The Kampo medicine Yokukansan (YKS) enhances nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells

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ABSTRACT

Accumulating evidence indicates that neurotrophic factor-like substances involved in the induction of neurotrophic factor synthesis may aid in the treatment of neurological disorders, such as Alzheimer's disease. Yokukansan (YKS), a traditional Kampo medicine, has been used for the treatment of anxiety and mood disorders. In the present study, we aimed to identify the signaling pathways associated with YKS-mediated enhancement of nerve growth factor (NGF)-induced neurite extension in rat pheochromocytoma (PC12) cells. Akt and extracellular-regulated kinase 1/2 (ERK1/2) phosphorylation levels were assessed by western blot analysis, in the presence of YKS and following the treatment with TrkA inhibitor, K252a. YKS treatment (NGF+YKS 0.5 group) enhanced NGF-induced neurite outgrowth and phosphorylation/activation of Akt and ERK1/2 in PC12 cells. Moreover, YKS-induced effects were inhibited by the treatment with the TrkA receptor antagonist K252a (NGF+YKS 0.5+K252a group); no significant difference in neurite outgrowth was observed between K252a-treated (NGF+YKS 0.5+K252a group) and NGF-K252a-treated cells (NGF+K252a group). However, neurite outgrowth in K252a-treated cells (NGF+K252a and NGF+YKS 0.5+K252a group) reached only one-third of the level in NGF-treated cells (NGF group). NGF-mediated Akt phosphorylation increased by YKS was also inhibited by K252a treatment (NGF+YKS 0.5+K252a group), but no significant difference in ERK1/2 phosphorylation was observed between NGF-YKS-K252a- and NGF-treated cells (NGF group). Our results indicate that YKS treatment enhanced NGF-induced neurite outgrowth via induction of Akt and ERK1/2 phosphorylation, following the binding of NGF to the TrkA receptor. These findings may be useful in the development of novel therapeutic strategies for the treatment of Alzheimer's disease.

KEY WORDS: NGF; Neurite outgrowth; Kampo; Yokukansan; Akt; ERK1/2; PC12 cells; YKS; TrkA inhibitor; nerve growth factor

INTRODUCTION

Yokukansan (YKS) is a traditional Kampo medicine (Kampo) composed of seven dried medicinal herbs: *Atractylodes lancea* (*A. lancea*) rhizome, *Poria cocos* (*P. cocos*) sclerotium, *Cnidium* rhizome, *Uncaria* hook, Japanese *Angelica* root, *Bupleurum* root, and *Glycyrrhiza* [1]. YKS has been used for the treatment of anxiety disorders and insomnia, as well as night-time crying, emotional distress, and agitation in infants [1-5]. A recent meta-analysis [6,7] of randomized controlled trials reported that YKS improved behavioral and psychological symptoms associated with dementia (e.g., hallucinations, agitation, aggressiveness, and anxiety) in patients with Alzheimer's disease (AD), Lewy body dementia, and other forms of age-related cognitive decline. In addition, non-clinical studies [8] have reported that YKS exerts a neuroprotective effect against oxidative stress and endoplasmic reticulum stress [9]. Moreover, Kubota et al. demonstrated that YKS elicits a nerve growth factor (NGF)-like effect *in vitro* [10]. Further studies have documented neuropharmacological acitivity of YKS, including the promotion of neuroplasticity; regulation of neurotransmitter release in serotonergic, glutamatergic, cholinergic, dopaminergic, and gamma-aminobutyric acid neurons; and attenuation of stress and inflammation-induced damage [8,11-15]. However, the

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mechanisms underlying these neuropharmacological actions of YKS remain to be determined.

NGF is a member of the neurotrophin family [16] that regulates cell proliferation and differentiation within specific neural tissues, under both physiological and pathological conditions [17]. As NGF is associated with neuronal growth and maintenance, it is hypothesized to play an important role in the pathogenesis and treatment of neurodegenerative diseases, including AD, Parkinson's disease, and depression [18,19]. However, up until now, no *in vitro* analysis has demonstrated enhancement of NGF-mediated neurite extension following treatment with YKS.

Rat pheochromocytoma cells (PC12 cells) represent a useful *in vitro* model of neuronal differentiation, activity within adrenergic, cholinergic and dopaminergic systems, and signaling associated with various neurobiochemical and neurobiological processes [20-23]. Exposure to NGF causes PC12 cells to differentiate into sympathetic neuron-like cells that exhibit increased neurite outgrowth. NGF plays pivotal roles in the proliferation and differentiation of PC12 cells by inducing the phosphorylation and activation of protein and lipid kinase pathways, such as those involving extracellular-regulated kinase 1/2 (ERK1/2) and protein kinase B (PKB or Akt) [19,24,25]. Activation of ERK1/2 and Akt is known to regulate the survival of cells and cholinergic activity [26-28].

