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Original Article

Carboxymethylated chitosan promotes Schwann cell proliferation and expression of neurotrophic factors by activation of PKA and PKC signaling pathways

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Abstract: This present study was conducted to investigate the effects of Carboxymethylated chitosan (CMCS) on Schwann cells (SCs) proliferation, biosynthesis of neurotrophic factors and its possible involvements of protein kinases A (PKA) and protein kinase C (PKC) signaling pathways. SCs were treated with CMCS alone or in the presence of PKA inhibitor (H89) or PKC inhibitor (Staurosporine). Cell proliferation was assessed by MTT and immunocytochemistry of BrdU staining. The contents and activities of cAMP, PKA and PKC were analyzed by ELISA assay. Western blot analysis and immunofluorescence staining were used to detect the expression of CREB and p-CREB. Real-time PCR was used to determine the expression of NGF, CNTF, BDNF and GDNF mRNA. The results showed CMCS could promote SCs proliferation, promote PKA and PKC activation, increases cAMP and expression of NGF, CNTF, BDNF and GDNF mRNA. These stimulating effects were inhibited by H89 and Staurosporine. Taken together, Data from this present study indicated CMCS may stimulate proliferation and biosynthesis of cultured SCs by activation of PKA and PKC signaling pathways.

Keywords: Schwann cells, proliferation, carboxymethylated chitosan, protein kinase A, protein kinase C

Introduction

Schwann cells (SCs) play the critical role in repair of injured nerves by formation of bands of Büngner at the distal ends in injured nerves [1]. SCs proliferation also could provide a guide for regenerating axons [2]. SCs are the major source of neurotrophic factors, such as nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), these factors play the critical role in response to nerve injury and successful axonal elongation [3, 4].

In mammalian cells, the activity of cyclic AMP (cAMP) were affected by hormones and growth factors in proliferation has been well studied. Activated protein kinase A (PKA) will phosphorylate proteins that bind to the cAMP responsive element binding protein (CREB) [5]. The activation of CREB is phosphorylated by either PKA or protein kinase C (PKC). Many researchers indicated that PKA and PKC signaling pathways play the important role in SCs proliferation [6, 7].

Chitosan, a natural cationic biopolymer, is derived from chitin by N-deacetylation [8]. Chitosan has been shown to have the desirable biocompatible and biodegradable properties [9], such as neuroprotective effects [10, 11]. Carboxymethylated chitosan (CMCS) is the soluble derivative of chitosan, possesses many desirable physiochemical and biological features: biocompatibility [12], promoting effect of cell proliferation [13], and enhancement of rat sciatic nerve repair [14].

In our previous studies, we have found CMCS could suppress the degeneration of cartilage in osteoarthritis and protect chondrocytes and nucleus pulposus cells from oxidative stress-induced apoptosis [15-17]. We have also found CMCS could promote proliferation of SCs by activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK), phosphatidylinositil-3 kinase/protein kinase B

(PI3K/Akt) and Wnt/β-catenin signaling pathways, and also protect SCs from oxidative stress induced apoptosis [18-20]. However, up to data, little has been known about the effect of PKA and PKC in CMCS induced proliferation and biosynthesis of neurotrophic factors in SCs. The purpose of this study is to investigate the roles of PKA and PKC in CMCS induced proliferation and synthesis in SCs.

Materials and methods

Animals and materials

New born (3~5 days old) Sprague-Dawley (SD) rats were supplied by Experimental Animal Center of Wuhan University (Wuhan, China), and all the procedures were supported by the Ethics Committee of Wuhan University. CMCS powder (purity > 99%) was provided by the Institute of Chemical and Molecular Science of Wuhan University. Primers were provided by Invitrogen Biotech Company (USA). MTT and BrdU were provided by Sigma-Aldrich (USA). PKA inhibitor H-89 and PKC inhibitor Staurosporine were obtained from the Beyotime Institute of Biotechnology Company (Haimen, China). ELISA assay kits for cAMP, PKA and PKC were provided by Roche Company (Basel, Switzerland). Antibodies were obtained from Cell Signaling Technology (Beverly, MA) and included: anti-CREB (cAMP responsive element binding protein, CREB), anti-p-CREB. Anti-S-100 and anti GAPDH antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 647 labeled fluorescent secondary antibody was provided by R&D Company (USA). Other high-purity reagents and materials were purchased locally.

Cell isolation and culture

SCs culture was referenced to the previous study [21]. In this study, all the procedures were carried out in accordance with the relevant guidelines and regulations for the disposal of animals, and all the experimental protocols were approved by the Ethics Committee of Wuhan University. Briefly, bilateral sciatic nerves were separated digested by trypsin (0.25%) and collagenase type-2 (0.03%) at 37°C for 30 min, the digestive cells were centrifuged at 1000 rpm for 5 min, then the cells were cultured in Dulbecco's Modified Eagle Media/Ham's F-12 (DMEM/F-12). Cell purification was achieved by arabinoside (Ara, 5 μg/ml) and different collagenase digestions [22].

