Botanical Drug Puerarin Coordinates with Nerve Growth Factor in the Regulation of Neuronal Survival and Neuritogenesis via Activating ERK1/2 and PI3K/Akt Signaling Pathways in the Neurite Extension Process

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Keywords

ERK1/2; Neuritogenesis; Nrf2/heme oxygenase-1; PI3K/Akt; Puerarin.

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Received 19 August 2014; revision 12

September 2014; accepted 13 September

2014

SUMMARY

Aim: Nerve growth factor (NGF) regulates neuronal survival and differentiation by activating extracellular signal-regulated-kinases (ERK) 1/2 and phosphoinositide-3-kinase (PI3K)/Akt pathways in two distinct processes: latency process and neurite extension process. This study was designed to investigate whether botanical drug C-glucosylated isoflavone puerarin coordinates with NGF to regulate neuritogenesis via activating ERK1/2 and PI3K/Akt in neurite extension process. **Methods:** We investigated the neuroprotective and neurotrophic activities of puerarin in MPTP-lesioned mice and dopaminergic PC12 cells. The effects of puerarin on ERK1/2, Akt, Nrf2, and HO-1 were assessed by Western blotting. The neurite outgrowth was assayed by neurite outgrowth staining kit. **Results:** Puerarin protected dopaminergic cells and ameliorated the behavioral impairments in MPTP-lesioned mice. Puerarin potentiated the effect of NGF on neuritogenesis in PC12 cells by >10-fold. Mechanistic studies revealed: (1) puerarin rapidly activated ERK1/2 and Akt, leading to the activation of Nrf2/heme oxygenase-1 (HO-1) pathways; (2) ERK1/2, PI3K/Akt, and HO-1 inhibitors attenuated the neuritogenic activity of puerarin. Notably, puerarin enhanced NGF-induced neuritogenesis in a timing-dependent manner. **Conclusion:** Puerarin effectively coordinated with NGF to stimulate neuritogenesis via activating ERK1/2 and PI3K/Akt pathways in neurite extension process. These results demonstrated a general mechanism supporting the therapeutic application of puerarin-related compounds in neurodegenerative diseases.

doi: 10.1111/cns.12334

Introduction

Parkinson's disease (PD) is the second common neurodegenerative disease hallmarked by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [1,2]. The pathogenesis of PD has been attributed to several factors including

oxidative stress, inflammation, mitochondrial disruption, protein aggregation, excitotoxicity, and autophagic-lysosomal alterations [3,4]. Oxidative stress disrupts the functions of neurotrophins including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) and their tyrosine kinase receptors (Trks), thereby suppressing the survival and differentiation of neuronal

cells [5,6]. Consistently, the levels of BDNF and NGF were markedly decreased in the nigrostriatal regions and ventricular and lumbar cerebrospinal fluid of patients with PD [7,8]. The reduction of neurotrophins was also observed in PD models induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) [7]. These results highlight the pivotal roles of neurotrophins in neuronal functions, survival, and regeneration [9,10]. Genetic and direct introduction of various neurotrophins improved the parkinsonian symptoms in several animal PD models and reassured the growth of neurons in cell culture [11,12]. For example, NGF stimulates neuronal growth and differentiation and consequently delays neurodegeneration in PD [13,14]. The neuroprotective and neuritogenic activities of NGF are mainly mediated by the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways upon binding to its high affinity receptor TrkA [15–17]. Continuous NGF stimulation is critical to sustain the activation of ERK and subsequent induction of various transcriptional factors such as c-fos. Such stimulation induces a panel of NGF target genes to support neuronal differentiation [18–20]. A recent study revealed that NGF induced the neuritogenesis in dopaminergic cells via two distinct processes, namely, the early ERK-driven and transcription-dependent latency process, and the later ERK- and PI3K/Akt-driven and transcription-independent neurite extension process [21].

