Tenuifoliside A promotes neurite outgrowth in PC12 cells via the PI3K/AKT and MEK/ERK/CREB signaling pathways

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Abstract. Previous studies have demonstrated the neuroprotective effect of tenuifoliside A (TFSA) on corticosterone-induced neuron damage in SH-SY5Y cells, however, the effect of TFSA on the promotion of neurite outgrowth remains to be elucidated. PC12 cells were treated with TFSA or nerve growth factor, and the levels of proteins were evaluated by western blotting. In addition, for pharmacological experiments, inhibitors of the PD98059 mitogen-activated protein kinase kinase (MEK) and phosphatidylinositol 3-kinase (PI3K; LY294002) were added into the culture medium. The present study demonstrated that TFSA significantly increased the percentage of neurite-bearing cells and promoted neurite extension in PC12 cells. In addition, TFSA-treated PC12 cells also expressed increased levels of the 43 kD growth-associated protein (GAP-43) neural marker, comparable to those in nerve growth factor-treated cells. The present study also demonstrated that TFSA enhanced the phosphorylation of extracellular signal-regulated kinase (ERK) and Akt, which are important signaling molecules involved in neural differentiation in PC12 cells. Co-treatment of the PC12 cells with the PD98059 MEK inhibitor and LY294002 PI3K inhibitor inhibited the neurite outgrowth induced by TFSA. In addition, treatment with TFSA also promoted the phosphorylation of cyclic AMP response element-binding protein (CREB), which was inhibited completely by treatment with PD98059. In conclusion, the results of the present study demonstrated that TFSA induces neurite extension of PC12 cells and suggested that activation of the MEK/ERK/CREB and PI3K/Akt signaling pathways is involved in this process.

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Introduction

The roots of *Polygala tenuifolia* Willd are used widely as a traditional medicine in China and Korea for the treatment of neurasthenia, amnesia and depression (1-3). Previous studies have reported that oligosaccharide esters, including tenuifolisid A (TFSA) and 3,6-disinapoyl sucrose are the predominant active components of *Polygala* (4), and Liu *et al* demonstrated that it exerted a neuroprotective effect on corticosterone-induced neuron damage in SH-SY5Y cells (3). However, the effect of TFSA on the promotion of neurite outgrowth remains to be elucidated.

PC12 cells, which are widely used as a model for investigating neurite extension, are from a cell line derived from the rat pheochromocytoma of the adrenal medulla (5). Upon treatment with nerve growth factor (NGF), PC12 cell neurite extension occurs, differentiating into morphologically and functionally neuron-like cells that express neuronal specific genes, including 43 kD growth-associated protein (GAP-43), Tuj1 and synapsin I. This process is associated with the activation of the tropomyosin-related kinase (TrkA)-dependent mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (6-8). The present study aimed to investigate the effect of TFSA on neurite outgrowth in PC12 cells, and to determine the potential involvement of the MAPK kinase (MEK)/extracellular-signal regulated kinase (ERK)/cyclic AMP response element-binding protein (CREB) and PI3K/Akt signaling pathways. Thus, the neuritogenic effects of TFSA may suggest that TFSA could be used as a potential treatment for CNS injury, including spinal cord injury.

Materials and methods

Cell culture. Rat PC12 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in Roswell Park Memorial Institute 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated horse serum (Invitrogen Life Technologies), 5% fetal bovine serum (FBS; Invitrogen Life Technologies and 100 U/ml penicillin/streptomycin (Invitrogen Life Technologies) under 5% CO_2 at 37°C. The medium was replaced every 3 days. For the differentiation experiments, the PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 media

