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Chitosan oligosaccharides suppressant LPS binding to TLR4/MD-2 receptor complex

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ABSTRACT

Lipopolysaccharide, a potent activator of the immune system, elicits the production of pro-inflammatory mediators in immunocytes. Toll-like receptor 4 (TLR4) and myeloid differentiation factor (MD) 2 receptor complex is required for recognition and signaling of LPS. Previous study suggested water soluble chitosan decreased secretion of pro-inflammatory cytokines TNF-α and IL-6. However, no studies have provided direct target and molecular mechanism of anti-inflammatory effect of chitosan oligosaccharide on LPS-stimulated cells. In this study, we found that chitosan oligosaccharides significantly inhibited binding of LPS to TLR4/MD-2 receptor complex, thus attenuated activation of mitogen-activated protein kinases (MAPKs) and decreased nuclear translocation of nuclear factor-κB (NF-κB). Finally, chitosan oligosaccharides reduced the production of pro-inflammatory mediator, such as IL-1β and nitric oxide (NO) in LPS-stimulated RAW 264.7 cells. Therefore, chitosan oligosaccharides are potential inhibitive effector of LPS.

1. Introduction

Bacterial lipopolysaccharide (LPS), one of the principal components of the outer membrane of Gram-negative bacteria, has been recognized as a key molecule in the pathogenesis of sepsis and septic shock. It is composed of O-antigen, core oligosaccharide and lipid A. Monocytes, macrophages and other types of immune cells can be activated by LPS, inducing secretion of TNF-α, IL-1β, IL-6 and production of free radicals, such as nitric oxide (NO) and reactive oxygen species (ROS) (Glauser, Zanetti, Baumgartner, & Cohen, 1991). Over-expression of these pro-inflammatory mediators may result in fever, severe damage of tissues or septic shock (Parrillo, 1993). Toll-like receptor 4 (TLR4) and differentiation factor (MD) 2 form a functional complex in the recognition and signaling of LPS (Park et al., 2009; Yamada et al., 2006; Yamazoe et al., 2008). The most effective LPS-neutralized antibiotic, polymyxin B (PMB), can bind to the lipid A moiety, but is toxic when given systemically (Moriyama et al., 2006).

Chitosan, an N-deacetylated derivative of chitin, is a naturally abundant mucopolysaccharide which is distributed in the shell of crustaceans, in the cuticle of insects and the cell walls of some fungi and microorganisms (Park, Je, & Kim, 2003). Chitosan oligosaccharides (COS) are oligosaccharides derived from degradation and deacetylation of chitosan. They have a lower molecular weight, better solubility and safety than chitosan. More and more studies have demonstrated that COS have versatile biological functions, including anti-tumor (Jeon & Kim, 2002; Wu, Yao, Bai, Du, & Lin, 2008; Xu et al., 2007), anti-oxidation (Je, Park, & Kim, 2004; Liu et al., 2009; Xing et al., 2005), and against bacterial infection (Fernandes et al., 2008; Hernandez-Lauzardo et al., 2008; Okawa et al., 1988; Tikhonov et al., 2006). However, no studies have provided direct target or molecular mechanism of biological functions of COS, including the anti-inflammatory effect on LPS-stimulated cells.

Activation of mitogen-activated protein kinases (MAPKs) signaling and nuclear translocation of NF-κB are two key pathways in the LPS-induced signal transductions (Hsu & Wen, 2002; O'Sullivan, Wang, & Redmond, 2009). While the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase are three major kinases involve in MAPKs. Previous findings have shown that inhibition of MAPKs and NF-κB prevented cytokine production in LPS-stimulated cells (Scherle et al., 1998).

Abbreviations: TLR4, Toll-Like receptor 4; MD-2, myeloid differentiation factor 2; COS, chitosan oligosaccharides; LPS, lipopolysaccharide; PMB, polymyxin B; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-κB.

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In our report, we provided evidences that COS suppressed LPS binding to TLR4/MD-2 receptor complex. Furthermore, it decreased activation of MAPKs, nuclear translocation of NF-κB and reduced the production of pro-inflammatory mediator IL-1β and NO in LPS-stimulated inflammatory processes.

