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Curcuminoids Promote Neurite Outgrowth in PC12 Cells through MAPK/ERK- and PKC-Dependent Pathways

Kuo-Kai Liao,[†] Ming-Jiuan Wu,[‡] Pei-Yi Chen,[§,∥] Szu-Wei Huang,[†] Shu-Jun Chiu,[⊥] Chi-Tang Ho,[#] and Jui-Hung Yen*[,†,§]

[†]Department of Molecular Biology and Human Genetics, Tzu Chi University, Hualien 970, Taiwan

[‡]Department of Biotechnology, Chia-Nan University of Pharmacy and Science, Tainan 717, Taiwan

[§]Institute of Medical Science, Tzu Chi University, Hualien 970, Taiwan

[∥]Center of Medical Genetics, Buddhist Tzu Chi General Hospital, Hualien 970, Taiwan

[⊥]Department of Life Science, Tzu Chi University, Hualien 970, Taiwan

[#]Department of Food Science, Rutgers University, 65 Dudley Road, New Brunswick, New Jersey 08901-8520, United States

Supporting Information

ABSTRACT: Curcuminoids, the predominant polyphenolic compounds in the rhizome of *Curcuma longa* Linn., consist of curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC). They exhibit multiple desirable characteristics for a neuroprotective agent including antioxidant, anti-inflammatory, and antiamyloid activities. In this work, we report the first investigation of the neurotrophic action and mechanism of curcuminoids in PC12 cells, which respond to nerve growth factor (NGF) and therefore serve as a model system for primary neuronal cells. The percentages of neurite-bearing cells for those treated with 20 μM curcumin, DMC, and BDMC for 72 h reached 21.6 ± 2.0%, 16.3 ± 2.4%, and 19.9 ± 2.5%, respectively, and were significantly higher than that of the negative control (2.0 ± 0.3%, $p < 0.05$). In parallel, increased expression of the neuronal differentiation markers, growth-associated protein-43 (GAP-43), and neurofilament-L (NF-L) was found in curcuminoid-treated cells. All three curcuminoids (20 μM) activated extracellular signal-regulated protein kinase 1/2 (ERK1/2) and protein kinase C (PKC) signalings, and inhibition of these kinases with the respective pharmacological inhibitors effectively attenuated curcuminoid-induced neurite outgrowth. Furthermore, our results show that both curcumin and DMC, but not BDMC, induced phosphorylation of cAMP response element-binding protein (CREB) and CRE-reporter gene activity significantly ($p < 0.05$). These inductions were markedly attenuated by the addition of MEK/ERK or PKC inhibitor; as a consequence, ERK- and PKC-dependent pathways may be involved in curcuminoid-mediated neuritogenesis in PC12 cells. Moreover, activation of CREB coupling with CRE-dependent gene transcription may play a vital role for curcumin- or DMC-induced PC12 differentiation.

KEYWORDS: *curcuminoids, PC12 cells, neurite outgrowth, ERK1/2, PKC, CREB*

INTRODUCTION

Neurotrophic factors, such as nerve growth factor (NGF), are a family of secreted proteins that play vital roles in promoting neural growth and survival during development, and are crucial for maintaining the integrity of neurons throughout an individual's entire lifetime.[1] There is growing evidence that reduced neurotrophic support is a significant factor in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS).[2] Therefore, neurotrophic factors are attractive candidates for therapeutic agents in chronic neurodegenerative diseases and acute injuries including trauma and stroke. However, therapeutic application of neurotrophic factors is severely restricted by their poor penetration of the blood–brain barrier (BBB) and undesirable apoptotic effect through interaction with the $p75^{NTR}$ receptor.[3] Thus, there remains the need for identification of small molecules that are safe, nontoxic, and can mimic the neurotrophic action as an alternative therapy approach.[4]

Several food-derived polyphenols, such as epigallocatechin-3-gallate (EGCG),[5] nobiletin,[6] luteolin,[7] and garcinol[8] have been demonstrated to be neuroprotective in vitro. Recent literature strengthens the perception that diverse molecular signaling pathways, participating in the neuroprotective activity of polyphenols, renders this type of dietary phytochemicals as potential agents to reduce the risk of various neurodegenerative diseases.[9,10] These effects include selective actions on a number of protein kinase and lipid kinase signaling cascades, most notably the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen activated protein kinase (MAPK) pathways that regulate pro-survival transcription factors and gene expression.[11]

Curcuma longa is a rhizomatous herbaceous perennial plant of the ginger family Zingiberaceae indigenous to South and Southeast Asia. Turmeric from the rhizome of this plant has a long history of use in Asian cookery, medicine, cosmetics, and fabric dying for more than 2000 years.[12] Curcuminoids are the active components responsible for the majority of the medicinal properties of turmeric, and they consist of a mixture of curcumin

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(75−80%), demethoxycurcumin (DMC, 15−20%), and bisdemethoxycurcumin (BDMC, 3−5%) (Figure S1 in the Supporting Information).[13,14] Curcuminoids have multiple desirable characteristics for a neuroprotective drug, including antioxidant, anti-inflammation, and antiprotein-aggregate activities.[15] Their pleiotropic activities were derived from complex chemistry as well as the ability to influence multiple signaling pathways, including survival pathways such as those regulated by Nuclear Factor-κB (NF-κB), Akt, growth factors, and cytoprotective pathways dependent on NF-E2-related factor 2 (Nrf2).[12,16]

Curry consumption has been reported to be associated with better cognitive functions in elderly Asians.[17] Some reports have suggested that curcumin may transverse the blood−brain barrier (BBB) and possess possible beneficial effects on the experimental models of AD.[18,19] An in vitro study also demonstrated that curcumin protected PC12 cells, a rat pheochromocytoma cell line, from 1-methyl-4-phenylpyridinium ion-induced apoptosis,[20] amyloid-β-protein (Aβ) 25−35-induced neurotoxicity,[21] and inhibited activity of β-secretase, which mediates Aβ generation by cleavage of β-amyloid precursor protein (APP).[22] It has been characterized that neurotrophic agents, such as NGF, cAMP, flavonoids, and other natural phenolic compounds could promote neuronal differentiation in PC12 cells. However, there is scarce information regarding whether curcuminoids possess this kind of neurotrophic activity. In the present study, we aim to examine the effects and underlying mechanisms of curcumin, DMC, and BDMC on the promotion of neurite outgrowth and neuronal differentiation in PC12 cells.

MATERIALS AND METHODS

Chemicals. Curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) were purified by HPLC and provided by Sabinsa Corporation (Piscataway, NJ). Poly-L-lysine hydrobromide and dimethyl sulfoxide (DMSO) as well as other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated. U0126 [1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio)-butadiene], a selective and potent inhibitor of MAPK/ERK kinase1/2 (MEK1/2), was purchased from Promega (Madison, WI). Bisindolylmaleimide I (BIM), a protein kinase C inhibitor, was purchased from Cayman chemical (Ann Arbor, MI).

Cell Culture. PC12 cell, a rat adrenal pheochromocytoma cell line, was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). The floating cells were maintained in RPMI-1640 medium (Sigma-Aldrich) 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) and 5% fetal bovine serum (FBS) (Biological Industries, Kibbutz Haemek, Israel) in a 5% CO_2 incubator at 37 °C.

Analysis of Cell Viability by MTT Assay. Cell viability was measured by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to purple formazan. Briefly, cells were incubated with MTT solution (1 mg/mL final concentration) for 4 h at 37 °C followed by centrifugation at 8,000*g* for 4 min. The medium was carefully removed by aspiration, and formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The extent of the reduction of MTT was determined by measurement of the absorbance at 550 nm.

Analysis of Neurite Outgrowth of PC12 Cells. Before starting the experiment, 6-well culture plates (Corning Inc., NY) were coated with poly-L-lysine as follows: 1 mL of a sterile aqueous solution of poly-L-lysine hydrobromide (0.1 mg/mL) was added to each well. After rocking gently to ensure coating of the well surface for 1 h, the solution was removed by aspiration. The surface of the dish was rinsed with sterile water and dried for at least 2 h before introducing cells and medium. PC12 cells (passage number <10) were seeded in poly-L-lysine-coated 6-well plates at a density of 3×10^5/mL with normal serum medium, and after 24 h, cells were changed to low serum (1% horse serum and 0.5% FBS) medium and treated with vehicle (0.1% DMSO) or indicated reagents for 72 h. PC12 cells were photographed by an inverted microscope (Olympus IX71) using phase-contrast objectives and examined later by counting the neurite-positive cells. Images of two fields per well were taken with an average of 100 cells per field. The number of differentiated cells was determined by visual examination of the field and counting cells that had at least one neurite with a length equal to the cell body diameter and expressed as a percentage of the total cells in the field. The neurite length was also measured for all identified neurite-bearing cells in a field by tracing the longest length of neurite per cell using Image J 1.42 software (NIH Image software). Each experiment was conducted in triplicate.