Many natural products are highly active neuroprotective and neuritogenic agents for promoting neuronal differentiation and maturation or potentiating the actions of NGF with low cost and few side effects [22–24]. Interestingly, various flavonoids can potentiate the effect of NGF on neuritogenesis and exhibit the antiparkinsonian activities [25–27]. Puerarin (Figure 1) is a C-glucosylated isoflavone derived from herbal medicine *Radix Puerariae* (the dried root of *Pueraria lobata* (Wild.) Ohwi). Puerarin is well known for a range of pharmacological activities such as vasodilatory, anticonvulsive, retinoprotective, cardioprotective, and neuroprotective activities [28–30]. Recent studies have demonstrated that puerarin activates MEK/ERK and PI3K/Akt pathways in many cell types including neuronal cells [31,32].

In this study, we tested the hypothesis that puerarin may coordinate with NGF to induce neuritogenesis by activating ERK and PI3K/Akt pathways in a timing-dependent manner. We investigated the effect of puerarin on the survival of the dopaminergic neurons in the SNpc and the deficits in motor performance in MPTP-induced parkinsonian model in mice. We carefully examined the timing dependence of the activation of ERK1/2 and PI3K/Akt pathways in the effect of puerarin on NGF-induced neuritogenesis through three different protocols.

Materials and Methods

Materials

Antibodies against ERK1/2, phospho-ERK1/2, Akt, phospho-Akt, β 3-tubulin, microtubule-associated protein 2 (MAP2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Alexa Fluor 594-conjugated goat anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Heme oxygenase 1 (HO-1) antibody was purchased from Stressgene (Ann

Arbor, MI, USA). Antityrosine hydroxylase (TH) antibody was purchased from BOSTER (Wuhan, China). Anti-Nrf2, antilamin B, and anti-rabbit horseradish peroxidase (HRP)-conjugated IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit HRP-conjugated IgG secondary antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Immunohistochemical DAB Detection Kit (Streptavidin-Biotin) was purchased from MAIXIN Biotechnology (Fuzhou, China). Sn(IV) protoporphyrin IX dichloride (SnPP) was obtained from Frontier Scientific (Logan, UT, USA). Puerarin, NGF-2.5S, collagen I, and other biochemicals were purchased from Sigma-Aldrich unless indicated otherwise.

Animals and Drug Intervention

Adult male C57BL/6 mice (8 weeks, 22–25 g) were supplied by the Laboratory Animal Center of Fujian University of Traditional Chinese Medicine (Fuzhou, Fujian, China) and housed in a temperature- and humidity-controlled environment on a 12 h light–dark cycle, and fed with a standard laboratory mice chow and free-drinking water. All animal experimentation procedures were conducted in compliance with the guidelines of the Animal Ethics Committee of Fujian University of Traditional Chinese Medicine on the Care and Use of Animals for Laboratory Research. Prior to the drug intervention in mice, 1.5 g puerarin was dissolved in 100 mL of 50% 1, 3-propanediol saline solution (v/v) to give the final concentration of 15 mg/mL. The solution was sterilized by passing through a 0.22- μ m filter. Mice were randomly divided into four experimental groups ($n = 6$): control group; MPTP group; MPTP + puerarin (L) group, and MPTP + puerarin (H) group. Mice were administered with MPTP (25 mg/kg/day in 100 μ L saline) through intraperitoneal (i.p.) injection every afternoon for seven consecutive days, whereas control animals received seven injections of the same volume of saline containing 50% 1,3-propanediol. Puerarin (50 mg/kg/day or 150 mg/kg/day in 100 μ L saline containing 50% 1,3-propanediol) or the same volume of saline containing 50% 1,3-propanediol was administered via i.p. injection every morning over 7 days before MPTP injection. The mice were sacrificed at 24 h after the last MPTP injection.

Behavioral Assessment in Forced Swim Test

The effects of drugs on animal behavior were assessed in a blind fashion by experienced examiners who were not involved in the design of the experiments. The mice were forced to swim in an acrylic plastic cylinder filled with water as described [33]. Briefly, mice were placed individually into plastic cylinders (height, 25 cm; diameter, 10 cm) filled with 10 cm of water, conditioned at $21 \pm 2^\circ\text{C}$ and left there for 5 min. A mouse was classified to be immobile when it floated in an upright position and only made small movements to keep its head above water. The duration of immobility was recorded during the 5-min testing period.

Immunohistochemical Staining

Immunohistochemical examination of TH expression in mice midbrain was investigated as previously described [28,34].

