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Chitosan oligosaccharides suppress production of nitric oxide in lipopolysaccharide-induced N9 murine microglial cells *in vitro*

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Abstract Chitosan oligosaccharides (COS) have been reported to exert many biological activities, such as antioxidant, antitumor and anti-inflammatory effects. In the present study, we examined the effect of COS on nitric oxide (NO) production in LPS induced N9 microglial cells. Pretreatment with COS (50~200 μg/ml) could markedly inhibit NO production by suppressing inducible nitric oxide synthase (iNOS) expression in activated microglial cells. Signal transduction studies showed that COS remarkably inhibited LPS-induced phosphorylation of p38 MAPK and ERK1/2. COS pretreatment could also inhibit the activation of both nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). In conclusion, our results suggest that COS could suppress the production of NO in LPS-induced N9 microglial cells, mediated by p38 MAPK and ERK1/2 pathways.

Keywords Chitosan oligosaccharides · Microglial cell · Inflammation · MAPKs · NF-κB · AP-1

Abbreviations

AP-1:	Activator protein-1
COS:	Chitosan oligosaccharides
DAF-FM DA:	3-Amino 4-aminomethyl-2′,7′-diflurescein, diacetate
FACS:	Fluorescence-activated cell sorting
HC:	Hydrocortisone
iNOS:	Inducible nitric oxide synthase
LPS:	Lipopolysaccharide
MAPK:	Mitogen-activated protein kinase
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO:	Nitric oxide
NF-κB:	Nuclear factor-κB
RT-PCR:	Reverse Transcription-Polymerase Chain Reaction.

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Introduction

Microglial cells are the major cellular elements with immune function inside the central nervous systems, and play a role of the resident innate immune cells in the brain [1, 2]. They are considered to participate in a number of pathological conditions, such as inflammation, trauma, neurodegenerative diseases, brain tumors, HIV dementia and multiple sclerosis [3]. Activated microglial cells may be intended to protect neurons at first, including removing cell debris and

myelin fragments, secretion of neurotrophins and cytokines capable of supporting survival of injured neurons [4]. However, under most conditions, activation of microglial cells and their inflammatory products may be involved in neuronal destruction in various neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [5]. Nitric oxide (NO), as one of the pivotal inflammatory factors released from activated microglial cells, has been shown to be the major autocrine mediator in microglial cells apoptosis during the inflammation process. Research by my colleagues illustrated that mitogen-activated protein kinases (MAPKs) are involved in lipopolysaccharide (LPS)-induced NO and iNOS production in microglial cells [6]. Therefore, drugs, which have the effects against the LPS-induced activation through attenuating MAPK and NF-κB in microglial cells, may be candidates for therapeutic use in neurodegenerative diseases.

Chitosan, which is derived from chitin by chemical or enzymatic deacetylation, is composed of β-1-4-linked 2-acetamido-D-glucose and β-1-4-linked 2-amino-D-glucose units with the proportion of latter usually exceeding 80 % [7]. It has been demonstrated that chitosan oligosaccharides (COS, Fig. 1a), obtained by hydrolysis or degradation of chitosan, have antimicrobial, antifungal, antioxidant, and antitumor activities [8]. Recently, more attention has been focused on the neuroprotective effects of COS. It has been found that COS could promote peripheral nerve regeneration in both rabbit and rat nerve crush injury models [9]. COS could also exert neuronal differentiation [9] and protection [10] effects. In this study, we focused on the anti-inflammatory effect of COS in activated microglial cells, which may provides a new way of further application of COS against neuronal diseases.

We aimed at investigating the inhibitory effect of COS on LPS-induced over-expression of NO in microglial cells. To explore the underlying mechanisms of COS, we evaluated the involvement of MAPKs signaling pathway, and the roles of transcription factor NF-κB and activator protein-1 (AP-1).

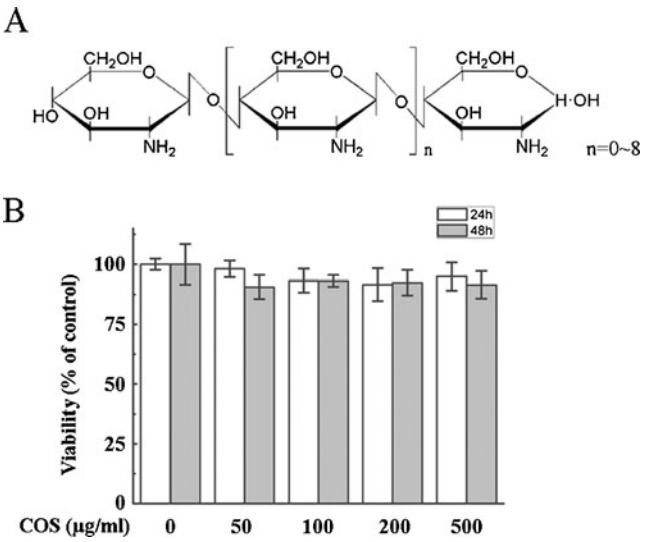

Fig. 1 Effect of COS on N9 microglial cells viability. **a** The molecular structure of COS. **b** N9 microglial cells were treated with different concentrations (50–500 μg/ml) of COS for 24 h or 48 h. After treatment, cell viability were determined by MTT assay (n=6) as described in Material and methods. Values are normalized to untreated controls. Results are expressed as the mean±SD

