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Original Article

Cortex Mori Radicis Extract induces neurite outgrowth in PC12 cells activating ERK signaling pathway via inhibiting Ca^{2+} influx

Nina Yin*, Xiaoping Hong*, Yongming Han, Yanjun Duan, Yanhong Zhang, Zebin Chen

Department of Anatomy, Basic Medical College, Hubei University of Chinese Medicine, Wuhan 430065, China.
**Equal contributors.*

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Abstract: Cortex Mori Radicis is a traditional Chinese herbal medicine which has a long history of use for the treatment of headaches, cough, edema and diabetes. However, its function and mode of action within nervous system remain largely unclear. In the present study, we have attempted to determine the effects of Cortex Mori Radicis Extract (CMRE) on neuronal differentiation. Here, we reported that CMRE induces the neurite outgrowth in pheochromocytoma PC12 cells and primary cortical neuron. Following the generation of neurite outgrowth, extracellular Ca^{2+} influx was inhibited and intracellular Ca^{2+} decreased. In addition, CMRE induced the extracellular signal-regulated kinase 1/2 (ERK1/2) activation and also stimulated the Rap1-GTP expression, which is closely linked to neuritogenesis. Moreover, the neurite outgrowth induced by CMRE was antagonized to a marked degree by suppressing activation of p-ERK1/2 with the specific ERK1/2 inhibitor (PD98059), suggesting the involvement of Rap1-GTP and ERK1/2 in CMRE-induced neurite outgrowth. Taken together, these results demonstrate that CMRE induces neurite outgrowth of PC12 cells through Rap1-ERK signaling pathway via inhibiting Ca^{2+} influx, and provide a novel insight into the manner in which CMRE participates in neuritogenesis.

Keywords: Cortex Mori Radicis Extract, PC12 cells, ERK signaling pathway, Rap1, Ca^{2+} influx

Introduction

The central nervous system consists of neurons and glial cells, and neurons play a central role in signal transduction by releasing neurotransmitters [1]. The cause of many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis can be ascribed to the loss of functional neurons. Induction of neurite outgrowth by administration of endogenous trophic factors has been proposed as one of several new therapeutic strategies [2, 3]. Medications to alleviate these neurodegenerative diseases can only provide benefits for several years but are not effective as the diseases progress. Some undesired side effects associated with these drugs include hallucinations, dyskinesia, nausea and constipation [4]. In this regard, complementary and alternative medicine which is now gaining momentum may be a promising way for prevention and protection against such neurodegenerative diseases [5].

Cell cultures derived from nervous system tissue have proven to be powerful tools for elucidating cellular mechanisms of nervous system function. The effect of chemicals, drugs, natural products or even growth factors on neurite outgrowth can be quantified by enumerating the number of cells that bear neurites using in vitro cell line model. pheochromocytoma (PC12) cells, originated from a rat adrenal medullary tumour (pheochromocytoma) have been widely employed as a model of neuronal differentiation and neurite outgrowth [6]. In response to nerve growth factor (NGF), these cells extend neurites and develop characteristics of sympathetic neurons [7]. In line, some studies have shown that Rap1 signaling is also involved in various aspects of neuronal differentiation, like the establishment of neuronal polarity or axonal growth cone movement [8, 9]. Differentiation of PC12 cells through the cAMP-Epac-Rap1 axis leaded to activation of B-Raf, phosphorylation of ERK, and accompanying downstream events [10]. In addition, neurite outgrowth from neu-

rons and neuronal cell lines induced by stimulation of neural cell adhesion molecules, such as L1, or by neurotrophins, such as NGF, absolutely requires the activation of ERK [11].

The traditional Chinese herbal medicine Cortex Mori, the root bark of Morus alba L, has a long history of use for the treatment of headaches, cough, edema, cutaneous inflammation, pulmonary asthma and diabetes [12]. According to recent studies, extracts of Cortex Mori Radicis have shown antiinflammatory, antitumor, and hypoglycemic effects [13]. However, its function and mode of action within nervous system remain largely unclear.

In the present study, we aimed to investigate the effects of Cortex Mori Radicis Extract (CMRE) on neuronal differentiation. The results showed that exposure of PC12 and primary cortical neuron to CMRE induces the outgrowth of neurites, and extracellular Ca^{2+} influx was inhibited with CMRE treatment. In addition, CMRE induced the ERK1/2 activation and enhanced the expression of Rap1-GTP. Moreover, the outgrowth of neurites was prevented by PD98059. These results suggest that CMRE may be a novel therapeutic candidate for the treatment of neurodegenerative disease.

Materials and methods

Preparation of cortex mori radicis extract (CMRE)

Cortex Mori Radicis was purchased from Long Pulse medicines Ltd. (Gansu, China). 100 g Cortex Mori Radicis was sliced thinly with scissors soaked in 500ml of distilled water at 50°C for 3 h, and concentrated using a rotary evaporator (Rotary evaporator, BUCHI B-480, Switzerland). The concentrated extracts were lyophilized by Freeze dryer (EYELA FDU-540, Tokyo, Japan). After the lyophilization, yellow-brownish active powder was obtained (yield = 10.8 g) and was dissolved in dimethyl sulfoxide (DMSO) as a stock at a 100 mg/ml concentration and stored at 4°C, and the stock solution (Cortex Mori Radicis Extract, CMRE) was then diluted with medium to the desired concentration prior to use.

