Bioorganic & Medicinal Chemistry Letters 19 (2009) 6655–6658

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Factors affecting anti-inflammatory effect of chitooligosaccharides in lipopolysaccharides-induced RAW264.7 macrophage cells

Sang-Hoon Lee [a], Mahinda Senevirathne [b], Chang-Bum Ahn [b], Se-Kwon Kim [a], Jae-Young Je [b,*]

[a] Department of Chemistry, Pukyong National University, Busan 606-737, Republic of Korea
[b] Division of Food Science and Aqualife Medicine, Chonnam National University, Yeosu 550-749, Republic of Korea

ARTICLE INFO

Article history:
Received 23 July 2009
Revised 15 September 2009
Accepted 2 October 2009
Available online 7 October 2009

Keywords:
Chitooligosaccharides
Lipopolysaccharide
Cyclooxygenase-2
Inducible nitric oxide synthase
Cytokines

ABSTRACT

In this study, factors affecting anti-inflammatory effect of chitooligosaccharides (COSs) in lipopolysaccharides (LPS)-induced RAW264.7 macrophage cells were investigated. The inhibition of NO secretion by COSs revealed that 90-COSs (90% N-deacetylation) significantly inhibited NO secretion than those of 50-COSs (50% N-deacetylation), and 90-HMWCOS (5000–10,000 Da) in the 90-COSs showed the highest inhibition activity. Furthermore, 90-HMWCOSs also found to inhibit LPS-stimulated production of PGE_2, TNF-α and IL-6, as well as the expression of iNOS, COX-2, TNF-α, and IL-6. These results suggested that 90-HMWCOS may have anti-inflammatory effect via down-regulation of transcriptional and translational expression levels of TNF-α, IL-6 and iNOS and COX-2.

Macrophages play pivotal roles in inflammation and host defense. One of the most significant mechanisms is the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS). Increased NO mediates many physiological functions, such as nonspecific host defense, antimicrobial defence, and antitumor activities, as well as pathological processes which include the pathogenesis of septic shock and organ destruction in some inflammatory and autoimmune diseases.[1] NO is generated via the oxidation of terminal guanidine nitrogen of L-arginine by NOS, and is involved in inflammation and carcinogenesis.[2] Thus, the inhibition of NO overproduction by blocking iNOS expression may be a useful strategy for the treatment of various inflammatory disorders.

The conversion of arachidonic acid to prostaglandin H_2 which is a precursor of a series of important biological mediators called prostanoids including prostaglandin (PG) E_2, prostacyclin, and thromboxane A_2 is catalyzed by cyclooxygenase (COX).[3] Three isoforms of COX are currently known: COX-1, COX-2, and COX-3. COX-3 is a splice variant of COX-1which retains intron one and has frameshift mutation, hence some referred it as COX-1b or COX-1 variant.[4] Different tissues express varying levels of COX-1 and COX-2. Even though, both enzymes act primarily in the same fashion, COX-1 catalyzes the synthesis of prostaglandins for normal physiological functions and found in most mammalian cells.[5] On other hand, COX-2 is not detectable in most normal tissues. In the presence of variety of proinflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and lipopolysaccharide (LPS), COX-2 catalyze the synthesis of high amount of PGE_2 and implicated in the pathogenesis of sepsis and inflammation.[6,7]

Chitosan, the N-deacetylated derivative of chitin, is an important functional biomaterial because of its biodegradability, biocompatibility, non-toxicity, and adsorption properties. It has been reported that chitosan could mediate cell differentiation, proliferation and cytokine production.[8,9] Chitooligosaccahrides (COSs) are derivative of chitosan and it can be obtained by either enzymatic or chemical hydrolysis of chitosan. COSs are regarded as physiologically bioactive substances since they possess versatile biological activities such as antitumor,[10] immuno-stimulating,[11] antioxidant[12] and antimicrobial characteristics.[13] In this study, as part of our ongoing investigation on COSs biological activities, we prepared COSs with different degree of deacetylation (DD) and molecular weight (MW) in order to carry out an evaluation of their factors affecting anti-inflammatory effects in LPS-induced RAW264.7 macrophage cells.