Figure 1 *In vivo* neuroprotective and neurotrophic activities of puerarin against MPTP neurotoxicity in a murine model of PD. **(A)** Chemical structure of puerarin. The structure was generated by ChemSketch software (<http://www.acdlabs.com>). **(B)** Amelioration of MPTP-induced behavioral impairments. A total of 24 mice were divided into four groups ($n = 6$): Untreated, no treatment; MPTP, 25 mg/kg MPTP (i.p.) per day; MPTP+Puerarin (L), 50 mg/kg of puerarin (i.p.) and 25 mg/kg MPTP (i.p.) per day; MPTP+Puerarin (H), 150 mg/kg of puerarin (i.p.) and 25 mg/kg MPTP (i.p.) per day. The immobility of mice in forced swim test was recorded as an indicator of behavioral performance. **(C)** Reduction of MPTP neurotoxicity on tyrosine hydroxylase positive (TH⁺) dopaminergic neurons. The animals were treated as described in Panel B. TH protein was immunostained with specific antibody and visualized as dark brown stains produced by the HRP conjugate of secondary antibody. **(D)** Data analysis of TH immunostaining. Following detection described in Panel C, TH expression levels were determined by reading the optical density of stains. The mean values of the optical intensity for each group of animals ($n = 6$) were analyzed by one-way ANOVA (** $P < 0.01$, Puerarin + MPTP group vs MPTP group). **(E)** Recovery of MPTP-induced loss of TH expression. The animals were treated as described in Panel B. TH protein was determined by Western blotting with specific antibody. **(F)** Data analysis of TH expression. The optical density of TH bands in Panel C was determined by software Quantity One (Bio-Rad). The mean values of the optical intensity for each group of animals ($n = 6$) were analyzed by Student's *t*-test (** $P < 0.01$, Puerarin + MPTP group vs MPTP group).

Briefly, the paraffin sections of mouse midbrain were deparaffinized and hydrated in Millipore water. The antigenic sites were exposed by 20 min of incubation in 10 mM citrate buffer (pH 6.0) at 90°C. Endogenous peroxidase activity was quenched with 3.0% hydrogen peroxide solution. The sections were sequentially blocked in 5% BSA in PBST (PBS with Tween-20) for 30 min and incubated with rabbit anti-TH (1:100) overnight at 4°C. The bound antibodies were tracked with biotinylated

goat anti-rabbit secondary antibody for 30 min and horseradish peroxidase-labeled streptavidin for 30 min. The bound horseradish peroxidase was assayed with 3,3'-N-diaminobenzidine tetrahydrochloride (DAB). The sections were restained with hematoxylin. The numbers of positive cells from each animal were counted within three nonoverlapping areas (total number of about 300 cells) at 400-fold magnifications on an Olympus microscope (Olympus Corp., Tokyo, Japan).

Cell Culture and Drug Treatments

Rat pheochromocytoma cell line PC12 was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum (HS), 5% heat-inactivated fetal bovine serum (FBS), and 1% penicillin/streptomycin on collagen I-coated dishes at 37°C under a humidified atmosphere of 5% CO₂ and 95% air. For drug treatments, the cells were seeded at the density of 2×10^4 cells/mL in 6-well plate for 24 h, incubated in differentiation medium (DMEM + 1% HS + 1% FBS) for 24 h and then treated with puerarin and NGF, alone or in combination.

Assay of Neurite Outgrowth

Neurite outgrowth was measured by Neurite Outgrowth Staining Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, PC12 cells were seeded at 4×10^4 cells per well in a collagen I-coated 6-well plate for 24 h, cultured in differentiation medium for another 24 h, and then treated with puerarin and NGF, alone or in combination. The cells were incubated with cell membrane stain and cell viability indicator for 20 min at 37°C, washed twice with PBS, and subsequently incubated with background suppression dye. The images were acquired by a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The cells were scored at different times for the proportion of neurite-bearing cells relative to the cell monolayer per view or alternatively by measuring the average neurite length in the indicated cell cultures. Triplicate wells were scored, and counts represent means $\pm$ SD.