Materials

Rat β-NGF was purchased from R&DS ystems (Minneapolis, MN, USA). MTT and LY294002 were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). PD98059 and SB203580 were purchased from Calbiochem (SanDiego, CA, USA). SP600125 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). Anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Rap1B antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

Timed pregnant Sprague-Dawley rats were purchased from the Laboratory Animal Services Center of Hubei University of Chinese Medicine (Wuhan, China). The animals were fed in the new environment (room temperature, 20 ± 1°C; relative humidity, 55 ± 15%; 12 h light/12 h dark illumination cycle). Food and water were provided ad libitum throughout the experiments. All animals were treated in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The day of insemination and the day of birth are designated as embryonic day 0.5 (E0.5).

Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT) supplemented with 10% heat-inactivated horse serum (Gibco, Grand Island, NY), 5% heat-inactivated FBS (Gibco, Grand Island, NY), and antibiotics (100 units/ml streptomycin and 100 units/ml penicillin) (Gibco, Grand Island, NY) at 37°C in a 5% CO_2 incubator. Cells were changed with fresh medium three times per week, and were split at a 1:3 ratio twice per week.

Primary neuronal culture

Neuronal cultures were taken from frontal cortices of Sprague-Dawley rat embryonic (E16-18) pups. Cultures are grown in neurobasal medium (invitrogen, Carlsbad, CA) supplemented with B27, 0.5 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY). Neurons were seeded at a density of 500,000 cells per well of 6-well plates.

MTT assay

PC-12 cells were seeded into 96-well plates (200 μl/well) at a density of 5×10^4 cells/ml. At

the end of the drug incubation period, MTT (Sigma, MO, USA) working solution (0.5 mg/ml) was added to each well, and the plates incubated for an additional 4 h at 37°C. After centrifugation at 350 g for 5 min, the medium was replaced with dimethyl sulfoxide (Sigma, MO, USA). The absorbance of reduced MTT at 570 nm was measured with a plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The inhibitory rate of PC12 cells proliferation (%) = (OD value of control group-OD value of test group) × 100%/OD value of control group. Based on the IC_{50} of CMRE in PC12 cells, one selective concentration of CMRE (50 μg/ml) was adopted for the following test.

Evaluation of neurite outgrowth

PC12 cells (1×10^5 cells/ml) were seeded onto 24-well plates and were cultured for 1 day, after which time drugs were added and the cells cultured for an additional 2 days. The cells were then fixed with 4% paraformaldehyde (Sigma, MO, USA) in phosphate-buffered saline (PBS), and cell morphology was assessed under a phase-contrast microscope. Neurite extension from PC12 cells was regarded as an index of neuronal differentiation. Processes with a length equivalent to one or more diameters of the cell body were regarded as neurites. The differentiation of PC12 cells was evaluated by examining the proportion of neurite-bearing cells to total cells in randomly selected fields. The mean differentiation score was obtained for more than a 100 PC12 cells in each well. In some experiments, images of the cells were captured, and the total length of the neurite extension per positive cell and average length of neurites in positive cells were determined in randomly chosen fields using Motic Images Plus software (version 2.0 S; Motic Instruments Inc., Richmond, Canada).

Electron microscopy

For electron microscopy, monolayers of cortical neurons were used. Cultures were grown on slides were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at for 2 h, rinsed, and post fixed in 1% osmium tetroxide for 1 h at room temperature. They were then dehydrated in graded ethanol series. After triple rinsing in 100% Freon, the specimens were vigorously shaken for a few seconds to dry. The samples were introduced into a sputter coater (Polaron ES100), coated with gold, and examined with a TESCAN.VEGA3LMU (TESCAN, Brno, Czech Republic) at an accelerating voltage of 10 kV. Photographs of selected fields have been taken at different magnifications.

Cytosolic Ca^{2+} measurements

Ratiometric imaging of intracellular Ca^{2+} using cells loaded with fura-2 was measured as previously described. All cells for these experiments were grown in round coverslips (30 mm) under normal tissue culture conditions, except were specified. Coverslips with cells were placed in a cation-safe solution composed of (in mM): 107 NaCl, 7.2 KCl, 1.2 $MgCl_2$, 11.5 glucose, 20 Hepes-NaOH, pH 7.3 and loaded with fura-2/AM (2 μM final concentration) for 30 min at 37°C. Cells were washed, and de-esterification was allowed for a minimum of 15 min. Ca^{2+} measurements were made using a Leica DMI 6000B fluorescence microscope controlled by the Slidebook software (Intelligent Imaging Innovations; Denver, CO). Fluorescence emission at 505 nm was monitored while alternating excitation wavelengths between 340 and 380 nm at a frequency of 0.5 Hz; intracellular Ca^{2+} measurements are shown as 340/380 nm ratio obtained from groups [15-25] of single cells. External solutions were (in mM): 135 NaCl, 5.4 KCl, 10 HEPES, 0.02 NaH_2PO_4, 2 Mg^{2+}, 10 glucose, pH 7.4. Measurements shown are representative of a minimum of three independent experiments.