2. Materials and methods

2.1. Reagents

Chitosan oligosaccharides are products of our lab with 95% of deacetylation, The weight percentages with DP (degree of polymerization) 2–6 in the mixture of COS are 3.7%, 16.1%, 28.8%, 37.2% and 14.2% respectively, which have been described previously (Dou et al., 2007; Xu et al., 2007). Possible LPS contamination was excluded using *Tachypleus* amebocyte lysate assay (obtained from Chinese Horseshoe Crab Reagent Manufactory, CO., Ltd, Xiamen, China), with the LPS concentration lower than 0.2 EU/mg. LPS (*Escherichia coli* 055:B5), FITC-LPS (*E. coli* 055:B5), DAPI and polymyxin B (PMB) were purchased from Sigma–Aldrich (St. Louis, MO). RPMI 1640 and fetal bovine serum were obtained from Invitrogen-Gibco. Rabbit anti ERK, p38, phospho-p38, JNK and phospho-JNK polyclonal antibodies, were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-phospho-ERK monoclonal antibody, rabbit anti-NF-κB p50 polyclonal antibody, rhodamine-conjugated goat anti-rabbit IgG, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from Santa Cruz (Santa Cruz, CA, USA).

2.2. Cell culture

Mouse macrophage cell line, RAW 264.7 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated endotoxin free fetal bovine serum, 2 mM L-glutamine, and 100 U/ml streptomycin and 100 U/ml penicillin under a humidified 5% CO_2 atmosphere at 37 °C. HEK 293T cell line was cultured in DMEM (Sigma) supplemented with 10% heat-inactivated endotoxin free fetal bovine serum, 100 U/ml streptomycin and 100 U/ml penicillin.

2.3. Tachypleus amebocyte lysate assay for measurement of LPS

A quantitative chromogenic *tachypleus* amebocyte lysate (TAL) assay was performed for the determination of LPS-neutralizing activities of COS. The concentrations of LPS were detected according to the instructions provided by the manufacturer. In brief, 100 μl standard samples of LPS or LPS mixed with COS were added in triplicate to 100 μl of TAL in a pyrogen-free tube. After incubation at 37 °C for 8 min, 100 μl of prewarmed substrate solution was added, followed with 100 μl of stop buffer 6 min later. The absorbance was measured at 545 nm. One nanogram of endotoxin is equivalent to 2.5 endotoxin units.

2.4. Plasmid construction and transfection

Full-length of MD-2 and TLR4 were amplified by PCR from mouse bone marrow cDNA library (Clontech, San Diego, CA) and inserted into pcDNA3 vector (Invitrogen). Both constructs were confirmed by sequencing. All transfections were carried out using LipofectAMINE2000 (Invitrogen) according to the manufacturer's instructions.

2.5. Flow cytometry analysis

For analysis the binding of FITC-conjugated LPS to RAW 264.7 cells, cells were harvested and re-suspended in RPMI 1640 medium for the binding assay. Different concentrations of COS were prepared in 20 μl PBS containing 10 μg/ml FITC-conjugated LPS. After 15 min incubation at 37 °C, cells were centrifuged and washed twice in ice-cold PBS. HEK 293T cells were co-transfected with TLR4 and mMD-2 plasmids, and cells transfected with empty vector served as the control. Transfected cells were harvested 48 h later, incubated with FITC-LPS or the FITC-LPS/COS mixture and then subjected to FACS for analysis.

2.6. Western blot

RAW 264.7 cells were seeded at a density of 2×10^5 cells/well in 6-well plates for 24 h, and treated with indicated reagents for 30 min. Cells were scraped from the plates into RIPA lysis buffer with protease inhibitor and phosphatase inhibitor. After centrifugation the supernatant was harvested and the protein concentration was determined by the bicinchoninic acid (BCA). Equal amounts of 20 μg protein were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane. The membrane was blocked of 5% skim milk or 3% BSA in TBST for 2 h at room temperature, and then incubated for 2 h at room temperature with specific primary antibody in incubation media. After being washed in TBST three times, the membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Membrane-bound HRP-labeled protein bands were monitored with enhanced chemiluminescent reagents and chemiluminescent signals were detected using X-ray film.

