg/mL DA-9801. Hence, in differentiation experiments using PC12 cells, the concentration of DA-9801 was kept below 30 µg/mL. DA-9801 showed no effect on neurite outgrowth in the absence of NGF (Fig. 2B), but modestly induced neurite outgrowth in PC12 cells cultured in low serum condition and in the presence of NGF (2 ng/
mL; Figs. 2A, B). A longer incubation (7 d) with DA-9801 was shown to have no effect on neurite outgrowth in the absence of NGF (data not shown). These findings suggest that DA-9801 itself does not elicit the observed neurotrophic effect, but rather enhances NGF signal transduction through the ERK1/2 pathway to activate CREB. This NGF-potentiating effect was also observed in PC12 cells stimulated by suramin in the presence of 1 ng/mL of NGF. Sialic acid derivative MCC-257, known to potentiate the action of neurotrophins on central and peripheral neurons, protects the neuronal cells from a number of fatal conditions. Upon exposure to NGF, PC12 cells cease division, extend neurites, become electrically excitable, and express neuronal markers. In this study, DA-9801 increased both proliferation and neurite outgrowth in the presence of NGF. In a previous study, the presence of NGF in the media improved the viability of PC12 cells and increased neurite length. Curcuminoids have multiple characteristics desirable in a neuroprotective drug, including antioxidant, anti-inflammatory, and anti-protein aggregation activities. Curcuminoids were previously shown to increase cell proliferation and neurite outgrowth in PC12 cells.

NF-L is a neuron-specific protein which exhibits elevated synthesis and axonal fast-transport during nerve regeneration and can serve as a useful indicator of PC12 cell differentiation. Upon exposure to NGF, PC12 cells cease division, extend neurites, become electrically excitable, and express neuronal markers. In this study, DA-9801 increased both proliferation and neurite outgrowth in the presence of NGF. In a previous study, the presence of NGF in the media improved the viability of PC12 cells and increased neurite length. Curcuminoids have multiple characteristics desirable in a neuroprotective drug, including antioxidant, anti-inflammatory, and anti-protein aggregation activities. Curcuminoids were previously shown to increase cell proliferation and neurite outgrowth in PC12 cells.

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in the regulation of cell survival, growth, and proliferation.\(^{58}\)
Similarly, our data indicate that DA-9801 induced ERK1/2 phosphorylation within its modulatory effect on neurite outgrowth (Fig. 5). The DA-9801-induced process formation and branching of neurons were significantly reduced by the treatment with ERK1/2 inhibitor (Fig. 4).

Previous studies have shown that treatment of PC12 cells with NGF rapidly activates p38 MAP kinase and JNK. The p38 MAP kinase pathway is also critically involved in NGF-induced neuronal differentiation of PC12 cells.\(^{59-62}\) In our current study, p38 MAP kinase pathway was not involved in DA-9801-induced neurite outgrowth in PC12 cells (Fig. 4D).

Several signaling pathways, including those involving ERK and protein kinase C (PKC), have been associated with the regulation of \textit{de novo} protein synthesis in the context of synaptic plasticity, converging on the phosphorylation of CREB.
at Ser133 residue. Phosphorylated CREB protein recruits the transcriptional activator CREB-binding protein (CBP) to stimulate the transcription of CRE-regulated genes involved in the neurogenesis and neuritogenesis. DA-9801 enhanced the levels of Ser133-phosphorylated CREB protein. Additionally, concurrent treatment with DA-9801 and NGF elicited a higher phosphorylation of CREB compared to incubation with NGF alone (Fig. 6). DA-9801 was observed to sustain the phosphorylation of CREB.

We demonstrated that DA-9801 elicits its beneficial effects of stimulating neurite outgrowth through the ERK1/2-CREB pathway. However, further studies are needed to elucidate the exact molecular pathways involved in the effect of DA-9801. We conclude that DA-9801, a novel botanical drug, could therefore be a therapeutic option for the management or prevention of DPN.
Acknowledgment This work was supported by the MOTIE (Ministry of Trade, Industry and Energy) R&D program (Grant no. 10039303).

Conflict of Interest The authors declare no conflict of interest.

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