JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Neurotrophic Action of 5-Hydroxylated Polymethoxyflavones: 5-Demethylnobiletin and Gardenin A Stimulate Neuritogenesis in PC12 Cells

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Supporting Information

ABSTRACT: Polymethoxyflavones (PMFs) exhibit a broad spectrum of biological properties, including anticancer, antiatherogenic, and neuroprotective effects. The aim of this study is to investigate the neurotrophic effects of 5-demethylnobiletin, a hydroxylated PMF found in citrus plants, and gardenin A, a synthetic PMF analogue, on neurite outgrowth and neuronal differentiation in PC12 cells. The results of this study showed that 5-demethylnobiletin and gardenin A (10−20 μM) potently induce neurite outgrowth in PC12 cells, accompanied by the expression of neuronal differentiation and synapse formation marker proteins, growth-associated protein-43 (GAP-43), and synaptophysin. We observed that the addition of K252a, a TrKA antagonist, significantly inhibited NGF-induced neurite outgrowth in PC12 cells, but 5-demethylnobiletin- or gardenin A-induced neurite outgrowth was not affected. Treatment with 5-demethylnobiletin and gardenin A markedly induced the phosphorylation of both cyclic AMP response element-binding protein (CREB) and CRE-mediated transcription, which was suppressed through the administration of the inhibitor 2-naphthol AS-E phosphate (KG-501) or using CREB siRNA. Furthermore, our results showed that MAPK/ERK kinase 1/2 (MEK1/2), protein kinase A (PKA), and protein kinase C (PKC) inhibitors blocked the CRE transcription activity and neurite outgrowth induced through 5-demethylnobiletin or gardenin A. Consistently, increased ERK phosphorylation and PKA and PKC activities were observed in PC12 cells treated with 5-demethylnobiletin or gardenin A. These results reveal for the first time that 5-demethylnobiletin and gardenin A promote neuritogenesis through the activation of MAPK/ERK-, PKC-, and PKA-dependent, but not TrkA-dependent, CREB signaling pathways in PC12 cells.

KEYWORDS: *polymethoxyflavones, 5-demethylnobiletin, gardenin A, PC12 cells, neurite outgrowth*

INTRODUCTION

Neurotrophic factors, such as nerve growth factor (NGF), play critical roles in neuronal development and survival and in the maintenance of synaptic connection and plasticity.[1] The loss of neurotrophic support is a critical factor in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.[2] Therefore, the administration of neurotrophic agents to stimulate neurogenesis, neuritogenesis, neuronal differentiation, and synaptogenesis has been considered to be a potential therapeutic strategy for patients with neurodegenerative diseases. Recently, it was shown that several phytochemicals, such as polyphenolics and flavonoids, possess high neurotrophic potency and might penetrate the blood−brain barrier (BBB), influencing numerous neuronal functions within the brain.[3] These small molecules mimic the neurotrophic ability of NGF and might contribute to the development of important prevention strategies or therapeutics to combat neurodegenerative disorders.

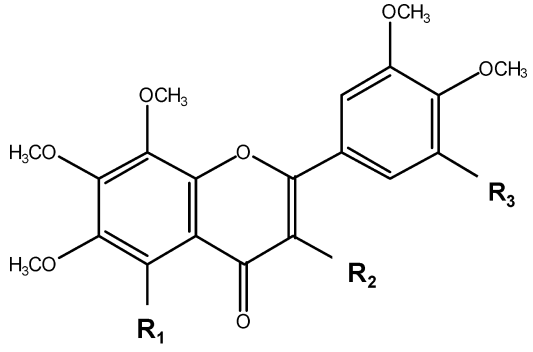
The rat pheochromocytoma cell line PC12 is widely used as a cell culture model for neuronal differentiation and survival.[4] PC12 cells exhibit the cell surface expression of the NGF-specific receptor tyrosine kinase (TrkA). Upon exposure to NGF, PC12 cells exhibit neurite outgrowth, neuronal differentiation, and the formation of synaptic connections, associated with the increased expression of neuronal-specific genes such as growth associated protein-43 (GAP-43), type III β-tubulin, and synaptophysin.[5] NGF induces rapid TrkA dimerization, autophosphorylation, and the activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) dependent signal pathway. It has been reported that the NGF-mediated activation of MAPK/ERK induces phosphorylation of the cAMP response element binding protein (CREB) at Ser[133], which further enhances the transcription activity of protein-coding genes or microRNA, associated with neuronal differentiation, synapse formation, synaptic plasticity, and long-term memory.[6−9] In addition to the MAPK/ERK-dependent pathway, several individual signal transduction cascades are also involved in the

Received: June 7, 2013
Revised: August 28, 2013
Accepted: September 4, 2013
Published: September 4, 2013

activation of CREB, including cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), calcium/calmodulin-dependent protein kinase (CaMK), and phosphatidylinositol 3-kinase (PI3-K)/Akt.[10]

Polyphenolic compounds such as flavonoids, derived from fruits or vegetables, exhibit diverse pharmacological properties that stimulate neuritogenesis and neuroprotective activities. Recent studies have shown that these naturally derived compounds, such as curcuminoids,[11] epigallocatechin 3-gallate (EGCG),[12] fisetin,[13] and luteolin,[14] interact with critical diverse signaling molecules that regulate neurotrophic activity. Citrus fruits contain a wide range of flavonoids. Polymethoxyflavones (PMFs) are present in the peels of citrus fruits such as sweet oranges (*Citrus sinensis* (L.) Osbeck) and mandarin oranges (*Citrus reticulate* Blanco).[15] The citrus PMFs display a broad spectrum of biological activities, including anti-inflammatory, anticancer, antiangiogenesis, antiatherogenic, and neuroprotective properties.[16−20] The well-studied citrus PMF nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone) and its metabolites have been reported to stimulate neuritogenesis through MAPK/ERK- and CREB-dependent pathways in vitro and to enhance or improve impaired memory in animal models with neurological disorders.[21−23] Recently, we also demonstrated that the hydroxylated PMF, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-OH-HxMF), isolated from citrus peel extract, is a potent promoter of neurite outgrowth in PC12 cells through cAMP/PKA/CREB pathways.[24]

In the present study, we selected two structurally related 5-hydroxylated PMFs, 5-demethylnobiletin (5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone) and gardenin A (5-hydroxy-6,7,8,3′,4′,5′-hexamethoxyflavone) (Figure 1), to examine their effects on neuritogenesis in PC12 cells. 5-Demethylnobiletin can be isolated from the extract of aged orange peels and derived from the autohydrolysis of nobiletin during long-term storage.[15] Gardenin A (GA) is a synthetic derivative of 5-hydroxylated PMFs.[25] The only structural difference between 5-demethylnobiletin and gardenin A is on the 5′-position of the B-ring moiety with −H and $-OCH_3$, respectively. Recent studies have compared the structure-relative activities of hydroxylated PMFs with their permethoxylated counterparts. It has been reported that 5-hydroxylated PMFs, such as 5-demethylnobiletin, possess much stronger anticancer properties compared with their PMF counterparts, suggesting the critical role of the hydroxyl group at the 5-position in the enhanced inhibitory effects on the growth of cancer cells.[25] Thus, we investigated the effectiveness of the 5-hydroxylated PMFs, 5-demethylnobiletin, and gardenin A on the neurite outgrowth and neuronal differentiation in PC12 cells.



5-Demethylnobiletin	R_1= OH	R_2=H	R_3=H
Gardenin A	R_1= OH	R_2=H	R_3= OCH_3
Nobiletin	R_1= OCH_3	R_2=H	R_3=H
5-OH-HxMF	R_1=OH	R_2=OCH_3	R_3=H

Figure 1. Chemical structures of 5-demethylnobiletin and gardenin A.

MATERIALS AND METHODS

Chemicals. 5-Demethylnobiletin (5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone) was purified as previously described.[26] Gardenin A (GA), RPMI-1640 medium, poly-L-lysine, dimethyl sulfoxide (DMSO), forskolin, KN-62, H-89, 2-naphthol AS-E phosphate (KG-501), Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS), and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA),unless otherwise indicated. U0126, PD98059, and LY294002 were purchased from Promega (Madison, WI, USA). Bisindolylmaleimide I (BIM) was purchased from Cayman Chemical (Ann Arbor, MI, USA). TrkA antagonist K252a was purchased from Enzo Life Sciences (Ann Arbor, MI, USA). The mouse 7S nerve growth factor (NGF) was purchased from Millipore (Billerica, MA, USA).