Western blot

PC12 cells were collected, washed once with ice-cold PBS, and lysed with 0.1-0.2 ml RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.25% NaN_3, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate, and 1mM NaF). Lysates were centrifuged for 10 min at 12,000 × g and supernatants were analyzed for protein concentration using a BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against phosphor-ERK1/2 and ERK1/2, respectively. Immunoreactive bands were visualized by enhanced chemiluminescence. Bands were

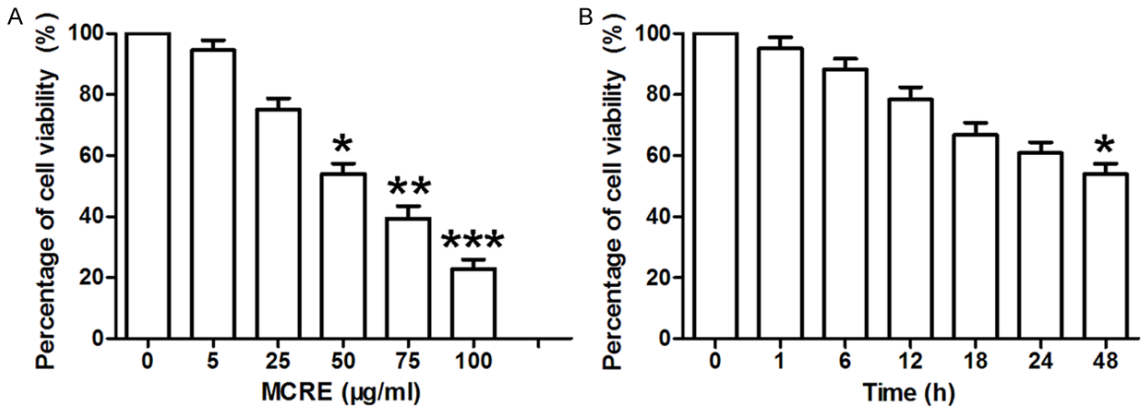

Figure 1. CMRE inhibits cell proliferation in rat pheochromocytoma PC12 cells. PC12 cells were seeded in a 96-well plate at an initial density of 1×10^4 cells per well. After 24 h of stabilization, cells were treated with various concentrations of CMRE for 48 h (A) or incubated with the indicated concentration of CMRE (50 μg/ml) for various periods (B). Cell viability was determined by MTT assay. Results are expressed as the percentage of the vehicle treated control ± SEM of three separate experiments. A Student's t-test was used for the determination of significance. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, comparison with the untreated control.

Table 1. The inhibitory rates of MCRE treatment on the proliferation of PC12 cells

MCR (μg/ml)	OD 570 (Mean ± S.D.)	Inhibitory rate (%)
0	0.995 ± 0.008	0
5	0.960 ± 0.013	5.416 ± 0.603
25	0.638 ± 0.026	24.980 ± 1.276
50	0.586 ± 0.017	46.018 ± 1.305
75	0.367 ± 0.012	60.642 ± 2.038
100	0.252 ± 0.008	77.182 ± 2.229

scanned using a scanner (HP Scanjet 7400C, Hewlett-Packard), and their intensities were quantified with the Image J program (NIH).

Immunoprecipitation

Pull-down assay for activated GTP-Rap1 levels was assessed using GST-RalGDS-RBD, which was expressed in *E.* coli, and bound to GST-beads. Briefly, NGF-stimulated PC12 cells were collected, washed once with ice-cold PBS, and lysed in buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 5% glycerol, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF. Samples were incubated on ice for 5 min followed by centrifugation (16,000 × g, 15 min, 4°C). Equivalent amounts of supernatants were incubated with GST-RalGDS-RBD with GST-beads for GTP-Rap1 for 1 h at 4°C. The beads were washed 3 times with lysis buffer and bound proteins were eluted with 2 × Laemmli sample buffer by boiling. Samples were electrophoresed and analyzed by western blotting with anti-Rap1B antibodies.

Statistical analysis

All results are expressed as the means ± SEM of data obtained from triplicate experiments. Data in two groups were analyzed by Student's t-test. Multiple comparisons of the data were done by ANOVA followed by Tukey's test. Differences at $P < 0.05$ were considered statistically significant.

Results

CMRE treatment inhibits cell proliferation in rat pheochromocytoma PC12 cells

To examine the growth-inhibitory effects of CMRE, MTT assay was used to detect cell viability of PC12 cells. After PC12 cells were treated with various concentrations of CMRE for 48 h, the proliferation of PC12 cells was inhibited with a dose-dependent manner (**Figure 1A**). The inhibitory rates of CMRE in different concentration on PC12 cells were described in **Table 1**. The IC_{50} of CMRE in PC12 cells was 48.993 μg/ml, so CMRE (50 μg/ml) was adopted for the following test. As shown in **Figure 1B**, a timedependent decrease in cell viability was observed after CMRE treatment. The results showed that CMRE induced reduction of viabil-