2.7. Immunofluorescent staining

Immunofluorescent staining was performed to examine the NF-κB intranuclear translocation in RAW 264.7 cells following LPS stimulation. Briefly, RAW 264.7 cells (4×10^4) cultured on glass coverslips were plated into 24-well for 24 h, and then treated with indicated reagents for 1 h. Upon the completion of drug treatment, glass coverlips were washed with ice-cold PBS and fixed in 4% paraformaldehyde at room temperature for 30 min, then incubated with 0.3% Triton X-100 in PBS for 10 min. After washing, coverslips were blocked with 10% goat serum for 1 h, and then incubated with the rabbit polyclonal antibody against NF-κB p50 overnight at 4 °C, followed with incubation with rhodamine-conjugated rabbit IgG for 1 h. After washing, coverslips were incubated with DAPI at room temperature for 10 min. Finally, coverslips were washed with PBS and mounted with aqueous mounting media. Fluorescence signals were analyzed by Fluoview microscopy (OLYM-PUS, Japan). Nuclear stains were masked to measure the relative p50 mean fluorescence intensity (MFI) within the nuclei. Percentage of nuclear NF-κB at least 70 cells from each experimental group was analyzed by analysis of variance (ANOVA) using IPP 6.0 software.

2.8. RNA isolation and RT-PCR

RAW 264.7 cells were treated with indicated reagents for 6 h. The cells were harvested and total RNA was isolated by TRIzol according to the manufacturer's instructions. For amplification of IL-1β, the following primers were used: IL-1β primer forward 5′ ATG GCA ACT GTT CCT GAA CTC 3′; reverse 5′ TTA GGA AGA CAC AGA TTC CAT GG 3′. RT-PCR analysis was performed with RT System (TaKaRa), as described (Xu et al., 2007). Briefly, 2 μg of total RNA was performed with the following cycles: 30 °C for 10 min, 42 °C for 30 min, 99 °C for 5 min. For PCR amplification, the following conditions were used; 94 °C for 30 s (denaturation), 56 °C for 30 min (annealing) and 72 °C for 1 min (extension) for 28 cycles. The amplified PCR products were separated with 1.2% agarose gel, and then stained with ethidium bromide.

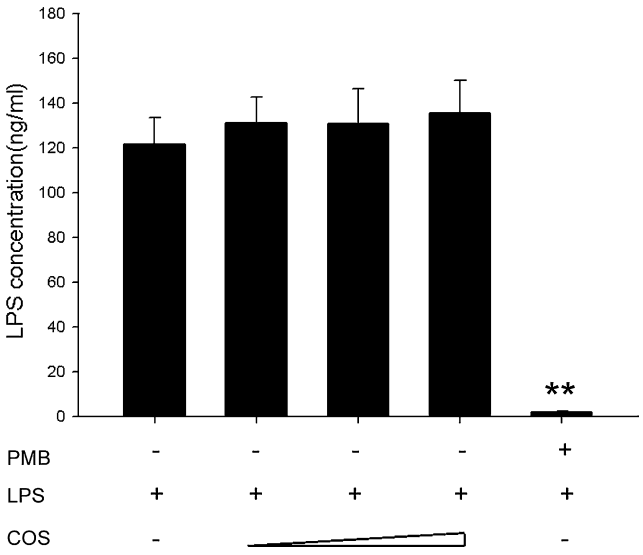

Fig. 1. LPS-neutralizing assay of COS *in vitro*. LPS (100 ng/ml) was incubated with various concentrations of COS (0.1, 0.2 and 0.4 mM) at 37 °C for 30 min, and then was detected by TAL assay. The results were presented as mean ± S.E.M. of data from three independent experiments.

2.9. Measurement for cytokine

RAW 264.7 cells were seeded at a density of 2×10^5 cells/well in 6-well plates and treated with indicated reagents for 4 h. Cells were lysed by repeated freeze-thaw cycles. The concentration of intracellular of pro-IL-1β was determined by ELISA (R&D, Minneapolis, MN), following the manufacturer's instructions.

2.10. NO estimation

NO_2^- accumulation was used as an indicator of NO production as previously described (Dou et al., 2007). Briefly, RAW 264.7 cells were plated at 2×10^5 cells/well and incubated with indicated reagents at 37 °C for 6 h. Aliquot of the incubation supernatant (50 μl) was transferred to 96-well plates and nitrite was determined spectrophotometrically using the Griess reagent (0.8% sulfanilamide, 0.75% N-(naphthyethylene)diamine in 0.5 M HCl) by mixing a 100 μl of the Griess reagent. After 15 min incubation at room temperature, the nitrite concentrations were measured at 540 nm using a microplate reader.