Reverse Transcription Quantitative PCR (RT-Q-PCR) Analysis of GAP-43 and Neurofilament-L mRNA Expression. PC12 cells (1×10^6/mL) were seeded in poly-L-lysine-coated 6-well plates in normal medium for 24 h. The cells were then shifted to low serum (1% horse serum and 0.5% FBS) for 24 h prior to exposure to vehicle (0.1% DMSO) or indicated reagents for 72 h. Total cellular RNA was prepared using Total RNA mini Kit (Geneaid, Taipei, Taiwan). Reverse transcription of 2 μg of RNA was performed using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed with 2 μL of cDNA obtained as described above in 25 μL containing 200 nM primers [GAP-43, 5′-CTAAGGAAAGTGCCCGACAG-3′ (forward) and 5′-GCAGGAGAGACAGGGTTCAG-3′ (reverse); neurofilament-L, 5′-TAGCGCCATGACGGGACACAATC-3′ (forward) and 5′-TCTTCCTGGACGTGGCTGGTAT-3′ (reverse); β-actin, 5′-CCTCTGAACCCTAAGGCCAA-3′ (forward) and 5′-AGCCTG-GATGGCTACGTACA-3′ (reverse)][7,23] and Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Burlington, CA). Amplification was conducted in an ABI Prism 7300 Real-Time PCR System. PCR conditions were as follows: 94 °C for 4 min, 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 was used for data analysis of GAP-43 and neurofilament-L mRNA expression estimated in triplicate samples and normalized to β-actin expression levels.

Western Blotting Analysis of GAP-43, ERK, Phospho-ERK, CREB, and Phospho-CREB Proteins. PC12 cells (1×10^6/mL) were seeded in poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h, then shifted to low serum medium (1% horse serum and 0.5% FBS) for 24 h prior to exposure to vehicle (0.1% DMSO) or indicated reagents for the indicated periods. Cells were washed with phosphate buffer saline (PBS), scraped in ice cold RIPA buffer (Thermo Fisher Scientific, Inc., Rockford, IL), and incubated on ice for 15 min. The cellular debris was removed by centrifugation (8,000*g* for 15 min) at 4 °C, and the cell lysate was carefully transferred to the microcentrifuge tube. The protein concentration was measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

The cell lysate (30 μg) was separated on 10% SDS−PAGE and transferred onto the PVDF membrane (PerkinElmer, Waltham, MA) at 25 V overnight at 4 °C. The membranes were blocked at 4 °C in blocking buffer PBST (1% nonfat dried milk in PBS containing 0.1% Tween-20) for 8 h. Blots were incubated with the appropriate antibodies: anti-GAP-43 (1:1000) (Millipore, Billerica, MA), anti-β-actin (1:8000) (Sigma-Aldrich), anti-CREB (1:1000), antiphospho-CREB (Ser133) (1:1000), anti-p44/p42 MAPK (ERK1/2) (1:1000), and antiphospho-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204) (1:1000) (Cell Signaling Technology, Beverly, MA) overnight at 4 °C. After three washes with PBST, the blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:10,000) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The blots were washed with PBST, and the proteins were detected by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) according to the manufacturer's instructions, and then the chemiluminescence signal was visualized with Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK).

Reporter Gene Assay of Cyclic AMP Response Element (CRE)-Mediated Transcription Activity. PC12 cells (2×10^5/well) were seeded in poly-L-lysine-coated 24-wells plate in normal serum

medium for 24 h. Cells were then shifted to serum-free medium and cotransfected with the pCRE-Luc Cis-reporter plasmid (Stratagene, La Jolla, CA, USA) and *Renilla* luciferase vector (Promega) using Lipofectamine 2000 (Invitrogen) for 4 h. Cells were then changed into DMEM supplemented with 20% horse serum and 10% FBS medium. Twenty-four hours after transfection, cells were changed to low serum medium and treated with vehicle (0.1% DMSO) and curcuminoids (20 μM) for 8 h and harvested by using Passive Lysis Buffer (Promega). Luciferase activities were determined by Dual-Luciferase Reporter Assay System Kit (Promega) according to the manufacturer's instructions. The intensity of the luciferase reactions measured in the lysates of the transfectants was normalized to their *Renilla* luciferase activity, which was used as an internal control.

Statistical Analysis. All experiments were repeated at least three times. All values are expressed as the mean ± SD. Statistic calculations were performed using SPSS 12.0 software. One-way ANOVA with Dunnet's posthoc test was used to determine the significant differences between means of compound-treated groups and vehicle. Two-sided Student's *t*-test was used to assess significant differences between means of specific inhibitor-treated and -untreated groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effects of Curcuminoids on the Cell Viability and Neurite Outgrowth of PC12 Cells. To evaluate the effects of curcuminoids on PC12 cell viability, the MTT assay was performed as described in Materials and Methods. It is found that all three compounds (5−20 μM) stimulated cell proliferation to different extents: hormetic response curves were noted for DMC and BDMC; however, a dose-dependent increase was found for curcumin (Figure 1). Nevertheless, none of the compounds exhibited cytotoxicity in the PC12 cells after treatment of all three curcuminoids for 72 h in low serum medium.

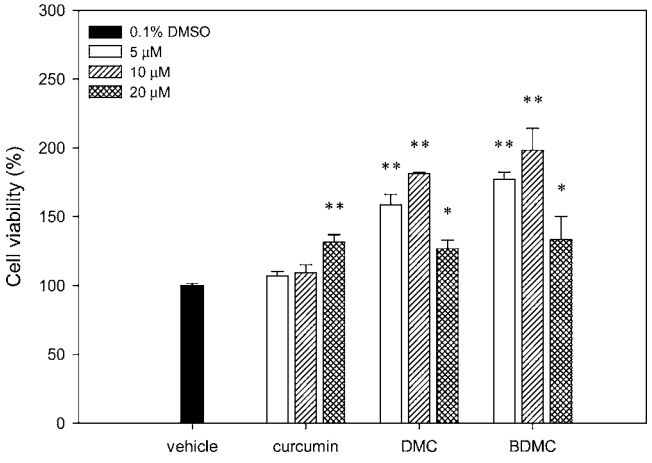

Figure 1. Effects of curcuminoids on the cell viability of PC12 cells. PC12 cells were seeded in 24-well plates in low serum medium (1% horse serum and 0.5% FBS) and exposed to vehicle (0.1% DMSO) as a negative control or indicated agents for 72 h. Cell viability was determined by the MTT assay as described in Materials and Methods. Values are expressed as the percentage of vehicle-treated control. Data represent the mean ± SD from three independent experiments. *$p < 0.05$ and **$p < 0.01$ represent significant differences compared with vehicle-treated cells (negative control) (ANOVA followed by Dunnet's posthoc test).