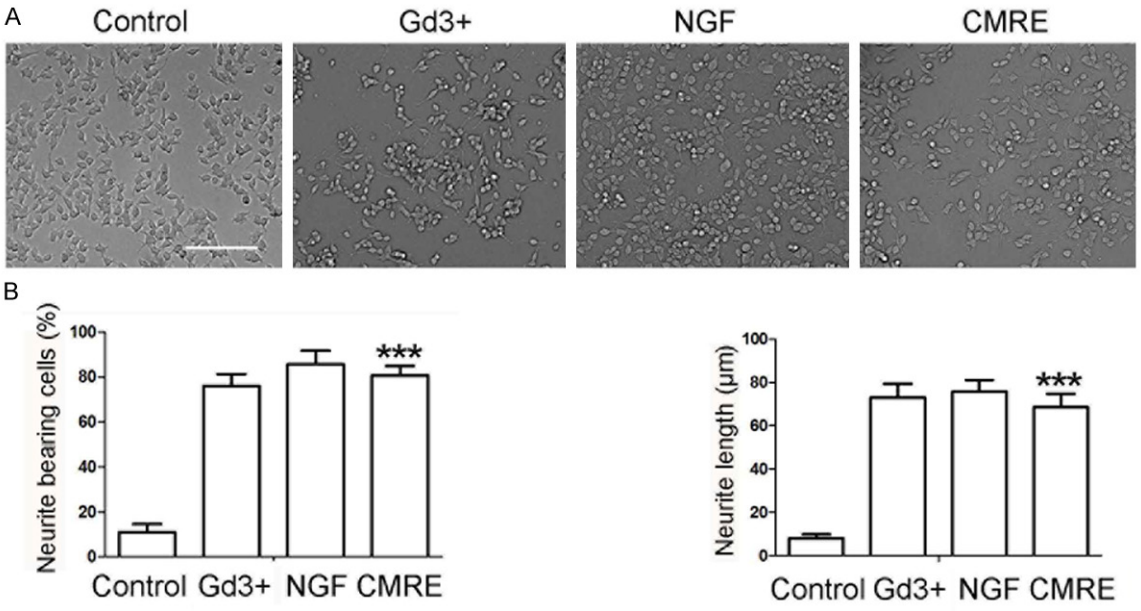

Figure 2. CMRE induces the neurite outgrowth in PC12 cells. PC12 cells (1×10^5 cells/ml) were seeded onto 24-well plates and were cultured for 24 h, after which time drugs were added and the cells cultured for an additional 48 h. A. Morphological change in PC12 cells treated by Gd3+ (100 μM), NGF (50 ng/ml) and CMRE (50 μg/ml) was observed, respectively. Scale bar is 100 μm. B. The percentage of neurite bearing cells was calculated from 5 random fields in one well, and the data are expressed as a mean percentage of 4 wells in one representative experiment. Data are presented as mean ± SEM. $^{***}P < 0.001$, comparison with the untreated cells. C. Length of neurite bearing cells was calculated, and CMRE significantly promoted neurite outgrowth of PC12 cells. Data are presented as mean ± SEM. $^{***}P < 0.001$, comparison with the untreated control.

ity in a dose- and time-dependent manner in PC12 cells.

CMRE induces the neurite outgrowth in PC12 cells and *cortical neuron*

Following CMRE (50 μg/ml) treatment, the generation of neurite outgrowth with morphological change in PC12 cells (**Figure 2A**) and cortical neuron was observed (**Figure 3A**). Both the percentage of neurite bearing cells and the length of the branches were determined to have marked increased as the result of CMRE stimulation in PC12 cells (**Figure 2B** and **2C**). In addition, CMRE significantly increased spine density of cortical neuron with the morphological changes in the scanning electron microscope (**Figure 3B**). NGF, a well-known differentiating factor, was employed in an effort to induce the neurite outgrowth, and was used as a positive control (**Figures 2** and **3**). Gd3+, an inhibitor of store operated calsium channel (SOCC), was also found to be able to induce neurite outgrowth in PC12 cells and cortical neuron (**Figures 2** and **3**).

Inhibition of extracellular Ca^{2+} influx is involved in CMRE-induced neurite growth

To examine the mechanism of CMRE-signaling, cytosolic Ca^{2+} was measured by the fura 2 assay (**Figure 4A**). CMRE induced inhibition of Ca^{2+} influx in a time-dependent manner in PC12 cells, which was consistent with the effect of Gd3+. Interestingly, NGF induced the neurite outgrowth following the more increased Ca^{2+} influx compared with CMRE-induced neurite growth (**Figure 4B**). These results suggested that CMRE-induced neurite growth might involve the different signaling pathway unlike NGF.