2.11. Statistic analysis

Statistical evaluation was carried out using SPSS 10.0 package (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± S.E.M. of at least three independent experiments. ANOVA and Student's *t*-test were performed to determine statistical significance. Differ-

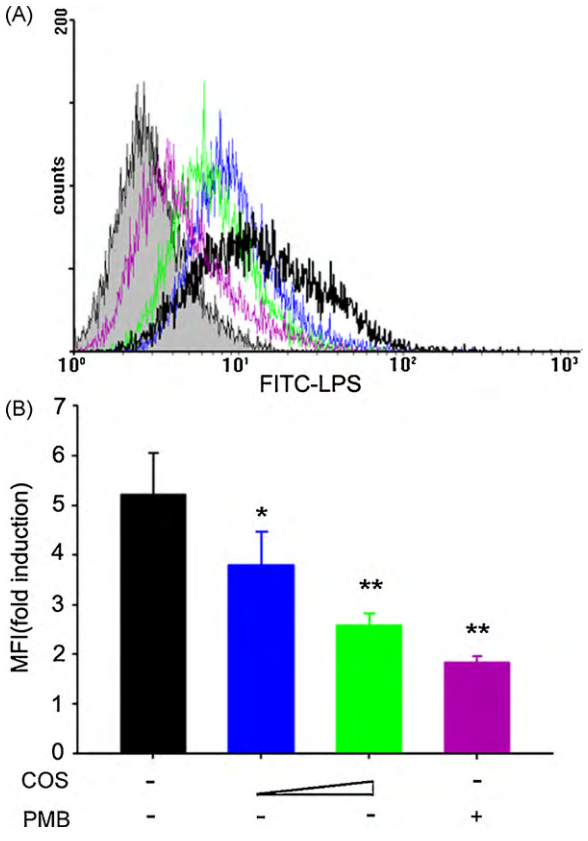

Fig. 2. Effect of COS on binding of FITC-LPS to RAW 264.7 cells. (A) Cells were incubated with 10 μg/ml FITC-LPS in different concentrations of COS (0.2 and 0.4 mM) or PMB (1 μg/ml). Background fluorescence is shown as a gray profile. The binding of LPS to the cell surface was analyzed by FACS. (B) The results from FACS analysis are expressed as mean fluorescence intensity (MFI) fold induction, calculated by dividing the MFI values of stimulated cells with the values of control cells. The results were presented as mean ± S.E.M. of data from three independent experiments, $^*p < 0.05$, $^{**}p < 0.01$.

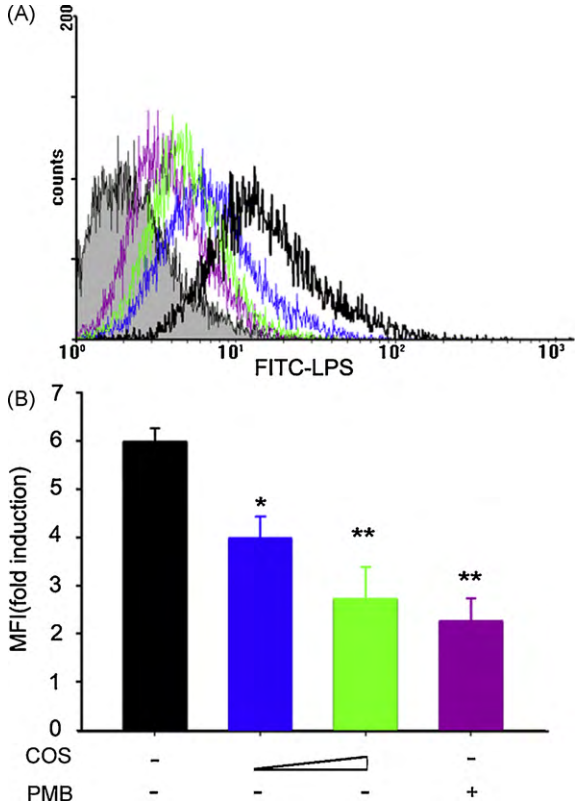

Fig. 3. Effect of COS on binding of FITC-LPS to HEK 293T. (A) HEK 293T cells were transiently co-transfected with TLR4 and MD-2 constructs 48 h later, then cells were collected, washed and incubated with 10 μg/ml FITC-LPS in the absence (thick lines, black) or presence (thin line) of COS (0.2 and 0.4 mM) or PMB (1 μg/ml) and analyzed by FACS. Cells transfected with pcDNA3 empty vector alone served as the background control. (B) The results from FACS analysis are expressed as mean fluorescence intensity (MFI) fold induction, calculated by dividing the MFI values of stimulated cells with the values of control cells. The results were presented as mean ± S.E.M. of data from three independent experiments, $^*p < 0.05$, $^{**}p < 0.01$.

ences between groups were considered to be significant at p-values <0.05.

