

# Chitooligosaccharides in combination with interferon- $\gamma$ increase nitric oxide production via nuclear factor- $\kappa$ B activation in murine RAW264.7 macrophages

Guan James Wu, Guo Jane Tsai \*

Department of Food Science, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 202, Taiwan

Received 13 March 2006; accepted 25 July 2006

## Abstract

A low-molecular weight chitosan (LMWC) with a molecular mass of 20 kDa and a chitooligosaccharide mixture (oligomixture) which is composed of sugars with a degree of polymerization (DP) of 1–6 were isolated from the chitosan hydrolysate. The effects of the chitosan hydrolysate, LMWC and oligomixture on the production of nitric oxide (NO) in RAW 264.7 macrophages were evaluated, and their effects on NF- $\kappa$ B activation and the gene expression of inducible NO synthase (iNOS) were further investigated. None of the tested 3 samples of hydrolysate, LMWC and oligomixture alone affected the NO production in RAW 264.7 macrophages. However, treatment of macrophages with a combination of hydrolysate/oligomixture and interferon- $\gamma$  (IFN- $\gamma$ ) significantly induced NO production in a dose-dependent manner, whereas a combination of LMWC and IFN- $\gamma$  inhibited NO production. These effects on NO synthesis were evidenced via regulating the iNOS gene expression. Both hydrolysate and oligomixture promoted the migration of NF- $\kappa$ B into the nucleus and enhanced its DNA binding activity. MG132, a specific inhibitor of NF- $\kappa$ B, eliminated the NO synthesis in IFN- $\gamma$  plus hydrolysate/oligomixture-induced RAW264.7 macrophages. The treatment of RAW264.7 macrophages with anti-CD14, anti-TLR4, and anti-CR3 antibodies significantly blocked NO production induced by IFN- $\gamma$  plus hydrolysate/oligomixture. These results demonstrated that the oligomixture, which is the main functional component in the chitosan hydrolysate, in combination with IFN- $\gamma$ , synergistically induced NF- $\kappa$ B activation and NO production through binding with the receptors of CD14, TLR4 and CR3 in RAW264.7 macrophages. © 2006 Elsevier Ltd. All rights reserved.

**Keywords:** Chitooligosaccharides; Chitosan hydrolysate; Inducible NO synthase; Low-molecular weight chitosan; NF- $\kappa$ B; Nitric oxide; RAW264.7 macrophages

## 1. Introduction

Nitric oxide can mediate many biological function, including vascular homeostatis, neurotransmission and host defense (Moncada et al., 1991; Nathan, 1992). NO is synthesized by a family of NO synthase (NOS) enzymes (Nathan, 1992). There are two types of NOS. One type (cNOS) is constitutively present in some cells including neurons and endothelial cells (Nathan, 1992), and the second type (iNOS) is present in various cell types including macrophages (Xie et al., 1992). The expression of iNOS in activated macrophages is responsible for the profound production of NO that function of killing tumor cells

*Abbreviations:* AP-1, activating protein-1; cNOS, constitutive NO synthase; CR3, complement receptor type 3; DP, degree of polymerization; EMSA, electrophoretic mobility shift assay; GM-CSF, granulocyte macrophage colony stimulating factor; IFN- $\gamma$ , interferon- $\gamma$ ; IL-1, interleukin-1; IL-6, interleukin-6; iNOS, inducible NO synthase; IRF-1, interferon regulatory factor-1; LMWC, low-molecular-weight chitosan; L-NMMA, *N*<sup>G</sup>-monomethyl-L-arginine acetate salt; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinases; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NO, nitric oxide; STAT-1 $\alpha$ , signal transducer and activator of transcription-1 $\alpha$ ; TLR4, toll-like receptor type 3; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

\* Corresponding author. Tel.: +886 2 24622192x5150; fax: +886 2 24624113.

E-mail address: [B0090@mail.ntou.edu.tw](mailto:B0090@mail.ntou.edu.tw) (G.J. Tsai).

and pathogens (Green et al., 1991; Karupiah et al., 1993). However, excessive NO production has been implicated in many pathophysiological conditions including inflammation, and atherosclerosis (Kolios et al., 2004; Naseem, 2005). Thus, the effect on NO production by some immune-enhancers of food origin, such as chitosan and chitoooligomers that have been shown potential as immunotherapeutic agents (Tokoro et al., 1989; Kobayashi et al., 1990; Ueno et al., 1999) should not be neglected.