The Rap1-ERK signaling pathway is involved in the CMRE-induced neurogenesis

To better understand the signal pathways involved in neurogenesis, we attempted to assess the effects of CMRE on the activity of several signaling pathways. The phosphorylation of ERK1/2 was increased by CMRE treatment (**Figure 5A** and **5B**). To confirm the involve-

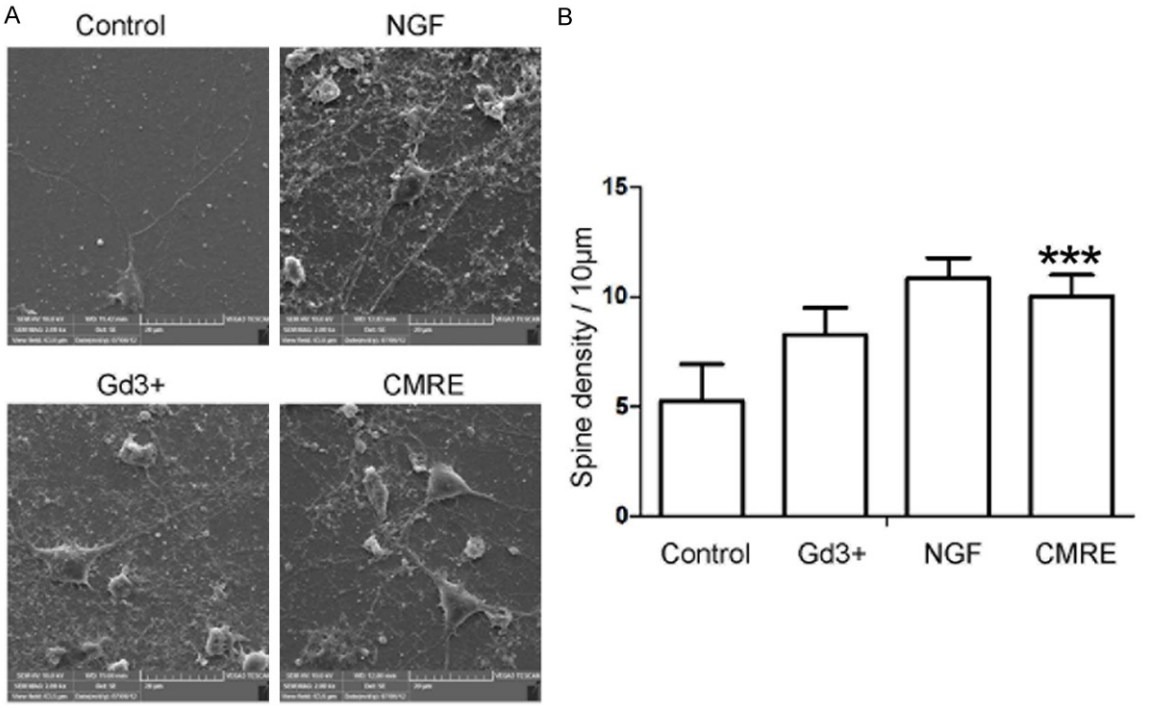

Figure 3. CMRE increases cortical neuron spine density with the morphology in the scanning electron microscope. Neuronal cultures were taken from frontal cortices of SD rat embryonic (E16-18) pups. Cortical neurons were seeded at a density of 5 × 10^5 cells per well of 6-well plates and were cultured for 2 weeks, after which time drugs were added and the cells cultured for an additional 48 h. A. Neuron body and dendrite were observed following treatment with Gd3+ (100 μM), NGF (50 ng/ml), CMRE (50 μg/ml), respectively. B. The average spine density was calculated, and CMRE markedly increased neurite outgrowth of cortical neuron. Data are presented as mean ± SEM and one representative experiment is shown. $^{***}P < 0.001$, comparison with the untreated neuron.

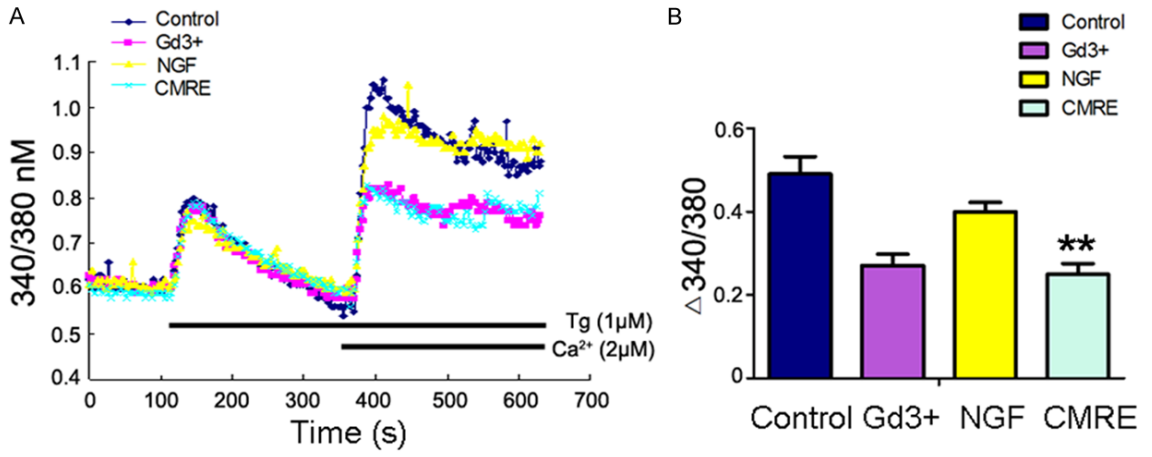

Figure 4. Inhibition of extracellular Ca^{2+} influx is involved in CMRE-induced neurite growth. Cytosolic Ca^{2+} was measured in Fura-2-loaded PC12 cells plated to glass coverslips. A. Following treated with Gd3+ (100 μM), NGF (50 ng/ml), CMRE (50 μg/ml), respectively, and representative traces of intracellular Ca^{2+} changes (measured as F340/F380) in PC12 cells was observed. B. Involvement of inhibiting extracellular Ca^{2+} influx in CMRE-induced neurite growth. Al data shown are from individual experiments representative of at least three independent replicates. Data are expressed as mean ± SEM. $^{**}P < 0.01$, comparison with the untreated control.

