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(−)-3,5-Dicaffeoyl-*muco*-quinic acid isolated from *Aster scaber* contributes to the differentiation of PC12 cells: through tyrosine kinase cascade signaling

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Abstract

Aster scaber T. (Asteraceae) has been used in traditional Korean and Chinese medicine to treat bruises, snakebites, headaches, and dizziness. (−)-3,5-Dicaffeoyl-*muco*-quinic acid (DQ) isolated from *A. scaber* induced neurite outgrowth in PC12 cells. It has been reported that the activation of the extracellular signal regulated kinase 1/2 (Erk 1/2) and phosphoinositide 3 (PI3) kinase plays a crucial role in the NGF-induced differentiation of PC12 cells. This study showed that the effect of DQ on neurite outgrowth is mediated via the Erk 1/2 and PI3 kinase-dependent pathways like NGF. Furthermore, DQ stimulated the phosphorylation of Trk A. Overall, DQ elicited the differentiation of PC12 cells through Trk A phosphorylation followed by Erk 1/2 and PI3 kinase activation.

Keywords: (−)-3,5-Dicaffeoyl-*muco*-quinic acid; PC12; Neurite outgrowth; Tyrosine kinase receptor; Erk 1/2; PI3 kinase

Neurite genesis and retraction is deeply associated with the development and pathogenesis of the nervous system. Neurotrophic factors are mainly responsible for neurite outgrowth. Therefore, attention has been given to the availability of the neurotrophic factors as therapeutic agents. In particular, recent experiments have suggested that the nerve growth factor (NGF), which was first identified to be part of the neurotrophic factor family [1], might be a potential therapeutic agent to prevent the degeneration of the cholinergic neurons in Alzheimer's disease patients [2,3]. However, there are some limitations to the application of NGF to medicine. These include: (1) inability to penetrate the blood–brain barrier and (2) its destabilization by peptidases when administered peripherally. Recently, several synthetic compounds and natural products have been found to enhance the action of NGF or show a similar NGF mode of action in the PC12 cells that generate neurite outgrowth in response to the NGF [4,5].

The NGF binds to the Trk receptors followed by dimerization and then by the stimulation of their intrinsic tyrosine kinase activity, leading to the phosphorylation of several tyrosine residues. Tyrosine phosphorylation is essential for the activation of the catalytic activity of the receptor as well as for the recruitment of the signaling proteins, such as PLCγ, PI3 kinase, the shc protein, and the extracellular signal regulated kinase (Erk) [6,7]. This signal cascade has been reported to be responsible for inducing neurite outgrowth.

Aster scaber, which is widespread in Korea, has been used as a traditional medicine to treat bruises, headaches, and dizziness. Some triterpene glycosides and volatile compounds have been isolated from the

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A. scaber [8,9]. In a previous study, although it was demonstrated that quinic acid derivatives isolated from *A. scaber* contribute to the differentiation and survival of PC12 cells [10], the neuroprotective mechanism was not reported. This study investigated the mechanism underlying (−)-3,5-dicaffeoyl-*muco*-quinic acid (DQ), which is the most effective among the quinic acids isolated from *A. scaber*, in promoting the neurite outgrowth in PC 12 cells.

Materials and methods

Reagents. RPMI 1640, horse serum (HS), fetal bovine serum (FBS), penicillin–streptomycin (PS), and NGF were purchased from Gibco-BRL (Gland island, NY, USA). PD9805, k252b, and LY294002 were obtained from New England Biolab (UK) and Calbiochem (USA), respectively.

Cell culture. The PC12 pheochromocytoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI 1640 supplemented with 10% HS, 5% FBS, and 1% PS in a humidified incubator at 37 °C and 5% CO_2.