Cell Culture. The PC12 cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were maintained in RPMI-1640 medium containing 2 mM glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1 mM sodium pyruvate, supplemented with 10% heat-inactivated horse serum (Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS) (Biological Industries, Kibbutz Haemek, Israel) in a 5% CO_2 incubator at 37 °C.

Cell Viability Analysis. The cell viability was measured through the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan. The cells were incubated with MTT solution (1 mg/mL final concentration) for 4 h at 37 °C. The formazan crystals were dissolved in DMSO, and the extent of the reduction of MTT was measured as the absorbance at 550 nm.

Analysis of Neurite Outgrowth in PC12 Cells. The quantification of neurite outgrowth in PC12 cells was performed as previously described.[27] Briefly, the cells (2×10^5/mL) were seeded onto poly-L-lysine-coated six-well plates with normal serum medium. After 24 h, the cells were changed to low serum (1% horse serum and 0.5% FBS) medium and treated with vehicle (0.1% DMSO) or the indicated compounds for 48 h. Changes in the morphology of PC12 cells were observed and photographed under an inverted microscope (Olympus IX71), and subsequently the neurite-positive cells were counted. The neurite-bearing cells were analyzed from at least 10 randomly selected microscopic fields with an average of 100 cells per field. The number of differentiated cells in the field was visually examined, and cells showing at least one neurite, with a length equal to the cell body diameter, were counted. The data are expressed as a percentage of the total number of cells in the field. Each experiment was conducted in triplicate.

RT Quantitative PCR (RT-Q-PCR) Analysis of GAP-43. PC12 cells (1×10^6/mL) were seeded onto poly-L-lysine-coated six-well plates in normal serum medium for 24 h. The cells were subsequently shifted to low serum medium for 24 h prior to exposure to vehicle (0.1% DMSO) or the indicated compounds for 48 h. Total cellular RNA was isolated using the total RNA minikit (Geneaid, Taipei, Taiwan). The reverse transcription of 2 μg of RNA was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed with 2 μL of cDNA in a 25 μL reaction containing 200 nM primers [GAP-43, 5′-CTAAGGAAAGTGCCCGACAG-3′ (forward) and 5′-GCAGGAG-AGACAGGGTTCAG-3′ (reverse); β-actin, 5′-CCTCTGAACCCT-AAGGCCAA-3′ (forward) and 5′-AGCCTGGATGGCTACGTACA-3′ (reverse)] and Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Burlington, CA). The amplification was conducted using an ABI Prism 7300 real-time PCR system. The following PCR conditions were used: 94 °C for 4 min, followed by 40 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. The $\Delta\Delta C_t$ method

dx.doi.org/10.1021/jf4024678 | J. Agric. Food Chem. 2013, 61, 9453−9463

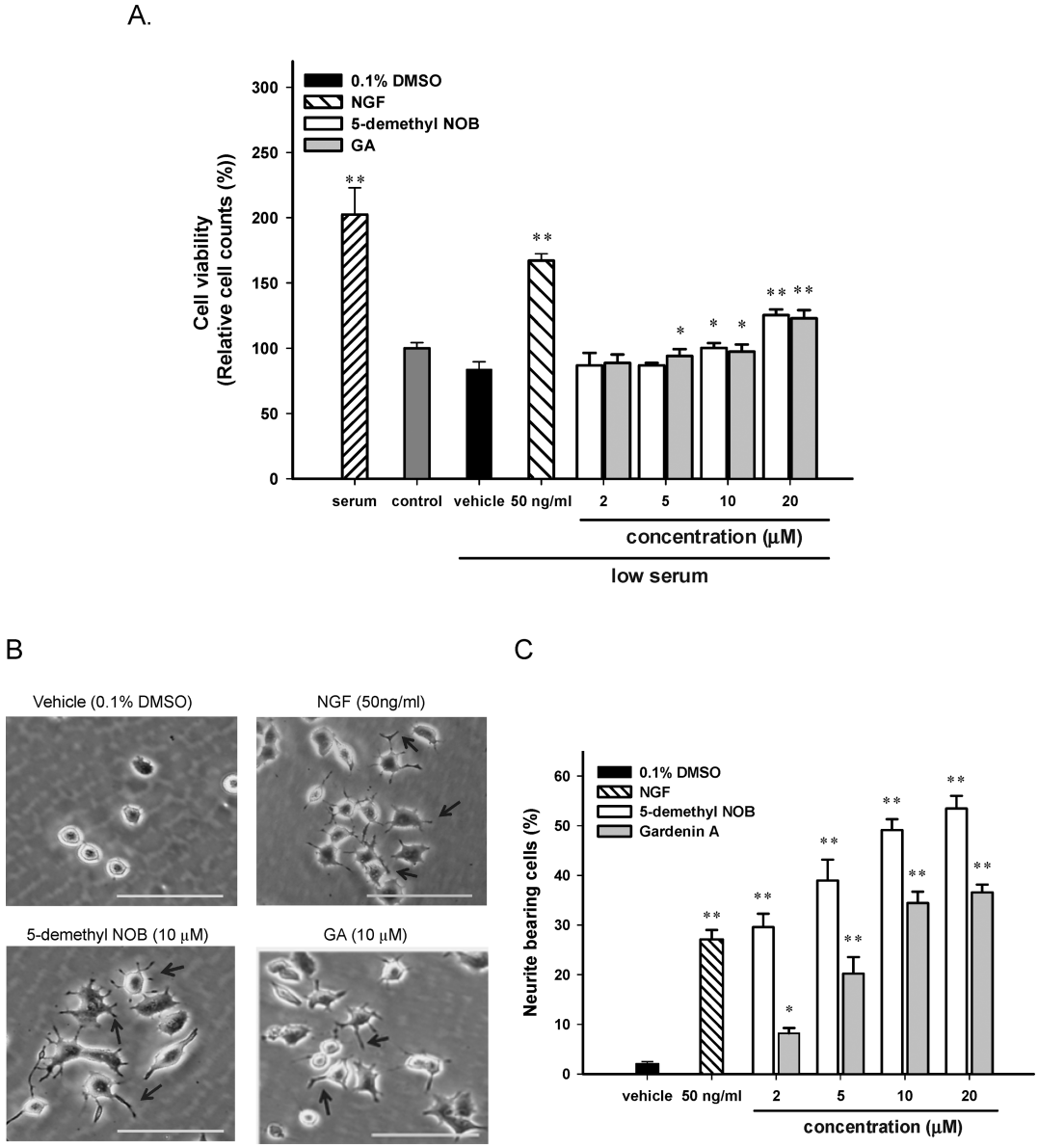


Figure 2. Effects of 5-demethyl NOB and GA on cell viability and neurite outgrowth in PC12 cells. (A) PC12 cells were seeded onto 24-well plates in normal (10% horse serum and 5% FBS) or low-serum (1% horse serum and 0.5% FBS) medium and subsequently exposed to vehicle (0.1% DMSO), NGF (as a positive control), 5-demethyl NOB, or GA (2−20 μM) for 48 h. The cell viability was measured using an MTT assay. The data were expressed as a percentage of the control group, which represents the cell counts prior to medium change. The data represent the mean ± SD from three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences compared with control group cells. (B) PC12 cells were seeded onto poly-L-lysine-coated six-well plates in normal serum medium for 24 h and subsequently shifted to low-serum medium and exposed to vehicle (0.1% DMSO), NGF (50 ng/mL), 5-demethyl NOB, or GA (10 μM) for an additional 48 h. The cell morphology was observed and photographed as described in Materials and Methods. The arrowheads indicate neurite-bearing cells. (C) Neurite-bearing cells were analyzed as described in Materials and Methods. The data represent the mean ± SD from three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences compared with vehicle-treated cells.

was used for the analysis of GAP-43 mRNA expression, estimated in triplicate samples and normalized to β-actin expression level.