Two kinds of COSs (90-COSs and 50-COSs) were prepared from 90% and 50% deacetylated chitosan as described in our previous report, and further fractionated into three kinds of COSs using an ultrafiltration membrane system.[14] COSs were designated based on their molecular weights as high molecular weight COSs (5000–10,000 Da: 90-HMWCOSs and 50-HMWCOSs), medium molecular weight COSs (1000–5000 Da: 90-MMWCOSs and 50-MMWCOSs) and low molecular weight COSs (below 1000 Da: 90-LMWCOSs and 50-LMWCOSs).

Prior to evaluation anti-inflammatory effects of COSs, non-toxic concentrations of COSs were selected following cytotoxicity testing

* Corresponding author. Tel.: +82 61 659 3416; fax: +82 61 659 3419.
E-mail address: jjy1915@chonnam.ac.kr (J.-Y. Je).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.10.007

to study further the signaling pathways of their activities. RAW264.7 macrophage cells treated with COSs were stimulated with LPS to assess the effect of COSs on the production of NO secretion, and then highly effective COSs further study the effect on the production of proinflammatory mediators such as TNF-α, IL-6 and PGE_2 mainly involved in chronic inflammation and transcriptional mRNA levels of TNF-α and IL-6. Finally, to identify the inhibitory effect of COSs on iNOS and COX-2, which are responsible for LPS-induced NO and PGE_2 production, LPS-treated RAW264.7 macrophage cells were assessed for their protein levels, using appropriate antibody, in Western blotting. All experiments were performed using the standard manufacturer's protocols.

The overproduction of NO can be harmful and may cause various inflammatory and autoimmune diseases.[15,16] Thus, pharmacological interferences in the reduction of the NO production cascade reveal an interesting strategy for therapeutic intervention in inflammation disorders. Incubation of RAW264.7 macrophage cells with LPS alone for 24 h caused a significant increased in NO production. However, cells pretreated with 90- and 50-COSs at different concentrations showed a reduction in the production of NO after induction with LPS (Fig. 1A and B). 90-COSs showed higher inhibitory activities than those of 50-COSs against NO production. Among 90-COSs, 90-HMWCOSs significantly (p <0.05) inhibited the NO secretion in dose-dependent way. These results suggest that the inhibition of NO secretion in LPS-induced RAW264.7 macrophage cells by COSs was dependent on DD and MW of COSs, that is, high DD value (90%) and MW (5000–10,000 Da) are major factors affecting the inhibition of NO secretion in our tested samples.

Considering the results of NO secretion, 90-HMWCOS was tested further study on the signaling pathway. In the same way, incubation of RAW264.7 macrophage cells with LPS alone for 24 h caused a significant increased in PGE_2 production which is an indication of COX-2 activation, and it was a threefold increment compared with that of the untreated RAW264.7 cells (Fig. 2). However, this increase was markedly inhibited by the different concentrations of 90-HMWCOSs in dose-dependent manner. Kim et al.[17] have shown that the prevention of LPS-induced PGE_2 production is related with the block of NF-κB translocation and activation. Overproduction of PGE_2 derived from COX-2 is an important pathophysiological factor contributing inflammation. Hence, increased productions of PGE_2 and COX-2 expression contribute to immune dysfunction.[18] However, treatment with selective COX-2 inhibitor or PGE_2 receptor antagonist exhibited a beneficial effect, and improved survival in a murine model of burn infection or trauma, suggesting that modulation of COX-2 or PGE_2 pathway may play an important role in the pathogenesis of burn injury and inflammation.[19]

Proinflammatory cytokines are formed mostly by activated macrophage cells, and are involved in the up-regulation of inflammatory reactions. TNF-α is proinflammatory cytokine, and is known to be a key mediator for the induction of apoptosis but TNF-α has disadvantageous effect such as inducing tissue injury at high concentration.[20] IL-6 is multifunctional cytokine which is upregulated by numerous signals such as bacterial LPS and viruses, and high serum IL-6 levels are observed in many pathological conditions such as inflammation, trauma and autoimmune diseases.[20,21] To investigate the effects of COSs on cytokines production, RAW264.7 macrophage cells were treated with LPS only or LPS with different concentrations of 90-HMWCOSs. The LPS-induced production of TNF-α and IL-6 were dose-dependently