Sample treatment. The cells (1×10^4 cells/well) were plated to a 6-well plate and the medium was changed with RPMI 1640 containing 2% HS and 1% FBS. The 6-well plates were pre-coated with poly-D-lysine (5 mg/ml). After 24 h, the medium was changed and DQ or NGF was treated. PD98059, a MEK 1/2 inhibitor, k252b, a Trk A inhibitor, and LY 294002, a PI3 kinase inhibitor, were pre-treated 2 h prior to the addition of DQ or NGF. PD98059, k252b, and LY 294002 were prepared in a 20 mM solution in DMSO and diluted with medium.

Neurite outgrowth. The neurite length per cell was quantified after 48 h of incubation. The instrument used was an Olympus optical inverted phase-contrast microscope (Model CK-2; 100× magnification). The processes with a length equivalent of one or more cell body diameters were scored as neurites and measured using the Optimas 6.5 program (Media Cybernetics, USA). The differentiation of the PC12 cells was scored as follows: cells without neurite outgrowth were scored as 0. The cells bearing neurites as long as one cell diameter were scored as 1. The cells bearing neurites twice or three times the length of one cell diameter were scored as 2, 3, and the cells with a synapse-like neurite were scored as 4.

Western blot analysis. The PC12 cells were washed with PBS and harvested by a trypsin–EDTA solution. The pellet was washed and lysed with a buffer (50 mM Tris–Cl, pH 8.0, 0.1% SDS, 150 mM NaCl, 1% NP-40, 0.02% sodium azide, and 0.5% sodium deoxycholate). The protein content in the supernatant was determined using a Bio-Rad protein assay kit with BSA as the standard. The supernatant was boiled with a SDS-sample buffer. Eighty micrograms of the protein was subjected to electrophoresis on a 1.2% SDS–PAGE gel and transferred to the PVDF membrane. The membrane was blocked using 5% non-fat milk. The membrane was incubated with polyclonal antibodies (Erk 1/2, Akt, Trk A and phosphor-ErkA/2, phospho Akt, phospho TrkA, 1:1000, and anti-rabbit, Cell signaling) overnight at room temperature. After removing the primary antibody, the membrane was washed with the PBS buffer and then incubated with an HRP-conjugated rabbit anti-mouse IgG (secondary antibodies 1:10000 dilution, Amersham–Pharmacia) for 1 h. The immunoreactive proteins were detected with ECL reagent (Amersham–Pharmacia Biotech, UK).

PI3 kinase assay. The PI3 kinase activity was measured by immunoprecipitation with the anti-phosphotyrosine antibodies. Immunoprecipitation was done as follows: After washing twice with ice-cold PBS, the control and DQ-treated cells were dissolved in 1 ml lysis buffer (20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM Na_3VO_4, 2 mM EDTA, 1% NP-40, 10% glycerol, 5 μg/ml leupeptin, and 1 mM PMSF) and incubated for 30 min. The cell lysates were then centrifuged at 15,000 rpm for 15 min at 4 °C and the supernatant was harvested after centrifugation. For immunoprecipitation, 20 μl of the anti-phosphotyrosine antibody agarose was incubated with the supernatant containing 500 μg of the protein for 1 h at 4 °C. The immunoprecipitates were washed three times with PBS containing 1% NP-40 and 100 μM Na_3VO_4, three times with 100 mM Tris–HCl, pH 7.5, containing 500 mM $LiCl_2$ and 100 μM Na_3VO_4, and finally twice with 25 mM Tris–HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 100 μM $NaVO_4$. In order to determine the PI3 kinase activity, the immunoprecipitate was resuspended in 100 μl of a kinase assay buffer (20 mM Tris–HCl, pH 7.6, 75 mM NaCl, and 10 mM $MgCl_2$) and 200 μg/ml phosphatidylinositol sonicated in 10 mM Tris–Cl, pH 7.6, 1 mM EGTA, and 10 μCi [γ-^{32}P]ATP, and then incubated for 20 min at room temperature with constant shaking. The reaction was quenched by adding a solvent mixture of 100 μl of 1 N HCl and 200 μl $CHCl_3$–methanol (1:1). The samples were centrifuged, and the lower organic phase was harvested and applied to a silica gel TLC plate (Merck, Darmstadt, Germany) coated with 1% potassium oxalate. The TLC plates were developed in $CHCl_3$–CH_3OH–H_2O–NH_4OH (60:47:11.3:2), dried, and visualized by autoradiography.