ment of the ERK signaling pathway, we pre-treated the cells with various inhibitor including PD98059, LY294002, SB203580 and SP600125 prior to CMRE treatment, and

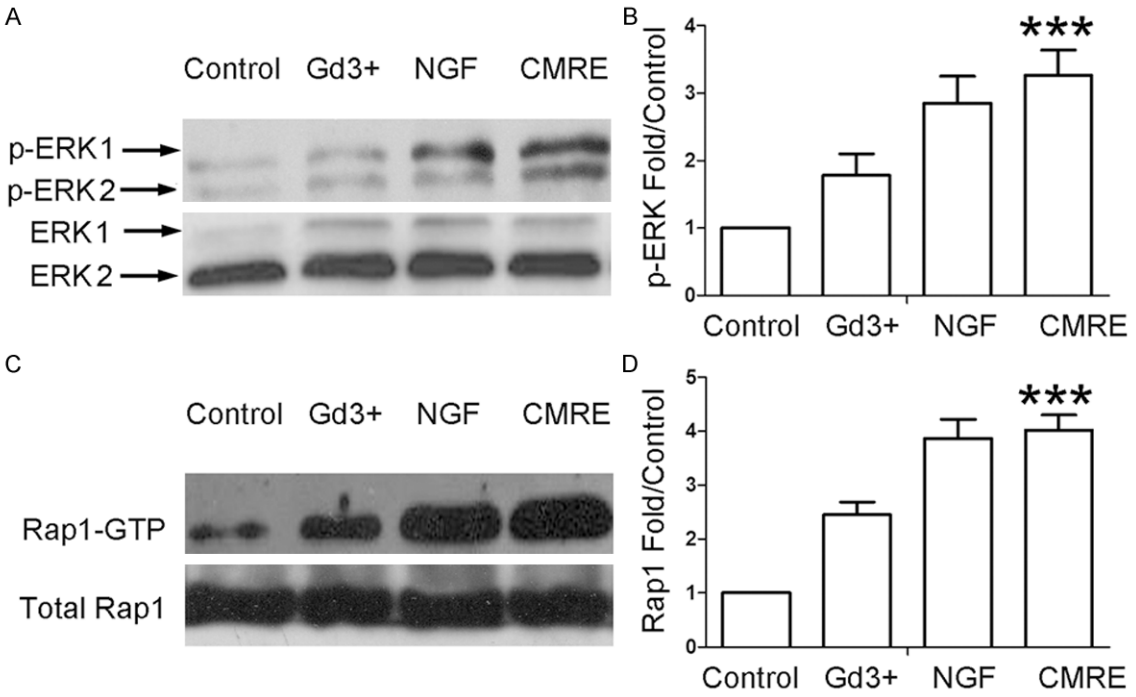

Figure 5. The Rap1-ERK signaling pathway is involved in the CMRE-induced neurogenesis. PC12 cells were seeded in a 6-well plate at an initial density of 5×10^5 cells per well and were cultured for 24 h, then treated with Gd3+ (100 μM), NGF (50 ng/ml) and CMRE (50 μg/ml) for an additional 48 h, respectively. A and C. The cell lysates were analyzed via immunoblotting with anti-phospho ERK1/2, anti-ERK1/2, and anti-Rap1B antibodies. A. CMRE induces p-ERK1/2 activation following neurite outgrowth in PC12 cells, C. CMRE induces Rap1 expression in treated PC12 cells. B and D. The expression levels of p-ERK and Rap1 in different group were assessed with semi-quantitative analysis. All experiments were done in triplicate. Data are expressed as mean ± SEM. $^{***}P < 0.001$, comparison with the untreated control.

then assessed neurite growth of PC12 cells. The results showed that the neurite outgrowth induced by CMRE was suppressed significantly by PD98059 pretreatment (**Figure 6A**), but not the PI3K inhibitor (LY294002), the p38MAPK inhibitor (SB203580) and the JNK inhibitor (SP600125) (**Figure 6**).

To elucidate the molecular mechanisms underlying CMRE-induced neuronal differentiation, we then attempted to characterize the effects of CMRE on the generation of Rap1, as this was known to perform a critical function in neurite outgrowth and proliferation. Levels of Rap1-GTP were shown to have increased upon stimulation with CMRE (**Figure 5C** and **5D**). These results suggested that the Rap1-ERK pathway might be associated with CMRE-induced neurogenesis.

Discussion

In the present study, we showed that CMRE promotes neurite growth and differentiation of PC12 cells, which is connected with increased expression of Rap1-GTP. Of note, CMRE induces neurogenesis by activating of the ERK signaling pathways, as well as inhibiting Ca^{2+} influx.