As NGF plays a key role in the proliferation, differentiation, and regulation of neural stem cells, several studies have indicated the clinical potential of NGF treatment in the regeneration of nerve cells following injury [29,30]. For example, a recent clinical trial has reported promising results regarding the use of exogenous NGF to induce trophic responses in damaged neurons of the central nervous system in patients with AD [31]. However, as NGF displays numerous actions and affects various biochemical pathways, the pharmacological effects of this small molecule are difficult to control [32-34]. Based on previous findings, we hypothesized that treatment with YKS would enhance NGF-related functions. Therefore, in the present study, we aimed to identify the potential signaling pathways associated with YKS-mediated enhancement of NGF-induced neurite extension. Our findings suggested that this signaling mechanism may also account for the clinical effects of YKS on psychiatric and neurological symptoms.

MATERIALS AND METHODS

Materials

Murine NGF 2.5S (NGF derived from mouse submaxillary glands) was obtained from Alomone Labs (Jerusalem, Israel). K252a was purchased from LC Laboratories (Woburn, MA, USA). YKS was obtained as a powdered extract made from a mixture of *A. lancea* rhizome (4.0 g, rhizome of *A. lancea*

de Candolle, family Compositae), *P. cocos* (4.0 g, sclerotium of *P. cocos* Wolf, family Polyporaceae), *Cnidium* rhizome (3.0 g, rhizome of *Cnidium officinale* Makino, family Umbelliferae), *Uncaria* hook (3.0 g, hook of *Uncaria rhynchophylla* Miquel, family Rubiaceae), Japanese *Angelica* root (3.0 g, root of *Angelica acutiloba* Kitagawa, Umbelliferae), *Bupleurum* root (2.0 g, root of *Bupleurum falcatum* Linnaeus, family Apiaceae), and *Glycyrrhiza* (1.5 g, root and stolon of *Glycyrrhiza uralensis* Fisher, Leguminosae) [Tsumura & Co., Japan, lot number: 2110054010]. To prepare YKS stock solution, YKS was suspended in Dulbecco's Modified Eagle Medium supplemented with nutrient mixture F-12 (DMEM/F12; Gibco, Life Technologies, Carlsbad, CA, USA) and 1% (v/v) penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan) and shaken at room temperature overnight. On the following day, the suspension was centrifuged ($2500 \times g$ for 20 minutes), and the resulting supernatant was filtered through a membrane with a pore size of 0.22 μm (EMD Millipore Corporation, Billerica, MA, USA).

Cell culture

PC12 cells obtained from the Riken Cell Bank (Ibaraki, Japan) were maintained in DMEM/F-12 supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Life Technologies) and 1% (v/v) penicillin-streptomycin. The cells were then incubated at 37°C in an atmosphere of 5% CO_2/95% air.

Cell viability assay

PC12 cells were seeded into 96-well plates (Corning, NY, USA) at a density of 1.0×10^4 cells/well for 24 hours. The cells were then incubated with various concentrations of YKS (0.001-2.0 mg/mL) for additional 24 hours, following which the cell viability was assessed using the cell proliferation reagent WST-1 (Roche Applied Science, Mannheim, Germany), in accordance with the instructions provided by the manufacturer. Briefly, culture medium was removed from wells following treatment, and 100 μL of medium containing 10 μL of WST-1 was added to each well. After additional 4 hours, the absorbance was determined at 450 nm.

Evaluation of morphological differentiation (neurite outgrowth) in PC12 cells

PC12 cells were seeded into 60-mm culture plates coated with type I collagen (Iwaki, Tokyo, Japan), at a density of 1.5×10^5 cells/plate in DMEM/F-12 containing 10% FBS. Following 24 hours of incubation, PC12 cells were treated with differentiation medium (DMEM/F-12 containing 5% FBS plus 50 ng/mL NGF with or without YKS at 0.01-0.5 mg/mL). In addition, the cells were treated with various concentrations of NGF (5-500 ng/mL) in the presence or absence of YKS

(0.5 mg/mL). Neurite length was measured 24 hours after the administration of treatment regimens. The cells were incubated for additional 24 hours, and then the images were obtained with a phase-contrast microscope (ECLIPSE TS100, Nikon, Tokyo, Japan) equipped with a digital camera (Digital Sight DS-L2 system, Nikon, Tokyo, Japan).

Images containing an average of 10-15 PC12 cells within each field were obtained from five randomly selected fields per culture plate. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to measure the degree of neurite extension from cells in each field. For every field, the average neurite length was calculated by dividing the total neurite length by the total number of cells in the field. Finally, the average neurite length per treatment condition was obtained by averaging the results from all fields for a specific condition (n = 5). Maximal response (E_{max}) values indicative of maximum length of neurite outgrowth and the concentration of NGF producing half E_{max} (EC_{50}) were determined using KaleidaGraph software version 4.5.1 (Synergy Software, Reading, PA, USA), based on the E_{max} model for dose–response curve: $E = E_{max} \times ([NGF]/(EC_{50}+[NGF]))$, where E represents the neurite length. Unless otherwise specified, data are presented as mean ± standard deviation (SD), 95% confidence interval (95%, CI), or coefficient of determination (R^2) of mean values, where appropriate.