Statistical analysis. All the data are expressed as means ± SD. The statistical significance was determined using a Student's *t* test. A P value <0.05 was considered significant.

Results

DQ-induced neurite outgrowth from PC 12 cells

The effect of (−)-3,5-dicaffeoyl-*muco*-quinic acid (Fig. 1) on the neurite outgrowth in the undifferentiated PC 12 cells was examined. The PC12 cells treated with DQ (1, 5, and 10 μM) showed neurite outgrowth in a dose-dependent manner (Figs. 2A–F). NGF (50 ng/ml), which was used as the positive control, also triggered the differentiation of PC12 cells. The potency of DQ at 10 μM was similar that of the NGF at 50 ng/ml. This result suggests

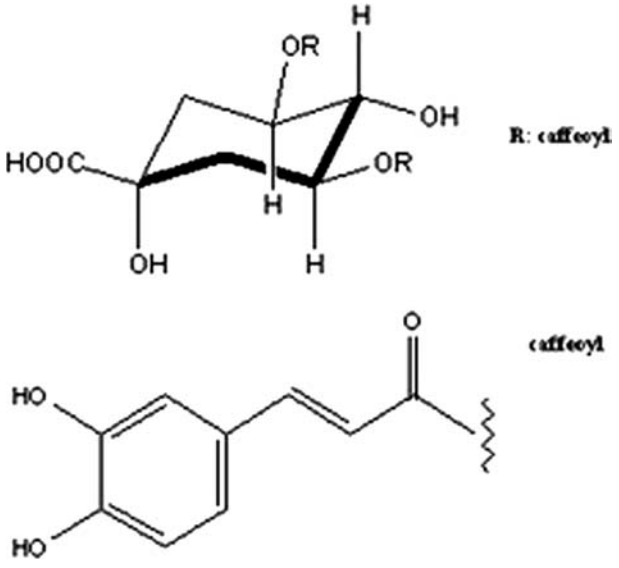

Fig. 1. Structure of (−)-3,5-dicaffeoyl-*muco*-quinic acid (DQ) isolated from *Aster scaber* T. (Asteraceae).