3. Results

3.1. LPS-neutralizing assay of COS in vitro

Previous reports have introduced several LPS antagonists (Iwagaki, Porro, & Pollack, 2000; Liu et al., 2005; Yamada et al., 2006). The most potent antagonist is PMB, a cationic, cyclic peptide antibiotic that inhibits biological activities of LPS through a high-affinity binding to the lipid A moiety (Iwagaki et al., 2000). Since PMB and COS both carry cationic charge, we first hypothesize that COS could neutralize LPS in the same way. In an attempt to investigate whether COS could inhibit the biological activities of LPS, we examined LPS-neutralizing effect of COS by *Tachypleus* amebocyte lysate (TAL) assay, which is an extremely sensitive indicator to test the presence of free, non-neutralized LPS. However, results indicated that COS did not inhibit the procoagulant activity of LPS as PMB, suggesting that COS did not neutralize biological activity of LPS (Fig. 1).

3.2. Effect of COS on binding of FITC-LPS to RAW 264.7 macrophages

Previous studies have shown that recognition of LPS by immune cells depends upon the proper function of the TLR4/MD-2 receptor complex (Kim, Park, et al., 2007; Park et al., 2009), which is expressed mainly on monocyte and macrophages (Lin et al., 2008; Nagaoka et al., 2001). We therefore employed different kinds of cells to detect whether COS could interfere with LPS binding to cell surface by flow cytometry. Mouse macrophage cells RAW 264.7, were incubated with 10 μg/ml FITC-conjugated LPS. Cells with unlabeled LPS served as background control. FITC-LPS obviously bound to cells compared with control. However, additional administration of COS strongly inhibited the binding of FITC-LPS to RAW 264.7 cells (Fig. 2). Similar results were also obtained from primary $CD14^+$ monocyte and peritoneal macrophage (data not shown).

3.3. Effect of COS on binding of FITC-LPS to HEK 293T which transiently co-transfected with TLR4 and MD-2 constructs

To further investigate COS interfere with LPS binding to receptor complex, HEK 293T cells were transiently co-transfected with TLR4 and MD-2 constructs. As shown in Fig. 3, FITC-conjugated LPS obviously bound to TLR4/MD-2 co-transfected HEK 293T cells compared with cells transfected with empty vector. Consistent with above observation, the binding of FITC-conjugated LPS was also inhibited by the presence of COS.

3.4. Effect of COS on LPS-induced MAPKs pathway activation

To further verify the nature of COS as a partial antagonist, we analyzed the activation of MAPKs signaling and the nuclear translocation of NF-κB which are critically responsible for LPS-induced inflammatory reaction. The extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase are three major kinases in LPS-initiated MAPKs pathways. Treatment of PMB served as the positive control. As shown in Fig. 4, we found that COS suppressed LPS-induced phosphorylation of ERK, JNK and p38 MAPK in a dose-dependent manner, which was consistent with above observations.

3.5. Effect of COS on LPS-induced nuclear translocation of NF-κB

To confirm that the inhibitory effects of COS on LPS-induced signaling, we next investigated whether COS regulate activation of NF-κB by LPS. As shown in Fig. 5, the nuclear translocation of NF-κB (p50) was also partly inhibited by COS administration.