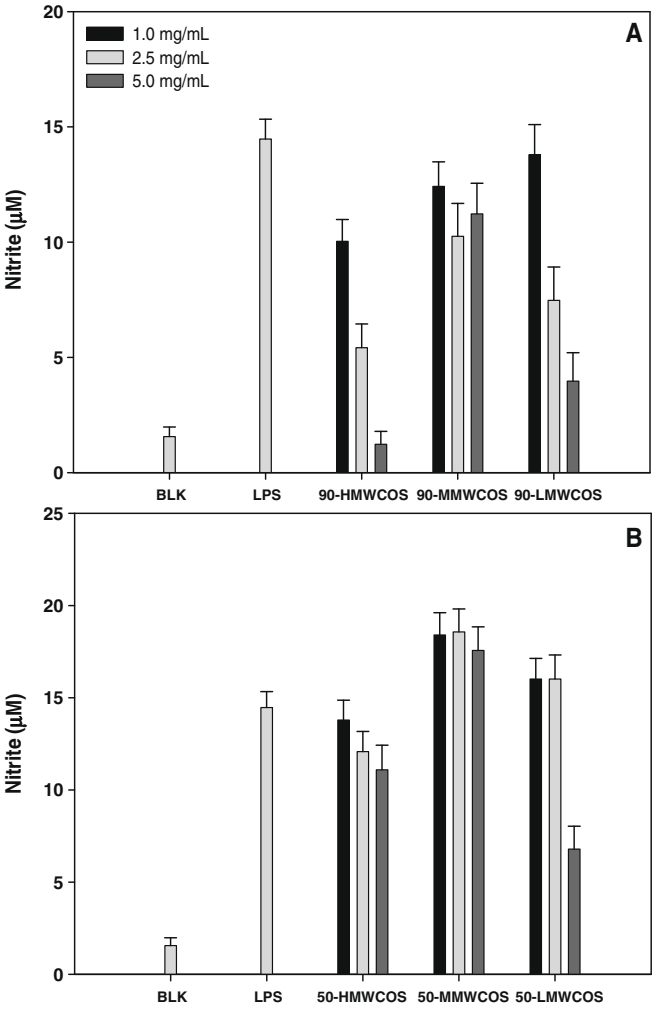

Figure 1. Effects of COSs on LPS-stimulated NO secretion in RAW264.7 macrophage cells. Data are the mean ± SD of triplicates. Cells were treated with 1 μg/mL LPS alone or with various concentrations of COSs for 24 h. The concentration of NO in macrophage cells were determined by Griess reagent at 550 nm. A: 90-COSs; B: 50-COSs.

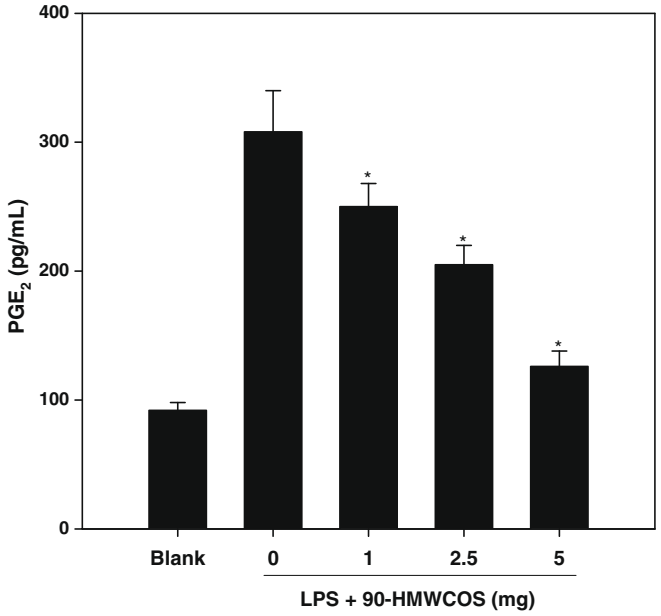

Figure 2. Effect of 90-HMWCOSs on LPS-stimulated PGE_2 production in RAW264.7 macrophage cells. Data are the mean ± SD of triplicates. Cells were treated with 1 μg/mL LPS alone or with different concentrations of 90-HMWCOSs for 24 h. The amount of PGE_2 was measured by the mouse PGE_2 enzyme-linked immunosorbent assay (ELISA) kit. Statistical comparison, $^*p$ <0.05.