Western blot analysis

PC12 cells were seeded into tissue culture plates coated with type I collagen, at a density of 1.5×10^5 cells/plate in DMEM/F-12 containing 10% FBS. After 24 hours of incubation, PC12 cells were treated with differentiation medium (DMEM/F-12 containing 5% FBS plus 50 ng/mL NGF with or without YKS at 0.5 mg/mL). The cells were harvested in ice-cold Tris-buffered saline (TBS; Bio-Rad, Hercules, CA, USA) at various times following NGF treatment (0, 5, 10, 20, 30, and 60 minutes), and after that they were lysed with PathScan Sandwich ELISA Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) on ice for 5 minutes. The samples were then subjected to centrifugation to remove cellular debris, and the protein concentration of each lysate was measured using a Bicinchoninic Acid Assay Kit (Pierce, Rockford, IL, USA). Protein samples containing 16 µg of total protein were separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (4-15%), and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Then, the PVDF membranes were blocked with bovine serum albumin [5%] (Wako Pure Chemical Industries, Osaka, Japan) in TBS containing Tween-20 (0.1%) at room temperature for 1 hour.

Immunoblotting was performed using primary antibodies against phospho-Akt [p-Akt] (Ser473; 1:1000, Cell Signaling Technology), Akt (1:1000, Cell Signaling Technology), p-ERK1 [T202/Y204]/ERK2 [T185/Y187] (1:2000, R&D Systems, Minneapolis, MN, USA), and ERK1/2 (1:2000, R&D Systems). The immunoreactivity was tested using horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and visualized with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech) and Fuji RX-U X-ray film (Fuji Film, Tokyo, Japan). Data were analyzed using ImageJ software.

Inhibition of tropomyosin receptor kinase A (TrkA)-receptor in PC12 cells

We evaluated the effect of TrkA receptor inhibition on NGF-mediated regulation of neurite extension. PC12 cells were pretreated with K252a (100 nM), a pharmacological inhibitor of the TrkA receptor, 30 minutes before the addition of NGF (50 ng/mL) with or without YKS (0.5 mg/mL). Following incubation with NGF for additional 24 hours, neurite extension was analyzed as described earlier. In addition, some cells were harvested 20 minutes after NGF treatment and subjected to Western blot analysis using the following primary antibodies: Anti-p-Akt, anti-Akt, anti-p-ERK1/2, and anti-ERK1/2.

Statistical analysis

Quantitative data, expressed as mean ± SD, were evaluated using analysis of variance (ANOVA) followed by Tukey's post hoc tests. The level of statistical significance was set at $p < 0.05$.

RESULTS

YKS enhances NGF-induced neurite outgrowth in PC12 cells

To evaluate the effects of YKS on PC12 cell viability the cells were analyzed with WST-1 assay, following incubation with YKS for 24 hours. YKS did not affect cell proliferation at concentrations <0.5 mg/mL, whereas cytotoxicity was observed at concentrations of 1.0 mg/mL or higher (Figure 1). Given these observations, we selected a YKS concentration range of 0.01-0.5 mg/mL for subsequent experiments.

To investigate the potential enhancement effects of YKS and NGF on neurite outgrowth, PC12 cells were cultured for 24 hours in the absence or presence of NGF and with increasing concentrations of YKS (0.01-0.5 mg/mL). In agreement with previous studies [35-37], the treatment with NGF alone significantly increased the total neurite outgrowth in PC12 cells; however, little neurite outgrowth was observed in vehicle controls (Figure 2A and B). NGF-induced neurite outgrowth was significantly enhanced by the treatment with YKS at 0.1 and 0.5 mg/mL (NGF+YKS 0.1 and 0.5, respectively). In contrast, no apparent differences in the neurite extension were observed

between the cells treated with YKS alone (0.5 mg/mL) and vehicle controls (Figure 2A and B). Furthermore, YKS significantly enhanced the NGF-induced outgrowth (NGF+YKS) at all tested NGF concentrations, i.e. 5-500 ng/mL (Figure 2C). The E_{max} responses differed significantly between NGF and NGF+YKS treatments (732.8 μm [95% CI: 680.9-785.1] and 974.0 μm [95% CI: 905.0-1043.0], respectively); however, the concentrations of NGF corresponding to EC_{50} values, for the two treatment conditions, were not significantly different (19.7 ± 5.1 ng/mL [95% CI: 14.3-25.7] and 13.9 ± 3.9 [95%, CI: 9.5-18.5] ng/mL, respectively). The curve fitting analysis of the observed plots using the E_{max} model showed comparably good coefficients of determination for both NGF and NGF+YKS groups (R^2 0.95822 and 0.94777, respectively). Overall, YKS increased the E_{max} for neurite outgrowth induced by NGF treatment, whereas no significant change was observed for the EC_{50} of NGF. These results suggest that YKS and NGF acted in a cooperative rather than additive manner to increase the neurite extension. That is, if YKS was an agonist, the E_{max} would remain unchanged.

YKS potentiates NGF-induced phosphorylation of Akt and ERK1/2

We then evaluated Akt and ERK1/2 phosphorylation in PC12 cells over several time points, in relation to NGF-induced neurite extension and in the presence and absence of YKS. The activation of Akt and ERK1/2 signaling was analyzed using western blotting. Following the addition of NGF in the absence of YKS, the levels of p-Akt gradually increased and peaked at 20 minutes (Figure 3A and B). Subsequently, p-Akt levels decreased to 43% of the peak value 60 minutes after the NGF treatment (Figure 3B). After the addition of both YKS and NGF (NGF + YKS 0.5) to PC12 cells, p-Akt levels also peaked 20 minutes after NGF treatment and decreased thereafter. However, the p-Akt levels in NGF+YKS 0.5 group were 1.9- to 2.4-fold higher than those observed in the absence of YKS, at all time points (Figure 3B).