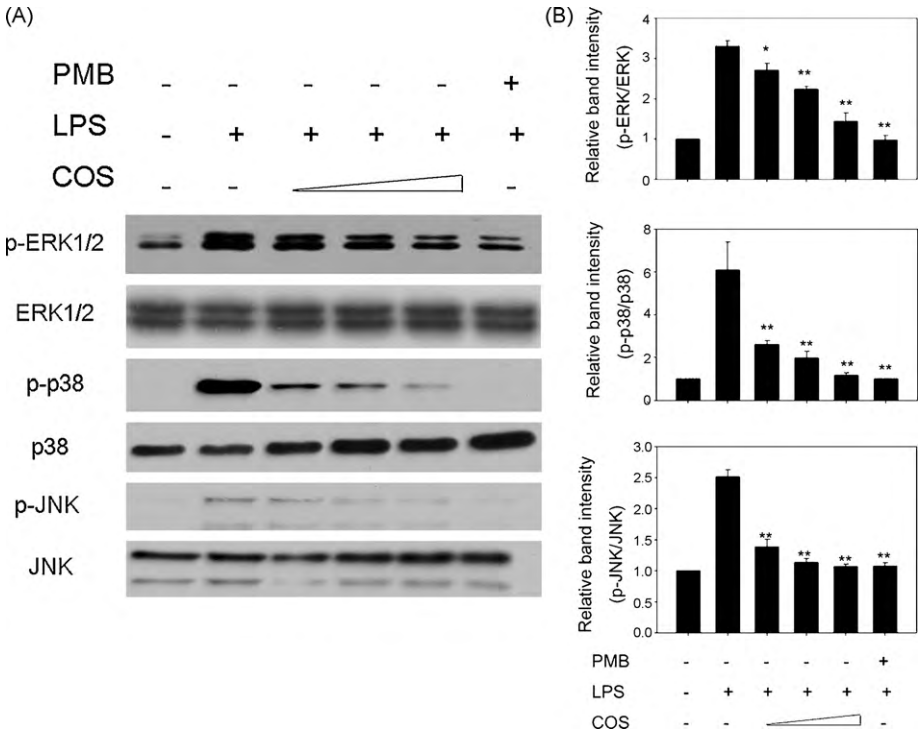

Fig. 4. Effect of COS on ERK, p38 and JNK signaling transduction in RAW 264.7 cells. (A) The cells were stimulated with LPS (100 ng/ml) alone or together with COS (0.1, 0.2 and 0.4 mM) at 37 °C for 30 min, LPS containing PMB (100 ng/ml) as a positive control. Protein samples were analyzed by western blot with phosphor-specific antibodies. The total ERK, p38 and JNK levels were used as an internal control. (B) The phosphorylation of ERK, p38 and JNK were quantified using an image analyzer. The results were presented as mean ± S.E.M. of data from three independent experiments, $^*p < 0.05$, $^{**}p < 0.01$.

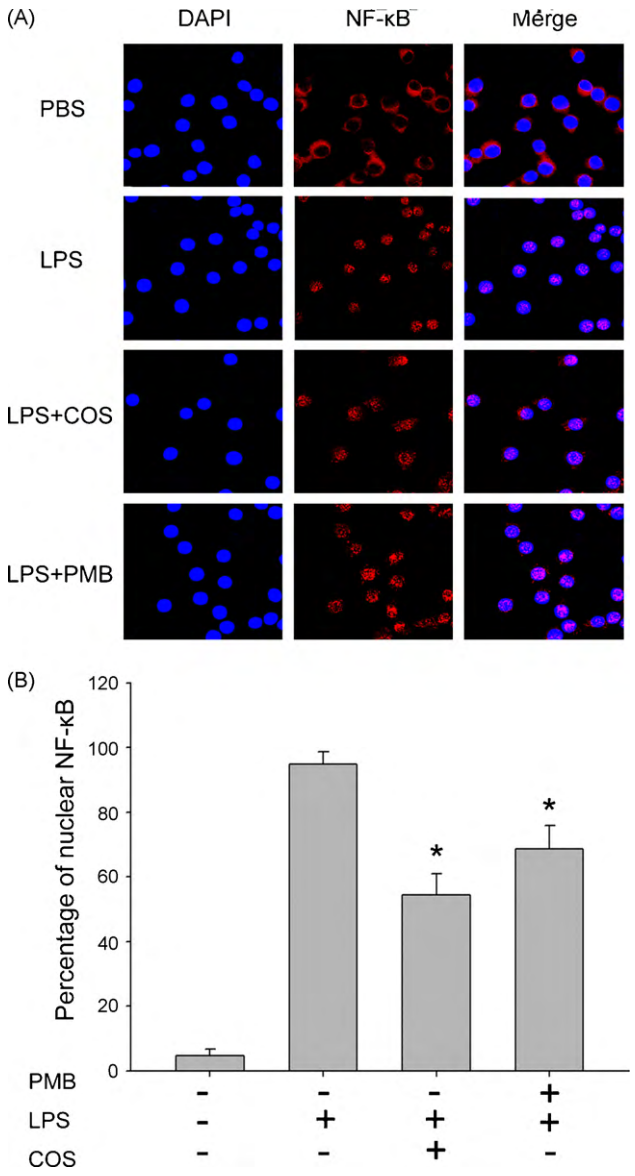

Fig. 5. Immunofluorescent staining assays for COS on intranuclear translocation of NF-κB in LPS-induced RAW 264.7 cells. (A) Cells were cultured for 1 h, fixed, permeabilized, and incubated with rabbit anti-p50 antibody followed by rhodamine-conjugated goat anti-rabbit IgG (red). The nuclei were visualized by DAPI staining (blue). (B) Percentage of nuclear NF-κB at least 70 cells from each experimental group was analyzed by analysis of variance (ANOVA) using IPP 6.0 software, $^{*}p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.6. *Effect of COS on LPS-induced inflammatory mediators production in vitro*