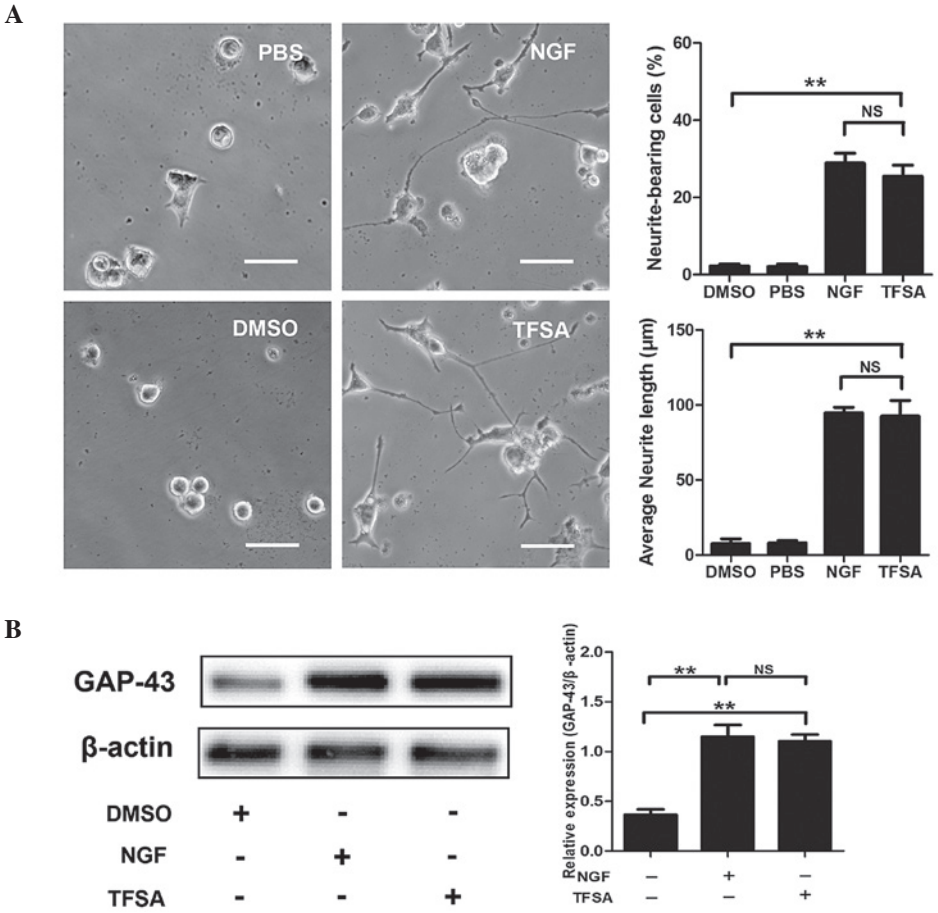

Figure 1. Effects of TFSA on neurite extension in PC12 cells. (A) Percentage of neurite-bearing cells and average neurite lengths in PC12 cells treated with PBS, DMSO, TFSA or NGF for 24 h. Scale bar=50 μm. (B) Relative expression levels of GAP-43 in PC12 cells treated with DMSO, TFSA or NGF. The data are expressed as the mean ± standard devosatopn from three independent experiments (**P<0.01). TFSA, tenuifoliside A; NGF, nerve growth factor, DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; GAP-43, growth-associated protein 43; NS, non-significant.

(Invitrogen Life Technologies) in the presence of 1% FBS and 100 ng/ml NGF (Sigma-Aldrich, St. Louis, MO, USA).

Measurement of neurite outgrowth. The PC12 cells ($8x10^3$) were plated in ploy-l-lysine coated 24-well plates. Neurite outgrowth was examined 48 h following treatment with NGF (100 ng/ml) or TFSA (10 μM). In certain experiments, the cells were cultured with PD98059 (10 μM; Sigma-Aldrich) or LY294002 (10 μM; Sigma-Aldrich). In each well, images were captured of 10 randomly-selected fields, each containing ~100 cells using an inverted phase contrast microscope (Nikon, Tokyo, Japan). The cells, which exhibited extension of at least one neurite with a length that was longer than the diameter of the cell body were identified as neurite-bearing cells. The neurite lengths of all neurite-bearing cells were measured using ImageJ software version 1.44p (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis. The cells were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing protease inhibitor cocktails and phosphatase inhibitor cocktails (Sigma-Aldrich). The protein contents were measured using a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracted protein samples (20 μg) were separated by 10% SDS-PAGE (KeyGen Biotech, Co., Ltd., Nanjing, China) and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). Following blocking in 5% bovine serum albumin (Sigma-Aldrich) in phosphate-buffered saline for 2 h at room temperature, the membranes were incubated with primary antibody at 4˚C overnight, and were subsequently incubated with horse anti-mouse (cat. no. 7076) or goat anti-rabbit (cat. no. 7074) IgG horseradish peroxidase-conjugated secondary antibodies (1:2,000, Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at room temperature. The primary antibodies were as follows: Rabbit anti-rat total-ERK1/2 (monoclonal, 1:1,000; cat. no. 4695; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-rat total-Akt (monoclonal, 1:1,000; cat. no. 4685; Cell Signaling Technology, Inc.), rabbit anti-rat total-CREB (monoclonal, 1:1,000; cat. no. ab32515; Abcam, Cambridge, UK), rabbit anti-rat phosphorlyated (p-) ERK1/2 (monoclonal, 1:1,000; cat. no. 4377; Cell Signaling Technology, Inc.), mouse anti-rat p-Akt (monoclonal, 1:1,000; cat. no. 4058; Cell Signaling Technology, Inc.), rabbit anti-rat p-CREB (monoclonal, 1:1,000; cat. no. ab32096; Abcam), mouse anti-rat β-actin (monoclonal, 1:2,000; cat. no. 4970; Cell Signaling Technology, Inc.) and rabbit anti-rat GAP43 (monoclonal, 1:1,000; cat. no. ab75810; Abcam). Finally, the