The amount of phosphorylated ERK1/2 (p-ERK1/2) was the highest 10 minutes after the addition of NGF, both in the presence and absence of YKS (Figure 3C and D). However, the treatment with NGF and YKS increased ERK1/2 phosphorylation approximately 2.3-fold, relative to that induced by NGF alone at the same time points (Figures 3C and D). These results demonstrated that the addition of YKS to NGF upregulated NGF-induced stimulation of Akt and ERK1/2 phosphorylation in PC12 cells.

Effects of a TrkA receptor antagonist on NGF-induced neurite outgrowth and phosphorylation of Akt/ERK1/2 following YKS treatment

We then examined the effect of K252a, a typical TrkA receptor antagonist, on YKS-mediated enhancement of NGF-induced neurite extension. PC12 cells were treated with K252a
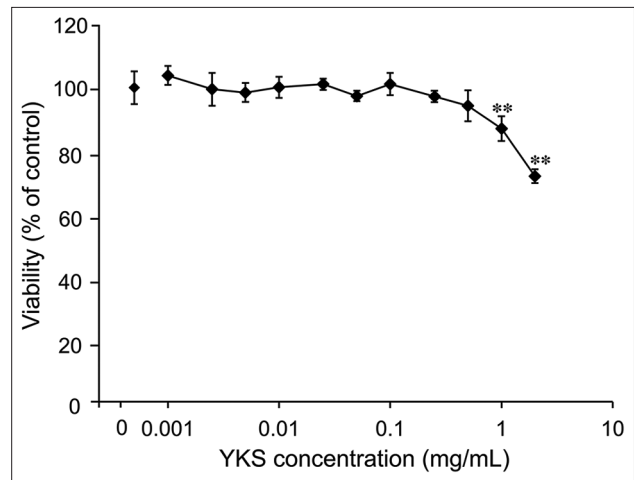

FIGURE 1. Viability of PC12 cells treated with Yokukansan (YKS). PC12 cells were treated with YKS for 24 hours. Cell viability was determined using WST-1 assay, and the results are expressed as a percentage of the control value (0 mg/mL). The experiments were repeated at least three times, and the values reported represent the mean of the values obtained from three experiments ± SD (**$p < 0.01$ versus control).

in the absence and presence of YKS (0.5 mg/mL) for 24 hours, following which they were incubated with NGF for additional 24 hours. As shown in Figure 2A and B, NGF evoked neurite extension in PC12 cells, and YKS enhanced this NGF-induced effect (Figure 4A and B). However, the effects of YKS were completely inhibited by the treatment with K252a (Figure 4A and B). K252a also attenuated the effects of YKS on NGF-induced phosphorylation of Akt (80% decrease) and ERK1/2 [91% decrease] (Figure 4C and D). Moreover, the treatment with K252a inhibited the potentiation of ERK1/2 phosphorylation observed following the addition of NGF plus YKS to a lesser extent (39% decrease) than for Akt phosphorylation. No significant differences in the levels of ERK1/2 phosphorylation were observed between the NGF treatment and combined treatment with K252a, NGF, and YKS (Figure 4C). The levels of Akt phosphorylation did not significantly differ between the cells treated with combined K252a, NGF, and YKS (88% decrease) and combined NGF and K252a, but were significantly lower compared to the levels observed in the cells treated with NGF alone (Figure 4D).

These findings suggest that the YKS enhances NGF-induced neurite outgrowth through interactions at TrkA receptors, and that these interactions potentiate Akt and ERK1/2 phosphorylation. However, the partial inhibition of ERK1/2 phosphorylation observed following the treatment with K252a suggests an additive interaction. Thus, signaling pathways other than TrkA receptor signaling are presumably affected by YKS.