Immunofluorescence staining

Cells were identified by S-100 immunofluorescence staining as previously reported [18-20]. Briefly, the cells were seeded on slides and cultured, then fixed with 4% paraformaldehyde for 40 min, then permeabilized by 3% Triton X-100. Rabbit anti-S-100, CREB and p-CREB monoclonal antibody (1:100) was added to incubate overnight at 4°C. Adding goat anti-rabbit secondary antibody connected to FITC (1:10,000) incubated for 1 h. Labeled cells were observed under an inverted fluorescence microscope. S-100 labeled cells were counted and treated by using Image-Pro Plus software.

MTT assay

Cell proliferation was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, purified SCs ($1×10^5$/well) were seeded in 96-well microplates and exposed to indicated reagents for 24 h at 37°C prior to the addition of MTT, 100 μl DMSO (Sigma-Aldrich) was added to dissolve the formazan crystals. Metabolic activity was quantified by light absorbance at 490 nm in microboplate reader (EL ×800 Absorbance Microplate Reader, USA). All assays were performed in triplicate.

BrdU incorporation assay

Cell proliferation was determined by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation into the DNA. 24 h after CMCS treatment, the cells were serum starved overnight, treated with 200 μM BrdU and incubated for about 16 h. The cells were fixed in freshly prepared 4% (v/v) paraformaldehyde for 10 min and permeabilized with 0.5% (v/v) Triton X-100, followed by incubation with DNase I (0.5 U/μl) for 30 min at 37°C. The cells were incubated with primary antibody against BrdU overnight at 4°C. Finally, nuclei were counter-stained with Hoechst 33342 for 15 min, rinsed with PBS for three times and visualized under fluorescent microscopy.

ELISA assay

The contents of cAMP, PKA and PKC released into the culture medium were determined by ELISA assay kits following manufacture's protocols. SCs were seeded in six-well plates. CMCS, PKA inhibitor H89 and PKC inhibitor Staurosporine were used. After treatment, cells were

Table 1. Primer sequences of target genes

Gene	Primer	Sequence	Product size (bp)
NGF	Forward	ACAGATAGCAATGTCCCAGAGG	150
	Reverse	AGTGATGTTGCGGGTCTGC	
CNTF	Forward	ACCTCTGTAGCCGTTCTATCTGG	175
	Reverse	AGTCGCTCTGCCTCAGTCATC	
BDNF	Forward	GCCTCCTCTGCTCTTTCTGC	139
	Reverse	TGTGACCCACTCGCTAATACTG	
GDNF	Forward	AAAGGTCACCAGATAAACAAGCG	177
	Reverse	CGTAGCCCAAACCCAAGTCA	
β-actin	Forward	CCCATCTATGAGGGTTACGC	150
	Reverse	TTTAATGTCACGCACGATTTC	

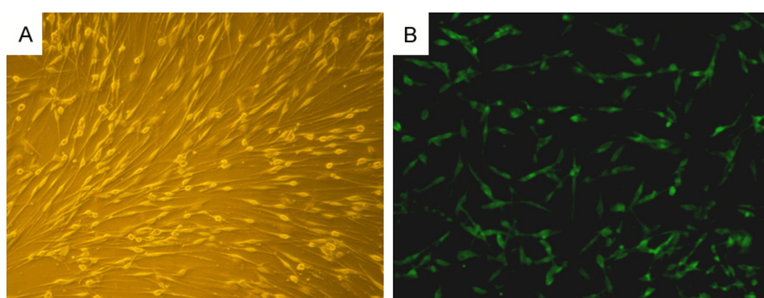

Figure 1. SCs culture and identification. A. SCs cultured for 7 days. B. Immunofluorescence of SCs by S-100 staining, green fluorescence was S-100 staining. Pictures were taken at 200× magnification.

lysed with 0.5 mL of 0.1 M HCl and Triton X-100 (0.1%) for 15 minutes. Cell lysates were subsequently collected and subjected to cAMP, PKA and PKC enzyme immunoassay according to the manufacturer's instructions. For all experiments, the amounts of solvent were normalized among the treatments and controls.