Material and methods

Reagents

Chitosan oligosaccharides (degree of deacetylation ≥ 95 %, average molecular weight: <1,000 Da) were prepared from enzymatic hydrolysis of chitosan in our lab [11]. The weight percentages of COS with degree of polymerization between 2 and 6 were 3.7 %, 16.1 %, 28.8 %, 37.2 % and 14.2 %, respectively, based on HPLC analysis. LPS from Escherichia coli 0127:B8, hydrocortisone (HC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iscove's modified dulbecco's medium (IMDM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). 3-Amino, 4-aminomethyl-2′,7′-diflurescein, diacetate (DAF-FM DA) and polyclonal antibody against NF-kB were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The other primary antibodies and secondary antibodies used in this article were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and drug treatment

The murine microglial cell line N9 was originally developed by Prof. P. Ricciardi-Castagnoli [12] and a generous gift from Professor Yun Bai (Third Military Medical University, Chongqing, China). These cells express typical markers of resting mouse microglia and have been extensively used as representative of primary mouse microglial cells [13, 14]. N9 cells were cultured in IMDM supplemented with 10 % heat-inactivated FBS, 50 μM β-mercaptoethanol, 100unit/ml penicillin and 100 μg/ml streptomycin under a humidified atmosphere of 5 % CO_2 and 95 % O_2 at 37°C.

For most experiments, after growing to sub-confluence, cells were pre-cultured with COS (50~200 μg/ml) in IMDM containing 10 % FBS for 24 h. After pretreatment, the supernatant was removed, and cells were washed with phosphate buffered saline (PBS, pH=7.4) twice. Then, the cells were challenged with or without LPS (1 μg/ml) for different time intervals at 37°C until further assay.

Cell viability assay

The cell viability analysis was based on the capacity of mitochondrial enzymes to transform MTT tetrazolium salt into formazan. N9 microglial cells were seeded in 96-well plates (5×10^3 cells/well) and incubated with 150 μl of IMDM with 10 % FBS and incubated overnight. After the cells were treated with different concentrations of COS (50~500 μg/ml) for the indicated time periods, the culture medium was removed and the cells were incubated with 100 μl of MTT (0.5 mg/ml) for 3 h at 37°C in CO_2 incubator. The formazan blue, which was formed in cells, was solubilized in 150 μl of DMSO. The optical densities were measured at 490 nm using an ELISA reader (Sunrise Remote, Tecan Austria GmbH, Grödig, Austria). The viability of N9 cells was presented as a percentage of the control cells.

Intracellular NO measurements by confocal microscopy and flow cytometry

DAF-FM DA was used as a fluorescent indicator of intracellular NO [15]. For confocal microscopy studies, N9 cells were grown on cover glass in 6-well plate and pre-treated with COS (50~200 μg/ml) for 24 h or HC (1 μM) for 2 h. Then the cells were treated with or without LPS (1 μg/ml) for 24 h. After treatment, the cells were washed three times with PBS (pH=7.4) and incubated with 5 μM of DAF-FM DA in PBS (pH=7.4) for 20 min at 37°C. After incubation, N9 cells were rinsed three times with PBS (pH=7.4). NO production was measured by using Laser Scanning Spectral Confocal Microscopy (Leica TCS SP2, Leica Microsystems GmbH, Germany).

Flow cytometry was used to quantitatively analyze the fluorescence of intracellular NO in LPS-induced N9 microglial cells. The cells were pre-incubated on a 6-well plate with COS or HC, and then treated with or without LPS for another 24 h as described above. Cells were harvested with 0.25 % EDTA-trypsin and resuspended with PBS containing DAF-FM DA (5 μM). After 20 min incubation at 37°C, cells were washed twice and resuspended in PBS (pH=7.4). The intracellular fluorescence of NO was measured by flow cytometry with a fluorescence-activated cell sorting (FACS) system (Becton Dickinson, San Jose, CS, USA) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm.

Nitrate assay

Levels of NO derivative nitrite were determined in the culture medium by Griess reaction as described previously [16]. N9 microglial cells grown in 96-well plates (5×10^3 cells/well) were pretreated with COS (50~200 μg/ml) for 24 h. After the cells were incubated with LPS (1 μg/ml) for another 24 h, the concentrations of NO_2^- and NO_3^- in culture supernatants were measured to assess NO production in microglial cells. Aliquots of 50 μl of supernatants were mixed with 50 μl of Griess reagent (1 % sulfanilamide and 0.1 % naphthylenediamine dihydrochloride in 5 % phosphoric acid) in 96-well plates and incubated for 10 min at room temperature. The absorbance at 540 nm was measured on an ELISA reader (Sunrise Remote, Tecan Austria GmbH, Grödig, Austria). Nitrite concentrations were calculated with reference to a standard curve of sodium nitrite generated by known concentrations.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed to determine changes of iNOS gene expression. Total RNA was extracted using cold TRIZOL reagent (Takara, Dalian, China) according to the manufacturer's protocol. Nucleotide sequences of the primers were designed on the basis of published cDNA sequences of mouse iNOS and β-actin (iNOS primer, 650 bp, forward 5′-TGG AGC GAG TTG TGG ATT GTC-3′, reverse, 5′-CCC TTT GTG CTG GGA GTC AT-3′; β-actin primer, 386 bp, forward 5′-GAT GGT GGG AAT GGG TCA GA-3′, reverse 5′-GGA GAG CAT AGC CCT CGT AGA T-3′). The PCR products were visualized by electrophoresis in 1 % agarose gel containing 1 % GoldView™. Band intensity was analyzed with ImageJ system (NIH, USA).