Immunofluorescence Staining of Neuronal Biomarker MAP2

PC12 cells were seeded at 4×10^4 per well onto collagen I-coated 35-mm confocal dishes for 24 h and treated with NGF and puerarin, alone or in combination, in differentiation medium for 72 h. The cells were fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized with 0.5% Triton X-100 for 30 min, and blocked in 5% normal goat serum in PBS for 2 h at room temperature. The cells were then incubated with anti-MAP2 antibody in 1% BSA at 4°C overnight. The bound antibodies were detected by Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody. At the end of detection, the cell nuclei were indicated by staining with 4'-6-diamidino-2-phenylindole (DAPI) for 15 min. The immunofluorescence images were acquired on Zeiss fluorescence microscopy (Carl Zeiss).

Inhibition of Neurotrophic Signaling Pathways

PC12 cells were pretreated with MEK inhibitors PD98059 (20 μ M) and U0126 (2 μ M), HO-1 inhibitor SnPP (20 μ M) for 1 h, and treated with puerarin and NGF, alone or a combination for 72 h. Neurite outgrowth was studied as described above.

Western Blot Analysis

At the end of drug treatment, mouse brain tissues and PC12 cells were lysed in ice-cold RIPA buffer (20 mM Tris-HCl [pH

7.5], 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, and 1 μ g/mL leupeptin). The cellular proteins were recovered by centrifugation at 14000 rpm for 15 min. The protein concentrations were determined with protein assay dye reagent from Bio-Rad (Hercules, CA, USA). The brain tissue and cellular proteins (30 μ g) were resolved by gel electrophoresis on 10% SDS-polyacrylamide gels and subsequently transferred onto PVDF membranes. Following blocking overnight with TBS-T (Tris-buffered saline with 0.2% Tween-20) buffer containing 5% nonfat milk powder or BSA at 4°C, the blots were probed with specific primary antibodies, detected with HRP-conjugated secondary antibody, and finally visualized by Amersham™ ECL™ Select Western blotting detection reagent from GE Healthcare Biosciences (Uppsala, Sweden).

Statistical Analysis

The results were presented as means $\pm$ SD and analyzed by one-way ANOVA test with SPSS 13.0 software (IBM, New York, NY, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Puerarin Protected Dopaminergic Neurons Against MPTP Neurotoxicity in Mice

To investigate the *in vivo* neuroprotective and neurotrophic activities of puerarin, we examined whether puerarin could ameliorate the impairments of motor functions in MPTP-lesioned mice. As shown in Figure 1B, compared with untreated controls, MPTP-treated mice became less active in swim test. Interestingly, puerarin at the doses of 50 and 150 mg/kg/day decreased the immobility time of mice in swim test. We further examined the effect of puerarin on dopaminergic neurons in the substantia nigra. TH levels in dopaminergic neurons were detected by immunohistochemical staining. As shown in Figure 1C,D, MPTP treatment caused dramatic loss of TH expression in mice. Puerarin at the doses of 50 and 150 mg/kg/day preserved TH expression in MPTP-treated mice. Western blot analysis of TH expression in mouse brains further supported that puerarin (150 mg/kg) significantly rescued MPTP-induced loss of TH expression (Figure 1E,F).

Puerarin Enhanced NGF-Induced Neuritegenesis

To explore the effect of puerarin on the neurotrophic activity of NGF, we treated PC12 cells with NGF (2 ng/mL) alone or in combination with puerarin at the concentrations of 10, 25, and 50 μ M. After 72 h of treatment, the neurite-bearing cells were stained and enumerated under a fluorescence microscope. As shown in Figure 2A,B, puerarin induced the formation and growth of neurites in a concentration-dependent manner and profoundly increased the length of neurites in the presence of NGF (2 ng/mL). Importantly, the combination of NGF (2 ng/mL) and puerarin (50 μ M) achieved better stimulation of neurite outgrowth than NGF (20 ng/mL) alone. These results indicated