Significant higher and longer neurite outgrowth was also observed in curcuminoid-treated cells (Figure 2). The percentage of neurite-bearing cells increased as the curcuminoid concentration increased. The percentage of neurite-bearing

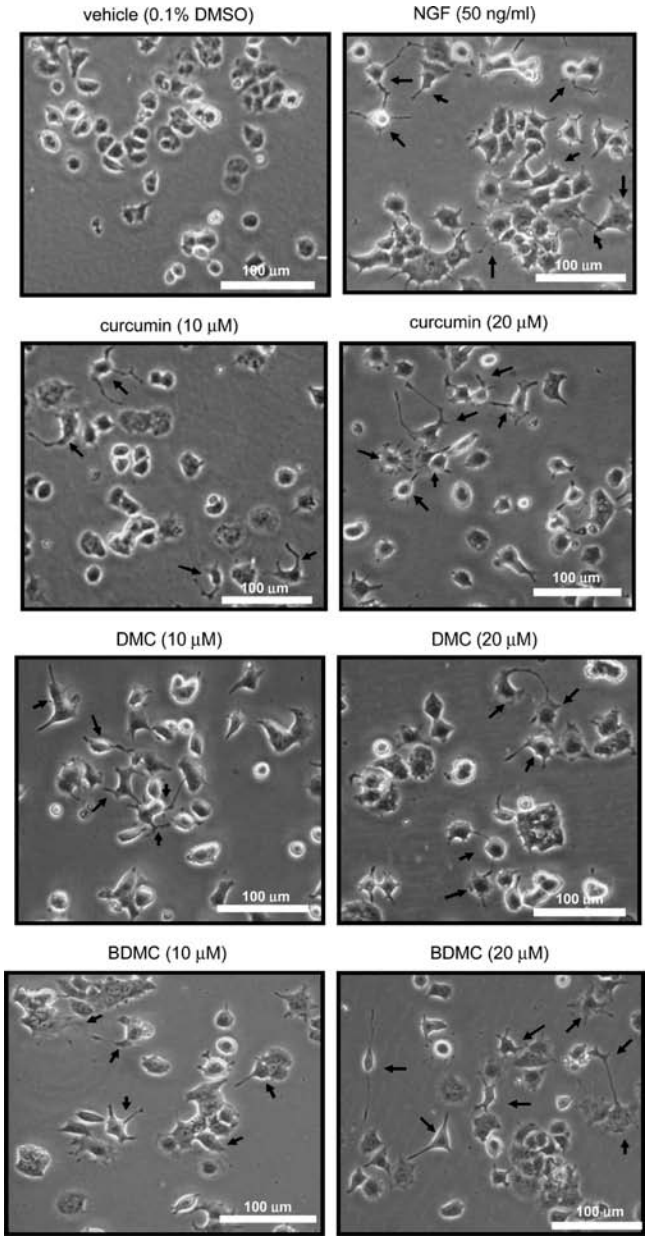

Figure 2. Effects of curcuminoids on the neurite outgrowth in PC12 cells. PC12 cells were seeded in poly-L-lysine-coated 6-well plates in normal serum medium for 24 h, then shifted to low serum medium (1% horse serum and 0.5% FBS), and exposed to vehicle (0.1% DMSO) as a negative control, nerve growth factor (NGF; 50 ng/mL) as a positive control, and curcumin, demethoxycurcumin (DMC), or bisdemethoxycurcumin (BDMC) (10−20 μM) for an additional 72 h. Cell morphology was observed and photographed as described in Materials and Methods. Arrowhead indicates the neurite-bearing cells in the vehicle-, NGF (50 ng/mL)-, or curcuminoid (10- 20 μM)-treated groups.

cells for cells treated with 20 μM curcumin, DMC, and BDMC reached 21.6 ± 2.0%, 16.3 ± 2.4%, and 19.9 ± 2.5%, respectively (Table 1). These were compatible with those treated with 50 ng/mL NGF (23.3 ± 1.9%) and significantly higher than those of negative control (2.0 ± 0.3%). In addition, the maximal neurite length also markedly increased in cells treated with curcuminoids as compared with those of the negative control, but no dose−response was found (Table 1). These data indicate that curcuminoids promote neurite outgrowth in PC12 cells.

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Table 1. Effects of Curcuminoids on Neurite Outgrowth in PC12 Cells[a]

compd treatment	neurite bearing cells (%)	neurite length (μm)
negative control		
vehicle	2.0 ± 0.3	14.8 ± 0.9
NGF		
50 ng/mL	23.3 ± 1.9**	45.2 ± 3.7**
curcumin		
10 μM	5.9 ± 1.1*	22.1 ± 3.6*
20 μM	21.6 ± 2.0**	23.5 ± 5.2*
DMC		
10 μM	13.5 ± 2.8**	28.5 ± 2.0**
20 μM	16.3 ± 2.4**	31.0 ± 2.2**
BDMC		
10 μM	11.0 ± 1.8**	20.6 ± 2.0*
20 μM	19.9 ± 2.5**	24.2 ± 2.6*

[a]PC12 cells were seeded in poly-L-lysine-coated 6-well plates in normal serum medium for 24 h, then shifted to low serum medium (1% horse serum and 0.5% FBS) and exposure to vehicle (0.1% DMSO) as a negative control or indicated agents for additional 72 h. Neurite bearing cells and neurite length were analyzed as described in Materials and Methods. Values are the mean ± SD from three independent experiments. *p < 0.05 and **p < 0.01 represent significant differences compared with negative control (ANOVA followed by Dunnet's posthoc test).

Effects of the Curcuminoids on the Expression of Neuronal Differentiation Markers in PC12 Cells. We further investigated the expression of neuronal differentiation markers, growth-associated protein-43 (GAP-43) and neurofilament-L (NF-L), in curcuminoid-treated PC12 cells. Table 2

Table 2. Effects of Curcuminoids on the GAP-43 and Neurofilament-L mRNA Expression in PC12 Cells[a]

	fold increase in mRNA expression	
compd treatment	GAP-43	neurofilament-L
negative control		
vehicle	1.0 ± 0.1	1.0 ± 0.0
curcumin		
10 μM	1.4 ± 0.1*	1.7 ± 0.4*
20 μM	1.5 ± 0.1*	2.4 ± 0.5**
DMC		
10 μM	1.6 ± 0.2*	2.0 ± 0.4*
20 μM	1.8 ± 0.3*	2.5 ± 0.3**
BDMC		
10 μM	1.8 ± 0.1*	1.4 ± 0.2*
20 μM	1.7 ± 0.3*	2.4 ± 0.1**

[a]Values are the mean ± SD of three independent experiments. p < 0.05 and p < 0.01 represent significant differences compared with the negative control (ANOVA followed by Dunnet's posthoc test).

shows that the relative mRNA levels of GAP-43 and NF-L increased to 1.5-, 1.8-, and 1.7-fold as well as 2.4-, 2.5-, and 2.4-fold, after the addition of 20 μM curcumin, DMC, and BDMC for 72 h, respectively, as compared with the vehicle-treated group (negative control). Western blot analysis also reveals that GAP-43 protein expression was significantly induced by curcuminoids after 72 h of treatment. GAP-43 protein levels were elevated 4.4-, 3.5-, 2.6-, and 4.2-fold in response to curcumin, DMC, BDMC (20 μM), and NGF (50 ng/mL), respectively, as compared to the vehicle-treated group (Figure 3). These results reveal that curcuminoids induce the gene expression

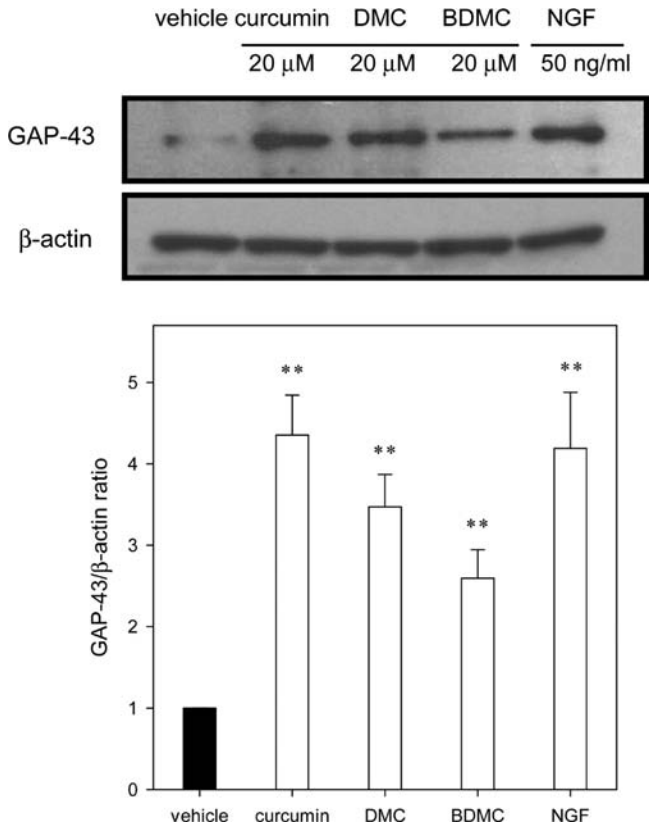

Figure 3. Effects of curcuminoids on the growth-associated protein-43 (GAP-43) protein expression in PC12 cells. PC12 cells were seeded in poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h and then shifted to low serum medium (1% horse serum and 0.5% FBS) for 24 h prior to exposure to indicated agents. Cells were treated with vehicle (0.1% DMSO) as a negative control, curcuminoids (20 μM), or NGF (50 ng/mL) for 72 h. GAP-43 protein expression was detected by Western blotting as described in Materials and Methods. The immunoblot experiments were replicated three times, and a representative blot was shown. Normalized intensity of GAP-43 versus β-actin is presented as the mean ± SD of three independent experiments. **p < 0.01 represent significant differences compared with vehicle-treated cells (negative control) (ANOVA followed by Dunnet's posthoc test).

of GAP-43 and NF-L associated with the differentiation of PC12 cells in the neuronal phenotype.