DISCUSSION

Previous studies have demonstrated a strong correlation between the loss of cholinergic neurons and memory impairment in patients with AD [38]. Many studies have
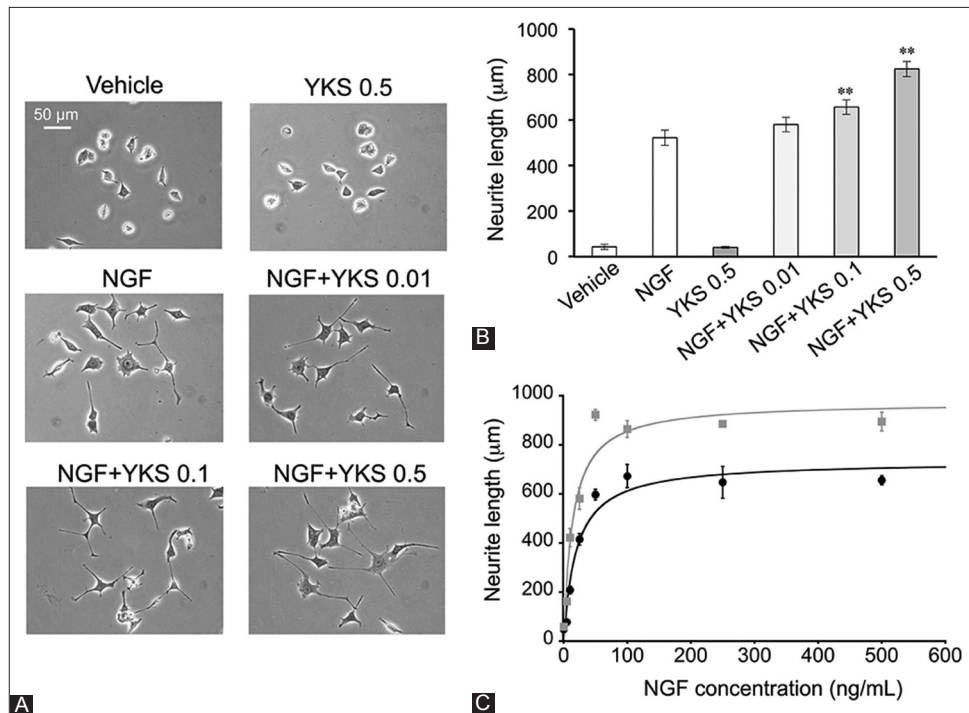
FIGURE 2. Effect of Yokukansan (YKS) on nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells. PC12 cells were treated for 24 hours with NGF, with or without YKS. (A) Images of the cultures at a magnification of ×400. Scale bars: 50 μm. (B) Neurite length was determined as indicated in the materials and methods section. The values represent mean ± standard deviation for all PC12 cells contained within five randomly selected fields for each condition. All experiments were repeated at least three times (**$p < 0.01$ versus NGF). (C) Neurite length in the presence of various concentrations of NGF (0-500 ng/mL).
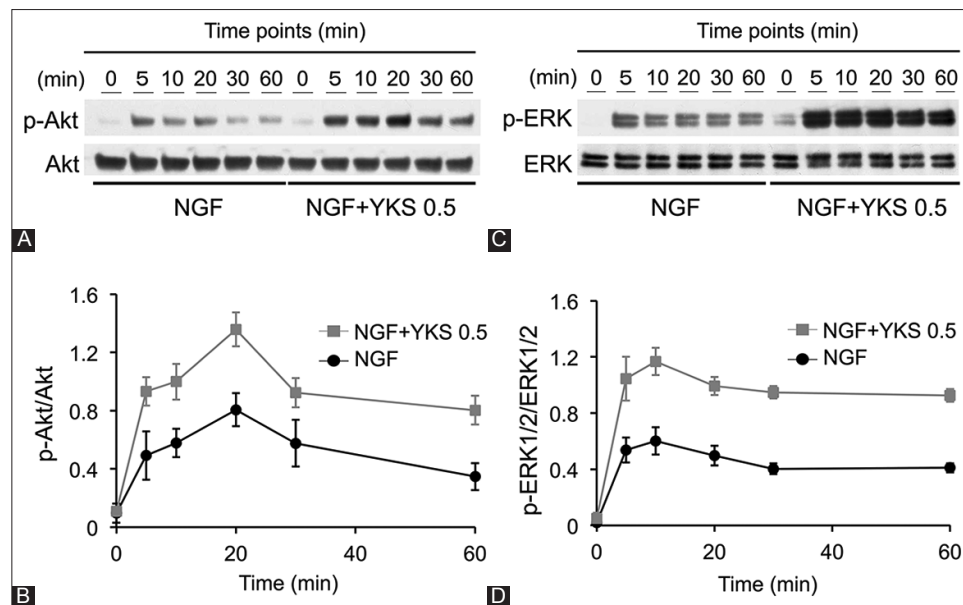

FIGURE 3. Effect of YKS on NGF-induced phosphorylation of Akt and ERK1/2 in PC12 cells. (A, C) PC12 cells were treated for various time periods (0, 5, 10, 20, 30, or 60 minutes) with NGF in the presence or absence of 0.5 mg/mL YKS; (A) the whole-cell extracts were subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and treated with primary anti-p-Akt and anti-Akt antibodies, or (C) with primary anti-p-ERK1/2 and anti-ERK1/2 antibodies. The extracts were then treated with horseradish peroxidase-conjugated secondary antibody. (B) The intensity of the p-Akt polypeptide band was normalized to the total Akt band (p-Akt/Akt), whereas (D) the intensity of the p-ERK1/2 polypeptide band was normalized to the total ERK1/2 band (p-ERK1/2/ERK1/2). The results are presented as the mean ± SD of three independent experiments. YKS: Yokukansan; NGF: Nerve growth factor; Akt or PKB: Protein kinase B; ERK1/2: Extracellular-regulated kinase 1/2; SDS: Sodium dodecyl sulfate; PVDF: Polyvinylidene fluoride; p: phospho.