Figure 2 Potentiation of NGF-induced neuritogenesis by puerarin. **(A)** Representative images of PC12 cells with or without neurites. PC12 cells were treated with puerarin alone or in combination with NGF (2 ng/mL). The cells were stained by neurite outgrowth staining kit and examined under a Zeiss fluorescence microscope. Scale bar, 100 μ m. **(B)** Puerarin potentiates NGF-induced neuritogenesis in a concentration-dependent manner. The neurite-bearing cells were enumerated with NIH Image J. Untreated PC12 cells were used as control. The values represent the means $\pm$ SD ($n = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$ (sample vs NGF 2 ng/mL). **(C)** Immunofluorescence staining of neuronal biomarker MAP2. The cells were treated with puerarin and NGF for 72 h as follows: **A**, control; **B**, NGF (2 ng/mL); **C**, puerarin (50 μ M); **D**, NGF (2 ng/mL) and puerarin (50 μ M). Following staining with anti-MAP2 and DAPI, the cells were examined under a Zeiss fluorescence microscope. **(D)** Western blot analysis of neuronal biomarker β 3-tubulin in PC12 cells. The cells were treated with vehicle control, NGF (2 ng/mL), puerarin (10 μ M) or combination of NGF (2 ng/mL) and puerarin (10 μ M) for 72 h. β 3-tubulin expression was analyzed by Western blotting using specific antibody, whereas GAPDH was analyzed as the control of protein loading.

that puerarin could enhance the activity of NGF on neuritogenesis by >10-fold. The expression of neuronal biomarkers MAP2 and β 3-tubulin in puerarin-treated cells was determined by immunocytochemical staining or Western blotting. As shown in Figure 2C, puerarin (50 μ M) marginally induced MAP2 expression, but effectively enhanced the effect of NGF on MAP2 induction. Furthermore, Western blot analysis revealed that β 3-tubulin expression was also increased by puerarin (10 μ M), NGF (2 ng/mL), or the combination of both reagents (Figure 2D). These results suggested that puerarin could effectively stimulate neuritogenesis even when NGF level was largely declined.

Puerarin Potentiated NGF-Induced Neuritogenesis via Activating ERK1/2, PI3K/Akt, and Nrf2/HO-1 Pathways

To investigate the mechanisms underlying the neurotrophic activity of puerarin, we initially examined the effect of puerarin on ERK and Akt. PC12 cells were treated with puerarin (50 μ M) for 0, 0.25, 0.5, 1, 3, or 6 h. The protein lysates were subsequently analyzed by Western blotting. As shown in Figure 3A, puerarin rapidly induced the phosphorylation of ERK1/2 and Akt. Interest-

ingly, the phosphorylation of ERK1/2 and Akt was detected with a peak at 15 min and sustained for approximately 3 h. We also verified the *in vivo* effects of puerarin on ERK1/2 and Akt pathways. Following drug treatment for consecutive 7 days, mouse brains were recovered and analyzed by Western blotting. As shown in Figure 3B, puerarin also activated ERK1/2 in MPTP-lesioned mice in a dose-dependent manner, whereas puerarin induced somewhat phosphorylation of Akt. Secondly, we investigated the effect of puerarin on Nrf2 nuclear translocation and subsequent HO-1 induction. After 6 h of treatment with or without puerarin (50 μ M), the nuclear and cytosolic fractions were prepared for Western blot analysis of Nrf2, whereas the cells exposed to drugs for 24 h were used for Western blot analysis of HO-1. Protein lamin B was detected as nuclear biomarker whereas GAPDH was examined as cytosolic biomarker. As shown in Figure 3C, puerarin effectively increased the level of nuclear Nrf2 and subsequent HO-1 induction. To further explore whether puerarin induced Nrf2 translocation and HO-1 expression via activating ERK1/2 and Akt, PC12 cells were cotreated with puerarin and MEK inhibitors (e.g., PD98059 and U0126) or PI3K inhibitor LY294002 (Figure 3C). Surprisingly, U0126 somewhat enhanced puerarin-induced increase in the level of nuclear Nrf2 and HO-1