Effects of Curcuminoids on the ERK Signaling Pathway and PC12 Cell Differentiation. We next investigated whether extracellular signal-regulated kinase 1/2 (ERK1/2) activation is essential for the neurite outgrowth induced by curcuminoids in PC12 cells. Figure 4A shows BDMC activated the ERK pathway with different kinetics from those of curcumin and DMC in PC12 cells. The phosphorylation of ERK1 (44 kDa) and ERK2 (42 kDa) (Thr202/Tyr204) by curcumin and DMC peaked at 15 min, and then gradually decreased after 60 min. In contrast, BDMC strongly induced ERK activation 15 min after treatment and remained at elevated levels for 60 min. Parallel blots were run and probed with an antibody that detected total levels of ERK1/2, demonstrating an equal amount of loading in the total amount of proteins. To further determine whether ERK1/2 activation relates to curcuminoid-mediated neurite outgrowth, a specific inhibitor of MEK1/2 was added. As shown in Figure 4B, inhibition of ERK phosphoryation by U0126 significantly attenuated the percentage of neurite-

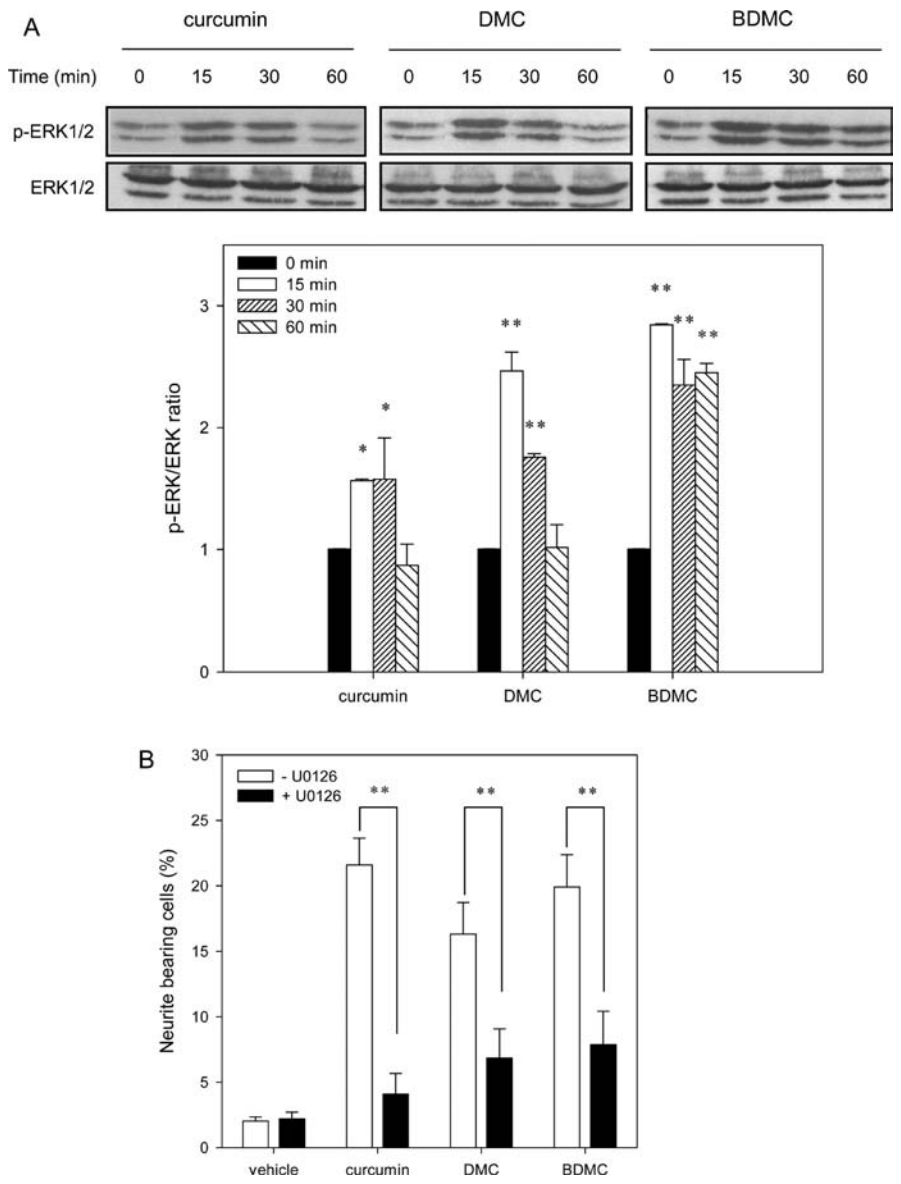

Figure 4. Effects of curcuminoids on the ERK1/2 phosphorylation in PC12 cells. PC12 cells were seeded in poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h and then shifted to low serum medium (1% horse serum and 0.5% FBS) for 24 h prior to exposure to curcumin, DMC, or BDMC (20 μM) for 0, 15, 30, 60 min. (A) Phospho-ERK1/2 (p-ERK1/2) and ERK1/2 were analyzed by Western blotting as described in Materials and Methods. The immunoblot experiments were replicated at least three times, and a representative blot was shown. 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 (ANOVA followed by Dunnet's posthoc test). (B) PC12 cells were seeded on poly-L-lysine-coated 6-well plates in normal medium for 24 h. Cells were then shifted to low serum medium (1% horse serum and 0.5% FBS) and then were pretreated with an inhibitor of MEK1/2, U0126 (10 μM), for 30 min. Cells were then exposed to vehicle (0.1% DMSO) or curcuminoids (20 μM) for 72 h. Neurite-bearing cells were analyzed as described in Materials and Methods. Data represent the mean ± SD from three independent experiments. **p < 0.01 represents significant differences compared with the respective U0126-untreated group (Student's t-test for comparison between groups with and without U0126 treatment).

bearing cells from the original 21.6 ± 2.4% to 4.1 ± 1.6%, 16.3 ± 2.4% to 6.8 ± 2.2%, and 19.9 ± 2.5% to 7.9 ± 2.5% for those treated with 20 μM curcumin, DMC, and BDMC, respectively. These results indicate that ERK signaling is involved in the curcuminoid-induced neuronal differentiation of PC12 cells.

Effects of Curcuminoids on the Activation of CREB in PC12 Cells. We continued to investigate the possible involvement of CREB (cAMP response element-binding protein) in curcuminoid-induced PC12 differentiation. As shown in Figure 5A, treatment of PC12 cells with curcumin and DMC (20 μM) increased the phosphorylation of CREB with time and remained elevated for 16 h. However, treatment of PC12 cells with BDMC did not significantly induce CREB phosphorylation.

To determine whether curcuminoid-increased CREB phosphorylation also activates CRE-mediated transcription, the pCRE-luciferase reporter plasmids and *Renilla* internal control vectors were cotransfected into the PC12 cells as described in Materials and Methods. Figure 5B confirms that when PC12 cells were treated with 20 μM curcumin and DMC, the luciferase activities were significantly increased by 6.2- and 7.6-fold, respectively, as compared to that of cells treated with the