Cell lysate preparation

For isolation of total cell extracts, cells were lysed in 200 μl of RIPA lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM PMSF). After incubated at 4°C for 20 min, the lysates were centrifuged at 12,000 g for 10 min. Cytoplasmic and nuclear extracts were prepared using Nuclear and Cytoplasmic Protein extraction kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Briefly, cells were washed twice with ice-cold PBS (pH 7.4), then the cells were collected and suspended in 200μl of lysis buffer A (10 mM HEPES with pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.4 % Igepal CA-630, 5μm leupeptin, 2μM pepstatin A, 1μM aprotinin and 1 mM phenylmethylsulfonyl fluoride) for 10 min at 4°C. the cell lysates were subjected to centrifugation at 12,000 g for 5 min and the supernatant was used for cytoplasmic proteins. The precipitate was washed once with lysis buffer A, and resuspended in 50μl of nuclear extraction buffer B (20 mM HEPES with pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride). The suspension was agitated

for 30 min at 4°C and centrifuged at 12,000 g for 10 min. the supernatant fraction was collected and used as nuclear proteins. The protein concentrations in cell lysates were determined using a Bicinchoninc Acid Protein Assay kit (Biomed Biotech Co., Ltd., Beijing, China) and all samples were stocked at −80°C until further study.

Western blot analysis

For Western blot analysis, an equal amount of protein from each sample was separated by 8~12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes at 300 mA. The membranes were blocked with 5 % skim milk in PBS with 0.1 % Tween 20 (PBST) at room temperature. After incubation, the membranes were sequentially incubated with primary antibodies (anti-iNOS, anti-p-p38, anti-p38, anti-ERK1/2, anti-p-ERK1/2, anti-NF-κB p65, anti-IκBα, anti-c-jun, anti-p-c-jun, anti-c-fos, anti-β-actin) in PBST at 4°C overnight and horseradish peroxidase-conjugated secondary antibodies in PBST at room temperature for 1 h. The membranes were developed using enhanced chemiluminescence reagents (ECL). Densitometric analysis was performed with the use of PDI Imageware System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analyses were performed by using the SPSS 10.0 package (SPSS Inc., Chicago, IL, USA). All data were presented as means±SD of at least three independent experiments. Statistical evaluations were performed with one-way ANOVA with Bonferroni's multiple comparison tests. P values less than 0.05 were considered to be statistically significant.

Results

COS show no inhibitory effect on N9 microglial cells viability

Effect of COS on N9 microglial cells viability was evaluated by MTT assay. As shown in Fig. 1b, the viability of N9 microglial cells was not significantly affected after incubation with different concentrations of COS (50~500 μg/ml) for 24 h or 48 h. Thus, for this study, we used COS at concentrations of 50~200 μg/ml, which cause no cytotoxicity to N9 microglial cells, for the further study of anti-inflammation and action mechanisms.

COS attenuate LPS-induced NO generation in N9 microglial cells

To study whether COS inhibit NO production in LPS-stimulated N9 microglial cells, we measured the intracellular and extracellular NO, respectively. The analysis of intracellular NO production in LPS-induced N9 microglial cells was performed by confocal microscopy and flow cytometry. For confocal microscopy analysis, few stained cells were observed in nonactivated N9 cells (Fig. 2b). In contrast, after LPS (1 μg/ml) treatment for 24 h, an obvious cytoplasmic staining was observed (Fig. 2c), which indicted a large amount of NO was generated after LPS stimulation in N9 microglial cells. Pretreatment with COS (50~200 μg/ml) could remarkably suppress the LPS-induced intracellular fluorescence of N9 cell (Fig. 2e–g), which provides a visualized proof of the effect of COS on LPS-induced N9 cells activation.

Flow cytometry analysis of intracellular NO induced by LPS in N9 cells is illustrated in Fig. 2h and i. Treatment with LPS (1 μg/ml) for 24 h induced a 2.4-fold increase of intracellular NO level compared with the untreated group ($p<0.05$). Pretreatment with COS (50~200 μg/ml) significantly reduced the fluorescence intensity of intracellular NO levels in a dose-dependent manner. These results were consistent with the confocal microscopy results.

The level of extracellular NO in LPS-induced N9 cells was measured by Griess reaction. As shown in Fig. 3, the NO concentration in culture medium of LPS-treated group was 24.9±0.3 μM after 24 h stimulation, which was significantly increased compared with 3.4±1.8 μM in the untreated group ($p<0.05$). Pretreated with COS for 24 h, the concentration of NO in supernatant decreased by 40 %~70 % compared with LPS treated group. HC has been reported to have the ability to effectively block NO production in activated microglia [17, 18], thus we used here as a positive control. As shown in Figs. 2 and 3, HC (1 μM) could exert its inhibitory effect on both intracellular and extracellular NO levels. These results suggested that COS (50~200 μg/ml) could effectively suppress the NO production in N9 cells and the effect was comparable to the positive control.

COS inhibit LPS-induced iNOS expression at mRNA and protein level in N9 microglia cells

We examined the effect of COS on LPS-induced iNOS expression at both the mRNA and protein expression levels in N9 microglial cells. As shown in Fig. 4a, LPS (1 μg/ml) significantly stimulated iNOS gene expression at 12 h by RT-PCR assay. After pretreatment with COS (50~200 μg/ml) for 24 h, a dose-dependent reduction of LPS-induced iNOS mRNA expression was observed in