Quantitative real-time polymerase chain reaction (QRT-PCR)

Total mRNA was extracted from cultured SCs by Trizol, ABI Prism 7500 real-time PCR system was used to detect the mRNA expression of target genes. The Primers of NGF, CNTF, BDNF, GDNF are as shown in **Table 1.**

For the RT analysis, 5 μl mRNA was revere transcribed to cDNA and amplified by specific reverse primers of targets genes using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, USA). The reaction mixture of PCR included 1 μl forward and reverse primers (10 μM), 1 μl SYBR Green I fluorescent dye and 1 μl cDNA, the PCR conditions were as following: initial step at 95°C for 2 min, then 40 cycles at 95°C for 20 s, 60°C for 40 s. The relative expressions of target genes were analyzed by comparative cycle threshold method, the data were standardized by β-actin. The results were obtained from three independent experiments.

Western blot

The CREB and p-CREB protein expression were detected by western blot analysis. Briefly, SCs were treated by cell lysates buffer after indicated treatment. The quantity of proteins was tested by using BCA protein assay kit. Same amount of protein was dissolved in the electrophoresis buffer and resolved by SDS gel electrophosresis. The proteins were transferred to the polyvinylidene difluoride (PVDF) membrane and washed by TBS-T (Tris-buffered saline and Tween-20) buffer and blocked by 5% non-fat milk at 4°C. The corresponding antibodies were added for incubation, then the appropriate secondary horseradish-peroxidase labeled antibodies were added for incubation. Immunodetection was performed with the enhanced chemiluminescence (ECL) and autoradiography on Kodak films. Images were processed by Geliance 200 Imaging system (Perkin-Elmer) and Gene Snap software (version 6.08.04, SYNGENE), and analyzed by the Gene Tools software (version 3.07.04, SYNGENE).

Statistical analysis

Data were presented as mean ± S.E.M and analyzed by One-way ANOVA assay using Statistical Package for Social Sciences (SPSS, version 17.0), $P<0.05$ was considered to be statistically significant. All the results were compared between CMCS treated SCs and control group.

Results

Isolation and identification of SCs

In this study, the SCs began to adhere to the culture plates and contacted each other with

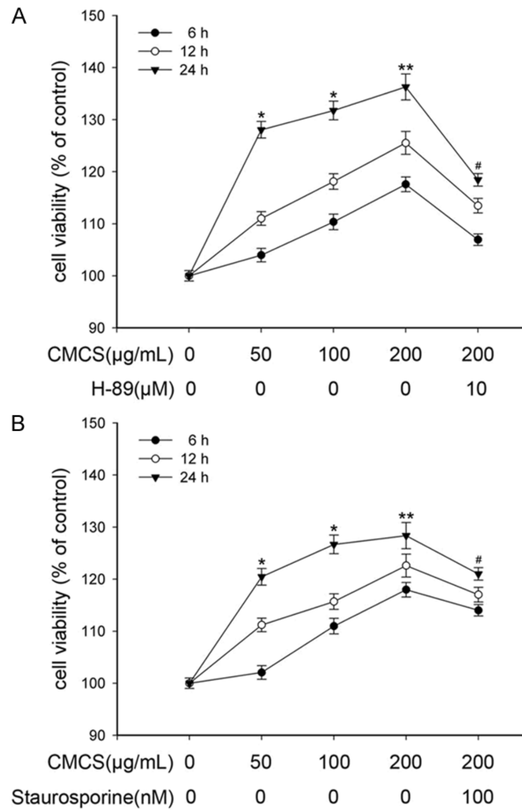

Figure 2. Effect of H-89 and Staurosporine on CMCS-induced SCs proliferation. A. SCs were induced by 50, 100, 200 μg/ml CMCS with or without 10 μM H-89. B. SCs were induced by 50, 100, 200 μg/ml CMCS with or without 100 nM staurosporine. **P*<0.05 vs. the control group; #*P*<0.05 vs. the 200 μg/ml CMCS treated group.

neuritis after 72 h, cultured for about 5~7 days, SCs displayed an appearance of bipolar with thin processes (**Figure 1A**). S-100 immunofluorescence results showed that almost all the cultured cells were S-100 labeled cells, the positive cells were cytoplasmic stained green (**Figure 1B**), the ratio of positive cells exceeding 95%.

Effects of PKA and PKC on CMCS induced SCs proliferation

Results of MTT assay: As shown in **Figure 2**, MTT results indicated CMCS (50, 100, 200 μg/ml) gradually increase cell proliferation with dose dependent manner, the maximum increase was observed at 24 h with 200 μg/ml CMCS exposure (3.66 ± 0.22 folder control group). After pretreatment with 10 μM H-89, the CMCS-induced proliferative activity was decreased (45.5 ± 4.4 percentage of 200 μg/ml CMCS treated group). After pretreatment with 100 nM Staurosporine, the proliferative activity was also decreased (34.5 ± 3.6 percentage of 200 μg/ml CMCS treated group). Above results indicated H-89 and Staurosporine could partially inhibit CMCS-stimulated SCs proliferation.