Small Rap guanosine-tri-phosphate (Rap-GTP) ases are crucially involved in many cellular processes, including cell proliferation, differentiation, survival, adhesion and movement. Furthermore, it has been shown that Rap signaling is involved in various aspects of neuronal differentiation [14]. Rap1 is a member of the Ras family of small GTPases that is activated by diverse extracellular stimuli in many cell types. Rap1 binds to either GTP or GDP, and the transition allows downstream effector molecules including B-Raf, c-Raf1 and AF-6 to discriminate between two states, GTP-form “on” and GDP-form “off” signals [15]. The small GTPase Rap1 regulates synaptic plasticity, learning and memory by integrating and relaying information about changes in neuronal depolarization or

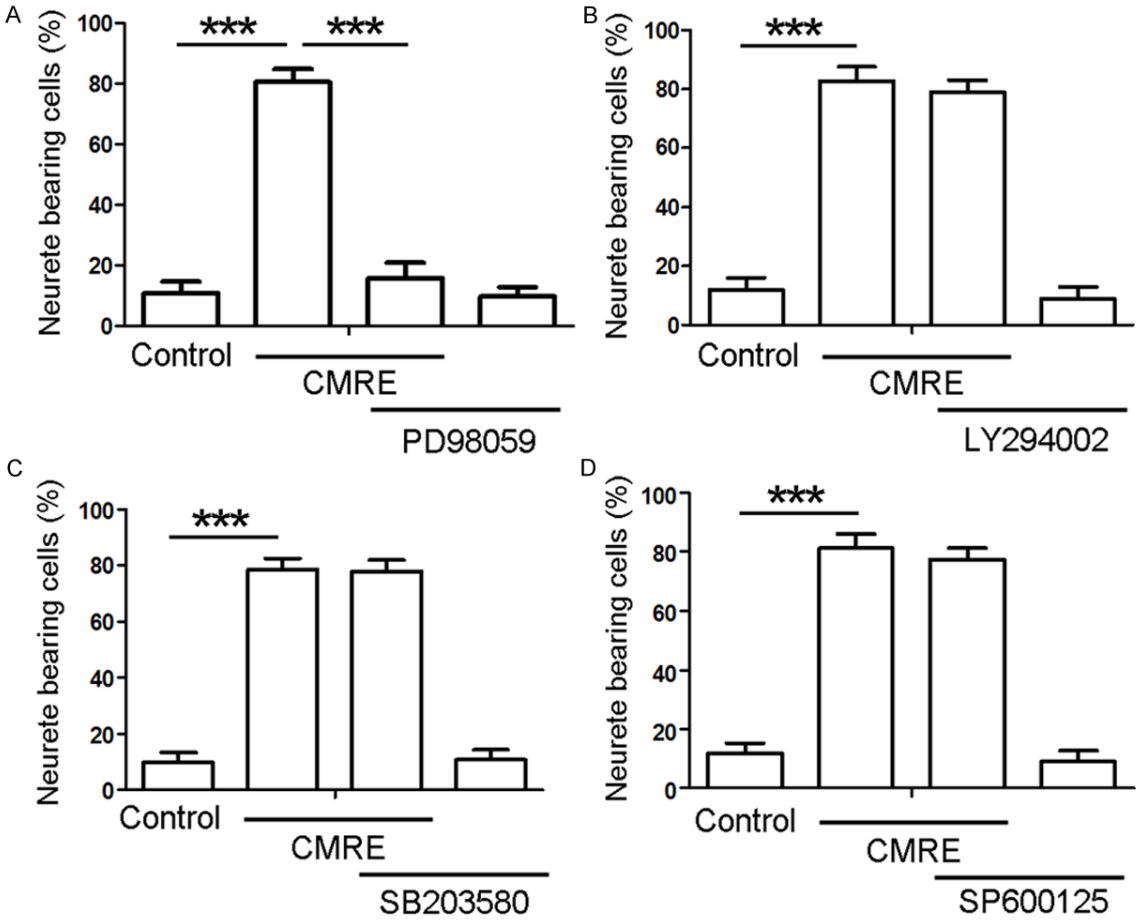

Figure 6. Effects of the specific inhibitors of ERK, PI3K, p38MAPK and JNK on CMRE-induced neurite growth of PC12 cells. PC12 cells (1×10^5 cells/ml) were seeded onto 24-well plates and were cultured for 24 h, after which time drugs were added and the cells cultured for an additional 48 h. The CMRE-induced neurogenesis in PC12 cells were antagonized by administration of the ERK1/2 inhibitor (PD98059; 10 μM), but not the PI3K inhibitor (LY294002; 10 μM), the p38MAPK inhibitor (SB203580; 10 μM), and the JNK inhibitor (SP600125; 10 μM). All experiments were done in triplicate. Data are expressed as mean ± SEM. $^{***}P < 0.001$, comparison with the CMRE-treated cells.