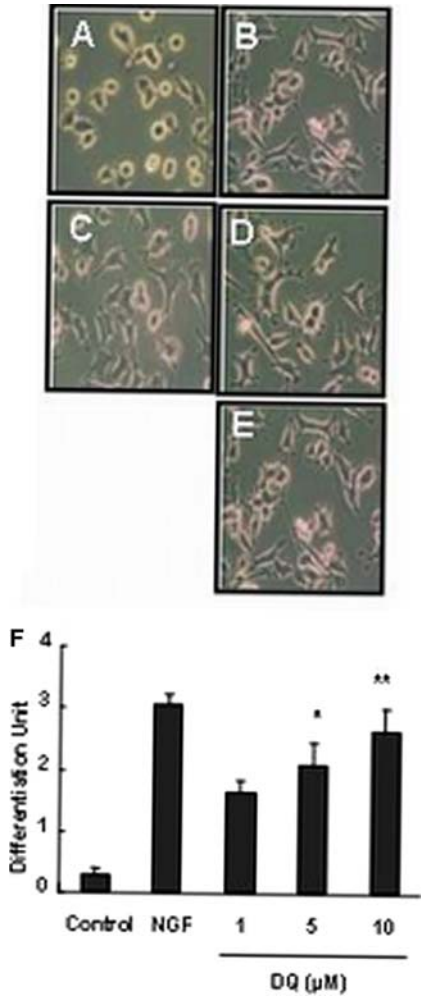

Fig. 2. Induction of PC12 cells neurite outgrowth by DQ. Forty-eight hours after treating the PC12 cells with the NGF (50 ng/ml) and DQ (1, 5, and 10 μM), randomly selected fields were observed using a camera attached a microscope (100×). (A) Vehicle; (B) NGF (50 ng/ml); (C) DQ (1 μM); (D) DQ (5 μM); and (E) DQ (10 μM). (F) Length of the PC12 cells' neurite outgrowth. The results are expressed as means ± SD of three independent experiments. The asterisks indicate a significant difference from the treatment with the vehicle (*$p < 0.05$, **$p < 0.01$). The differentiation of the PC12 cells was scored as follows: the cells without neurite outgrowth were scored as 0, cells bearing neurites as long as one cell diameter were scored as 1, cells bearing neurite twice the length of one cell diameter were scored as 2, and cells with a synapse-like neurite were scored as 4.

that the DQ had neuritogenic activity that induced the neuronal differentiation of the PC12 cells.

DQ stimulated the Trk A-mediated signaling pathways

It has been reported that the NGF-induced activation of the Ras-MAP kinase signal pathway plays a crucial role in the differentiation of PC12 cells [11]. We investigated whether or not the neurite outgrowth effect of DQ correlated with the intracellular signaling events. PD98059, which is a MEK 1/2 inhibitor, reversed the neurite outgrowth induced by DQ (Figs. 3A–G).