Results of BrdU incorporation assay: As shown in **Figure 3**, 200 μg/ml CMCS promoted DNA synthesis to the levels almost 2~3 fold than that observed in control cells. After pretreatment with 10 μM H-89 and 100 nM Staurosporine, the DNA synthesis in 200 μg/ml CMCS stimulated SCs was decreased compared with alone 200 μg/ml CMCS treated SCs.

Effects of CMCS on cAMP, PKA and PKC activity

As shown in **Figure 4**, ELISA results indicated CMCS could increase the cAMP, PKA and PKC contents compared with control group (P<0.05). After addition of H-89 and staurosporine, the contents of cAMP, PKA and PKC were significantly decreased compared with CMCS treated SCs (P<0.05).

Effect of CMCS on CREB activation

Western blot analysis: As shown in **Figure 5**, the expression of p-CREB was significantly increased in CMCS induced SCs compared with control group, the maximum expression was occurred at 200 μg/ml CMCS treated SCs (it was 4.3 ± 1.1 folder than control group). The activation of p-CREB was inhibited by pretreatment with H-89 (66 percent decrease than CMCS treated SCs).

Immunofluorescence staining

As shown in **Figure 6**, with Immunofluorescence staining, the expression of p-CREB was increased in CMCS induced SCs, after pretreatment of H-89 the fluorescence intensity of p-CREB was weaken.

Effects of PKA and PKC on CMCS induced biosynthesis

As shown in **Figures 7** and **8**, real-time PCR results indicated CMCS could increase NGF, CNTF, BDNF and GDNF mRNA expression with concentration dependent manner, the significant mRNA expression was observed in 200

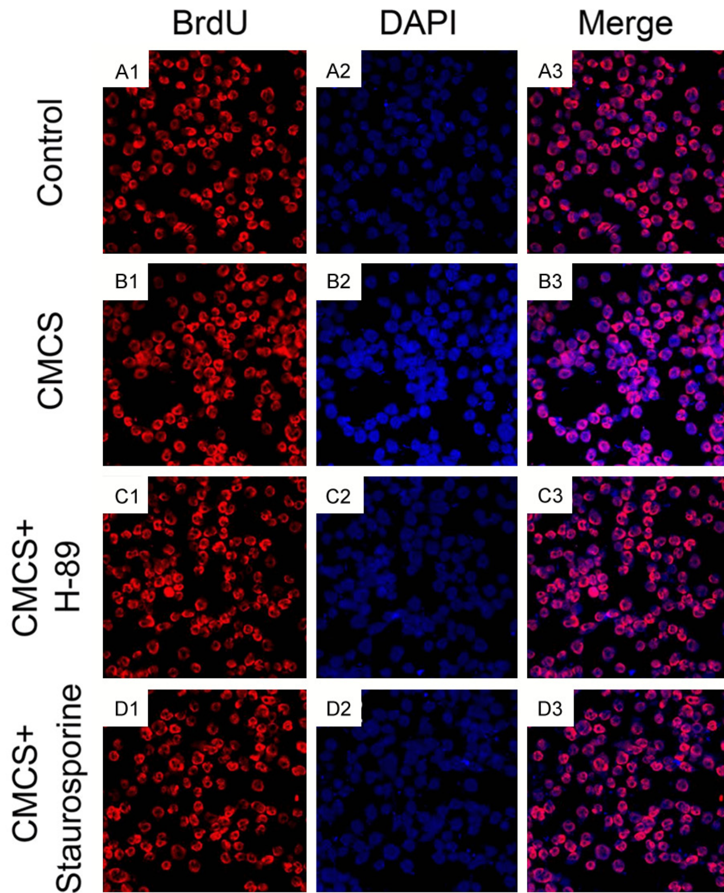

Figure 3. Effect of H-89 and Staurosporine on CMCS-induced SCs proliferation. Cell proliferation was assessed using BrdU and DAPI double staining. SCs were pretreated with 200 μg/ml CMCS with or without 10 μM H-89 and 100 nM staurosporine.

μg/ml CMCS treated group (*P<0.05). After addition of 10 μM PKA inhibitor H-89, the expressions of NGF, CNTF, BDNF and GDNF were significantly decreased compared with 200 μg/ml CMCS treated SCs ($^{\#}$P<0.05) (**Figure 7**). After addition of 100 nM PKC inhibitor staurosporine, the expressions of NGF, CNTF, BDNF and GDNF were also significantly decreased compared with 200 μg/ml CMCS treated SCs ($^{\#}$P<0.05) (**Figure 8**).