LPS stimulates macrophages to release inflammatory mediators. IL-1β is one of essential cytokines for mediating the pathogenesis of many inflammatory processes (Hsu & Wen, 2002; Scherle et al., 1998). RAW 264.7 cells were treated with LPS in the presence or absence of COS to assess the effect of COS on LPS-induced production of IL-1β. RT-PCR results showed that LPS induced significant increase in the IL-1β mRNA expression, in a dose-dependent manner (Fig. 6A). In accordance with this, ELISA analysis of pro-IL-1β production showed similar results (Fig. 6B).

As a strong activator of immune systems, LPS induce a large amount of NO production (Kim, Yoon, et al., 2007; Tsao et al., 2005). However, overproduction of NO can be oxidized to reactive nitrogen species that disrupt normal cell signaling and cause uncontrolled systemic inflammations (Kim, Johnson, Shin, & Sharma, 2004). In order to investigate the effect of COS on the production of NO after LPS stimulation, RAW 264.7 cells were treated with LPS alone or together with COS (Fig. 6C). The production of NO was increased by LPS and recovered by additional administration of COS, still in a dose-dependent manner.

4. Discussion

Our previous studies have shown that COS had pro-apoptotic effect on neutrophils from glycogen-induced peritonitis mice model (Dou et al., 2009). Besides, we found that COS possess anti-angiogenic activity (Wu et al., 2008), which might relate to anti-inflammatory effect of COS on different kinds of cells. In the present study, we demonstrated that COS were able to attenuate LPS-induced inflammation response in cells by suppressant LPS binding to TLR4/MD-2 receptor complex. Furthermore, we examined the regulative effect of COS on activation of ERK, JNK, p38 MAPK and NF-κB signaling, which are well known to be involved in the regulation of LPS-induced inflammatory cytokine productions (Emre et al., 2007; Guha & Mackman, 2001). As shown in our experiments, the rapid phosphorylation of ERK, JNK and p38 MAPK induced by LPS in RAW 264.7 cells were significantly reduced by COS treatment. The similar influence of COS shown in nuclear translocation of NF-κB.

Several reports have described natural and synthetic inhibitors of LPS-induced inflammatory responses (Gamal-Eldeen, Amer, Helmy, Talaat, & Ragab, 2007; Liu et al., 2005; Tsao et al., 2005). Such inhibitors could roughly be divided into two categories: receptor antagonists and LPS-neutralizing molecules. The former includes receptors monoclonal antibodies (Adachi et al., 1999) and cationic peptides (Nagaoka et al., 2001), while the latter includes PMB and synthetic peptides (Hwang et al., 2007; Lin et al., 2008). Chitosan, a polysaccharide larger than COS, has been reported to interact with LPS and produce a stable complex due to polycation and hydrophobic substituents (Davidova, Naberezhnykh, Yermak, Gorbach, & Solov'eva, 2006; Davydova et al., 2008; Yermak et al., 2006). But this interaction is much more weaker than PMB, and needs more than 18 h to form the complex (Yermak et al., 2006), and water soluble derivatives of chitosan with hydrophobic substituent and low substitution extent of amino group have better activity to binding endotoxin (Naberezhnykh, Gorbach, Likhatskaya, Davidova, & Solov'eva, 2008). In our study, through *Tachypleus* amebocyte lysate assay, we found COS, the cationic oligosaccharides derivative from chitosan, failed to neutralize biological activity of LPS. It is possibly due to less molecular weight, better soluble and less acyl substituents. Then, we found that COS interfered with LPS binding to TLR4/MD-2 receptor complex, however, this suppression was not completely blocked. As we know, chitosan is polydisperse with a mixture of chitosan oligomers of different molecular weights. Several previous studies demonstrated the binding of chitosan to immune cells via a variety of cell surface receptors including macrophage mannose receptor (MR) (Han, Zhao, Yu, Feng, & Yu, 2005; Hsu, Tsai, Lee, & Huang, 2004), natural killer (NK) cells lectin receptors NKR-P1A (Bezouska et al., 1997; Rohlenova, Ledvina, Saman, & Bezouska, 2005), whether COS or which particular component most effectively binding to TLR4/MD-2 receptor complex is interesting and needs further investigation.