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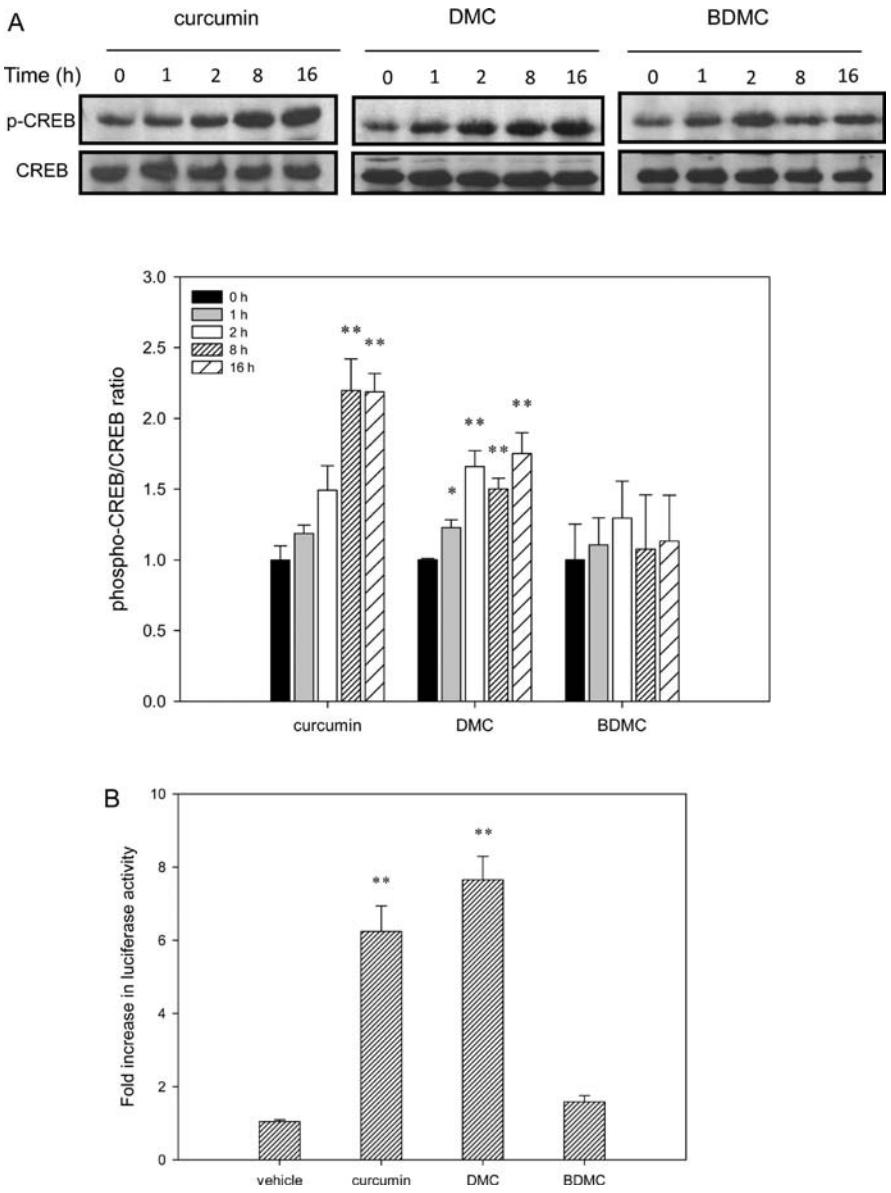

Figure 5. Effects of curcuminoids on the phosphorylation of CREB protein and CRE-mediated transcription in PC12 cells. PC12 cells were seeded on poly-L-lysine-coated 100 mm dishes in normal serum medium for 24 h and then shifted to low serum medium (1% horse serum and 0.5% FBS) for 24 h prior to exposure to indicated agents. (A) Cells were treated with vehicle (0.1% DMSO) as a negative control, curcumin, DMC, or BDMC (20 μM) for 1, 2, 8, and 16 h. Phospho-CREB (p-CREB) and CREB proteins were analyzed by Western blotting as described in Materials and Methods. The immunoblot experiments were replicated at least three times, and a representative blot is shown. 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 h group (ANOVA followed by Dunnet's posthoc test). (B) PC12 cells (2×10^5/well) were seeded on poly-L-lysine-coated 24-well plates in normal serum medium for 24 h. PC12 cells were transfected with a CRE-mediated luciferase reporter construct and *Renilla* luciferase control plasmid as described in Materials and Methods. After transfection, PC12 cells were treated with vehicle (0.1% DMSO), curcumin, DMC, or BDMC (20 μM) for 8 h. Cells were harvested, and luciferase activities were determined as described in Materials and Methods. The intensity of the luciferase reactions measured in the lysates of the transfectants was normalized to their *Renilla* luciferase control activity. Data represent the mean ± SD from three independent experiments. **$p < 0.01$ represent significant differences compared with the vehicle-treated cells (negative control) (ANOVA followed by Dunnet's posthoc test).

vehicle. However, treatment of PC12 cells with BDMC did not significantly increase CRE-mediated reporter activity. These results strongly indicate that curcumin and DMC induced CREB phosphorylation and in turn activated CRE-dependent transcription.

Effects of MAPK/ERK and Protein Kinase C (PKC) on the Curcuminoid-Mediated CREB Activation. To determine whether curcumin- and DMC-induced CREB activation/CRE-driven transcription are mediated by the MAPK/ERK signaling pathway, PC12 cells were transfected with reporter plasmids and treated with U0126 (10 μM) for 30 min, and then incubated with 20 μM curcuminoids before analyzing the luciferase activity of cells. Figure 6A shows that curcumin- and DMC-mediated CREB-transcription activities were markedly attenuated by U0126 ($p < 0.01$). Furthermore, Western blotting shows that inhibition of ERK activation with U0126 significantly abolished curcumin- or DMC-mediated CREB phosphorylation (Figure 6B). These results indicate that curcumin

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and DMC enhanced CRE-driven transcription and neurite outgrowth in PC12 cells via ERK-dependent CREB phosphorylation.

It has been demonstrated that protein kinase C (PKC) activation is involved in NGF-induced neurite outgrowth in PC12 cells;[24] therefore, we further investigated whether curcuminoid-induced neurite outgrowth is dependent on the activation of PKC signaling. Adherent PC12 cells maintained in low serum medium (1% horse serum and 0.5% FBS) were treated with PKC inhibitor, bisindolylmaleimide I (BIM, 5 μM), for 30 min prior to the addition of curcumin, DMC, and BDMC for 72 h. Figure 7A shows that inhibition of PKC signaling significantly attenuated the percentage of neurite bearing cells from the original 21.6 ± 2.0% to 6.2 ± 0.2%, 16.3 ± 2.4% to 7.0 ± 0.8%, and 19.9 ± 2.5% to 8.3 ± 0.3%, for those treated with 20 μM curcumin, DMC, and BDMC, respectively. Cells exposed to BIM (5 μM) also caused a dramatic reduction in the curcumin- or DMC-induced CRE-reporter gene activities (Figure 7B) and CREB phosphorylation (Figure 7C). These results indicate that curcumin and DMC induce neurite outgrowth via the ERK- and PKC-dependent CRE-mediated gene transcription. However, BDMC works through ERK- and PKC-dependent but CREB-independent pathways.

DISCUSSION

The neuroprotective effects of small molecules, such as natural phenolic compounds, have been attributed to their ability to not only exert antioxidant actions through their ability to scavenge reactive oxygen species or their possible influences on intracellular redox status but also modulate cell signaling and gene expression to their purported physiological benefits.[25] It becomes evident that polyphenolics such as flavonoids are able to stimulate neuronal regeneration and induce neurogenesis via their interactions with critical neuronal intracellular signaling pathways pivotal in controlling neuronal survival and differentiation.[26] Studies found that curcumin can transverse the blood–brain barrier and disrupt existing amyloid plaques in vivo.[27] Several cell culture and animal model studies also demonstrated that dietary curcumin has great potential for use in the prevention or treatment of age-related neurodegenerative disorders such as Alzheimer's disease.[28] Recent human trials have reported that curcumin is considered safe, well tolerated, and nontoxic in high single oral dose in human subjects.[29] However, the poor bioavailability of curcumin due to its low solubility, low gastrointestinal absorption, and rapid elimination continues to be highlighted as a major concern for its clinical application. Serum concentrations of curcumin usually peaked 1–2 h after the oral intake of curcumin, and average peak serum concentrations ranged from 0.51 μM at 4000 mg/day (10.9 mmol/day) to 1.77 μM at 8000 mg/day (21.7 mmol/day).[30] Numerous strategies which appear to provide longer circulation, better permeability, and resistance to metabolic processes have been developed.[31]

Because of the relative difficulty of studying signaling in neurons, neurotrophin signaling has been primarily studied using PC12 cells as a model system. Upon NGF exposure, PC12 cells cease mitosis, extend long, branching neurites, become electrically excitable, and express neuronal markers.[32] The extent of PC12 differentiation is therefore typically evaluated by counting the number of cells with expending neurites or by measuring neurite length. To evaluate the neurotrophic effects of NGF and/or test compounds, the culture medium was shifted to low serum medium to induce transition from a proliferative phase to differentiation stage. Our unpublished data showed that treatment of PC12 cells with NGF (25–100 ng/mL) in such a condition significantly induced differentiation in a dose- and time-dependent manner, and neurite-bearing cells