Western Blot Analysis of GAP-43, Synaptophysin, CREB, ERK1/2, and β-Actin Proteins. PC12 cells (1×10^6/mL) were seeded onto poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h and subsequently shifted to low serum medium for 24 h prior to exposure to vehicle (0.1% DMSO) or the indicated compounds for the indicated periods. The cells were harvested using RIPA buffer (Thermo Fisher Scientific, Inc., Rockford, IL) according to the manufacturer's instructions. The cell lysate (20 μg) was separated on 10% SDS−PAGE, and subsequently the proteins were transferred onto a PVDF membrane (PerkinElmer, Boston, MA, USA) at 25 V overnight at 4 °C. The membranes were blocked at 4 °C in PBST blocking buffer (1% nonfat dried milk in PBS containing 0.1% Tween-20) for 24 h. The blots were incubated with each of the following antibodies at a 1:1000 dilution: anti-GAP-43, anti-synaptophysin (Millipore, Billerica, MA, USA), anti-phospho-CREB (Ser-133), anti-CREB, anti-ERK1/2, anti-phospho-ERK1/2 (Cell Signaling Technology, Inc.) and monoclonal anti-β-actin (1:8000) (Sigma-Aldrich). The blots were incubated with the horseradish peroxidase conjugated secondary antibodies (1:10 000) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, and the proteins of interest were detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA, USA) according to the manufacturer's instructions. The chemiluminescence signal was visualized using Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, U.K.).

Immunofluorescence Staining of GAP-43 Proteins. PC12 cells were seeded onto poly-L-lysine-coated coverslips in normal serum medium for 24 h and subsequently shifted to low serum medium prior to

dx.doi.org/10.1021/jf4024678 | J. Agric. Food Chem. 2013, 61, 9453−9463

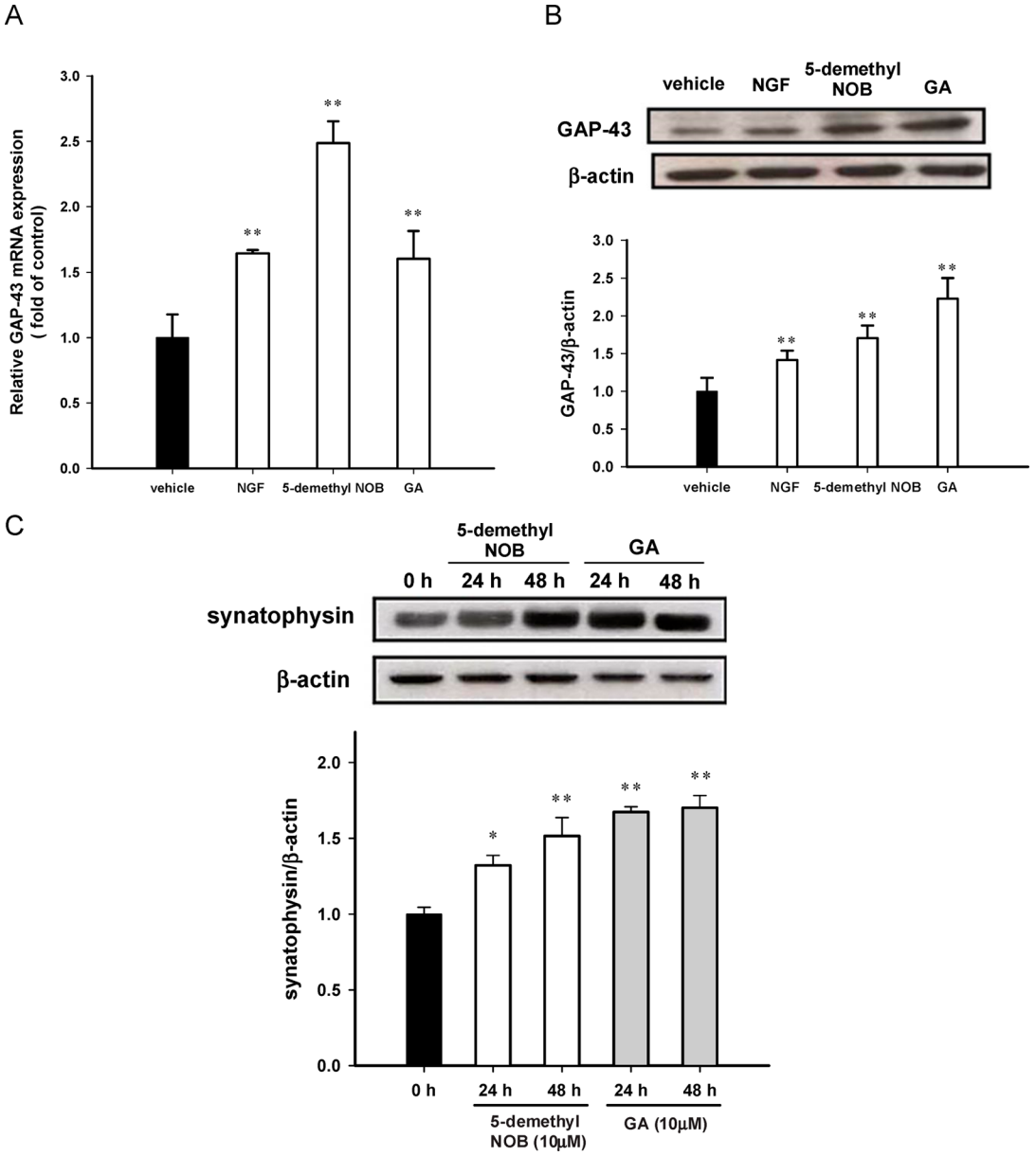


Figure 3. Effects of 5-demethyl NOB and GA on the growth associated protein-43 (GAP-43) and synaptophysin expression in PC12 cells. PC12 cells were seeded onto poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h and subsequently shifted to low-serum medium for 24 h prior to exposure to the indicated agents. The cells were treated with vehicle (0.1% DMSO), NGF (50 ng/mL), 5-demethyl NOB, or GA (10 μM) for 48 h. (A) The GAP-43 mRNA level was detected using RT-Q-PCR as described in Materials and Methods. The data represent the mean ± SD of three independent experiments. GAP-43 protein (B) or synaptophysin (C) protein expression was detected through Western blot analysis as described in Materials and Methods. The experiments were replicated three times, and a representative blot is shown. Normalized intensity of GAP-43 or synaptophysin versus β-actin is presented as the mean ± SD of three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences compared with vehicle-treated cells.

exposure to the indicated reagents. After 48 h of incubation, the PC12 cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 15 min, permeabilized with 0.1% Triton X-100, and soaked in blocking buffer (PBS containing 1% BSA) for 60 min at room temperature. Immunostaining was performed through incubation with the anti-GAP-43 antibody, washing, and subsequent incubation with the secondary Alexa Fluor 488 conjugated goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA) for 60 min. Subsequently, the cells were washed four times in PBS and incubated for 2 min with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. The coverslips were again washed, drained, and mounted with Fluoromount G (Andes import). The cells were observed and photographed on an inverted fluorescence microscope (Olympus IX71).

Analysis of Cyclic AMP Response Element (CRE) Mediated Transcription Activity. PC12 cells (2×10^5/well) were seeded onto a poly-L-lysine-coated 24-well plate in DMEM containing 10% horse serum and 5% FBS medium for 24 h. For transient transfection, cells were co-transfected with the pCRE-Luc Cis-reporter plasmid (Stratagene, La Jolla, CA, USA) and *Renilla* luciferase vector (Promega) using Lipofectamine 2000 reagent (Invitrogen). Twenty-four hours after transfection, the cells were changed to low-serum medium and treated with vehicle (0.1% DMSO) or indicated compounds for 8 h. Luciferase activities were determined by the dual-luciferase reporter assay system kit (Promega) according to the manufacturer's instructions. The intensities of the luciferase reactions measured in the lysates of the transfectants were normalized to their activity of *Renilla* luciferase, which was used as an internal control.

dx.doi.org/10.1021/jf4024678 | J. Agric. Food Chem. 2013, 61, 9453−9463

Transfection of Small Interference RNA (siRNA) Oligonucleotides. PC12 cells were seeded onto poly-L-lysine-coated six-well plates in normal serum medium. After 24 h, the cells were shifted to serum-free OPTI-MEM medium, followed by transfection with 2′-OMe modified siRNA, as a negative control, or rat-specific CREB siRNA duplexes [5′-GGAGUCUGUGGAUAGUGUA-3′ (forward) and 5′-UACACUA-UCCACAGACUCC-3′ (reverse)] (GeneDireX Inc., Las Vegas, NV, USA) at a final concentration of 150 pmol using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, the cells were changed to low-serum medium and treated with vehicle (0.1% DMSO), 5-demethylnobiletin, or gardenin A for further analysis.