In addition to LPS binding, excessively generated ROS is another possible regulatory mediator in inflammatory response. After exposition to LPS, cells quickly generate ROS, which can be effectively eliminated by the combined action of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and other endogenous antioxidant proteins (Nagai, Noguchi, Takeda, & Ichijo, 2007). Once the

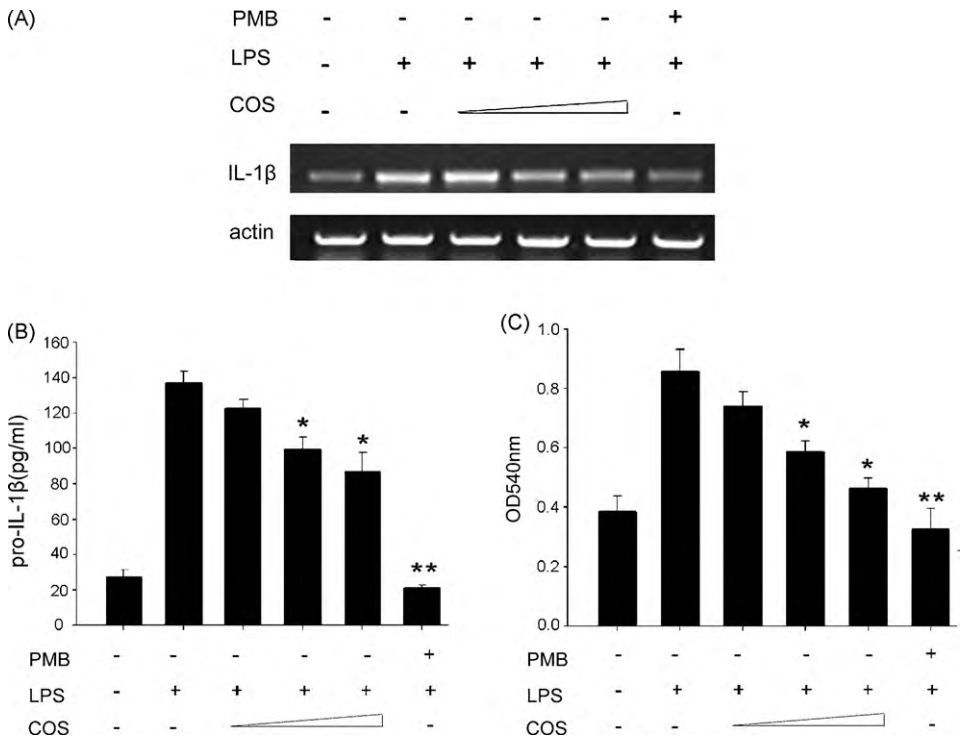

Fig. 6. Effect of COS on LPS-induced production of IL-1β and NO in RAW 264.7 cells. (A) Cells were treated with LPS (100 ng/ml) alone or together with COS (0.1, 0.2 and 0.4 mM) for 6 h, total RNA was extracted and subjected to RT-PCR. (B) The intracellular pro-IL-1β after LPS-stimulated for 4 h was detected by ELISA assay, LPS containing PMB (100 ng/ml) as a positive control. (C) NO in incubation supernatant was determined by Griess reagent. The results were presented as mean ± S.E.M. of data from three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$.

generation of ROS exceeds the capacity of the antioxidant proteins, cells would suffer from "oxidative stress", which is known to be a potential cause of many diseases such as neurodegenerative disorders and cardiovascular diseases (Nagai et al., 2007). Coincidentally, Our previous studies have shown that COS can effectively protect cells against oxidative stress, and restored the activities of endogenous SOD and GSH-Px in hydrogen peroxide-induced stress injury (Liu et al., 2009), which might also be involved in the control of inflammatory reaction.

Many studies have shown that COS and its derivatives have potent antibacterial activity *in vitro* (Fernandes et al., 2008). Our research shows that COS is a potential effective inhibitor of LPS. However, future studies should focus on how to improve the inhibition effect of COS or derivatives on LPS binding to TLR4/MD-2 receptor complex. Our results suggest that COS attenuated LPS-induced signals and inflammatory mediator production of macrophages providing a therapeutic strategy for concurrent multiorgan septic damage.

In conclusion, our findings clearly showed that COS can be potential inhibitive effector of LPS, decreasing LPS-induced production of inflammatory reaction and signaling through significantly inhibiting the binding of LPS to TLR4/MD-2 receptor complex.

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