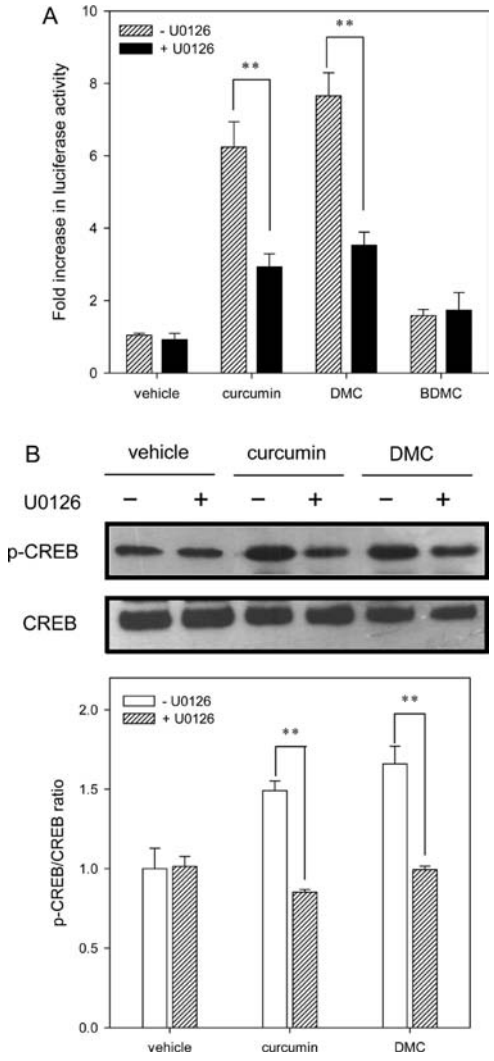

Figure 6. Effects of MEK1/2 inhibitor (U0126) on the curcuminoid-induced CREB activation in PC12 cells. (A) PC12 cells (2×10^5/well) were seeded on poly-L-lysine-coated 24-well plates in normal serum medium for 24 h. PC12 cells were transfected with a CRE-mediated luciferase reporter construct and *Renilla* luciferase control plasmid as described in Materials and Methods. After transfection, cells were pretreated for 30 min with inhibitor U0126 (10 μM) followed by exposure to vehicle (0.1% DMSO) as a negative control, curcumin, DMC, or BDMC (20 μM) for 8 h. The intensity of the luciferase reactions measured in the lysates of the transfectants was normalized to their *Renilla* luciferase control activity. Data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ represents significant differences compared with the respective U0126-untreated group (Student's *t*-test for comparison between groups with and without U0126 treatment). (B) Adherent PC12 cells were preincubated with U0126 (10 μM) for 30 min and then treated with vehicle (0.1% DMSO), curcumin, or DMC (20 μM) for 2 h. Phospho-CREB (p-CREB) and CREB proteins were analyzed by Western blotting as described in Materials and Methods. The immunoblot experiments were replicated at least three times, and a representative blot is shown. 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 the respective U0126-untreated group (Student's *t*-test for comparison between groups with and without U0126 treatment).

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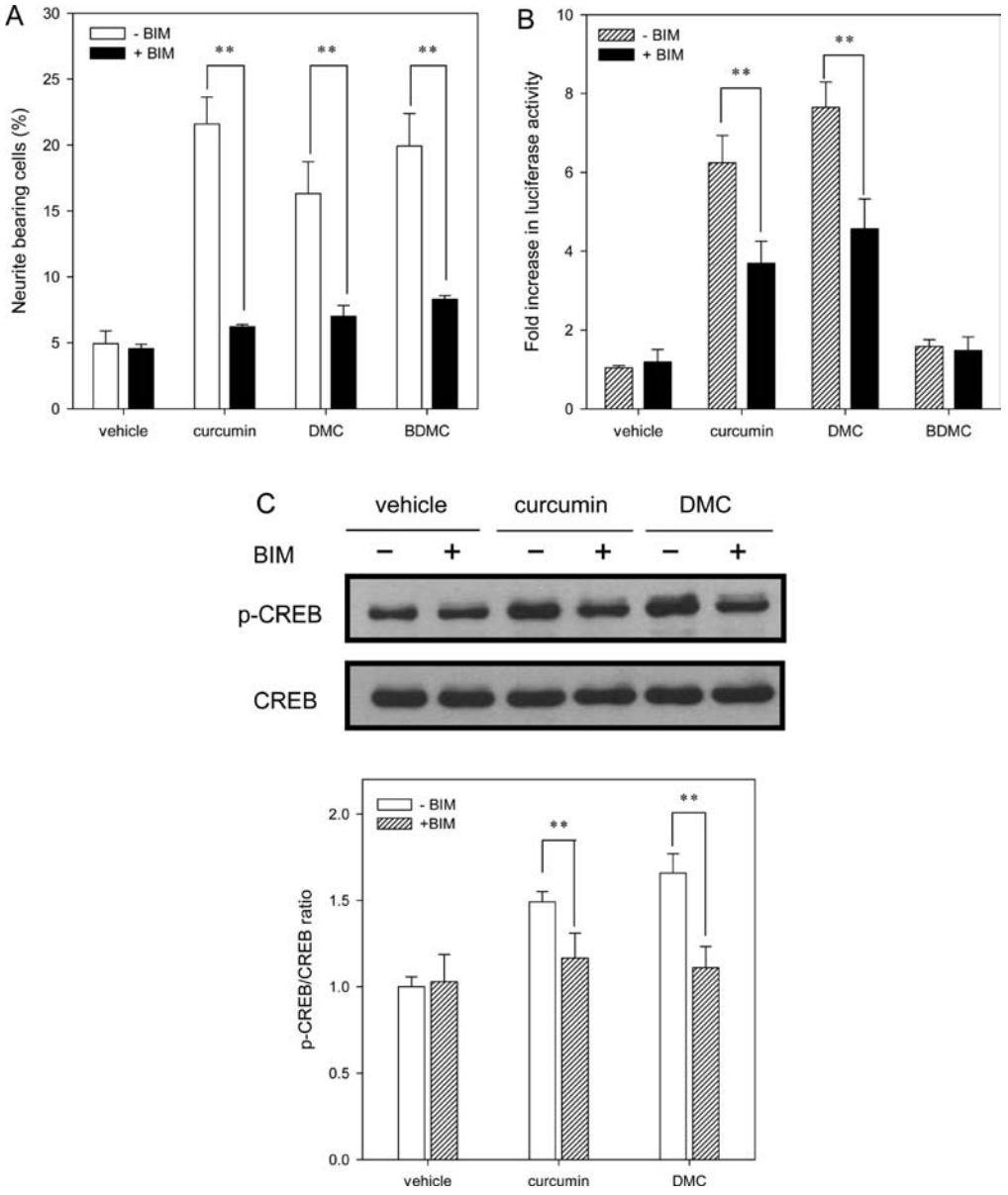

Figure 7. Effects of PKC inhibitor (bisindolylmaleimide I, BIM) on the curcuminoid-mediated neurite outgrowth and CREB activation in PC12 cells. (A) PC12 cells were seeded on poly-L-lysine-coated 6-well plates in normal serum medium for 24 h, then shifted to low serum medium (1% horse serum and 0.5% FBS) and exposure to vehicle (0.1% DMSO) as a negative control or curcuminoids (20 μM) for an additional 72 h. For treatment of cells with inhibitor, adherent cells were preincubated with bisindolylmaleimide I (BIM, 5 μM) for 30 min and then exposed to vehicle or curcuminoids (20 μM) for additional 72 h. Neurite-bearing cells were analyzed as described in Materials and Methods. (B) Reporter plasmid-transfected PC12 cells were pretreated with inhibitor BIM (5 μM) for 30 min followed by exposure to vehicle (0.1% DMSO), curcumin, DMC, or BDMC (20 μM) for 8 h. The intensity of the luciferase reactions was measured as described in Materials and Methods. (C) Adherent PC12 cells were preincubated with inhibitor BIM (5 μM) for 30 min and then treated with vehicle (0.1% DMSO), curcumin, or DMC (20 μM) for 2 h. Phospho-CREB (p-CREB) and CREB proteins were analyzed by Western blotting as described in Materials and Methods. The immunoblot experiments were replicated at least three times, and a representative blot is shown. 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 the respective BIM-untreated group (Student's *t*-test for comparison between groups with and without BIM treatment).

reached ~70% after treatment with 100 ng/mL NGF for 96 h. This data indicate that neuronal differentiation of PC12 cells can be detected robustly in our experimental system. In the current study, we demonstrated that curcuminoids (10–20 μM) modestly induced PC12 neurite outgrowth when they were cultured in this low serum condition (Figure 2).