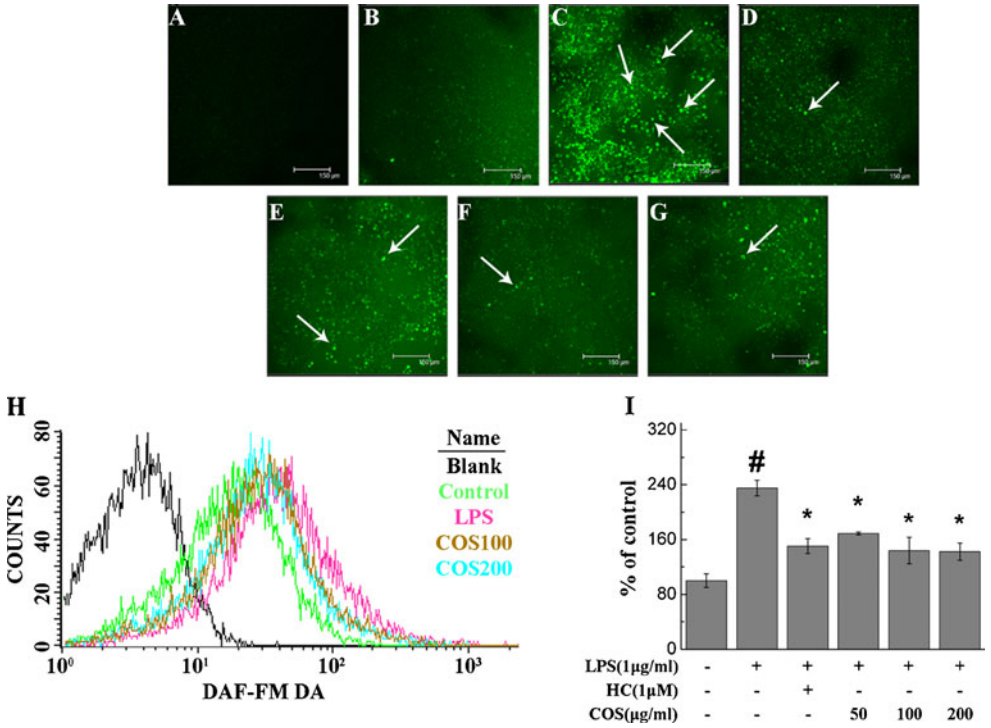

Fig. 2 Effect of COS on LPS-induced intracellular NO production in N9 microglial cells. Cells were pretreated with COS (50~200 μg/ml) for 24 h and then exposed to LPS (1 μg/ml) for 24 h. After treatment, intracellular NO levels were determined by Confocal microscopy (**a**–**g**) and flow cytometry (**h**, **i**) as described in Material and methods, respectively. (**a**–**g**) Representative of Confocal microscopy images of blank group (**a**), negative control group (**b**), LPS treated group (1 μg/ml) (**c**), HC pretreated group (**d**) and COS pretreated groups (50~200 μg/ml) (**e**–**g**), respectively. **h**. Representative of comparative flow cytometric analysis of LPS-induced NO production. (**i**). Fluorescent intensities of N9 microglial cells treated with COS and/or LPS by flow cytometric analysis. White arrows indicate the N9 cells with over-expression of NO. Values are normalized to the untreated controls. Data are expressed as mean±SD (n=3). # P<0.05 *versus* blank group. \ast P<0.05 *versus* LPS-treated group

N9 microglial cells (p<0.05). As shown in Fig. 4b, LPS (1 μg/ml) remarkably elevated iNOS protein expression at 16 h by Western blot analysis. Consistent with the RT-PCR results, pretreatment with COS (100~200 μg/ml) for 24 h significantly attenuated LPS-induced iNOS expression at protein level in a dose-dependent manner (p<0.05). These results indicated that LPS-induced production of iNOS in N9 microglial cells could be effectively suppressed by COS at both the transcription and translation levels.

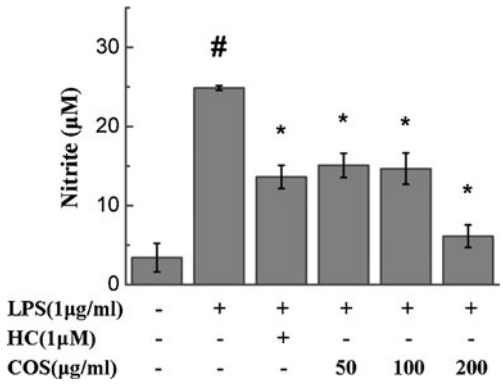

Fig. 3 Effect of COS on LPS-induced NO release in N9 microglial cells. Cells were pretreated with COS (50~200 μg/ml) for 24 h, and then exposed to LPS (1 μg/ml) for 24 h. Nitrite levels in the culture supernatants were measured by Griess reaction as described in Material and methods. Values are normalized to the untreated controls. Results are expressed as the mean±SD, n=6. # P<0.05 *versus* blank group. \ast P<0.05 *versus* LPS-treated group

COS inhibit MAPKs phosphorylation in N9 microglial cells

To determine the inhibitory effect of COS on MAPKs activation in LPS-activated microglial cells, cells were pretreated with COS (50~200 μg/ml) for 24 h before exposure to LPS (1 μg/ml) for 3 h. As shown in Fig. 5, both p38 MAPK and ERK1/2 were highly phosphorylated in N9 microglial cells when treated with LPS (1 μg/ml) alone. On the contrary, the increase of p38 MAPK and ERK1/2 phosphorylations were significantly inhibited by COS pretreatment (50~200 μg/ml). However, 100 μg/ml of COS exerted the most remarkable effect on the suppression of p38 MAPK phosphorylation (Fig. 5a). COS at higher concentrations (100~200 μg/ml) showed, however, a better suppression effect