Discussion

In the present study, SCs proliferation and neurotrophic factors biosynthesis induced by CMCS were investigated. In addition, these effects were correlated with evidence for the activation of PKA and PKC signaling pathways. SCs are the glial cells in the peripheral nervous system (PNS). They are essential for nerve regeneration as they provide a permissive environment for nerve regrowth and regeneration [23]. After peripheral nerve injuries, SCs proliferate and synthesize neurotrophic factors, such as NGF, CNTF, BDNF and GDNF, to provide trophic support for regenerating axons [24].

CMCS was extensively investigated based on its biological and pharmacological features. Recently, it has been reported that CMCS can be used for drug delivery [25], Postoperative peritoneal adhesion [26], promoting cell proliferation [13] and repairing injured nerves [14]. In previous study, we have found that CMCS can promote SCs proliferation by MEK/ERK, PI3K/Akt and Wnt/β-catenin signaling pathways, and protect SCs from oxidative stress induced apoptosis [18-20].

In this study, we established a culture system in which SCs were exposed to different concentrations (50, 100, 200 μg/ml) of CMCS and proliferating activity and mRNA expressions of neurotrophic factors were evaluated at the end of each culture cycle. The exposure to 200 μg/ml CMCS for 24 h affect proliferation obviously by MTT and BrdU assays. After addition of PKA inhibitor H-89 (10 μM) or PKC inhibitor staurosporine (100 nm), SCs showed decreased proliferation compared with CMCS induced group (**Figures 2** and **3**). The data obtained from MTT and BrdU labeled assay showed that inhibition of PKA pathway decreased proliferation of SCs. When PKC pathway was inhibited, CMCS induced cell proliferation was also inhibited. These results demonstrated both PKA and PKC pathways act in proliferative response to CMCS exposure.

The involvement of cAMP/PKA signaling pathway has long been accepted as the crucial element in proliferation, such as in mice pancreatic beta-cell [27], endometrial stromal fibroblasts [28], T lymphocytes [29] and brain vascular endothelial cells [30]. To investigate whether PKA-mediated system was also involved in CMCS induced cell proliferation and biosynthe-

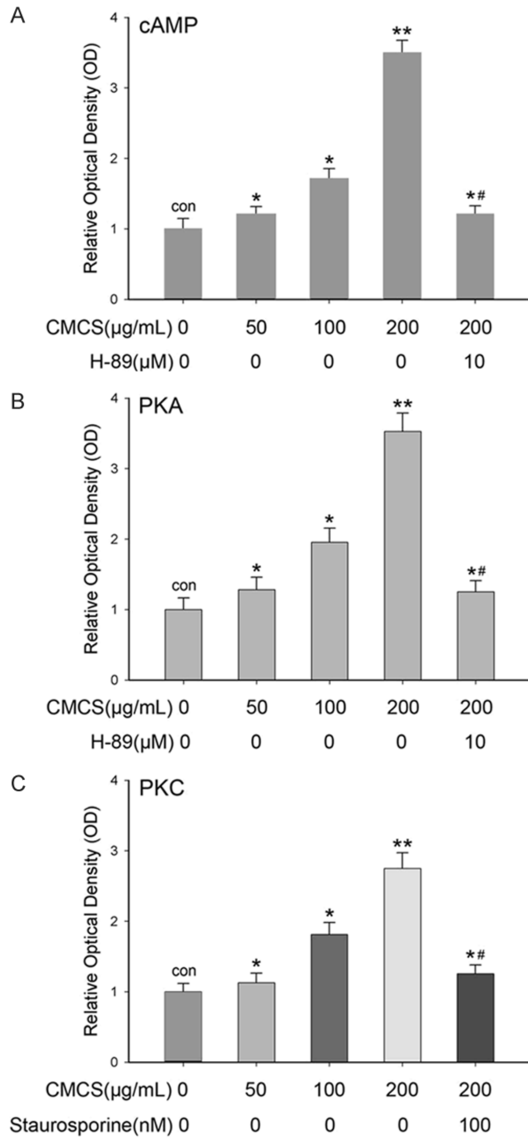

Figure 4. The activity of cAMP (A), PKA (B) and PKC (C) in CMCS induced SCs. SCs were pretreated with 50, 100, 200 μg/ml CMCS with or without 10 μM H-89 or 100 nM staurosporine. *P<0.05 vs. the control group; #P<0.05 vs. the 200 μg/ml CMCS treated group.