Growth-associated protein-43 (GAP-43) and neurofilament-L (NF-L) are neuron-specific proteins which exhibit elevated synthesis and axonal fast-transport during nerve regeneration and serve as useful indicators of PC12 cell differentiation.[33,34] The neuronal differentiation marker proteins are the abundant nerve proteins with the function of taking an incoming signal to the effectors and therefore regarded as signal proteins. Therefore, we examined GAP-43 and NF-L expression levels during curcuminoid-induced differentiation of PC12 cells. It is found that curcuminoids induced neurite extension associated with significant increases of neuronal differentiation markers GAP-43 and NF-L mRNA expression in a dose-dependent

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manner (Table 2). In addition, the GAP-43 proteins were induced significantly by curcuminoids (Figure 3). The elevated expression of differentiation makers confirmed that curcuminoids possess the ability to promote PC12 differentiation and may exert neurotrophic action in the nervous system.

It has been well revealed that extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation may activate various protein signaling cascades and influence a large variety of cellular processes, such as cell differentiation, survival, and cell-cycle regulation.[22] It has been reported that NGF interacts with a specific receptor tyrosine kinase (TrkA) and leads to neurite outgrowth and neuronal differentiation through phosphorylation of ERKs in PC12 cells. Several ERK-activating small molecules such as green tea polyphenols, fisetin, resveratrol, and luteolin possessed the activity of neurotrophic action or neuroprotection in in-vitro or in-vivo models. In this study, all three curcuminoids induced ERK phosphorylation (Figure 4A), which was further proven to be involved in the neurite outgrowth of PC12 cells (Figure 4B). The levels of phosphorylated ERK proteins decreased to basal level rapidly in the curcumin and DMC-treated cells; however, BDMC-induced ERK activation was persistent and lasted up to 1 h following exposure (Figure 4A). The different activation kinetics implied that a distinct signaling pathway downstream of ERK may account for BDMC-mediated neurotrophic effect. In addition to ERK, protein kinase C (PKC) is also involved in the curcuminoid-mediated neuritogenesis in PC12 cells. Herein, we found that the percentage of neurite bearing cells induced by curcuminoids was significantly attenuated by the addition of PKC inhibitor, bisindolylmaleimide I (BIM), in PC12 cells (Figure 7A). It has been known that PKC activity also regulates the levels of the GAP-43 mRNA in the PC12 cells and correlates with neurite outgrowth.[35]

Several signaling pathways, including ERK and protein kinase C (PKC), have been associated with regulation of de-novo protein synthesis in the context of LTP (long-term, synaptic plasticity and memory) and converge on the phosphorylation of CREB at Ser^{133}. Phosphorylated CREB protein recruits the transcriptional activator CREB binding protein (CBP) to stimulate transcription of CRE-regulated genes involved in the neurogenesis, neuritogenesis, and cognition function.[36] In this research, our results showed that curcumin and DMC enhanced the levels of Ser^{133}-phosphorylated CREB proteins and stimulated the CRE-mediated transcription activity (Figure 5). These inductions were significantly attenuated by the addition of a specific inhibitor of ERK or PKC signaling pathway (Figures 6A and 7B). However, BDMC did not induce CREB phosphorylation or CRE-mediated luciferase expression, although it exerted neurotrophic action in PC12 cells. The detail mechanisms underlying BDMC-induced CREB-independent neuritogenesis remain to be clarified.

In conclusion, we demonstrated that curcuminoids (10–20 μM), including curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC), can promote PC12 neurite outgrowth accompanied with the expression of neuronal differentiation markers. The involvement of the ERK and PKC signaling pathways in CREB phosphorylation and CRE-driven gene expression was found in response to both curcumin and DMC. However, ERK- and PKC-dependent but CREB-independent pathways were involved in the BDMC-mediated neuritogenic action of PC12 cells (Figure 8). The three curcuminoids usually occur in mixtures in natural foods, and these may be of interest to study, rather than individual compounds only. The bioavailability of curcuminods applied orally is low and difficult to reach the effective concentration for PC12 differentiation in vitro. To overcome the problem, modulation of the administration route such as injection or development of novel delivery strategies may increase its absorption toward the concentrations employed in this study.

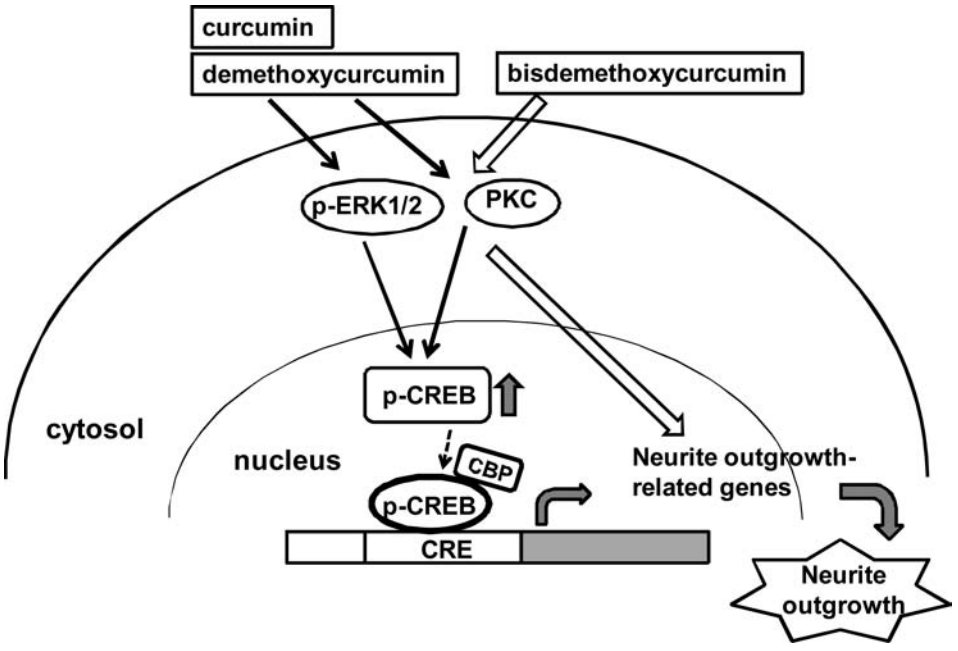

Figure 8. Hypothetic mechanism of curcuminoids in promotion of neurite outgrowth in PC12 cells. Curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) can promote neurite outgrowth associated with expression of neuronal differentiation markers (Figure 2 and 3). Curcumin and DMC induced neurite outgrowth through activation of MAPK/ERK-, PKC- and CREB-dependent pathways. However, BDMC stimulated neurite outgrowth through activation of MAPK/ERK- and PKC-dependent, but CREB-independent pathways in PC12 cells (Figure 4-7).

■ ASSOCIATED CONTENT

Supporting Information

Chemical structures of curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +886-3-856-5301 ext 2683. Fax: +886-3-856-1422. E-mail: imyenjh@mail.tcu.edu.tw.

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■ REFERENCES

(1) McAllister, A. K. Neurotrophins and neuronal differentiation in the central nervous system. *Cell. Mol. Life Sci.* **2001**, *58* (8), 1054–1060.

(2) Dawbarn, D.; Allen, S. J. Neurotrophins and neurodegeneration. *Neuropathol. Appl. Neurobiol.* **2003**, *29* (3), 211–230.

(3) Spedding, M.; Gressens, P. Neurotrophins and cytokines in neuronal plasticity. *Novartis Found Symp.* **2008**, *289*, 222–233; discussion 233–40.

(4) Price, R. D.; Milne, S. A.; Sharkey, J.; Matsuoka, N. Advances in small molecules promoting neurotrophic function. *Pharmacol. Ther.* **2007**, *115* (2), 292–306.

(5) Reznichenko, L.; Amit, T.; Youdim, M. B.; Mandel, S. Green tea polyphenol (-)-epigallocatechin-3-gallate induces neurorescue of long-term serum-deprived PC12 cells and promotes neurite outgrowth. *J. Neurochem.* **2005**, *93* (5), 1157–1167.