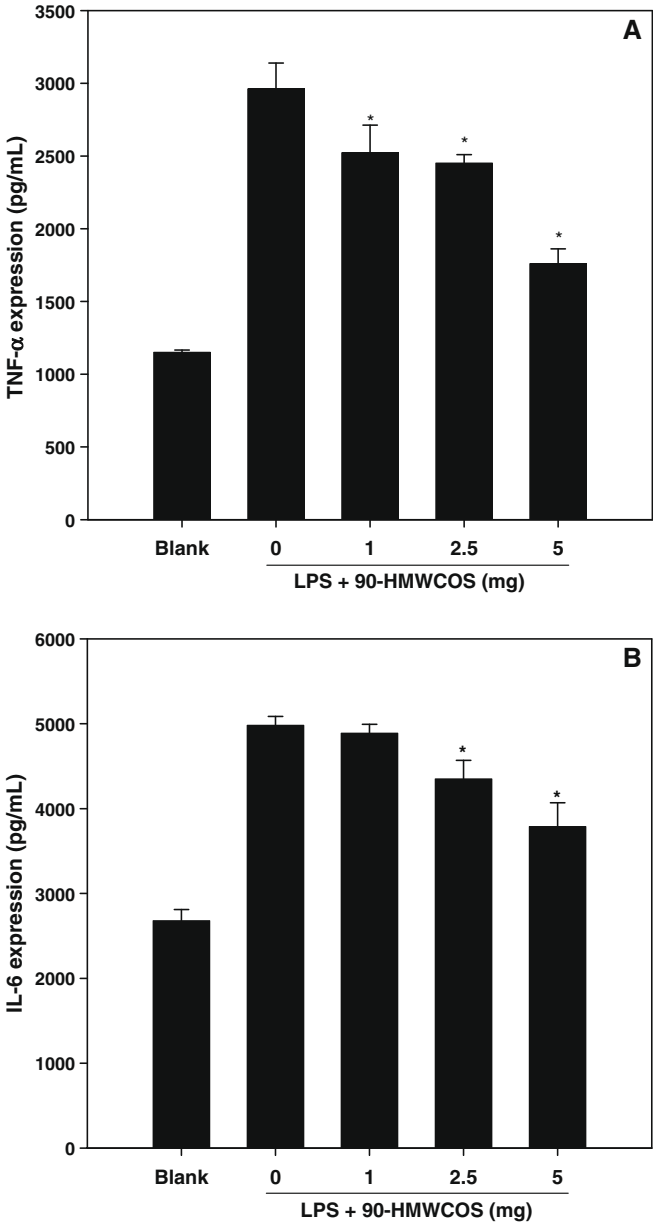

Figure 3. Effect of 90-HMWCOSs on LPS-stimulated TNF-α (A) and IL-6 (B) production in RAW264.7 macrophage cells. Data are the mean ± SD of triplicates. Cells were treated with 1 μg/mL LPS alone or with different concentrations of 90-HMWCOSs for 24 h. Following incubation, amounts of proteins produced were determined by their specific antibody-coated ELISA kit. Statistical comparison, $^{*}p < 0.05$.