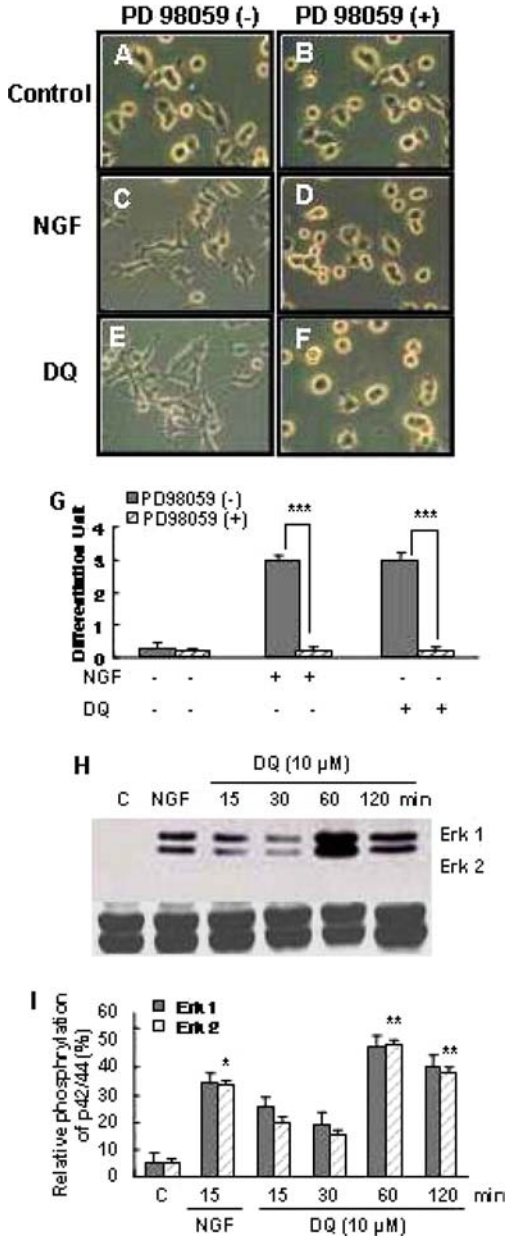

Fig. 3. Association of Erk 1/2 with the differentiation of PC12 cells by DQ. The PC12 cells were pre-incubated with PD98059 (5 μM), a MEK 1/2 inhibitor, for 2 h, and then NGF (50 ng/ml) or DQ (10 μM) was treated to PC12 cells. After 48 h, randomly selected fields were taken using a camera attached to a microscope (100×). (A) Vehicle alone; (B) vehicle + PD98059; (C) NGF alone; (D) NGF + PD98059; (E) DQ alone; and (F) DQ + PD98059. (G) Length of the PC12 cells' neurite outgrowth. The results are expressed as means ± SD. The three independent experiments were carried out. The asterisks indicate a significant difference from the treatment with the vehicle (***$p < 0.001$). The PC12 cells were stimulated for 15, 30, 60, and 120 min with DQ (10 μM) or NGF (50 ng/ml, 30 min). Western blot analysis was performed with phospho-Erk 1/2 and the total-Erk 1/2 antibody. (H) Phospho- and total-Erk 1/2 Western blot analysis. Each photograph is representative of three independent Western blot analyses. (I) Relative level of Erk 1/2 phosphorylation against total Erk 1/2. The results are expressed as means ± SD of three independent experiments. The asterisks indicate a significant difference from the treatment with the vehicle (*$p < 0.05$, **$p < 0.01$).