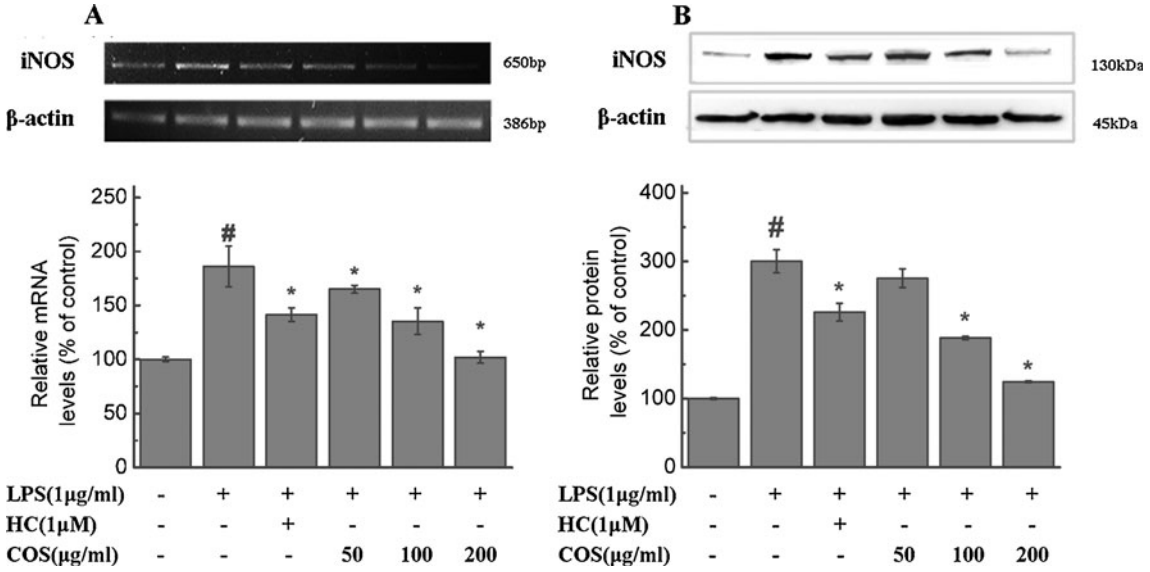

Fig. 4 Effect of COS on iNOS levels in LPS-induced N9 microglial cells. **a**. Cells were pretreated with COS (50~200 μg/ml) for 24 h, and exposed to LPS (1 μg/ml) for another 12 h. After treatment, the mRNA levels of iNOS were determined by RT-PCR analysis as described in Material and methods. **b**. Cells were pretreated with COS (50~200 μg/ml) for 24 h, and exposed to LPS (1 μg/ml) for 16 h. After treatment, the protein levels of iNOS were determined by Western blot analysis as described in Material and methods. Data are representative of three experiments. Values are normalized to the untreated controls and expressed as the mean±SD. # $P<0.05$ *versus* blank group. \ast $P<0.05$ *versus* LPS-treated group

on phosphorylation of ERK1/2 than that of 50 μg/ml ($p<0.05$). Based on the above results, we draw the conclusion that COS could suppress p38 MAPK and ERK1/2 phosphorylation in LPS-induced microglial cells.

COS block LPS-induced NF-κB translocation into nucleus in N9 microglial cells

As shown in Fig. 6, the significant elevation of NF-κB translocation into nucleus was detected after LPS (1 μg/

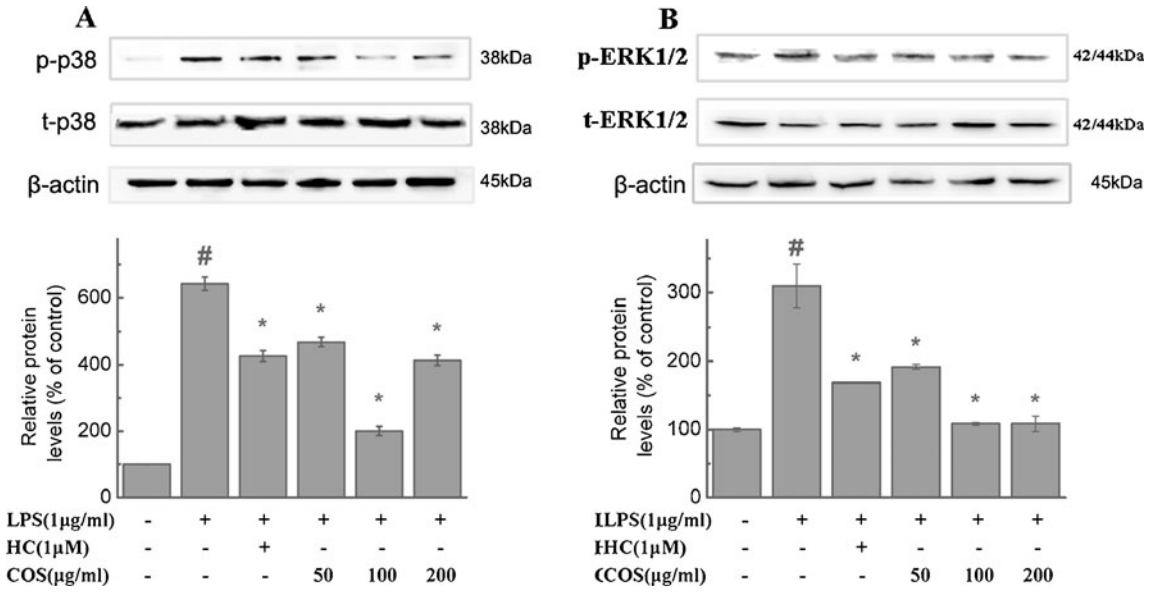

Fig. 5 Effect of COS on LPS-induced phosphorylation of p38 MAPK (**a**) and ERK1/2 (**b**) in N9 microglial cells. Cells were pretreated with COS (50~200 μg/ml) for 24 h and exposed to LPS (1 μg/ml) for 30 min. After treatment, total cell extract was prepared and the protein levels of phosphorylated p38 MAPK (p-p38), total p38 MAPK (t-p38), phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 (t-ERK1/2) were determined by Western blot analysis as described in Material and methods. Data are representative of three experiments. Values are normalized to the untreated controls and expressed as the mean±SD. # $P<0.05$ *versus* blank group. \ast $P<0.05$ *versus* LPS-treated group