Analysis of Protein Kinase C (PKC) and Protein Kinase A (PKA) Activity. Nonradioactive PKC and PKA activity assay kits (Enzo Life Sciences) were used to measure the PKC and PKA activity in the samples. PC12 cells (1×10^6/mL) were seeded onto poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h and subsequently shifted to low serum medium for 24 h prior to exposure to the indicated compounds for the indicated periods. The cellular proteins were harvested using RIPA buffer, and the protein kinase activity was measured according to the manufacturer's instructions. Briefly, the PKC or PKA substrate microtiter plates were soaked in kinase assay dilution buffer at room temperature. The cell lysates (50 ng) or standard (10 ng) were subsequently added, followed by the addition of ATP to initiate the reaction. After incubation at 30 °C for 90 min, the phosphospecific substrate antibody was added, and the mixture was incubated at room temperature for 1 h. The HRP-conjugated secondary anti-rabbit IgG was subsequently added to each well, and the mixture was incubated for an additional 30 min. The TMB substrate solution was added to each well, and the mixture was further incubated for 30 min. Finally, the stop solution was added, and the 96-well plate was read at 450 nm in a microplate reader.

Statistical Analysis. All experiments were repeated at least three times. All values are expressed as the mean ± SD. The results were analyzed using one-way ANOVA with Dunnet's post hoc test, and a p value of <0.05 was considered statistically significant.

RESULTS

Effect of 5-Demethylnobiletin (5-Demethyl NOB) and Gardenin A (GA) on Cell Viability and Neurite Outgrowth in PC12 Cells. PC12 cells were cultured in a low-serum media system (1% horse serum and 0.5% FBS) in the presence of the indicated concentration of compound for 48 h. The cell viability was analyzed using an MTT assay, and the values were expressed as percentage of vehicle-treated group (negative control). As shown in Figure 2A, NGF supported cell growth on PC12 cells under low-serum culture conditions. 5-Demethyl NOB and GA also showed the cell growth supporting effect at higher concentrations (10−20 μM) and did not exert detectable cytotoxicity on PC12 cells after 48 h of incubation in low serum medium. To investigate whether 5-demethyl NOB and GA induce neurite outgrowth in PC12 cells, the cells maintained in low-serum medium were treated with vehicle (0.1% DMSO), NGF (50 ng/mL), 5-demethyl NOB, or GA (2−20 μM). After 48 h, the neurite-bearing cells were photographed under an inverted microscope (Figure 2B), and the number of neurite-bearing cells was counted. The percentage of neurite-bearing cells reached 49.1 ± 2.2% and 53.5 ± 2.5% for 10 and 20 μM 5-demethyl NOB, respectively, and 34.4 ± 2.3% and 36.6 ± 1.5% for 10 and 20 μM gardenin A, respectively ($p < 0.01$) (Figure 2C). These values were significantly higher than those obtained from cells treated with 50 ng/mL NGF (27.1 ± 1.9%) and the negative control (2.1 ± 0.4%). These data indicate that 5-demethyl NOB and GA potently induce neurite outgrowth in PC12 cells.

Effects of 5-Demethyl NOB and GA on Marker Gene Expression Associated with Neuronal Differentiation and Synapse Formation. Growth associated protein-43 (GAP-43), a neuron-specific protein, exhibits increased synthesis and axonal transport during nerve regeneration. Thus, we further investigated the expression of GAP-43 in 5-demethyl NOB- and GA-treated PC12 cells. Figure 3A shows that the relative mRNA levels of GAP-43 were increased after addition of NGF (50 ng/mL, as positive control), 5-demethyl NOB, and GA (10 μM) for 24 h, respectively, compared with the vehicle-treated group. Western blot analysis also showed that the GAP-43 protein was significantly induced through treatment with 5-demethyl NOB and GA for 48 h. The GAP-43 protein levels were elevated in response to NGF (50 ng/mL), 5-demethyl NOB, and GA (10 μM), respectively, compared with the vehicle-treated group (Figure 3B). Immunofluorescence double labeling of cell specific markers with GAP-43 and DAPI indicated that 5-demethyl NOB- and GA-differentiated cells displayed strong GAP-43 expression in the neurites of PC12 cells (Figure S1 in Supporting Information). Furthermore, we analyzed the effects of 5-demethyl NOB and GA on the expression of a synapse formation marker, synaptophysin, in PC12 cells. Our data showed that treatment of NGF (50 ng/mL) for 24 h significantly increased the level of synaptophysin protein to 1.6-fold compared to the vehicle-treated group (Figure S2 in Supporting Information). As shown in Figure 3C, the level of synaptophysin significantly increased in 5-demethyl NOB- or GA-treated PC12 cells. These results indicate that 5-demethyl NOB and GA induce the expression of GAP-43 and synaptophysin, associated with the differentiation of PC12 cells into neuronal phenotypes.

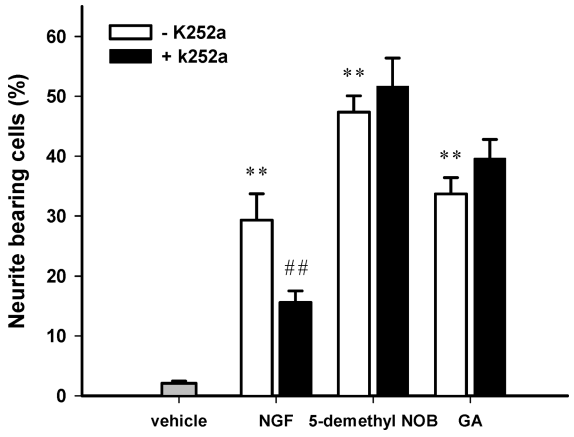


Figure 4. Effects of TrkA antagonist on the 5-demethyl NOB- or GA-induced neurite outgrowth of PC12 cells. PC12 cells were seeded onto poly-L-lysine-coated six-well plates in normal serum medium for 24 h and subsequently shifted to low-serum medium. PC12 cells were treated with K252a (100 nM) for 30 min prior to exposure to NGF (50 ng/mL), 5-demethyl NOB, or GA (10 μM) for 48 h, respectively. The neurite-bearing cells were determined as described in Materials and Methods. The data represent the mean ± SD from three independent experiments. (**) $p < 0.01$ represents significant differences compared with vehicle-treated cells. (##) $p < 0.01$ represents significant differences compared with the respective inhibitor-untreated group.

TrkA Is Not Involved in 5-Demethyl NOB- or GA-Mediated PC12 Neurite Outgrowth. NGF acts on tyrosine kinase receptor A (TrkA) to regulate neuronal differentiation. To evaluate whether 5-demethyl NOB and GA are agonists for receptor TrkA, PC12 cells were treated with 5-demethyl NOB, GA (10 μM), and NGF (50 ng/mL) alone or in combination with the TrkA antagonist K252a (100 nM) for 48 h to analyze the percentage of neurite-bearing cells. As shown in Figure 4, K252a significantly inhibited NGF-mediated neurite outgrowth; however, 5-demethyl NOB- or GA-treated cells were not affected.