further demonstrated that NGF improves cholinergic function [38,39] and prevents lesion-induced degeneration [40]. While the levels of NGF mRNA are reportedly unchanged in AD, TrkA expression is reduced. Furthermore, previous studies have shown clinically significant effects of YKS treatment on symptoms of dementia in patients with AD [6,7].
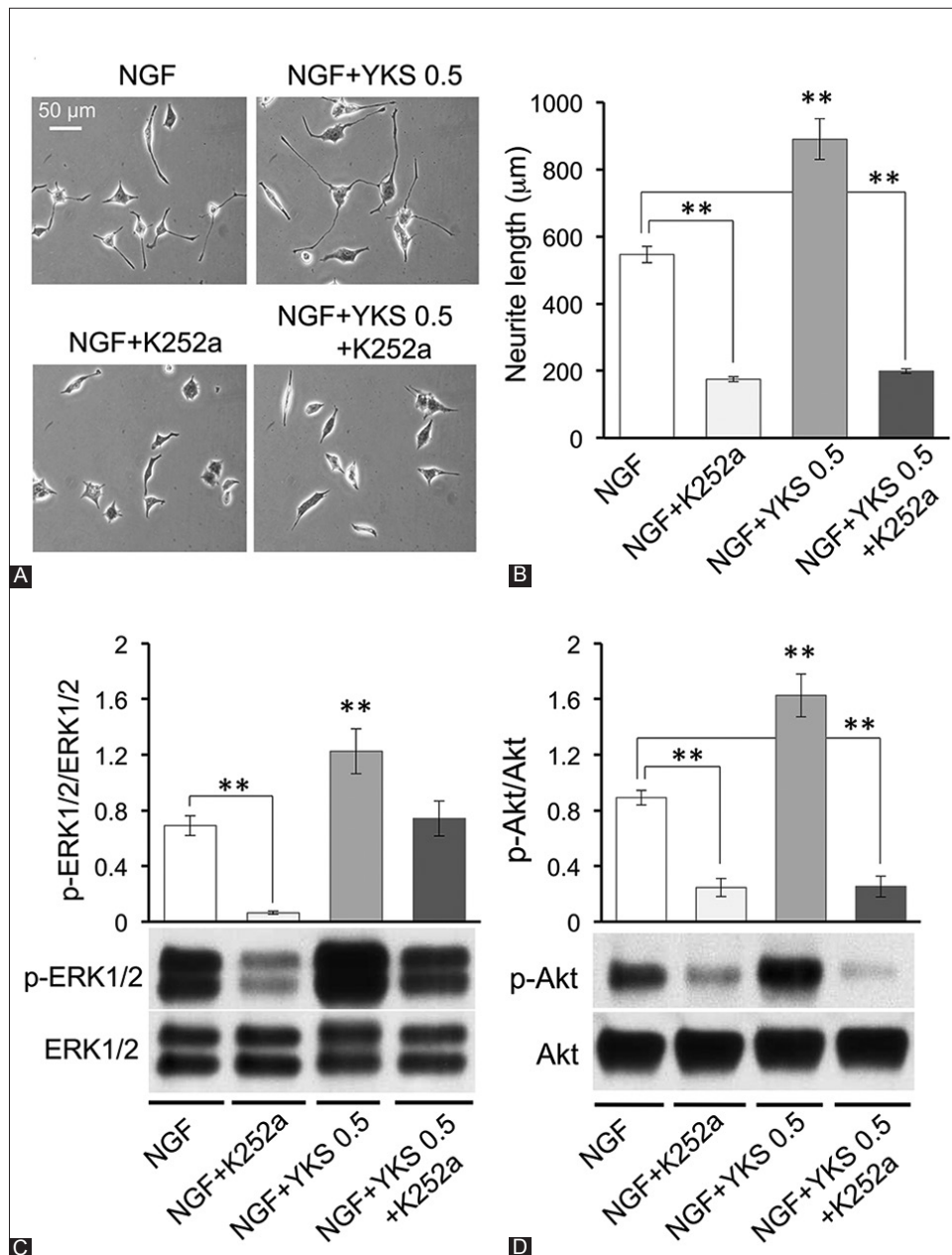
FIGURE 4. Effect of the TrkA inhibitor K252a on YKS-mediated potentiation of NGF-induced neurite outgrowth/Akt and ERK1/2 phosphorylation in PC12 cells. PC12 cells were preincubated in the presence or absence of K252a for 30 minutes. NGF and YKS were added, and the cells were incubated for additional 24 hours before neurite outgrowth assays or 20 minutes before western blot analysis. (A) Images of the cultures at ×400 magnification. Scale bars: 50 μm. (B) Neurite length was determined as detailed in the materials and methods section. For each condition, values are reported as the mean ± SD of the values for all PC12 cells included within five randomly selected fields (**$p < 0.01$ compared with NGF). The whole-cell extracts were subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and treated with (C) primary anti-p-ERK1/2 and anti-ERK1/2 antibodies, or (D) with primary anti-p-Akt and anti-Akt antibodies. The extracts were then treated with horseradish peroxidase-conjugated secondary antibody. (C) The intensity of the p-ERK1/2 polypeptide band was normalized to the total ERK1/2 band (p-ERK1/2/ERK1/2), whereas (D) the intensity of the p-Akt polypeptide band was normalized to the total Akt band (p-Akt/Akt). The values represent the mean ± SD of three independent experiments (**$p < 0.01$ compared with NGF). YKS: Yokukansan; NGF: Nerve growth factor; Akt or PKB: Protein kinase B; ERK1/2: Extracellular-regulated kinase 1/2; SDS: Sodium dodecyl sulfate; PVDF: Polyvinylidene fluoride; p: phospho.