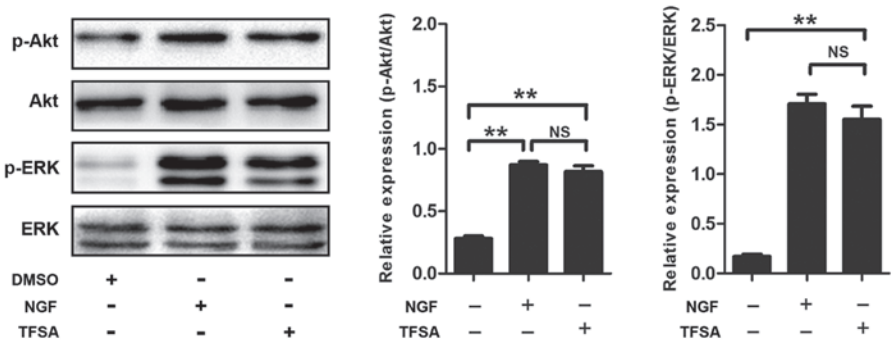

Figure 2. Effect of TFSA on the phosphorylation of ERK and Akt in PC12 cells. Phosphorylation of ERK anf Akt in PC12 cells treated with DMSO, NGF or TFSA were detected using western blot analysis. The data are expressed as the mean ± standard deviation from three independent experiments (**P<0.01). TFSA, tenuifoliside A; NGF, nerve growth factor; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated ERK; p-Akt, phosphorylated Akt; NS, non-significant.