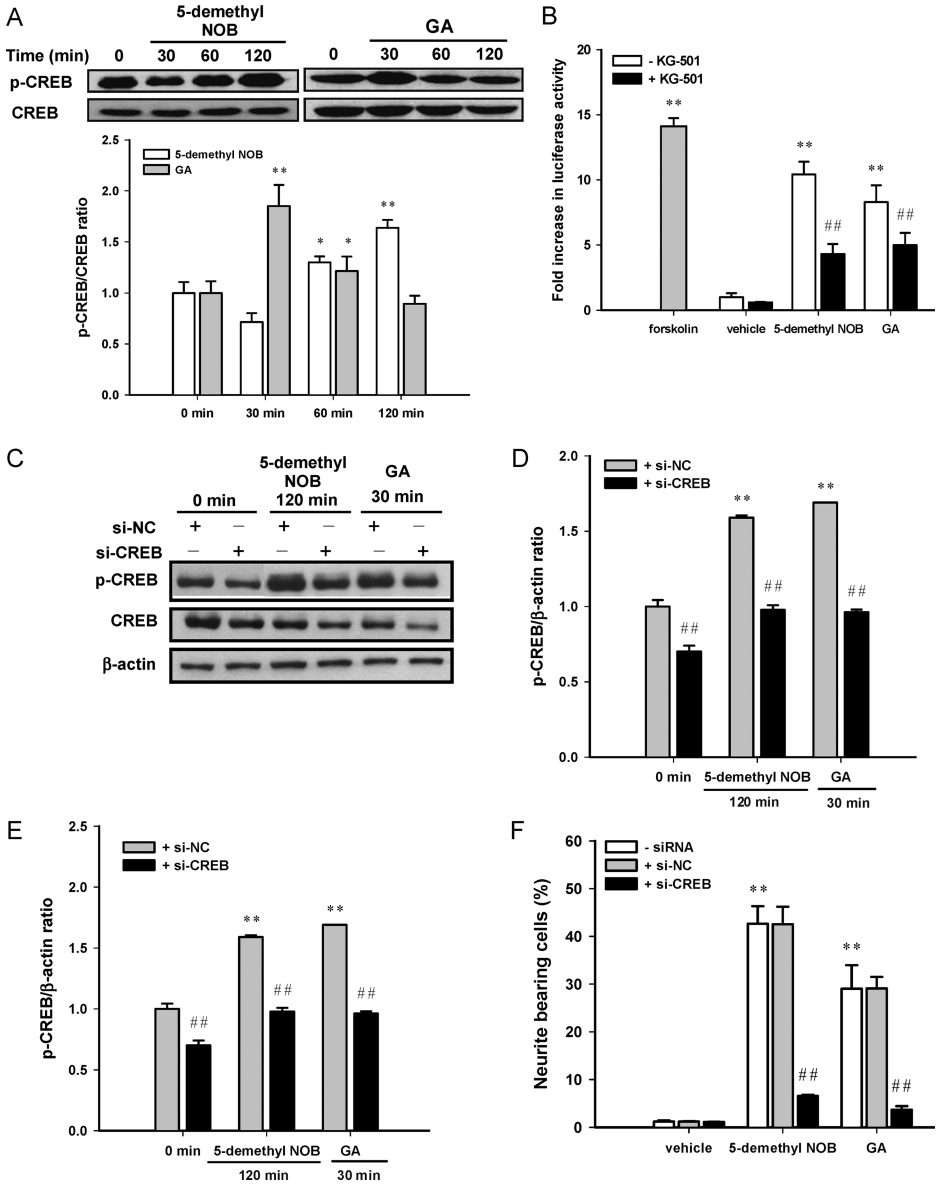


Figure 5. Effects of 5-demethyl NOB and GA on the CREB activation and CREB-mediated neurite outgrowth. (A) PC12 cells were cultured in normal serum medium for 24 h and subsequently shifted to low-serum medium for 24 h prior to exposure to 5-demethyl NOB or GA (10 μM) for 0−120 min. Phosphor-CREB-Ser^{133} (p-CREB) and CREB proteins were analyzed through Western blot analysis. The experiments were replicated at least three times, and a representative blot is shown. The normalized intensity of p-CREB versus CREB is presented as the mean ± SD of three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences compared with the 0 min group. (B) PC12 cells were transfected with a CRE-mediated luciferase reporter construct and *Renilla* luciferase control plasmid for 24 h, and the cells were subsequently treated with forskolin (2 μM, as a positive control), vehicle (0.1% DMSO), 5-demethyl NOB, or GA (10 μM) for 8 h. For treatment with inhibitor, the transfected cells were preincubated with KG-501 (10 μM) for 30 min prior to exposure to the indicated compounds for an additional 8 h. The luciferase activities were measured in the lysates of the transfectants and normalized to their *Renilla* luciferase control activity. The data represent the mean ± SD from three independent experiments. (**) $p < 0.01$ represents significant differences compared with vehicle-treated cells. (##) $p < 0.01$ represents significant differences compared with respective the KG-501 untreated group. (C) PC12 cells were transiently transfected with siRNA negative control (si-NC) or with CREB-specific siRNA (si-CREB) before treatment with vehicle (0.1% DMSO), 5-demethyl NOB, or GA (10 μM). Phosphor-CREB-Ser^{133} (p-CREB), CREB, and β-actin proteins were analyzed through Western blot analysis. The experiments were replicated at least three times, and a representative blot is shown. (D) The normalized intensity of p-CREB or (E) CREB versus β-actin is presented as the mean ± SD of three independent experiments. (F) Neurite-bearing cells were analyzed as described in Materials and Methods. The data represent the mean ± SD from three independent experiments. (**) $p < 0.01$ represents significant differences compared with vehicle-treated cells. (##) $p < 0.01$ represents significant differences compared with the siRNA negative control-transfected group.

These data suggest that 5-demethyl NOB and GA induce neurite outgrowth in PC12 cells via a TrkA-independent pathway.

5-Demethyl NOB and GA Promote CREB Phosphorylation and cAMP Response Element (CRE) Mediated Transcription. Several reports have suggested that cAMP response element binding protein (CREB) plays a role in neurogenesis.[28] To investigate whether 5-demethyl NOB and GA activate CREB, PC12 cells were treated with these two PMFs (10 μM) for the indicated periods, and Western blot analysis was performed with specific antibodies against phospho-CREB-Ser^{133} and pan-CREB. As shown in Figure 5A, 5-demethyl NOB treatment increased CREB phosphorylation with time, and a

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1.7-fold induction was observed after 120 min. However, GA transiently stimulated CREB activation, and a 1.9-fold increase was observed after 30 min. To determine whether 5-demethyl NOB- and GA-induced CREB phosphorylation also increases the transcription activity of cAMP response element (CRE), the pCRE-luciferase reporter plasmids and *Renilla* internal control vectors were co-transfected into PC12 cells, and subsequently the luciferase activity was measured. As shown in Figure 5B, treatment of PC12 cells with forskolin (2 μM, as positive control), 5-demethyl NOB, and GA (10 μM) markedly increased luciferase activity by approximately 14-, 10-, and 8-fold, respectively, compared with vehicle control ($p < 0.01$). The 5-demethyl NOB- or GA-mediated transcription activity was abolished after treatment with the specific CREB antagonist 2-naphthol AS-E phosphate (KG-501) (10 μM). This result suggests that the 5-hydroxylated PMFs, 5-demethyl NOB and GA, activate CREB and CRE-driven transcription.

Furthermore, we determined the effect of CREB knockdown on neurite outgrowth induced through these two 5-hydroxylated PMFs in PC12 cells. We have successfully achieved ~45% knockdown of the total CREB as well as phosphorylated CREB proteins in siRNA-transfected PC12 cells (si-CREB) compared with siRNA negative control (si-NC) (Figure 5C–E). 5-Demethyl NOB- or GA-induced neurite outgrowth was significantly attenuated in CREB siRNA-transfected cells (si-CREB) compared with siRNA negative control cells (si-NC) ($p < 0.01$) (Figure 5F). These results indicate that 5-demethyl NOB and GA induce neurite outgrowth through CREB activation in PC12 cells.