Figure 3 Potential mechanisms underlying the effect of puerarin on NGF-induced neuritogenesis. **(A)** Puerarin rapidly activates kinases ERK1/2 and Akt in PC12 cells. Following treatment with puerarin 50 μ M for the indicated times, phosphorylation of ERK1/2 and Akt was analyzed by Western blotting using specific antibodies. **(B)** *In vivo* effects of puerarin on ERK1/2 and Akt pathways. Following drug treatment for 7 days as described in Figure 1, phosphorylation of ERK1/2 and Akt in mouse brains was analyzed by Western blotting using specific antibodies. **(C)** Effects of puerarin on the nuclear translocation of Nrf2 and subsequent induction of HO-1. Following drug treatment as indicated, nuclear proteins and cytosolic proteins were isolated and analyzed for the levels of Nrf2 and HO-1 by Western blotting using specific antibodies. Protein kinase inhibitors were as follows: U, U0126 for MEK; PD, PD98059 for MEK; LY, LY294002 for PI3K. **(D)** Regulation of puerarin-enhanced neuritogenesis by kinase inhibitors and HO-1 inhibitor. Following drug treatment as indicated, the cells were stained by neurite outgrowth staining kit. The average neurite lengths were examined under a Zeiss fluorescence microscope and analyzed by NIH Image J. Untreated PC12 cells were used as control. The values represent the means $\pm$ SD ($n = 3$). * $P < 0.05$; ** $P < 0.01$ (sample vs NGF+Puerarin). **(E)** Representative images of PC12 cells with or without neurites. Scale bar, 100 μ m.

expression, whereas PD98059 and LY294002 showed strong inhibitory effect against the actions of puerarin on Nrf2/HO-1 pathway. Thirdly, we explored whether puerarin potentiated NGF-induced neurite outgrowth via activating ERK1/2, Akt, and HO-1 pathways. PC12 cells were cotreated with puerarin, NGF and MEK inhibitors (e.g., PD98059 and U0126), PI3K inhibitor LY294002 or HO-1 inhibitor SnPP. As shown in Figure 3D,E, the effect of puerarin and NGF on neurite outgrowth was effectively blocked by MEK inhibitor PD98059, PI3K inhibitor LY294002, and HO-1 inhibitor SnPP although MEK inhibitor U0126 showed relatively less inhibitory potency. The results confirmed that puerarin potentiated NGF-induced neuritogenesis via ERK1/2- and PI3K/Akt-dependent induction of HO-1 expression.

Puerarin Potentiated NGF-Induced Neurite Extension in a Timing-Dependent Manner

To clarify whether the activation of ERK1/2 and PI3K/Akt pathways by puerarin was sufficient to drive neurite extension, we treated PC12 cells by three different protocols: (1) transient stimulation with NGF and puerarin; (2) simultaneous addition of NGF and puerarin; and (3) sequential addition of two reagents with a time gap of 12 h (Figure 4A). As shown in Figure 4B,C, transient stimulation with NGF and puerarin provided enough signals to initiate the neurite extension process, but failed to sustain the continuous extension of neurites. It was not surprising that NGF at higher concentration induced

Figure 4 Timing dependence of the coordination between puerarin and NGF in neuritogenesis. **(A)** Experimental protocols for timing-dependent induction of neuritogenesis by NGF and puerarin. For transient stimulation, PC12 cells were incubated with NGF and puerarin for 1 h and differentiation medium for another 23 h; and for simultaneous stimulation, PC12 cells were incubated with NGF and puerarin for 24 h; for sequential stimulation, PC12 cells were sequentially incubated with NGF for 1 h, differentiation medium alone for 11 h, and then puerarin for 12 h. **(B)** Quantification of neuritogenesis regulated by NGF and puerarin. Following drug treatment as outlined in Panel A, the neurites were stained and measured as described in “Materials and Methods”. Untreated PC12 cells were used as control. The values represent the means $\pm$ SD ($n = 3$). **(C)** Representative images of PC12 cells following the treatments with NGF and puerarin. Following staining with neurite outgrowth assay kit, the images were acquired on a Zeiss fluorescence microscopy. Scale bar, 100 μ m.

better neurite extension. When NGF concentration was reduced to 2 ng/mL, little neurite outgrowth was actually promoted although the cells survived. No matter whether added simultaneously or sequentially, however, puerarin could effectively coordinate with NGF to drive neurite extension. The average neurite lengths were not significantly different in the cells treated with NGF and puerarin simultaneously or sequentially.

Structure-Activity Relationship in the Potentiation of NGF-Induced Neurite Outgrowth

To compare the neurotrophic effects of puerarin and its structural analogs (e.g., daidzein and genistein), we treated PC12 cells with NGF in combination with puerarin, daidzein, or genistein in a similar fashion. As shown in Figure 5, puerarin, daidzein, and genistein similarly potentiated NGF-induced neurite outgrowth.