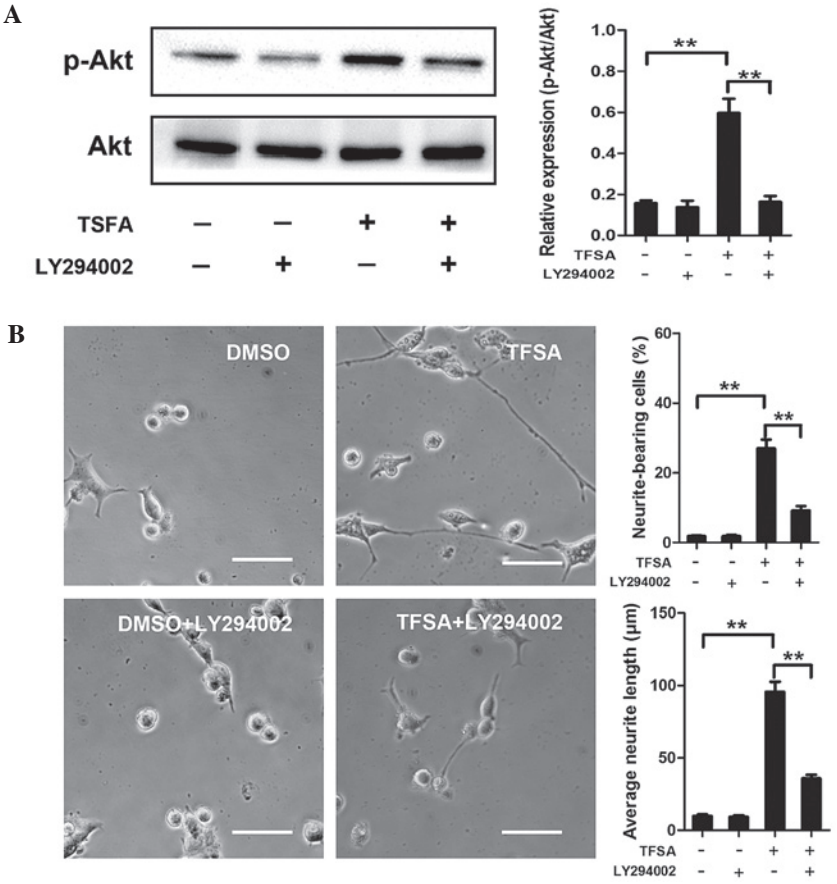

Figure 3. Effect of TFSA on PI3K/Akt activation and PI3K/Akt-mediated neurite outgrowth. (A) Phosphorylation of Akt in PC12 cells treated with DMSO, DMSO+LY294002, TFSA or TFSA+LY294002. Scale bar=50 μm. (B) Proportion of neurite-bearing cells and average neurite lengths in PC12 cells treated with DMSO, DMSO+LY294002, TFSA or TFSA+LY294002. The data are expressed as the mean ± standard deviation from three independent experiments (**P<0.01). TFSA, tenuifoliside A; PI3K, phosphatidylinositol 3-kinase; DMSO, dimethyl sulfoxide; p-Akt, phosphorylated Akt; NS, non-significant.

bands were measured using an enhanced chemiluminescent system (ECL Plus; Thermo Fisher Scientific).

Statistical analysis. All numerical data are presented as the mean ± standard deviation. Statistical differences were determined using one-way analysis of variance, combined with Scheffe's test for multiple comparisons. Data were analyzed using SPSS software version 11 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference. Each experiment was replicated at least three times.

Results

TFSA induces neurite outgrowth in PC12 cells. To investigate the effect of TFSA on neurite outgrowth in PC12 cells, the cells, which were maintained in low-serum medium, were treated with either vehicle (0.1% DMSO), NGF (100 ng/ml)