5-Demethyl NOB and GA Activate MAPK/ERK, PKA, and PKC in PC12 Cells. Next, we attempted to identify the signaling pathways activated through 5-demethyl NOB and GA in PC12 cells. As shown in Figure 6, the phosphorylation of ERK1/2 (Thr202/Tyr204) through 5-demethyl NOB was significantly increased at 15 min, peaked at 60 min, and remained at elevated levels for 120 min. However, GA induced ERK activation at 15 min after treatment, and this activity was subsequently reduced after 30 min. We also analyzed the effects of 5-demethyl NOB and GA on PKC and PKA activity. As shown in Figure 7A and Figure 7B, PKC and PKA activity peaked at 30 min and remained at elevated levels for 120 min after 5-demethyl NOB treatment ($p < 0.01$). The results shown in Figure 7C and Figure 7D indicate that similar PKC and PKA activity, with a peak at 15 min ($p < 0.01$), was observed in GA-treated PC12 cells.

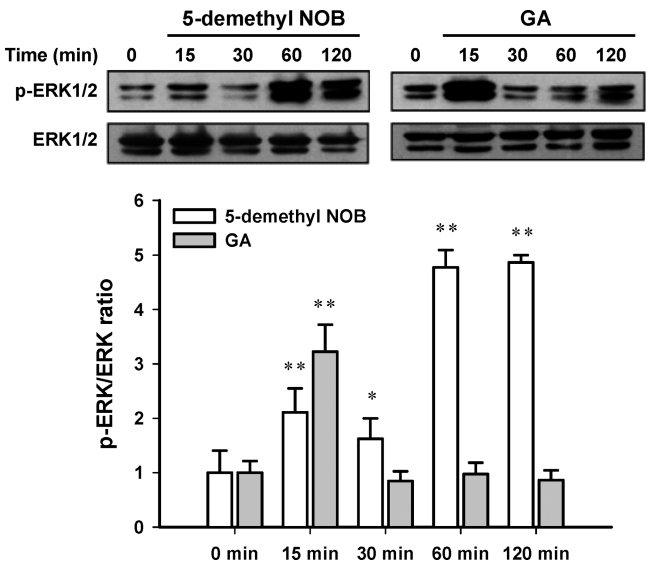

Figure 6. Effects of 5-demethyl NOB and GA on the ERK1/2 phosphorylation in PC12 cells. PC12 cells were cultured in normal serum medium for 24 h and subsequently shifted to low-serum medium for 24 h prior to exposure to 5-demethyl NOB or GA (10 μM) for 0, 15, 30, 60, and 120 min. Phospho-ERK1/2 (p-ERK1/2) and ERK1/2 were analyzed through Western blot analysis. The experiments were replicated at least three times, and a representative blot is shown. The normalized intensity of p-ERK1/2 versus ERK1/2 is presented as the mean ± SD of three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences compared with the 0 min group.

Next, we assessed whether these pathways are essential for the neurite outgrowth induced through 5-demethyl NOB and GA treatment in PC12 cells. PC12 cells were treated with KG-501(10 μM) or kinase-specific inhibitors, including MEK1/2 inhibitor (U0126; 10 μM), MEK1 inhibitor (PD98059; 20 μM), PKC inhibitor (bisindolylmaleimide I, BIM; 2.5 μM), two PKA inhibitors (H-89; 10 μM, and Rp-cAMPS; 200 μM), CaMK II inhibitor (KN-62; 10 μM), and PI3-K inhibitor (LY294002; 20 μM), for 30 min and subsequently incubated with 10 μM 5-demethyl NOB or GA before analyzing the percentage of neurite-bearing cells. As shown in Figure 8, the activation of CREB-, MAPK/ERK-, PKC-, and PKA-dependent pathways was involved in neurite outgrowth induced through both 5-demethyl NOB and GA.

Effects of MAPK/ERK, PKC, and PKA on 5-Demethyl NOB- and GA-Mediated CREB Activation. To examine whether crosstalk exists between MAPK/ERK, PKC, PKA, and CREB activation, PC12 cells were transfected with CRE reporter plasmids and the indicated inhibitors for 30 min and subsequently incubated with 10 μM 5-hydroxylated PMFs before analyzing the luciferase activity. As shown in Figure 9A, 5-demethyl NOB- and GA-mediated CREB transcription activities were significantly reduced after treatment with U0126, BIM, and H-89 ($p < 0.01$). Western blotting revealed that U0126, BIM, and H-89 significantly attenuated 5-demethyl NOB- and GA-mediated CREB phosphorylation (Figure 9B and Figure 9C). These results indicate that 5-demethyl NOB and GA induce CREB activation through MAPK/ERK-, PKC-, and PKA-dependent pathways. Furthermore, to assess whether PKC, PKA, and ERK pathways are coordinated, PC12 cells were treated with inhibitor BIM, H-89, or U0126 (as control) for 30 min followed by incubation with 10 μM 5-demethyl NOB or GA for indicated periods. As shown in Figure 9D, Western blotting showed that the inhibition of kinase activity by BIM or H-89 partially abolished 5-demethyl NOB- or GA-induced ERK activation. These results indicate that PKC/ERK- and PKA/ERK-dependent CREB pathways also partially involved in the 5-demethyl NOB- or GA-mediated neuritogenesis.

DISCUSSION

Citrus fruits are a rich source of flavonoids, including polymethoxyflavones (PMFs). Several studies have shown that citrus PMFs, such as nobiletin, 5-hydroxylated hexamethoxyflavone (5-OH-HxMF), and heptamethoxyflavone (HMF), exert potent effects on neuritogenesis, neurogenesis, and the promotion of cognitive functions and might enhance learning and memory in neuronal cell culture systems or animal models.[21,24,29,30] In the present study, we demonstrated that two 5-hydroxylated PMFs, 5-demethylnobiletin and gardenin A, markedly induce PC12 neurite outgrowth, accompanied by the expression of neuronal differentiation and synapse formation markers GAP-43 and synaptophysin. We also showed the involvement of the MAPK/ERK-, PKC-, and PKA-dependent signaling pathways in the activation of CREB, a transcription factor that specifically binds to the cAMP-response element (CRE) sites of promoters to