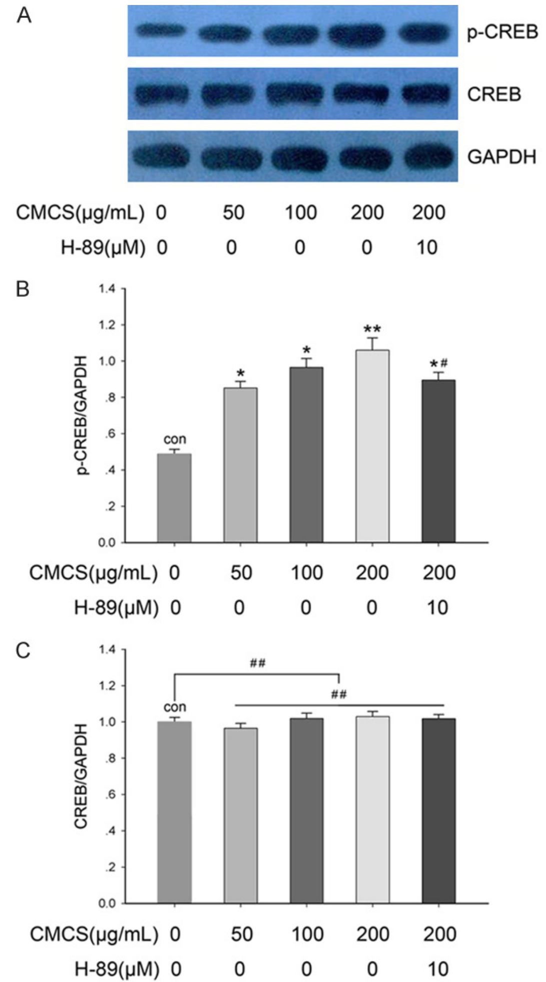

Figure 5. Expression of CREB and p-CREB in CMCS induced SCs. The expressions of CREB and p-CREB in different groups were detected by western blot (A). Quantitative analysis of signal strength was performed by the density. Relative expression of proteins was obtained from comparing with GAPDH which was used as internal (B, C). SCs were pretreated with 50, 100, 200 μg/ml CMCS with or without 10 μM H-89. The grouping of blot/gels were from different gels with same exposure *P<0.05 vs. the control group; #P<0.05 vs. the 200 μg/ml CMCS treated group.

sis in SCs, a specific inhibitor of PKA pathway, H-89, was applied. The results showed H-89 visibly blocked the CMCS-induced proliferation in the SCs. Our previous study showed MEK/ERK, PI3K/Akt and Wnt/β-catenin signaling pathways played the key roles in proliferation and biosynthesis of SCs after simulation with CMCS [18, 19]. In present study cAMP content and PKA activity were enhanced by CMCS induction accompanied with increased proliferation and neurotrophic factors biosynthesis in SCs. Furthermore, H-89 partially reversed stimulating effects of CMCS on SCs proliferation and biosynthesis. Our results were in accordance with previously published reports [6, 7].

The CREB is a transcriptional apparatus, which elicits different function in different tissues [31]. CREB and Fos/Jun family members form selective cross-family, and can be grouped into a superfamily of transcription factors, including CREB, CRE modulator, ATF, Jun and Fos [32]. In mammalian cells, stimulatory factors activate