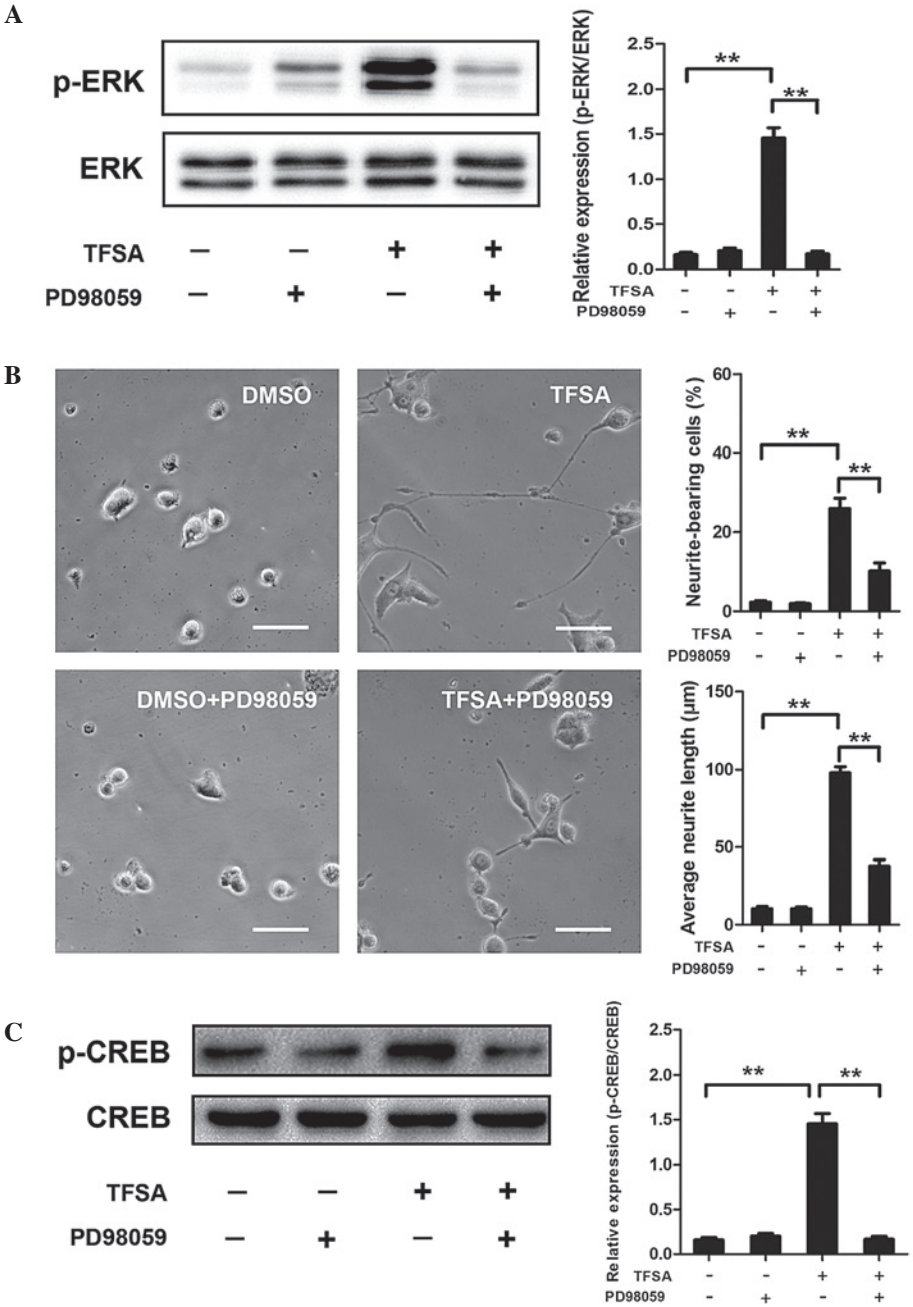

Figure 4. Effect of TFSA on MEK/ERK/CREB activation and MEK/ERK/CREB-mediated neurite outgrowth. (A) Phosphorylation of ERK in PC12 cells treated with DMSO, DMSO+PD98059, TFSA or TFSA+PD98059. Scale bar=50 μm. (B) Proportion of neurite-bearing cells and average neurite lengths in PC12 cells treated with DMSO, DMSO+PD98059, TFSA or TFSA+PD98059. (C) Phosphorylation of CREB in PC12 cells treated with DMSO, DMSO+PD98059, TFSA or TFSA+PD98059. The data are expressed as the mean ± standard deviation from three independent experiments (**P<0.01). TFSA, tenuifoliside A; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated ERK; MEK, mitogen-activated protein kinase kinase; CREB; cyclic AMP response element-binding protein; p-CREB, phosphorlyated CREB; DMSO, dimethyl sulfoxide; NS, non-significant.

or TFSA (10 μM). As shown in Fig. 1A, TFSA treatment led to the promotion of neurite extension of the PC12 cells 24 h post-treatment. The percentage of neurite-bearing cells reached 25.4+5.01% following treatment with 10 μM TFSA, which was significantly higher than percentage observed in the cells treated with the vehicle.

GAP-43, a marker of neurite outgrowth, is expressed in PC12 cells on differentiation towards a neuronal phenotype and exhibits increased synthesis and axonal transport during axonal regeneration (9). The present study investigated whether TFSA affected the expression of GAP43 in the PC12 cells. At 24 h post-treatment, the results of the western blot analysis demonstrated that TFSA induced a higher expression level of GAP-43, compared with that observed in the control group (Fig. 1B).

Activation of PI3K/Akt signalling with TFSA. Subsequently, the present study investigated the signaling pathway responsible for TFSA-induced neurite outgrowth. Previous studies have demonstrated the role of the activated PI3K/Akt pathway in inducing neural differentiation in PC12 cells. In the present study, the TFSA-treated cells demonstrated a high level of

phosphorlyation (p-Akt-S473), compared with the control cells (Fig. 2). To confirm the role of the PI3K/Akt signaling pathway in the neurite outgrowth induced by TFSA in the present study, LY294002., a specific pharmacological inhibitor of PI3K, was used. As shown in Fig. 3, LY294002 significantly inhibited TFSA-induced Akt phosphorylation and neurite outgrowth.

TFSA activates the MEK/ERK pathway. Previous studies have reported that signaling through MAPK classes is important in the neural differentiation of PC12 cells induced by NGF. The present study investigated whether the MEK/ERK pathway was involved in TFSA-induced neurite outgrowth in the PC12 cells. The results of the western blot analysis indicated that the phosphorylation of ERK1/2 was significantly increased in the PC12 cells, which were treated with TFSA (Fig. 2). In addition, the cells treated with PD98059, which is an MAPK/ERK kinase inhibitor, significantly inhibited TFSA-induced neurite formation (Fig. 4A and B).