dx.doi.org/10.1021/jf4024678 | J. Agric. Food Chem. 2013, 61, 9453–9463

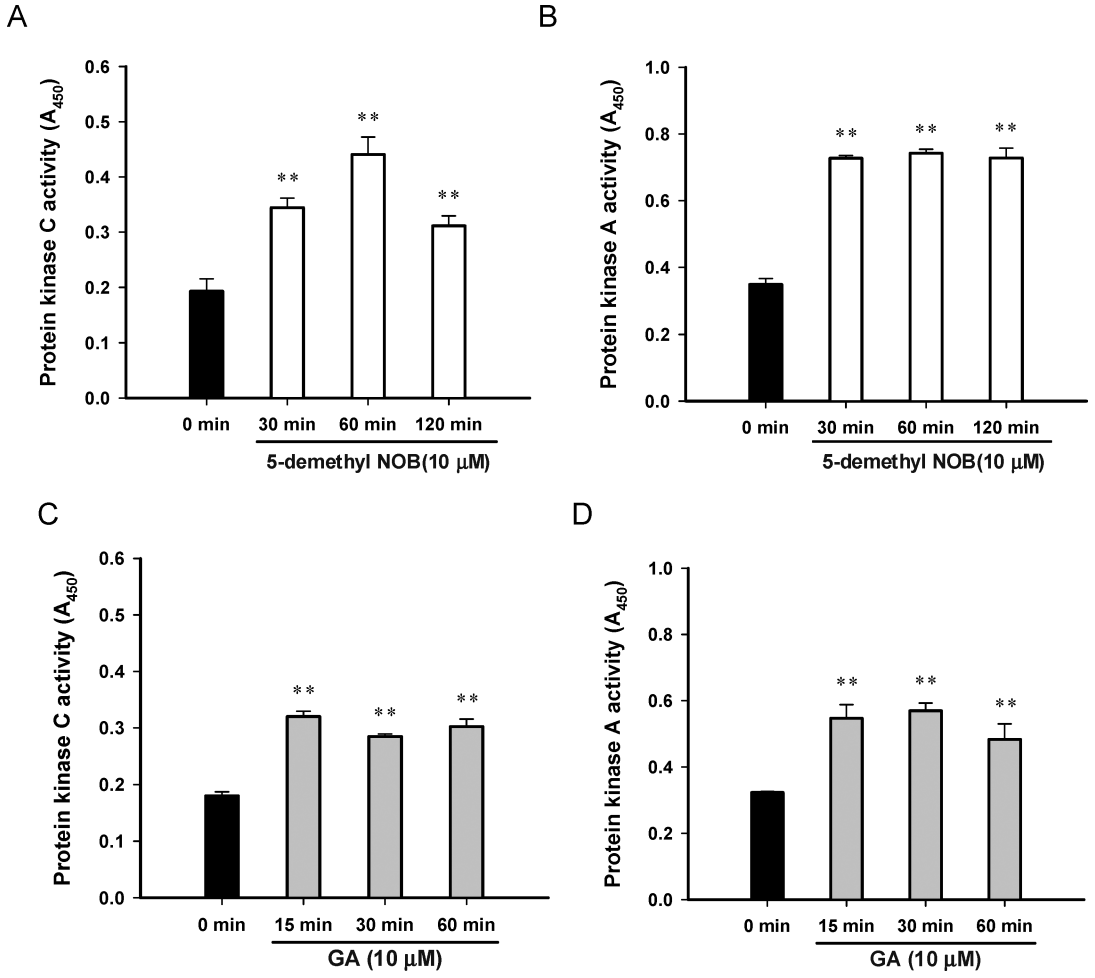

Figure 7. Effects of 5-demethyl NOB and GA on the PKC and PKA activity. Cells were incubated with 5-demethyl NOB or GA for the indicated periods. PKC (A, C) and PKA (B, D) activity was detected using an ELISA kit as described in Materials and Methods. The data represent the mean ± SD of three independent experiments. (**) $p < 0.01$ represents significant differences compared with the 0 min group.

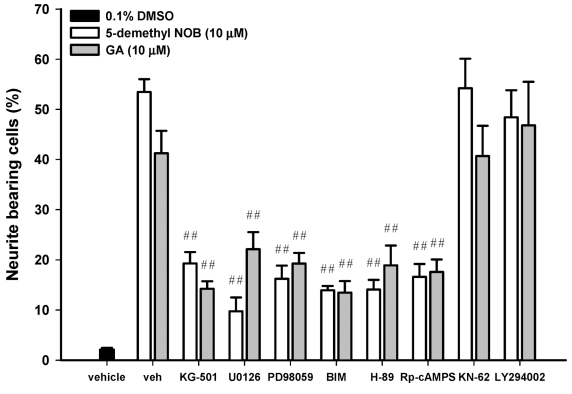

Figure 8. Involvement of CREB, ERK, PKC, and PKA signaling pathways in 5-demethyl NOB- and GA-mediated neurite outgrowth. PC12 cells were pretreated for 30 min with inhibitors KG-501 (10 μM), U0126 (10 μM), PD98059 (20 μM), BIM (2.5 μM), H-89 (10 μM), Rp-cAMPS (200 μM), KN-62 (10 μM), LY294002 (20 μM), or vehicle (0.1% DMSO), followed by exposure to 5-methyl NOB or GA (10 μM) for 48 h. The neurite-bearing cells were determined as described in Materials and Methods. The data represent the mean ± SD from three independent experiments. (##) $p < 0.01$ represents significant differences compared with the respective inhibitor-untreated group.

modulate transcription activity and enhance the neuritogenic actions of PC12 cells.

5-Demethylnobiletin is an abundant hydroxylated PMF in citrus peel extracts. The biological activities of this compound include anti-inflammatory, anticancer, and antiatherogenic properties.[16] Li et al. showed that among 15 PMFs and hydroxylated PMFs compounds, 5-demethylnobiletin exhibited the strongest activity for the antiproliferation and induction of apoptosis in HL-60 cells. However, gardenin A showed no inhibitory activity for cancer cell growth.[25] In the current study, we demonstrated that 5-demethylnobiletin and gardenin A, two hydroxylated PMFs with 5-OH in the A ring, effectively stimulated neurite outgrowth at low concentrations (2−5 μM) in PC12 cells. The neurotrophic actions of other structurally related PMFs and nobiletin and 5-OH-HxMF[24] (Figure 1) showed neuritogenic promotion in the order of 5-demethylnobiletin > gardenin A, 5-OH-HxMF > nobiletin (Table S1 in Supporting Information). These data indicate that the substitution of the methoxy group with a hydroxyl in the 5-position of PMFs might increase neurotrophic activities in PC12 cells.

PC12 cells can be differentiated with synaptic extensions when treated with extracellular stimuli such as NGF and cAMP. The up-regulation of neuronal differentiation marker GAP-43 mRNA and protein expression are associated with the differentiation of PC12 cells into the neuronal phenotype.[31] Synaptophysin, a presynaptic membrane protein, regulates synaptic vesicle fusion and neurotransmitter release. The levels of this protein increase with the formation of mature synapses in