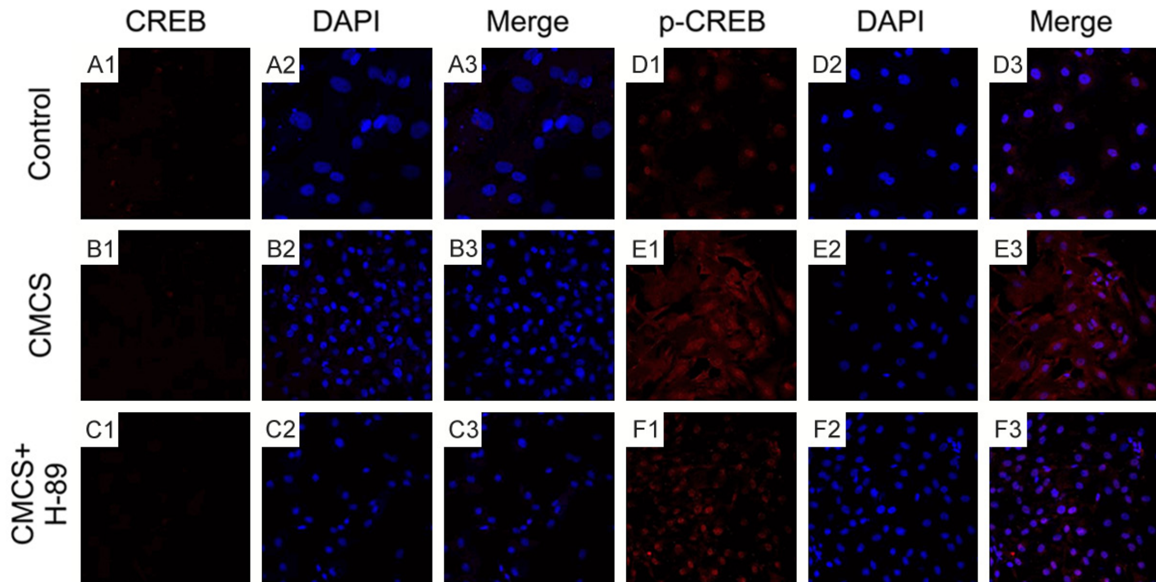

Figure 6. Expression of CREB and p-CREB in CMCS induced SCs. The distribution of CREB and p-CREB in different groups were detected by CREB, p-CREB and DAPI double staining. SCs were pretreated with 200 μg/ml CMCS with or without 10 μM H-89.

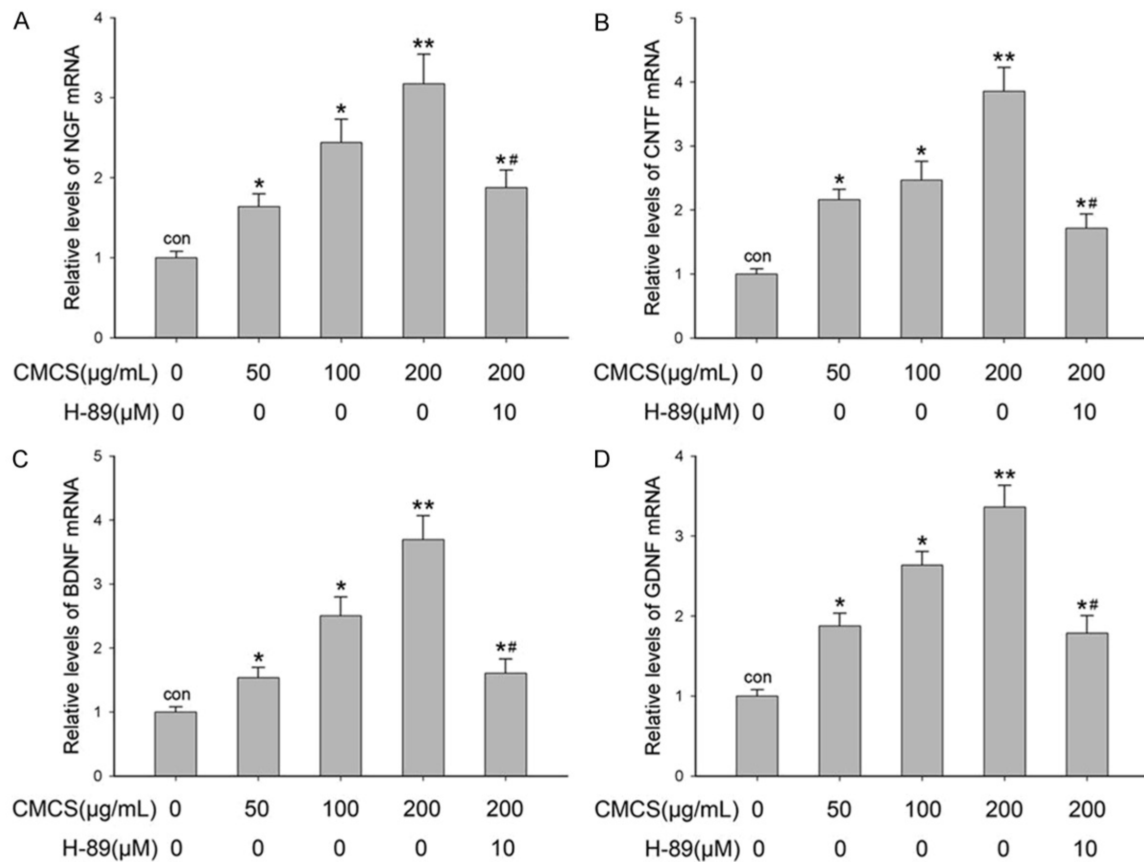

Figure 7. Effect of PKA signaling pathway on CMCS induced biosynthesis of neurotrophic factors. The expressions of NGF (A), CNTF (B), BDNF (C) and GDNF (D) mRNA were determined by real-time PCR. SCs were pretreated by 50, 100, 200 μg/ml CMCS with or without 10 μM H-89. **P*<0.05 vs. the control group; #*P*<0.05 vs. the 200 μg/ml CMCS treated group.