presence of growth factors [16]. Some studies have found that a dominant active Rap1 mutant (RapV12) attenuated the Ras-mediated ERK activation, probably via competitive interference with c-Raf1 activation by Ras [17, 18]. On the other hand, Rap1 was reported to stimulate the cell growth in another fibroblast line and to be capable of activating B-Raf independently of Ras in certain cell types [19]. The differential effects of Rap1 on c-Raf1 and B-Raf activation were suggested to be due to much stronger binding of Rap1GTP to the cysteine-rich domain of c-Raft than to that of B-Raf. Using SPA-1 gene-targeted mice, deregulated activation of endogenous Rap1 in hematopoietic progenitors resulted in enhanced proliferation leading to ERK activation, suggesting that Rap1GTP induced ERK activation independently of Ras possibly via B-Raf [20]. In PC12 cells, Rap1 can be activated by elevation of cyclic AMP (cAMP) and contributes to prolonged activation of ERK1/2 [21]. In addition, Influx of Ca^{2+} in platelets also activates Rap1 and consequently promotes platelet aggregation [22]. In neurons, Rap1 activated ERK1/2 through B-Raf and MEK, and Rap1 deletion increased calcium influx in axons through ERK1/2 [23]. In this study, CMRE treatment induces neurite outgrowth of PC12 and primary cortical neuron. Of note, CMRE enhanced the expression of Rap1 in PC12 cells following the progress of neural differentiation, suggesting the ERK1/2 signaling pathway involved in CMRE-induced neurogenesis.

The mitogen-activated protein kinase (MAPK) is a family of serine/threonine protein kinases that transduce extracellular stimuli into intracellular posttranslational and transcriptional responses [24]. The MAPK family mainly includes ERK, p38 MAPK, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and ERK5 [25]. The best characterized members of the MAPK family are the ERKs such as ERK1 and ERK2. The ERK kinase pathway is first discovered for its role in regulating the cell cycle. On the other hand, it is primarily a mediator of axonal growth and neuronal differentiation, although it also regulates cell proliferation and apoptosis [26]. Following stimulation of neuronal cells by growth factors, ERK is phosphorylated and activated by the dual-specificity kinase MEK (MAPK/ERK kinase), which is itself phosphorylated and activated by members of the Raf serine/threonine kinase family [27]. B-Raf has been shown to be the major Raf isoform activated by NGF in neuronal cells, leading to sustained activation of ERKs and cell differentiation [28]. In this study, CMRE treatment induced the p-ERK1/2 activation including increased expression of Rap1, which loaded in upstream of ERK signaling pathway. Following the activation of Rap1, it is possible to lead to sustained activation of B-Raf and ERK. On the other hand, the sustained activation of Rap1 would be able to suppress the activation of c-Raf1 by Ras, which involved in NGF-induced activation of ERK1/2 signaling pathway in cell differentiation. Moreover, the specific ERK1/2 inhibitor PD98059 significantly prevented the potentiation of CMRE-induced neurite outgrowth. Taken together, the Rap1-ERK signaling pathway is postulated to be involved in the mechanism of CMRE-induced neurogenesis in PC12 cells.

The intracellular Ca^{2+} is an important regulator of neurite outgrowth [29]. It has been reported that calcium signaling mediated by IP3 receptors resulted in neurite outgrowth, suggesting that IP3-mediated Ca^{2+} release from internal stores is necessary to maintain $[Ca^{2+}]_i$, within the optimum range of neurite outgrowth [30]. Several studies have reported that Ca^{2+} influx links extracellular signals to Ras-MAPK signaling pathway [31]. In some cases, Ca^{2+} influx activates Pyk2 and c-Src and results in activation of Shc (SH2/collagen protein) and MAPK [32]. In other cases, increase of intracellular Ca^{2+} activates several Ras guanylnucleotide-exchange factor (Ras-GEF) molecules, Ras-GRF1, and RasGRP/rbc7 and result in activation of Ras-MAPK signaling system [33]. Activity-dependent change in neuronal processes such as synaptic plasticity and neuronal survival are mediated in large part through elevations in intracellular calcium levels [34]. Many complicated downstream calcium dependent kinase signaling cascades were revealed, among which, ERK/MAPK is emerging as an important target for neuronal calcium signaling. ERK activation by neuronal calcium flux has been demonstrated both in vitro and in vivo [35, 36]. In present study, the results showed that CMRE inhibited intracellular calcium ion flux rapidly. Thus, in the context of ERK1/2 activation, inhibition of the calcium flux may be an important factor in determining signaling events.

In conclusion, our findings provide the first evidence of induction of neurite outgrowth in PC12 cells by CMRE known as a traditional Chinese herbal medicine. Additionally, we have demonstrated that Rap1-ERK signaling contributes, at least in part, to CMRE-induced neurogenesis via inhibiting Ca^{2+} influx. These results provide an important clue to look for therapeutic candidate for the treatment of neurodegenerative disease.

Disclosure of conflict of interest

None.

Abbreviation

CMRE, Cortex Mori Radicis Extract; NGF, nerve growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; DMSO, dimethyl sulfoxide.

Address correspondence to: Dr. Zebin Chen, Department of Anatomy, Basic Medical College, Hubei University of Chinese Medicine, 1 West Road, Hongshan District, Wuhan 430065, China. E-mail: chenzebin-hbtcm@outlook.com

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