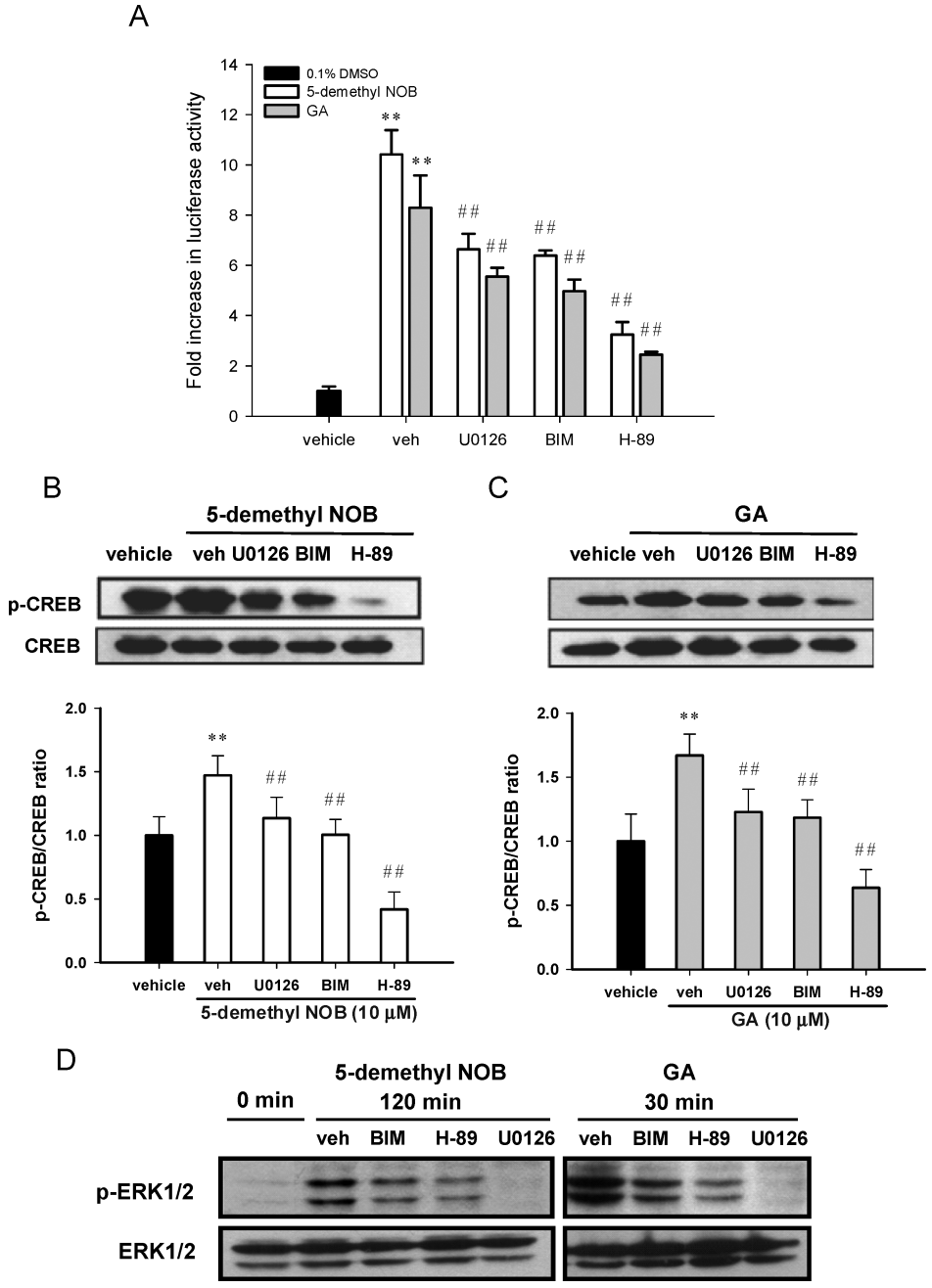

Figure 9. Involvement of ERK, PKC, and PKA signaling pathways in 5-demethyl NOB- and GA-mediated CREB activation. (A) Reporter plasmids-transfected PC12 cells were pretreated for 30 min with the following inhibitors: vehicle (0.1% DMSO), 10 μM U0126, 2.5 μM BIM, or 10 μM H-89, followed by exposure to the indicated reagents for 8 h. The intensities of the luciferase reactions, measured in the lysates of the transfectants, were normalized to their *Renilla* luciferase control activity. The data represent the mean ± SD from three independent experiments. PC12 cells were treated with the inhibitor U0126, BIM, or H-89 for 30 min prior to subsequent exposure to vehicle (0.1% DMSO) and 5-demethyl NOB for 120 min (B) or GA for 30 min (C). Phospho-CREB-Ser^{133} (p-CREB) and CREB were analyzed through Western blotting. The experiments were replicated at least three times, and a representative blot is shown. The normalized intensity of p-CREB versus CREB is presented as the mean ± SD of three independent experiments. (**) $p < 0.01$ represents significant differences compared with vehicle-treated cells. (##) $p < 0.01$ represents significant differences compared with the respective inhibitor untreated group. (D) PC12 cells were treated with the inhibitor U0126, BIM, or H-89 for 30 min prior to subsequent exposure to vehicle (0.1% DMSO) and 5-demethyl NOB(10 μM) for 60 min or GA (10 μM) for 15 min. The p-ERK1/2 and ERK1/2 were analyzed through Western blot analysis. The experiments were replicated at least three times, and a representative blot is shown.

neuronal cell culture.[32] In the present study, we demonstrated that 5-demethylnobiletin and gardenin A induced neurite outgrowth associated with significant increases in the expression of GAP-43 and synaptophysin. Thus, 5-demethylnobiletin and gardenin A promoted neuronal differentiation and mature synapse formation and might support neurotrophic actions in the nerve system. In the current study, gardenin A seems to be slightly better than 5-demethylnobiletin at inducing neuronal differentiation markers but poorer than 5-demethylnobiletin at inducing neurite outgrowth. It has been reported that GAP-43 protein may not be critical for initial extension and maintenance of neurites induced by neuritogenic agents; rather its role may

dx.doi.org/10.1021/jf4024678 | J. Agric. Food Chem. 2013, 61, 9453−9463

lie in growth cone function and in the manipulation of the presynaptic terminal.[33] This finding supported that the level of GAP-43 protein may be not a major factor for the maintenance of neurite extension in these two 5-hydroxylated-PMF-treated PC12 cells. The neuronal growth-associated molecules contribute to 5-demethylnobiletin- and gardenin A-mediated neuritogenesis, and maintenance of neurites in differentiated PC12 cells remains to be clarified.

It has recently been shown that the citrus PMFs, including nobiletin and 5-OH-HxMF, induce neurite outgrowth through TrkA-independent signaling pathways coupled with CREB-mediated gene transcription in PC12 cells.[24] The data obtained in the present study show that 5-demethylnobiletin- or gardenin A-mediated neurite outgrowth is significantly suppressed through treatment with the CREB:CBP antagonist KG501 but not the TrkA antagonist K252a. Furthermore, 5-demethylnobiletin and gardenin A treatment markedly enhanced the levels of phosphorylated CREB (Ser^{133}) and CRE-dependent transcription activity in PC12 cells. Moreover, the addition of KG-501 or siRNA-mediated knockdown of CREB significantly attenuated the promotion of neurite outgrowth through these two 5-hydroxylated PMFs. These results indicate that 5-demethylnobiletin and gardenin A exhibit similar neurotrophic activity, as 5-OH-HxMF or nobiletin, through CREB-dependent signaling pathways, which is not associated with receptor tyrosine kinase TrkA activation.

It has been reported that activation of other specific receptors such as NMDA receptors, calcium channels, or G-protein-coupled receptors (GPCRs) by external stimuli results in the activation of CREB.[34] Activation of the NMDA receptor, GPCRs, or calcium channel can increase intracellular calcium concentration, stimulate adenylate cyclase activity, and activate cAMP-dependent PKA pathway, resulting in increased CREB phosphorylation. In this study, 5-demethylnobiletin and gardenin A significantly increased PKA activity. The addition of H-89 or Rp-cAMPS to 5-demethylnobiletin- or gardenin A-treated PC12 cells for inhibition of cAMP-dependent PKA activity markedly decreased the percentage of neurite outgrowth and CREB activation. This result indicates that the cAMP-dependent PKA signaling pathway plays a critical role in the neurotrophic action of 5-demethylnobiletin and gardenin A. The mechanism by which 5-demethylnobiletin and gardenin A affect adenylate cyclase activity or cAMP activation is unclear. Whether it is through the activation of specific receptors, calcium channel, or adenylate cyclase alone, thereby elevating cAMP concentration to increase PKA activity, remains to be clarified.

In addition to PKA, the activation of other signaling transduction pathways, such as MAPK/ERK, PKC, CaMK, and PI3-K, converge at the level of CREB signaling.[35] In the present study, we observed that inhibitors of MEK1/2 and PKC significantly blocked the potentiation of 5-demethylnobiletin- or gardenin A-induced neurite outgrowth, CREB-Ser^{133} phosphorylation, and CRE-mediated transcription in PC12 cells. In contrast, CaMK II and PI3-K inhibitors did not affect the neurite outgrowth induced through these two 5-hydroxylated PMFs. These results suggest that the 5-demethylnobiletin and gardenin A induce neurite outgrowth in PC12 cells through MAPK/ERK- and PKC-dependent CREB signaling pathways. Herein, we also found that the level of ERK phosphorylation induced by 5-demethylnobiletin or gardenin A was partially attenuated by the addition of PKC or PKA inhibitor. These results reveal the involvement of cAMP/PKA/ERK/CREB- and PKC/ERK/CREB-dependent pathways in the 5-demethylnobiletin- and gardenin A-mediated neurite outgrowth of PC12 cells. It has been reported that nobiletin promoted neurite outgrowth through the activation of the cAMP/PKA/ERK/CREB pathway in PC12D cells.[20] However, the 5-hydroxylated PMF, 5-OH-HxMF, induces neurite outgrowth through cAMP/PKA/CREB-dependent, not ERK-dependent, signal transduction.[24] The structural difference between 5-demethylnobiletin and 5-OH-HxMF occurs at the 3-position of the C ring with $-H$ and $-OCH_3$, respectively. However, it is unclear whether 5-hydroxylated PMFs with an $-OCH_3$ group in the C ring play an essential role in the interference of ERK-mediated neurite outgrowth in PC12 cells.

In conclusion, these results demonstrate that 5-demethylnobiletin and gardenin A (2−20 μM) promote PC12 neurite outgrowth accompanied by the expression of neuronal differentiation and synapse formation markers. Furthermore, we demonstrated the involvement of MAPK/ERK-, PKC-, and PKA-dependent, but not TrkA-dependent, CREB signaling pathways in the neuritogenesis mediated through these two PMF compounds in PC12 cells.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 showing analysis results of GAP-43 through immunofluorescence assay in PC12 cells, Figure S2 showing that NGF induces synaptophysin expression in PC12 cells, and Table S1 listing the effects of nobiletin, 5-demethylnobiletin (5-demethyl NOB), gardenin A, and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-OH-HxMF) on neurite outgrowth in PC12 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This study was supported through Grants NSC-97-2320-B-320-011-MY3 and NSC 101-2313-B-320-001 (to J.-H.Y.) and Grant NSC-99-2320-B-041-005-MY3 (to M.-J.W.) from the National Science Council, Taiwan, Republic of China.

Notes

The authors declare no competing financial interest.

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