In the present study, using cell culture models, we showed that the enhancement of NGF-induced outgrowth by YKS and subsequent neurochemical changes were mediated by Akt and ERK1/2 and blocked by K252a. To the best of our knowledge, this is the first study to demonstrate an interaction between YKS and TrkA receptor-related signaling pathways in the potentiation of Akt and ERK1/2 phosphorylation and NGF-induced neurite outgrowth. In agreement with our findings, Hashimoto et al. [41] reported that the addition of K252a to cell cultures almost completely blocked the generation of neurites elicited by NGF [41]. Because YKS is a mixture of natural compounds, the observed effects may be due to the actions of a single or multiple components. Therefore, future studies should aim to identify the active compounds in YKS.
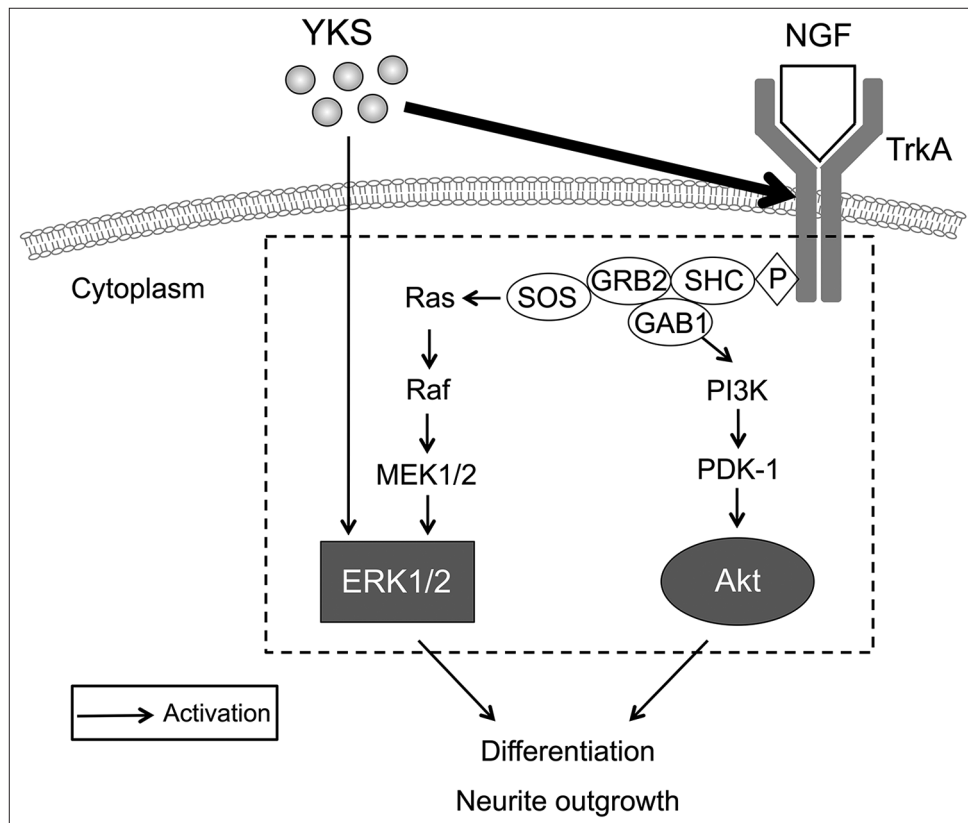
FIGURE 5. The proposed signaling mechanism involved in YKS-mediated potentiation of NGF-induced neurite outgrowth and TrkA/NGF signaling activation. YKS associates with TrkA receptors to activate the phosphorylation of downstream signaling molecules (e.g., Ras/ERK1/2 and PI3K/Akt) within the cytoplasm. In this model, YKS-mediated potentiation of NGF-induced neurite outgrowth occurs through the enhancement of the phosphorylation of Akt and/or ERK1/2. Enhanced phosphorylation of the upstream TrkA receptor may also play a role in the effect of YKS on NGF-induced neurite extension. YKS: Yokukansan; TrkA: Tropomyosin receptor kinase A; NGF: Nerve growth factor; Akt or PKB: Protein kinase B; ERK1/2: Extracellular-regulated kinase 1/2; PI3K: Phosphatidylinositol-3-kinase; SOS: Son of Sevenless; Grb2: Growth factor receptor-bound protein 2; P: Phosphorus; GAB1: GRB2-associated-binding protein 1; MEK or MAP2K: Mitogen-activated protein kinase kinase; PDK1 or PDPK1: Phosphoinositide-dependent kinase-1.