(6) Nagase, H.; Yamakuni, T.; Matsuzaki, K.; Maruyama, Y.; Kasahara, J.; Hinohara, Y.; Kondo, S.; Mimaki, Y.; Sashida, Y.; Tank, A. W.; Fukunaga, K.; Ohizumi, Y. Mechanism of neurotrophic action of nobiletin in PC12D cells. *Biochemistry* **2005**, *44* (42), 13683–13691.

(7) Lin, C. W.; Wu, M. J.; Liu, I. Y.; Su, J. D.; Yen, J. H. Neurotrophic and cytoprotective action of luteolin in PC12 cells through ERK-dependent induction of Nrf2-driven HO-1 expression. *J. Agric. Food Chem.* **2010**, *58* (7), 4477–4486.

(8) Weng, M. S.; Liao, C. H.; Yu, S. Y.; Lin, J. K. Garcinol promotes neurogenesis in rat cortical progenitor cells through the duration of extracellular signal-regulated kinase signaling. *J. Agric. Food Chem.* **2011**, *59* (3), 1031–1040.

(9) Macready, A. L.; Kennedy, O. B.; Ellis, J. A.; Williams, C. M.; Spencer, J. P.; Butler, L. T. Flavonoids and cognitive function: a review of human randomized controlled trial studies and recommendations for future studies. *Genes Nutr.* **2009**, *4* (4), 227–242.

(10) Spencer, J. P. The interactions of flavonoids within neuronal signalling pathways. *Genes Nutr.* **2007**, *2* (3), 257–273.

(11) Spencer, J. P. Beyond antioxidants: the cellular and molecular interactions of flavonoids and how these underpin their actions on the brain. *Proc. Nutr. Soc.* **2010**, *69* (2), 244–260.

(12) Hatcher, H.; Planalp, R.; Cho, J.; Torti, F. M.; Torti, S. V. Curcumin: from ancient medicine to current clinical trials. *Cell. Mol. Life Sci.* **2008**, *65* (11), 1631–1652.

(13) Aggarwal, B. B.; Sundaram, C.; Malani, N.; Ichikawa, H. Curcumin: the Indian solid gold. *Adv. Exp. Med. Biol.* **2007**, *595*, 1–75.

(14) Inoue, K.; Nomura, C.; Ito, S.; Nagatsu, A.; Hino, T.; Oka, H. Purification of curcumin, demethoxycurcumin, and bisdemethoxycurcumin by high-speed countercurrent chromatography. *J. Agric. Food Chem.* **2008**, *56* (20), 9328–9336.

(15) Ringman, J. M.; Frautschy, S. A.; Cole, G. M.; Masterman, D. L.; Cummings, J. L. A potential role of the curry spice curcumin in Alzheimer's disease. *Curr. Alzheimer Res.* **2005**, *2* (2), 131–136.

(16) Feng, J. Y.; Liu, Z. Q. Phenolic and enolic hydroxyl groups in curcumin: which plays the major role in scavenging radicals? *J. Agric. Food Chem.* **2009**, *57* (22), 11041–11046.

(17) Ng, T. P.; Chiam, P. C.; Lee, T.; Chua, H. C.; Lim, L.; Kua, E. H. Curry consumption and cognitive function in the elderly. *Am. J. Epidemiol.* **2006**, *164* (9), 898–906.

(18) Singh, M.; Arseneault, M.; Sanderson, T.; Murthy, V.; Ramassamy, C. Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. *J. Agric. Food Chem.* **2008**, *56* (13), 4855–4873.

(19) Balasubramanian, K. Molecular orbital basis for yellow curry spice curcumin's prevention of Alzheimer's disease. *J. Agric. Food Chem.* **2006**, *54* (10), 3512–3520.

(20) Chen, J.; Tang, X. Q.; Zhi, J. L.; Cui, Y.; Yu, H. M.; Tang, E. H.; Sun, S. N.; Feng, J. Q.; Chen, P. X. Curcumin protects PC12 cells against 1-methyl-4-phenylpyridinium ion-induced apoptosis by bcl-2-mitochondria-ROS-iNOS pathway. *Apoptosis* **2006**, *11* (6), 943–953.

(21) Park, S. Y.; Kim, H. S.; Cho, E. K.; Kwon, B. Y.; Phark, S.; Hwang, K. W.; Sul, D. Curcumin protected PC12 cells against beta-amyloid-induced toxicity through the inhibition of oxidative damage and tau hyperphosphorylation. *Food Chem. Toxicol.* **2008**, *46* (8), 2881–2887.

(22) Lin, R.; Chen, X.; Li, W.; Han, Y.; Liu, P.; Pi, R. Exposure to metal ions regulates mRNA levels of APP and BACE1 in PC12 cells: blockage by curcumin. *Neurosci. Lett.* **2008**, *440* (3), 344–347.

(23) Gopalakrishna, R.; Gundimeda, U.; Schiffman, J. E.; McNeill, T. H. A direct redox regulation of protein kinase C isoenzymes mediates oxidant-induced neuritogenesis in PC12 cells. *J. Biol. Chem.* **2008**, *283* (21), 14430–14444.

(24) Das, K. P.; Freudenrich, T. M.; Mundy, W. R. Assessment of PC12 cell differentiation and neurite growth: a comparison of morphological and neurochemical measures. *Neurotoxicol. Teratol.* **2004**, *26* (3), 397–406.

(25) Spencer, J. P. The impact of flavonoids on memory: physiological and molecular considerations. *Chem. Soc. Rev.* **2009**, *38* (4), 1152–1161.

(26) Spencer, J. P. The impact of fruit flavonoids on memory and cognition. *Br. J. Nutr.* **2010**, *104* (Suppl 3), S40–S47.

(27) Yang, F.; Lim, G. P.; Begum, A. N.; Ubeda, O. J.; Simmons, M. R.; Ambegaokar, S. S.; Chen, P.; Kayed, R.; Glabe, C. G.; Frautschy, S. A.; Cole, G. M. Curcumin inhibits formation of amyloid β oligomers and fibrils, binds plaques, and reduces amyloid in vivo. *J. Biol. Chem.* **2005**, *280* (7), 5892–5901.

(28) Cole, G. M.; Teter, B.; Frautschy, S. A. Neuroprotective effects of curcumin. *Adv. Exp. Med. Biol.* **2007**, *595*, 197–212.

(29) Epstein, J.; Sanderson, I. R.; MacDonald, T. T. Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. *Br. J. Nutr.* **2010**, *103* (11), 1545–1557.

(30) Cheng, A. L.; Hsu, C. H.; Lin, J. K.; Hsu, M. M.; Ho, Y. F.; Shen, T. S.; Ko, J. Y.; Lin, J. T.; Lin, B. R.; Ming-Shiang, W; Yu, H. S.; Jee, S. H.; Chen, G. S.; Chen, T. M.; Chen, C. A.; Lai, M. K.; Pu, Y. S.; Pan, M. H.; Wang, Y. J.; Tsai, C. C.; Hsieh, C. Y. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res.* **2001**, *21* (4B), 2895–2900.

(31) Anand, P.; Kunnumakkara, A. B.; Newman, R. A.; Aggarwal, B. B. Bioavailability of curcumin: problems and promises. *Mol. Pharmaceutics* **2007**, *4* (6), 807–818.

(32) Greene, L. A.; Tischler, A. S. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci.U.S.A.* **1976**, *73* (7), 2424–2428.

(33) Schimmelpfeng, J.; Weibezahn, K. F.; Dertinger, H. Quantification of NGF-dependent neuronal differentiation of PC-12 cells by means of neurofilament-L mRNA expression and neuronal outgrowth. *J. Neurosci. Methods* **2004**, *139* (2), 299–306.

(34) Costello, B.; Meymandi, A.; Freeman, J. A. Factors influencing GAP-43 gene expression in PC12 pheochromocytoma cells. *J. Neurosci.* **1990**, *10* (4), 1398–1406.

(35) Perrone-Bizzozero, N. I.; Cansino, V. V.; Kohn, D. T. Post-transcriptional regulation of GAP-43 gene expression in PC12 cells through protein kinase C-dependent stabilization of the mRNA. *J. Cell. Biol.* **1993**, *120* (5), 1263−1270.
(36) Spencer, J. P.; Vauzour, D.; Rendeiro, C. Flavonoids and cognition: the molecular mechanisms underlying their behavioural effects. *Arch. Biochem. Biophys.* **2009**, *492* (1−2), 1−9.