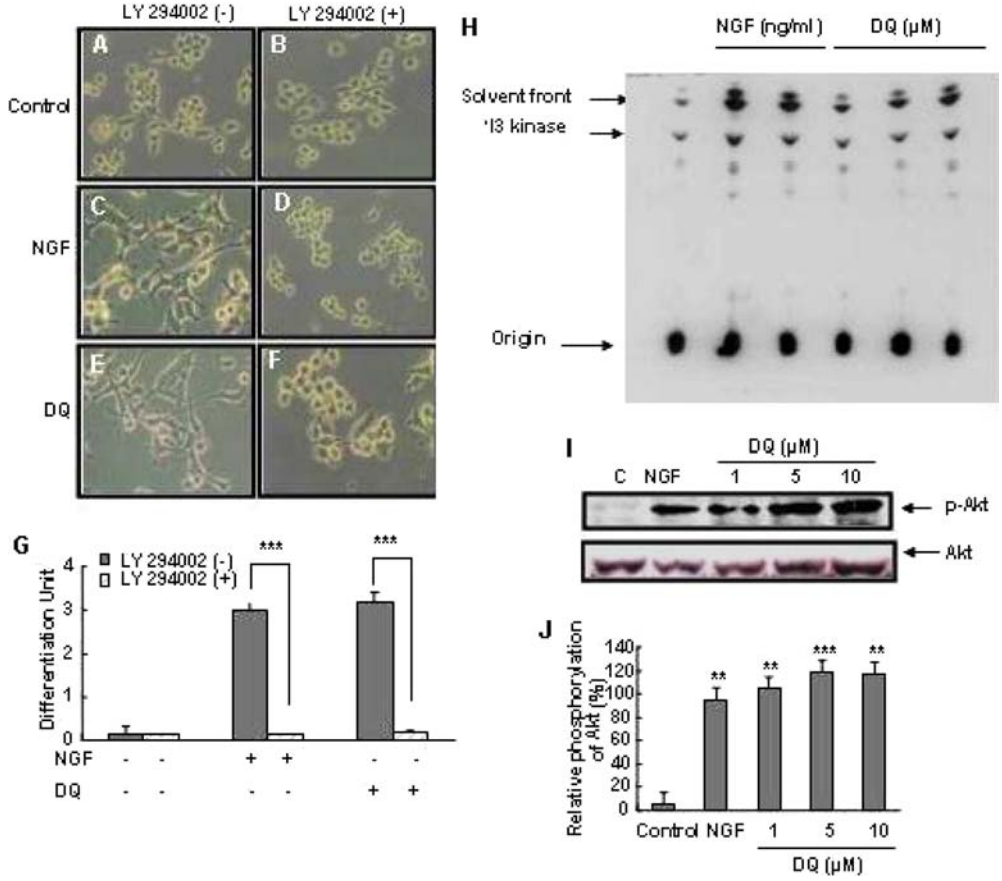

Fig. 4. Association of PI3 kinase with the differentiation of PC12 cells by DQ. The PC12 cells were pre-incubated with LY294002, a PI3 kinase inhibitor, for 30 min, which then was treated with NGF (50 ng/ml) or DQ (1, 5, and 10 μM). After 24 h, the PC12 cells were observed for neurite outgrowth. (A) Vehicle alone; (B) vehicle + LY294002; (C) NGF alone; (D) NGF + LY294002; (E) DQ alone; and (F) DQ + LY294002. (G) Length of the PC12 cells' neurite outgrowth. After 60 min treatment with either the NGF or DQ, the PC12 cells were harvested for PI3 kinase assay and Western blot analysis. (H) PI3 kinase assay; (I) phospho- and total-Akt Western blot analysis. Each photograph is representative of three independent experiments. (J) Relative level of phosphorylation of Akt against total Akt. These results are expressed as means ± SD of three independent experiments. The asterisks indicate a significant difference from the treatment with vehicle (**$p < 0.01$, ***$p < 0.001$).

In addition, Western blot analysis showed that DQ stimulated Erk 1/2 phosphorylation (Fig. 3H), suggesting that the activation of Erk 1/2 by DQ mediated the neurite outgrowth of the PC12 cells. The effect of DQ on the PI3 kinase was examined using the PI3 kinase inhibitor, LY294002. The inhibitors, which were similar to PD98059, blocked the neurite outgrowth in the DQ-treated PC12 cells (Figs. 4A–G). It was observed that DQ elevated the PI3 kinase activity as well as the phosphorylation of Akt, which is a substrate of PI3 kinase (Figs. 4H–J). This study further investigated whether or not DQ had a stimulatory effect on the upstream signaling pathway of Erk 1/2 and PI3 kinase in the PC 12 cells. Trk A, a specific NGF receptor, mediated both the Erk 1/2 and PI3 kinase signal pathways [12]. Therefore, it is possible that the activation of Erk 1/2 and PI3 kinase in the DQ-treated PC12 cells was due to the stimulation of Trk A activity by DQ. Treatment with the tyrosine kinase inhibitor, k252b, suppressed the differentiation triggered by DQ (Figs. 5A–G). Furthermore, the phosphorylation of 490-tyrosine in the tyrosine kinase was detected in the DQ-treated PC 12 cells (Figs. 5H and I).

Discussion

Previously, it was observed that DQ induced neurite outgrowth [10]. This study examined whether or not neurite outgrowth and the morphological differentiation of PC12 cells by DQ were related to the TrkA signal pathway. The treatment of the undifferentiated PC12 cells with DQ induced the outgrowth of the short neurite. The Erk 1/2 is associated with the neurite outgrowth of PC12 cells [13,14]. The blockade of neurite outgrowth by PD98059 and the elevation of Erk 1/2 phosphorylation in the DQ-treated PC12 cells suggested that the DQ activated phosphorylation of Erk 1/2 contributed to the differentiation of the PC 12 cells. It is well known that PI3 kinase survives the various cell types by