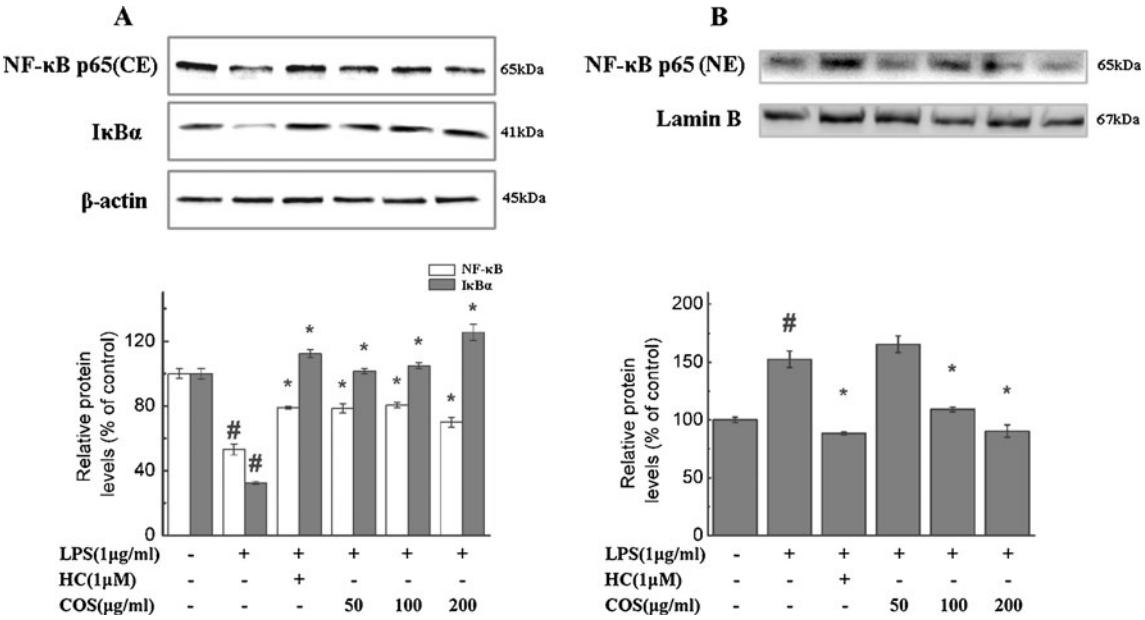

Fig. 6 Effect of COS on LPS-induced NF-κB translocation in N9 microglial cells. CE: cytoplasmic extracts; NE: nuclear extracts. **a**. Relative protein levels of NF-κB and IκBα in cytoplasm of N9 microglial cells. **b**. Relative protein levels of NF-κB in nucleus of N9 microglial cells. Cells were pretreated with COS (50~200 μg/ml) for 24 h, and exposed to LPS (1 μg/ml) for 3 h. After treatment, cytoplasmic and nuclear extracts were determined by Western blot analysis as described in Material and methods. Data are representative of three experiments. Values are normalized to the untreated controls and expressed as the mean±SD. # $P<0.05$ *versus* blank group. \ast $P<0.05$ *versus* LPS-treated group

ml) stimulation for 3 h in N9 microglial cells. The protein level of NF-κB in cytoplasm was significantly down-regulated to 53 %, and that in nucleus was up-regulated to 153 % compared to the control group. In contrast, pretreatment with COS for 24 h remarkably reversed this effect. The protein levels of NF-κB in cytoplasm were elevated to 70 % ~80 % (50~200 μg/ml) in cytoplasm and decreased to 90~110 % (100~200 μg/ml) in nucleus compared to the control group. Moreover, cytoplasmic IκBα protein levels were significantly higher in COS (50~200 μg/ml) pretreated group than that in LPS group ($p<0.05$), which should be consistent with the change of NF-κB in cytoplasm. In conclusion, COS administration could inhibit the degradation of IκBα, and prevent the translocation of NF-κB into the nucleus in N9 microglial cells.

COS inhibit LPS-induced AP-1 activation in N9 microglial cells

We investigated the suppressive effect of COS pretreatment on LPS-induced AP-1 activation in N9 microglial cells. As shown in Fig. 7a, LPS (1 μg/ml) stimulation for 3 h markedly promoted the phosphorylation of c-jun, and pretreatment with COS (50~200 μg/ml) for 24 h markedly attenuated this effect in a dose-dependent manner. As shown in Fig. 7b, c-fos protein, which upon translocation to the nucleus combines with pre-existing Jun proteins to form AP-1 dimer after stimuli, was elevated in LPS-induced N9 microglial cells at 3 h. Pretreatment with COS (50~200 μg/ml) for 24 h effectively inhibited the expression of c-fos in a dose-dependent manner. HC showed no significantly inhibitive effect on AP-1. These results indicated that pretreatment with COS inhibited the AP-1 activation and may lead to the suppression of NO production in microglial cells.