TFSA promotes the phosphorylation of CREB by activating MEK/ERK. Activation of the MEK/ERK pathway in PC12 cells leads to the phosphorylation of CREB, which is critical in neurogenesis (10,11). Therefore, the present study investigated the effect of TFSA on the phosphorylation of CREB. As shown in Fig. 4C, western blot analysis revealed that the phosphorylation level of CREB was significantly increased in the TFSA treated-group of cells. To further investigate the dependence of CREB phosphorylation on the activation of the MEK/ERK pathway, the effects of PD98059 on TFSA-induced phosphorylation of CREB was detected. The results indicated that the phosphorylation of CREB was almost completely inhibited following treatment with PD98059, suggesting that the TFSA-induced phosphorylation of CREB was MEK/ERK dependent (Fig. 4C).

Discussion

TFSA is an oligosaccharide ester in *Polygala*. As a widely used traditional Chinese medicine, *Polygala* can be used to treat diseases, including amnesia, neurasthenia, palpitation and insomnia (3,4). In the present study, the effect of TFSA on neuritogenesis in PC12 cells was investigated. The results revealed that treatment with TFSA induced neuronal differentiation and increased the expression of the neuronal marker, GAP43, in PC12 cells. Furthermore, the results demonstrated that the neurite formation induced by TFSA was associated with the activation of the PI3K/AKT and MEK/ERK/CREB signaling pathways.

PC12 cells are a cell line derived from the rat pheochromocytoma of the adrenal medulla. These cells have been extensively used as a neural cell model of neurite outgrowth (12-14). Undifferentiated PC12 cells do not produce neurites (<1%), as reported in a previous study (15). The results of the present study demonstrated that TFSA induced neurite outgrowth in the PC12 cells. In addition, no significant difference was observed between TFSA and NGF in the promotion of neurite outgrowth. To further investigate the neurite outgrowth-inducing effect of TFSA, the present study examined the expression of GAP43 in the PC12 cells. Western blot analysis revealed that treatment of the PC12 cells with TFSA was associated with significant increases in the expression levels of GAP-43. GAP-43 is a major protein kinase C substrate of growing axons, which has been demonstrated to be important in growth cone formation and neurite outgrowth (16,17).

The present study also investigated the potential signaling pathway responsible for TFSA-induced neurite outgrowth in the PC12 cells. Previous studies have demonstrated that NGF activates different extracellular and intracellular signaling pathways leading to neurite outgrowth, including the PI3K/Akt and MEK/ERK pathways (18-21). In the present study, treatment with TFSA led to a significant increase in the phosphorylation of Akt at Ser473. In addition, the LY294002 PI3K inhibitor suppressed TFSA-induced neurite outgrowth, indicating the involvement of the PI3K/Akt pathway in the neuritogenic effect of TFSA. It is also known that the binding of NGF to its receptor tyrosine kinase, TrkA, activates the ERK1/2 pathway, and activation of the ERK1/2 pathway has been demonstrated to be to critical in the differentiation of PC12 cells, as the PD98059 ERK inhibitor inhibits NGF-induced neuronal differentiation (22,23). To further investigate whether the TFSA-induced neurite outgrowth involved the activation of ERK1/2 MAPK, the present study assessed the phosphorylation state of ERK1/2 in the PC12 cells. The results of the western blot analysis revealed that the degree of ERK1/2 phosphorylation increased significantly, compared with that observed in the control group, and was comparable to that detected following treatment with NGF. In addition, the outgrowth of neurites induced by TFSA was significantly inhibited by application of the PD98059 MEK inhibitor (10 μM).

As previously demonstrated, activation of the MEK/ERK pathway, induced by NGF, leads to the phosphorylation of CREB, which in turn stimulates the ability of CREB to activate transcription in NGF-treated cells (24-26). In the present study, TFSA was observed to have a similar effect. Incubation of PC23 cells with TFSA resulted in an increase in the phosphorylation of CREB, which was comparable to that induced by NGF. To elucidate whether the phosphorylation of CREB resulted from MEK/ERK activation, the PD98059 MEK inhibitor was used, which almost completely inhibited the phosphorylation of CREB induced by TFSA. These results suggested that the activation of CREB may occur via the MER/ERK pathway.

In conclusion, the present study demonstrated that TFSA promoted neurite outgrowth in the PC12 cells via the activation of pathways involved in NGF-induced neuritogenesis. Treatment with TFSA activated the MEK/ERK and PI3K/Akt pathways and triggered the phosphorylation of CREB, followed by MEK/ERK activation, in the PC12 cells.

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