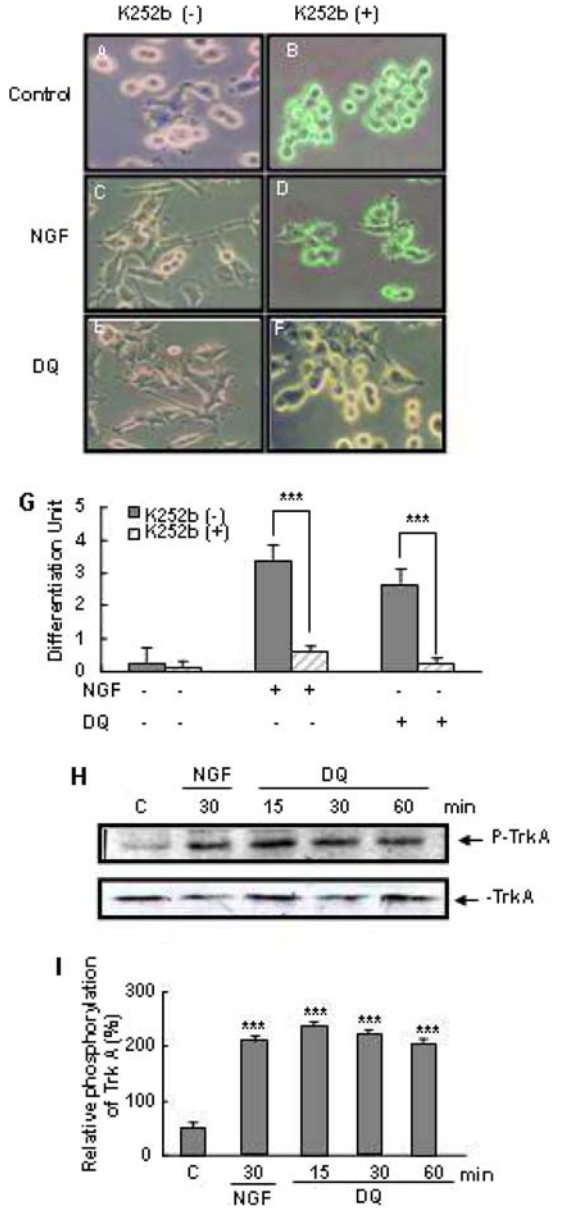

Fig. 5. Association of Trk A with the differentiation of PC12 cells by DQ. The PC12 cells were treated with K252b (10 μM), a tyrosine kinase inhibitor, for 24 h and then observed with the camera attached to a microscope (100×). (A) Vehicle alone; (B) vehicle + K252b; (C) NGF alone; (D) NGF + K252b; (E) DQ alone; and (F) DQ + K252b. (G) Length of the PC12 cells' neurite outgrowth. The PC12 cells were pre-incubated with K252b for 2 h and cells were then treated with the NGF (50 ng/ml, 30 min) or DQ (10 μM). After 15, 30, and 60 min, the cells were harvested for Western blot analysis. (H) Phospho- and total-Trk A Western blot analysis. (I) The relative phosphorylation against the total Trk A. These results are expressed as means ± SD of three independent experiments. The asterisks indicate a significant difference from the treatment with the vehicle (***$p < 0.001$).

virtue of Akt phosphorylation, which is known as protein kinase B (PKB), subsequently inactivates Bad, which is a member of the pro-apoptotic Bcl-2 family protein [15]. However, a recent report suggested that PI3 kinase plays a significant role in differentiation as well as in the survival of cell [16]. Akt/protein kinase B prevents the injury-induced motor neuron death and accelerates axonal regeneration [17]. The present study found that DQ enhanced the PI3 kinase activity and the phosphorylation of Akt, which is a substrate of PI3 kinase. Furthermore, the PI3 kinase inhibitor suppressed the DQ-triggered neurite outgrowth, which is in accordance with previous reports [10]. These results suggest that DQ exerts an effect on the differentiation of PC12 cells through both the Erk and PI3 kinase signal pathways. Neurotrophin binding to the Trk A receptors results in receptor dimerization and kinase activation. Trk A phosphorylation promotes the signaling cascades, including the Erk 1/2 pathway, the PI3 kinase/Akt pathway, and phospholipase C-γ1 (PLC-γ1) [18,19]. In light of the fact that the Erk 1/2 and PI3 kinase were downstream steps of Trk A, it has been suggested that the activation of Erk 1/2 and PI3 kinase required the activation of Trk A by DQ. This study found that K252b reversed the differentiation of the PC 12 cells by DQ. In addition, DQ triggered Trk A phosphorylation. Indeed, since an inhibitor entirely specific to TrkA was not available, it is possible that DQ might be related to the activity of other tyrosine kinases. However, the result showing that DQ elicited Trk A phosphorylation suggests that at least, the DQ neuritogenic activity was dependent on the Trk A signaling pathway. Overall, DQ elicited the activation of Erk 1/2 and PI3 kinase in the Trk A-dependent pathway, which leads to the differentiation of PC12 cells. Since the NGF has also been reported to induce these intracellular signaling pathways [20,21], DQ may mimic the NGF. Although the pharmacological research into DQ in vivo remains to be determined, DQ may have therapeutic possibilities in treating neurodegenerative disease if DQ can overcome the problems associated with using the NGF as a medicine.

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