Discussion

Microglial cells originating from monocytes are a type of glial cell and they are usually considered as the resident brain macrophages. Thus, microglial cells function as the first and main form of active immune defense in CNS [19]. When infectious agents are directly introduced to the CNS, microglial cells quickly respond to the infection decreasing the inflammation to protect the neural tissues. However, over-activated microglial cells can also be the active contributors to the neuronal damage in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, through secreting a variety of proinflammatory and neurotoxic factors. Thus, we can prevent the neuroinflammation in the CNS by suppressing the activation of microglial cells to provide us a considerable way of the neurodegenerative diseases therapy. N9 is a murine microglial cell line, which is widely used in the field of neurobiology, and has been generally considered a reliable *in vitro* model for microglial cells [20]; it facilitates the study of microglial function

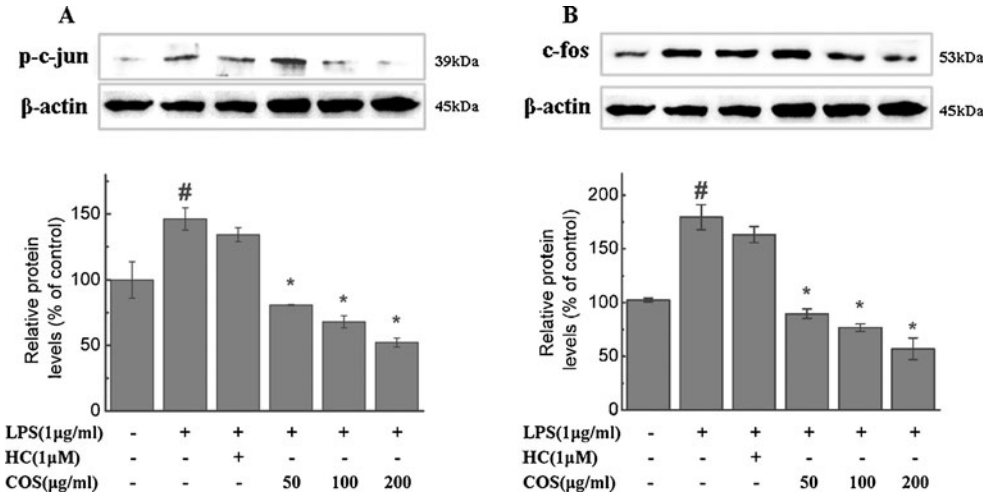

Fig. 7 Effect of COS on LPS-induced AP-1 activation in N9 microglial cells. Cells were pretreated with COS (50~200 μg/ml) for 24 h, and exposed to LPS (1 μg/ml) for 3 h. After treatment, total cell extract was prepared and the protein levels of phosphorylated c-jun (p-c-jun) (**a**), c-fos (**b**) and β-actin were determined by Western blot analysis as described in Material and methods. Data are representative of three experiments. Values are normalized to the untreated controls and expressed as the mean±SD. # $P<0.05$ *versus* blank group. \ast $P<0.05$ *versus* LPS-treated group

without the interference of possible responses from other type of cells present in the primary culture. Some agents, such as resveratrol, glucocorticoid and tetramethylpyrazine, have been confirmed to inhibit the production of NO and/or proinflammatory factors [6, 17, 21]. Here, for the first time, we demonstrate the anti-inflammatory effect of COS in LPS-induced N9 microglial cells, which contributes to a better scientific understanding of the potential therapeutic use of the COS for neurodegenerative diseases.

NO is a highly reactive free radical and it has been demonstrated to induce neuronal apoptosis as an inflammatory product [22]. NO, as a second messenger, participates in the cell signal transduction processes; it also plays a role in endothelial functions including angiogenesis and migration [23]. iNOS is a member of nitric oxide synthases (NOSs) family, which catalyze the production of NO from L-arginine, and function in most immune system as immune defense against pathogens. In our previous research, we have demonstrated that COS could inhibit the endothelial cell migration by decreasing the NO production to block the signal transduction pathway [24]. Some researchers also confirmed the inhibitory effect of COS on LPS-stimulated NO secretion from murine macrophage cells RAW264.7 [25]. This suggested that COS may inhibit activation of microglial cells, which function as resident brain macrophage. Consistent with these results, we revealed COS could attenuate the NO release and inhibit the iNOS expression at both mRNA and protein levels in LPS-induced N9 microglial cells. Hydrocortisone can suppress the production of NO and TNF-α in activated microglial cells [18]. Thus, we use 1 μM of HC as a positive control in this study. The results indicated that the effect of COS (50~200 μg/ml) was comparable to the positive control on NO production. In addition, the inhibitory effect was not due to the decrease in the number of N9 cells, because the incubation of N9 microglial cells with COS for 24 or 48 h did not affect the cells viability. On the contrary, COS could also promote the production of NO elsewhere. For example, in our previous studies, the production of NO in resting neutrophils was significantly increased after incubation with different concentrations of COS [26]. Moreover, we also demonstrated that COS could increase the NO and NOS levels in H_2O_2-induced ECV304 cells [16]. These contradictory biological effects of COS may be, at least in part, due to different types of cells, different stimuli that the cells are treated with and different oxidative circumstances the cells are in.

MAPKs are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities, such as inflammation, differentiation and apoptosis [27]. Two of the most extensively investigated MAPKs, which mediate intracellular signaling cascades in inflammatory responses are p38 MAPK and ERK1/2. It is well known LPS could activate MAPK signal transduction in immunocyte. Suppression of p38 MAPK activity by SB 203580, a specific p38 MAPK inhibitor, led to the down-regulation of NO and iNOS expression through the inhibition of the NF-κB and AP-1 activation in LPS-induced macrophages [28]. The duration of ERK1/2 activation influences the expression of Fra-1, Fra-2, c-Jun and JunB, whereas expression of c-Fos is strongly induced by even transient ERK activation [29]. In our previous research [6], it has been confirmed that MAPKs are up-regulated during LPS stimulation in N9 microglial cells, and are the upstream modulators for iNOS expression. Thus, we further investigated the suppressive effect of COS on MAPK phosphorylation. In the present study, we found that pretreatment with COS could

remarkably down-regulate the phosphorylated levels of p38 MAPK and ERK1/2 in LPS-induced N9 microglial cells. Interestingly, as for p38 MAPK phosphorylation, COS showed the best inhibitive effect at a concentration of 100 μg/ml, and a dose-dependent effect in ERK1/2 phosphorylation. Based on these results, we conclude that the inhibitory effect of COS on N9 microglial cells may be attributed to the inhibition of phosphorylated levels of both MAPKs.