Discussion

In the present study, we first validated that continuous puerarin treatment could protect TH-expressing neurons in the midbrain substantia nigra region and improve motor performance in MPTP-lesioned mice (Figure 1). Secondly, using dopaminergic PC12 cells as *in vitro* model, we found that puerarin potentiated NGF-induced neuritogenesis by more than 10-fold, possibly via activating ERK1/2 and PI3K/Akt pathways (Figures 2 and 3). According to a recent report [21], the actions of NGF on neuritogenesis can be divided into two different processes, namely, ERK-driven and transcription-dependent latency process and ERK- and PI3K/Akt-driven and transcription-independent neurite extension process. Thus, the aim of the present study was to investigate whether botanical drug puerarin could coordinate with NGF to induce neuritogenesis via activating ERK1/2 and PI3K/Akt pathways in the neurite extension process.

Figure 5 Structure-activity relationship in NGF-induced neuritogenesis. **(A)** Representative images of neuritogenesis induced by NGF and puerarin analogs. At the end of drug treatment with NGF and puerarin analogs, the neurites were stained and examined under a Zeiss fluorescence microscopy. Scale bar, 100 μm. **(B)** Quantification of neuritogenesis regulated by NGF and puerarin analogs. The average neurite lengths were determined by NIH Image J software. The values represent the means ± SD ($n = 3$). ** $P < 0.01$ (NGF + isoflavone vs NGF alone). **(C)** Chemical structures of the active isoflavones. The backbone structure was created by ChemSketch software ($R^1 = H, OH$; $R^2 = H, C\text{-glucosyl group}$). The solid lines indicate the shared structural properties, whereas the dashed lines indicate the variable structural properties.

Neurotrophins including NGF regulate neuronal survival, growth and differentiation through multiple mechanisms, especially, Raf/MEK/ERK and PI3K/Akt pathways [17]. Sustained activation of ERK1/2 is essential to support the actions of NGF on neurite extension [19,35,36]. The functional neurites are generated through a complex process involving three stages: (1) initiation of neurite formation; (2) elongation of neurites to meet the appropriate targets; and (3) synapse formation and functional maturation of the newly formed neuronal networks [37]. Many structural and signaling proteins are involved in modulating such a complex differentiation process [38]. It is now known that the ERK1/2 pathway governs two dispensable processes: transcription-involving latency process and neurite extension process [21]. In the transcription-involving latency process, neurotrophic factors activate ERK1/2 pathway and modulate the transcription of various genes for the initiation and elongation of neurites. On the other hand, in the neurite extension process, the activation of ERK1/2 and PI3K/Akt pathways triggers the extension of neurites, whereas gene transcription is no longer required for neurite outgrowth. Encouragingly, many small molecules are known to induce the activation of ERK1/2 and PI3K/Akt pathways [39,40]. Thus, it is of therapeutic interest to use these synthetic/natural small molecule drugs to mimic the actions of NGF on ERK1/2 and PI3K/Akt pathways and neurite extension.

Moreover, the activation of ERK1/2 and PI3K/Akt pathways often induces the nuclear translocation of transcriptional factor Nrf2 and subsequent HO-1 expression [41–43]. Upon the stimulation by electrophilic phase II inducers and oxidative stimuli, Nrf2 migrates into the nucleus, binds to antioxidant response element (ARE) in the promoter, and mediates the expression of various antioxidant enzymes including HO-1 [44–46]. HO-1 is a typical stress responsive enzyme that catalyzes the metabolism of heme into bilirubin, free iron, and carbon monoxide (CO), exhibiting antioxidant and cytoprotective activities. Previous studies suggest that Nrf2/HO-1 pathway plays a key role to stimulate neurite outgrowth [41,47]. It is also known that the activation of Nrf2 pathway triggers cross-talking between Nrf2 pathway and TrkA signaling pathway via inducing adaptor protein p62/ZIP [48]. The activation of Nrf2/HO-1 and Nrf2/p62/ZIP pathways could enhance NGF/TrkA-mediated neuronal differentiation. Collectively, a variety of synthetic and natural small molecules may potentiate the neurotrophic activity of NGF through the activation of ERK1/2, PI3K/Akt, and Nrf2 pathways.