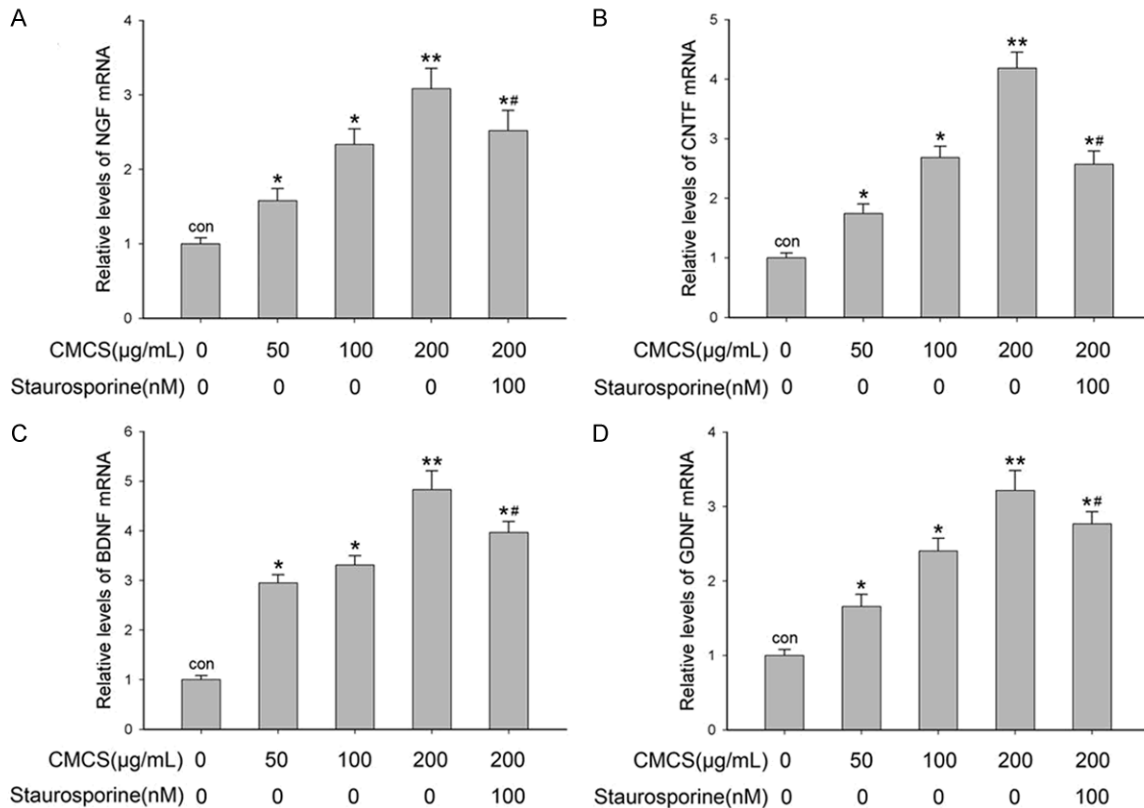

Figure 8. Effect of PKC signaling pathway on CMCS induced biosynthesis of neurotrophic factors. The expressions of NGF (A), CNTF (B), BDNF (C) and GDNF (D) mRNA were detected by real-time PCR. SCs were pretreated by 50, 100, 200 μg/ml CMCS with or without 100 nM staurosporine. **P*<0.05 vs. the control group; #*P*<0.05 vs. the 200 μg/ml CMCS treated group.

adenylyl cyclase (AC) to produce cAMP, which in turn activates PKA to phosphorylate and activate CREB, a constitutively expressed nuclear transcription factor that regulates the expression of genes involved in neuronal survival and function [33]. In this study, we found CMCS could increase expression of cAMP, and activate PKA activity, increase CREB and p-CREB expression by western blot and immunofluorescence staining, hence we can conclude that CMCS could activate cAMP/PKA/CREB signaling cascades to promote SCs proliferation.

Our this study also provide the insight into the mechanism of PKC signaling pathways involved in regulating proliferation and biosynthesis of neurotrophic factors in SCs. When SCs are treated with CMCS in the presence of PKC inhibitor staurosporine, the cell proliferation is further reduced as compared with treated by CMCS alone, thus the CMCS induced increase in cell proliferation and biosynthesis were inhibited, and similar observation has been made by BrdU incorporation assay. These results indicate PKC activity is necessary for proliferative signaling in SCs. This pro-proliferative effect of PKC were corroborated by several reports from other groups [34].

In conclusion, this study reveals CMCS promotes proliferation of SCs, and the enhancement of neurotrophic factors (NGF, CNTF, BDNF, GDNF) biosynthesis further confirms its stimulatory effect. In addition, we demonstrate that PKA and PKC mediated signal transduction systems regulate the CMCS-induced SCs proliferation and biosynthesis of neurotrophic factors. Further work will be needed to identify other signaling pathways and the precise molecular mechanisms of CMCS action in SCs.

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Disclosure of conflict of interest

None.

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