It is widely known that transcription factor NF-κB plays an important role in regulating cellular responses, and its predominant form is a heterodimer composed of p50 and p65 subunits. In unstimulated cells, the NF-κB heterodimer is combined with inhibitory proteins, such as IκBs, to form an inactive cytoplasmic complex [30]. NF-κB could be activated by various inducers including LPS, reactive oxygen species (ROS), tumer necrosis factor-α (TNF-α). After activation, the IκBs are rapidly degraded and free NF-κB dimmers translocate to the nucleus and activate target genes [31]. It has been well documented that both p38 MAPK and JNK are involved in the activation of NF-κB transcriptional activity [32]. In our previous research, we found that COS could suppress LPS-induced IL-6 expression in HUVEC through ERK1/2 but not p38 MAPK dependent NF-κB activation [33]. Thus, we assessed the effect of COS on the inhibition of NF-kB translocation from cytoplasm into nucleus. In this study, we revealed that, for NF-κB p65 level, LPS significantly led to a decrease in cytoplasm of N9 microglial cells and an increase in nucleus, which could be effectively inhibited by COS pretreatment. And we also found that COS could prevent the degradation of IκBα in cytoplasm, which may lead to the suppression of NF-κB translocation. Additionally, previous research also demonstrated that NO production could be suppressed by the inhibition of NF-κB activation in IFN-γ-induced microglial cells [4]. Together, these results suggest that the inhibitive effects of COS on the gene expression of iNOS are at least in part due to the inhibition of NF-κB activation.

AP-1 is another important transcription factor, and is comprised of a Jun family member (c-jun, v-jun, JunB or Jun-D) homodimerized with another Jun protein or heterodimerized with a Fos protein (c-fos, Fos-B, Fra-1 or Fra-2). AP-1 complex is involved in many physiological procedure, and has been confirmed to induce inflammation reaction in response to inflammatory cytokines [34]. Moreover, it has been well documented that MAPKs activation may lead to elevated AP-1 activity. The main nuclear target of ERK1/2 was Elk 1, which led to activation of serum response element on fos promoter. For p38 MAPK, the main targets in the nucleus were ATF2, MEF2A/C and SAP1a, which are involved in the formation of AP-1 transcription complex [35]. Both p38 MAPK and ERK1/2 were participating in the c-jun and c-fos expression in LPS-induced human gingival fibroblasts [36]. Some researchers confirmed that SB203580, which is p38 MAPK specific inhibitor, could inhibit the c-fos and c-jun expression in LPS-induced primary cortical glial cells [37]. It has also been found that Amyloid beta could induce iNOS expression through activation of AP-1, which regulated by upstream ERK and p38 MAPK in PC-12 cells [38]. Herein, we found that COS have the ability to inhibit the activation of AP-1 by not only blocking the phosphorylation of c-jun, but also attenuating the expression of c-fos, which may also participate in the inhibitive effect of COS on the gene expression of iNOS. The effect of COS on AP-1 expression might be due to the inhibition of both p38 MAPK and ERK1/2 activation. On the contrary, HC showed no inhibitive effect on AP-1, which suggested the anti-inflammatory mechanism might be different from COS.

COS, as an enzymatic hydrolysis from chitosan, has been proved that it could bind with the mannose receptor in rat macrophage RAW264.7 [39]. Some scientists have demonstrated that microglia, as the resident brain macrophages could also express mannose receptor [29, 40]. Mannose receptor could recognize complex carbohydrates located on glycoproteins and participate in macrophage endocytosis process. Thus, we inferred from these results that mannose receptor might also act as COS receptor on microglial cells. My colleague also demonstrated that COS could remarkably inhibit the binding of LPS to its receptor (TLR4/MD-2) in RAW264.7 macrophage [41]. This could at least partly explain the anti-inflammatory effect of COS in LPS-induced cell models. Hydrocortisone, which is one of the glucocorticoids, is proved to exert the protective effect in acute stress. Glucocorticoids are toxic at high concentration to neurons, and may contribute to the development of neurodegenerative diseases [42], while it also could inhibit activation of microglial cells. We used HC as the positive control in our study, and found HC could inhibit the NO production, repress the activation of NF-κB by inhibition of its translocation into nuclues, but could not inhibit the phosphorylation of c-jun and the expression of c-fos. These results suggested that the anti-inflammatory effects of HC in activated microglial cells might be in a NF-κB dependent and AP-1 independent pathway, which is different from COS. The differences between HC and COS may be partly due to the different receptors they use on microglial cells and the different signal pathways.

Conclusions

In conclusion, our findings demonstrate that COS could inhibit the inflammatory responses in N9 microglial cells through the suppression of NO production and down-regulation of iNOS production at both transcription and

translation levels. The molecular mechanisms may be performed through the inhibition of the NF-κB translocation and/or the activation of AP-1 by blocking p38 MAPK and ERK1/2 pathways. Our study provides an insight into some mechanisms of the inhibitory effect of COS on the microglial cells inflammation, and may shed light on future application of COS in the therapy of neurodegenerative diseases.

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