The present study not only revealed that puerarin rapidly activated ERK1/2 and PI3K/Akt, leading to the activation of Nrf2/HO-1 pathway, but also demonstrated that puerarin potentiated NGF-induced neuritogenesis in ERK1/2-, PI3K/Akt-, and Nrf2/HO-1-dependent manner. MEK inhibitor PD98059 and PI3K inhibitor

LY294002 attenuated the actions of puerarin on the Nrf2/HO-1 pathway. These results support the role of ERK and PI3K pathways in the regulation of HO-1 expression [49]. Moreover, MEK, PI3K, and HO-1 inhibitors U0126, PD98059, LY294002, and SnPP diminished the effect of puerarin on NGF-induced neurite outgrowth. The results also suggest that cross talks between ERK1/2, PI3K/Akt, and Nrf2/HO-1 pathways may mediate the coordination between puerarin and NGF in the induction of neurite outgrowth. The present study verified that puerarin regulated the differentiation of PC12 cells into neuron by immunostaining and Western blot analysis of common neuronal biomarkers (e.g., MAP2 and β 3-tubulin) [50]. Highly consistent with several previous studies [26,41,51], puerarin increased the expression of neuronal biomarkers MAP2 and β 3-tubulin. By transient, simultaneous and sequential stimulations of PC12 cells with NGF and puerarin, we for the first time observed that puerarin (50 μ M) effectively coordinated with NGF (2 ng/mL) in driving neurite outgrowth in three stimulation experiments (Figure 4). It is critical to maintain NGF at a sufficiently high level (e.g., 20 ng/mL) to effectively stimulate the extension of neurites. NGF at lower concentrations (e.g., 2 ng/mL) could not drive the extension of neurites. In the presence of NGF (2 ng/mL), however, puerarin (50 μ M), added either simultaneously or sequentially, could drive neurite outgrowth to a comparable extent that was achieved by NGF at higher concentrations. These results consolidated the pharmacological potential of puerarin to maintain neuronal differentiation when NGF level is low.

Natural products are increasingly recognized for the promotion of neuronal survival and neurogenesis against neuronal injury and chronic neurodegeneration [51,52]. Isoflavones including daidzein and genistein are commonly classified as phytoestrogens with the potential to enhance NGF-induced neurite outgrowth, although the underlying mechanisms remain elusive [53–55]. After assaying puerarin, daidzein, genistein, calycosin, onion, and naringin in parallel, we found that puerarin, daidzein, and genistein could similarly potentiate NGF-induced neurite outgrowth (Figure 5). In terms of the chemical structures of these isoflavones (Figure 5C), daidzein represents the backbone structure for all three testing compounds, whereas puerarin bears a unique C-glucosyl moiety and genistein is modified by one additional 5-hydroxyl group. These isoflavones shared the common structural characteristics (solid lines) and differ in two minor variable substitutions (dashed lines). Presumably, these analogs may activate ERK1/2 and PI3K/Akt to the similar extent via similar or different mechanisms. These results shed light on structural optimization to potentiate the neurotrophic activity of NGF with better *in vivo* potency and efficacy.

In conclusion, the present study for the first time demonstrated that pharmacological activation of ERK1/2 and PI3K/Akt pathways could effectively drive the neurite extension process following NGF-initiated latency process (Figure 6). The results of this study may suggest a general mechanism underlying the

Figure 6 Schematic illustration of the coordination between puerarin and NGF to regulate neuritogenesis. NGF induces Signal-1 to prime the cells to the ready-to-go differentiation state, whereas puerarin effectively promotes the activation of protein kinases ERK1/2 and Akt, leading to nuclear translocation of Nrf2 as Signal-2. Ultimately, the cross talks between Signal-1 and Signal-2 drive the neurite outgrowth.

neurotrophic application of puerarin and related small molecule compounds in the therapy of PD.

Acknowledgments

This project was supported by General Research Fund (GRF) (HKU 775812M) from the Research Grants Council of Hong Kong and the Seed Fund for Basic Research Programme, the University of Hong Kong (to J. R.).

Conflict of Interest

The authors declare no conflict of interest.

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