Several previous studies have examined the mechanisms underlying NGF-induced neurite outgrowth in PC12 [35,37,42]. During PC12 cell differentiation, Ras and phosphatidylinositol-3-kinase (PI3K) signaling plays a key role in the regulation of NGF-induced neurite extension [17,43,44]. Following the extension, Akt and ERK1/2 are activated downstream of Ras and PI3K, respectively [45,46]. In the present study, we observed a significant increase in neurite outgrowth following the addition of NGF (Figure 2), along with an increase in the phosphorylation/activation of Akt and ERK1/2 (Figures 3 and 4). Previous studies have indicated that the PI3K inhibitor Wortmannin inhibits NGF-induced activation of Akt [47], and that the Ras inhibitor lovastatin blocks NGF-induced activation of ERK1/2 and neurite outgrowth [48,49]. Together with our previous findings [37], these results suggest that the promotion of neurite outgrowth by NGF in PC12 cells is linked to the activation of PI3K, Akt, and ERK1/2 signaling pathways.

In the present study, YKS treatment enhanced NGF-induced neurite outgrowth and the phosphorylation of Akt and ERK1/2 in PC12 cells. However, the neurite outgrowth and activation of Akt and ERK1/2 were negligible following 24 hours of incubation with YKS in the absence of NGF (data not shown). This is in contrast to the results reported by Kubota et al., which demonstrated neurite extension following long-term treatment (6 days) with YKS in the absence of NGF [10]. Our findings indicated that YKS enhanced NGF-induced phosphorylation of Akt and ERK1/2 in a cooperative manner, rather than directly binding to TrkA receptors as an NGF agonist. Moreover, the pharmacodynamic effects of YKS showed that YKS changed the E_{max} (i.e. efficacy) but not the EC_{50} (i.e. potency) of NGF for the induction of neurite outgrowth. Thus, we propose that YKS increases E_{max} by increasing the number of NGF-TrkA receptor complexes and/or cellular response. Our data also suggested that the affinity of NGF for the TrkA receptor was unchanged by YKS, as we observed no change in the EC_{50} of NGF in the presence of YKS. The proposed mechanism is consistent with our finding that K252a treatment completely inhibited the NGF-induced neurite outgrowth in both the presence and absence of YKS. Kubota et al. showed that K252a completely inhibited NGF-induced, but not YKS-induced, neurite outgrowth in

PC12 cells [10]. They reported only a minor inhibitory effect of K252a on YKS-induced Akt and ERK1/2 activation; while, K252a significantly inhibited Akt and ERK1/2 activation when NGF was applied in the absence of YKS [10]. On the contrary, our findings showed that the YKS-enhanced effects of NGF at Akt receptors were markedly inhibited by the K252a treatment. We further observed that the activation of ERK1/2 after the treatment with both YKS and NGF was only moderately inhibited by K252a (Figure 4C and D). These results indicated that YKS enhanced the NGF-induced neurite outgrowth by increasing the Akt and ERK1/2 activation through the TrkA receptor. Kim et al. [47] reported that treatment of PC12 cells with the selective Akt inhibitor 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine (ML-9) completely blocked NGF-stimulated Akt phosphorylation and neurite outgrowth but did not substantially affect ERK phosphorylation [47]. This indicates that NGF-induced differentiation and neurite outgrowth in PC12 cells are not only regulated by the ERK signaling pathway but are also affected by the PI3K/Akt signaling pathway. We observed varying levels of inhibition of the Akt and ERK1/2 activation following the treatment with K252a, suggesting the existence of an additive interaction between YKS and ERK1/2 phosphorylation that is not coupled to the TrkA receptor (Figure 5). In accordance with the findings of Kubota et al. [10] and as suggested by our results, long-term treatment with YKS alone may promote neurite outgrowth through the activation of ERK1/2 and via TrkA-independent pathway in PC12 cells. However, further studies are required to clarify the mechanism of this direct effect of YKS on ERK1/2 activity.

In the present study, we demonstrated that combined treatment with YKS and NGF significantly enhances neurite outgrowth. Previous studies have suggested that the potentially active components of YKS include geissoschizine methyl (GM) ether and hirsuteine (HTE). GM and HTE are derived from *Uncaria* hook and have been shown to improve aggressive and social behavior by acting as partial agonists at serotonin 1A (5-HT$_{1A}$) receptors [50,51]. An *in vivo* pharmacokinetic study revealed that GM, HTE, and 18β-glycyrrhetinic acid from radix glycyrrhizae were detected in the plasma and brain after oral YKS administration. These findings support the notion that such ingredients are the components responsible for the psychotropic effects of YKS [52-54].

The involvement of the actin cytoskeleton during NGF-stimulated neurite extension in PC12 cells further supports the role of NGF/TrkA signaling in this process. In particular, myosin VI is known to interact with TrkA through the GAIP-interacting protein C terminus in neurons [32,55]. In addition, administration of flavonoid troxerutin to D-galactose-injected mice attenuates cognitive dysfunction and oxidative stress within the brain through the activation of the NGF/TrkA

signaling pathway [56]. These findings suggest that activation of the TrkA signaling pathway may play an important role in the treatment of AD.

CONCLUSION

The present study demonstrated that YKS treatment enhanced NGF-induced neurite outgrowth and that the molecular mechanism underlying this effect involves the enhancement of Akt and ERK1/2 phosphorylation/activation. Our data further indicated that such phosphorylation/activation processes are triggered by the binding of NGF to the TrkA receptor. As reduced TrkA expression has been associated with AD, identifying the components of YKS that act to increase signaling associated with TrkA receptor activation may lead to improved efficacy of treatments and the development of novel therapeutic strategies for AD.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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