The expression of iNOS in macrophages is induced in response to many cytokines and bacterial lipopolysaccharides (LPS) through activation of the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) (May and Ghosh, 1998; Kleiner et al., 2003). In quiescent cells, NF- $\kappa$ B is located in the cytosol as a dimer of protein components known as Rel family members (e.g. p50, p65), which is bound to an inhibitor I $\kappa$ B to prevent its nuclear localization. Upon activation, I $\kappa$ B is phosphorylated and released from the complex, and the Rel proteins migrate into the nucleus and bind to specific consensus DNA elements on the promoter of the target genes to initiate the production of various mediators (Karin and Ben-Neriah, 2000).

Chitosan, a partially deacetylated chitin (poly- $\beta$ -(1  $\rightarrow$  4) *N*-acetyl-D-glucosamine) has been used as one of the constituent in many healthy foods (or dietary supplements) due to its various biological activities, including hypocholesterolemic activity (Sugano et al., 1988; Lehoux and Grondin, 1993), antitumor activity (Seo et al., 2000), and immune-enhancing effects (Petillo et al., 1994). Chitosan has been reported to stimulate macrophages to produce the IL-1, IL-6, TNF- $\alpha$ , nitric oxide (NO), and granulocyte macrophage colony stimulating factor (GM-CSF) (Nishimura et al., 1987; Petillo et al., 1994; Seo et al., 2000; Yu et al., 2004). These functions were initiated by the binding of chitosan to the specific receptors on macrophages (Otterlei et al., 1994; Shibata et al., 1997; Feng et al., 2004). Both the chemical structure and the molecular size of chitosan might affect this binding efficacy.

Although chitosan has strong biological functions, its high molecular weight and high viscosity may limit its use in vivo. The water-soluble chitoooligomers – in particular, the haxmers of *N*-acetyl chitohexaose and chitohexaose – have recently attracted much attention for their immune-enhancing effects (Suzuki et al., 1986; Mori et al., 1998). In our previous study a water-soluble chitosan hydrolysate with immunoactivity was obtained by cellulase degradation of chitosan (Wu and Tsai, 2004). A low-molecular-weight chitosan (LMWC) and a chitoooligosaccharide mixture isolated from this chitosan hydrolysate were shown to have different stimulatory effects on the cell proliferation and IgM secretion of the human hybridoma HB4C5 cells (Wu and Tsai, 2004). In this study we found that the oligomixture in combination with IFN- $\gamma$  enhanced NO production and iNOS expression in murine macrophages RAW 264.7. The surface binding with cell receptors, and the involvement of NF- $\kappa$ B activation by this oligomixture were also demonstrated.

## 2. Materials and methods

### 2.1. Reagents

Mouse recombinant interferon- $\gamma$  (IFN- $\gamma$ ), sulfanilamide, *N*-1-naphthylethylenediamine, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), leupeptin, bovine serum albumin (BSA), dithiothreitol (DTT), Hepes, aprotinin, *N*<sup>G</sup>-monomethyl-L-arginine acetate salt (L-NMMA), Z-Leu-Leu-al (GM132), octylphenyl-polyethylene glycol (Igepal CA-630), polymyxin B, *N*-acetyl glucosamine, and glucosamine were purchased from Sigma Chemical Company (St. Louis, MO, USA). *N*-acetyl chitohexaose and chitohexaose were purchased from Seikagaku Corporation (Chuo-Ku, Tokyo, Japan). Rabbit anti-NF- $\kappa$ B p65 (Rel A) IgG and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG were purchased from Rockland (Gilbertsville, PA, USA). Rabbit anti-iNOS IgG, goat anti-CD14, goat anti-CR3 (integrin  $\alpha$ M), goat anti-TLR4, NF- $\kappa$ B binding oligonucleotide, and salmon sperm DNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RPMI 1640 medium and phenol red-free RPMI1640 medium were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Fetal calf serum (FCS) was obtained from PAA Laboratories (Linz, Austria).

### 2.2. Preparation of chitosan

Chitin was isolated from shrimp (*Solenocera prominentis*) shells (Tsai and Su, 1999). The chitin powder was deacetylated with 50% NaOH (1 g of chitin per 13 mL of NaOH) and heated at 140 °C in an oil bath for 1 h to obtain chitosan with a 95% degree of deacetylation (DD 95), as measured by a colloid titration method (Tōei and Kohora, 1976).

### 2.3. Production of chitosan hydrolysate and component separation

According to the protocol of Wu and Tsai (2004), in a 5-L-fermentor (CMCF-5, Chin Chi Co., Taipei, ROC) containing 2000 mL acetic acid-bicarbonate buffer (pH 5.2) 100 g DD95 were added, followed by the addition of cellulase (10 U/mL). After digestion at 55 °C with 125 rpm for 9 h the hydrolysate was neutralized and centrifuged (12,000 $\times$ g, 30 min). The supernatant (designated as chitosan hydrolysate in this study) was added with an equal volume of methanol and fractionated into two fractions: the upper aliquot chitoooligosaccharide mixture and a low-molecular-weight chitosan (LMWC) in the precipitate (Wu and Tsai, 2004). Chitosan hydrolysate, LMWC, and the chitoooligosaccharide mixture were lyophilized and examined as endotoxin-free by using the Pyrotell<sup>®</sup> Limulus Amebocyte Lysate kit (Associates of Cape Cod Inc., E. Falmouth, MA, USA).