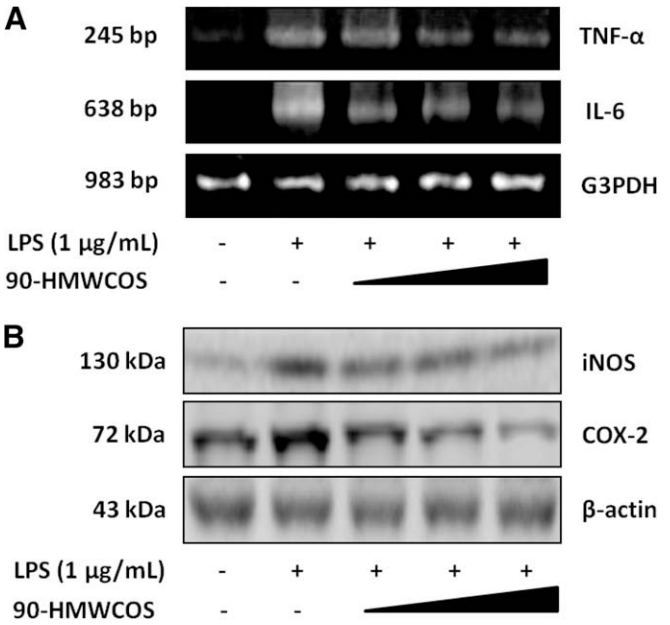

Figure 4. RT-PCR analysis for TNF-α and IL-6 mRNA expression (A), and Western blot analysis of iNOS and COX-2 protein expression (B) in RAW264.7 macrophage cells treated with different 90-HMWCOSs. Cells were treated with 1 μg/mL LPS alone or with various concentrations of 90-HMWCOSs for 24 h. Total RNA was collected and specific TNF-α and IL-6 primers were used to polymerize the reverse transcribed cDNA, which was visualized by ethidium bromide staining followed by electrophoretic separation. Respective G3PDH mRNA expression levels were used to confirm the equal amount of RNA used for cDNA synthesis. Equal amounts of protein in the cell lysates were electrophoresed and the levels of iNOS and COX-2 were determined using specific antibodies for iNOS and COX-2. Respective protein levels of actic were used to confirm the equal amount of protein used for electrophoresis.

recovered by the COSs (Fig. 3A and B). To identify the gene expression of TNF-α and IL-6, the TNF-α and IL-6 mRNA level were studied with reverse transcription-polymerase chain reaction (RT-PCR). As shown in Figure 4A, RT-PCR with mRNA of TNF-α and IL-6 showed that the amount of TNF-α and IL-6 secretion in the incubation media recovered with the concentration of 90-HMWCOSs. Hence, these results indicated that the 90-HMWCOSs markedly reduced the production of proinflammatory cytokines such as TNF-α and IL-6 in LPS-mediated RAW264.7 macrophage cells through transcriptional mechanism.

To confirm the inhibitory effects of 90-HMWCOSs on COX-2 and iNOS, LPS-treated RAW264.7 macrophage cells were assessed for their protein levels by Western blotting (Fig. 4B). The expression of COX-2 protein was very small in control cells, but the treatment of LPS caused a significant increase of COX-2. However, the protein expression of COX-2 could be reduced dose-dependently with the pretreatment of different concentrations of 90-HMWCOSs compared to that of LPS-treated RAW 264.7 macrophage cells revealing that the activity was attenuated through the translational mechanism. A similar result was observed by Western blot analysis performed to determine whether the inhibition of NO production resulted from reduced iNOS protein expression. Figure 4B showed the dose-dependent inhibition in iNOS protein expression revealing that the increased concentrations of 90-HMWCOSs decreased the expression of iNOS. NO is an important regulatory and effector molecule with variety of biological functions, and the production of NO is initiated by NOS, which convert L-arginine to L-citruline.[22,23] Over expression of iNOS is generally accompanied by inflammatory disorders, in which various inflammatory cytokines such as IL-6 and TNF-α are responsible for many acute and chronic responses to inflammatory diseases.[24]

In conclusion, we have shown that 90-HMWCOCs inhibit NO, PGE_2, TNF-α, and IL-6 production, and iNOS and COX-2 expression in RAW264.7 macrophage cells mediated by LPS. Also, data revealed that the effects shown in this study depends on molecular weight and degree of deacetylation. Understanding the mode of action of 90-HMWCOSs in mediating proinflammatory cytokines in immune cells such as macrophage will be important for the development of therapeutic potential compounds which can be used in chronic inflammatory disease.

Acknowledgment

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2008-331-F00070).

References and notes

1. Petros, A.; Bennett, D.; Vallance, P. *Lancet* **1991**, *338*, 1557.
2. Nathan, C. *FASEB J.* **1992**, *6*, 3051.
3. Smith, W. L.; Garavito, R. M.; Dewitt, D. L. *J. Biol. Chem.* **1996**, *271*, 33157.
4. Chandrasekharan, N. V.; Dai, H.; Roos, K. L.; Evanson, N. K.; Tomsik, J.; Elton, T. S.; Simmons, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13926.
5. O'Neill, G.; Ford-Hutchinson, A. W. *FEBS* **1993**, *334*, 156.
6. Hammond, R. A.; Hannon, R.; Frean, S. P.; Armstrong, S. J.; Flower, R. J.; Bryant, C. E. *Am. J. Vet. Res.* **1999**, *60*, 426.
7. Futaki, N.; Tajahashi, S.; Katagawa, T.; Yamakawa, Y.; Tanaka, M.; Higuchi, S. *Inflamm. Res.* **1997**, *46*, 496.
8. Mori, T.; Okumura, M.; Matsuura, M.; Ueno, K.; Tokura, S.; Okamoto, Y.; Minami, S.; Fujinaga, T. *Biomaterials* **1997**, *18*, 947.
9. Pae, H. O.; Seo, W. G.; Kim, N. Y.; Oh, G. S.; Kim, G. E.; Kim, Y. H.; Kwak, H. J.; Yun, Y. G.; Jun, C. D.; Chung, H. T. *Leukocyte Res.* **2001**, *25*, 339.
10. Jeon, Y.-J.; Kim, S.-K. *J. Microbiol. Biotechnol.* **2002**, *12*, 503.
11. Tokoro, A.; Tatewaki, N.; Suzuki, K.; Mikami, T.; Suzuki, S.; Suzuki, M. *Chem. Pharm. Bull.* **1988**, *36*, 784.
12. Je, J.-Y.; Park, P.-J.; Kim, S.-K. *Food Chem. Toxicol.* **2004**, *42*, 381.
13. Jeon, Y.-J.; Park, P.-J.; Kim, S.-K. *Carbohydr. Polym.* **2001**, *44*, 71.
14. Park, P. J.; Je, J. Y.; Jung, W. K.; Ahn, C. B.; Kim, S. K. *Eur. Food Res. Tech.* **2004**, *219*, 529.
15. Southan, G. J.; Szabo, C. *Biochem. Pharmacol.* **1996**, *51*, 383.
16. Guzik, T. J.; Korbut, R.; Adamek-Guzik, T. *J. Physiol. Pharmacol.* **2003**, *54*, 469.
17. Kim, Y. A.; Kim, G. Y.; Park, K. R.; Choi, Y. H. *J. Med. Food* **2007**, *10*, 218.
18. Mackrell, P. J.; Daly, J. M.; Mestre, J. R.; Stapleton, P. P.; Howe, L. R.; Subbaramaiah, K. *Surgery* **2001**, *130*, 826.
19. Shoup, M.; He, L. K.; Liu, H.; Shankar, R.; Gamelli, R. *J. Trauma* **1998**, *45*, 215.
20. Yoon, H. J.; Moon, M. E.; Park, H. S.; Im, S. Y.; Kim, Y. H. *Biochem. Biophys. Res. Commun.* **2007**, *358*, 954.
21. Van Snick, J. *Ann. Rev. Immunol.* **1990**, *8*, 253.
22. Seyidova, D.; Aliyev, A.; Rzayev, N.; Obrenovich, M.; Lamb, B. T.; Smith, M. A.; de la Torre, J. C.; Perry, G.; Aliev, G. *Intl. J. Exp. Clin. Pathophysol. Drug Res.* **2004**, *18*, 325.
23. MacMicking, J.; Xie, Q. W.; Nathan, C. *Ann. Rev. Immunol.* **1997**, *15*, 323.
24. Blancke, F.; Claeys, M. J.; Jorena, P.; Vermeiren, G.; Bosmans, J.; Wuyts, F. L.; Vrints, C. J. *Mediators Inflamm.* **2005**, *14*, 385.