### 2.4. Cell cultures

RAW264.7, a murine macrophage cell line, was obtained from the Bioresource Collection and Research Center (BCRC 60001), Taiwan. Cells were cultured in RPMI1640 supplemented with 10% FCS at 37 °C in an atmosphere of 5% CO<sub>2</sub>. The phenol red-free RPMI 1640 medium was used instead of RPMI1640 medium when the NO content in the cell culture was analyzed.

### 2.5. Nitrite assay

RAW264.7 macrophages ( $5 \times 10^5$  cells/mL) were cultured in 96-well plates with 200  $\mu$ L of medium (phenol red-free RPMI 1640 medium supplemented with 10% FCS) containing samples and IFN- $\gamma$  (10 ng/mL) at 37 °C for 24 h. The nitrite content in the cell cultures was measured by the Griess reaction (Ding et al., 1988). In this assay, 50  $\mu$ L of supernatant from each culture was incubated with an equal volume of Griess reagent (60 mM sulfanilamide in 3 N HCl and 4 mM *N*-1-naphthylethylenediamine in H<sub>2</sub>O) at room temperature for 10 min. The absorbance at 540 nm was determined by an automated plate reader ( $\mu$ Quant<sup>™</sup>, BIO-TEK

Instrument, Inc., Winooski, VT). Nitrite concentrations were determined by using a standard curve derived from known concentrations of NaNO<sub>2</sub> prepared in phenol red-free RPMI 1640 medium.

## 2.6. Western blotting (Park et al., 2003)

RAW264.7 macrophages ( $2 \times 10^6$  cells) were incubated with IFN- $\gamma$  alone, or IFN- $\gamma$  plus the tested samples for 12 h, and then, the cells were collected, disrupted in a cold lysis buffer [10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 10  $\mu$ g/mL leupeptin] for 20 min and then centrifuged (10,000 $\times$ g at 4 °C for 20 min). The protein concentration in the supernatant was determined with the detergent compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as the standard. Cellular proteins (20  $\mu$ g in total) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose (NC) membrane using a Labconco semi-dry blotter (Labconco, Kansas, MO, USA). The membrane was then blocked with 5% BSA in TBST buffer (10 mM Tris, 75 mM NaCl, 1 mM EDTA, pH 7.4, containing 0.1% Tween 20) overnight at 4 °C, then rinsed and incubated with rabbit anti murine iNOS or NF- $\kappa$ B p65 antibodies (1:500 in TBST) for 1 h at room temperature. After washing with TBST buffer three times, the blot was incubated with peroxidase conjugated goat anti rabbit-IgG (1:3000 in TBSB) for 1 h, and the antibody-specific proteins were visualized by the enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

## 2.7. Preparation of cytoplasmic and nuclear proteins (Chen and Wang, 1999)

RAW264.7 macrophages ( $5 \times 10^6$  cells) were incubated with IFN- $\gamma$  alone, or IFN- $\gamma$  plus the tested samples for 1 h, and then, the cells were collected, added with 400  $\mu$ L of an ice-cold homogenizing buffer (10 mM Hepes-KOH containing 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF, pH 7.9), and left on ice for 10 min. After adding 0.2  $\mu$ L of Igepal CA-630 and followed by vortexing, the cell lysate was centrifuged at 250 $\times$ g for 10 min at 4 °C. The supernatant was collected for cytoplasmic proteins and stored at -80 °C. The pellet was re-suspended in 100  $\mu$ L of an ice-cold suspension buffer (40 mM Hepes-KOH containing 0.4 M KCl, 1 mM DTT, 10% glycerol, 0.1 mM PMSF and 0.1% aprotinin, pH 7.9), left on ice for 45 min, vortexed, and centrifuged at 15,000 $\times$ g for 20 min at 4 °C. The aliquots of the supernatant containing nuclear proteins were stored at -80 °C.

## 2.8. Oligonucleotide labelling

The DNA sequence of the double-stranded consensus oligonucleotide specific for NF- $\kappa$ B binding was 5'-AGT TGA GGG GAC TTT CCA GGC-3'. Based on the protocol described by Chen and Wang (1999), the oligonucleotide was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Biosciences, Piscataway, NJ, USA) using T4 polynucleotide kinase (Promega, Madison, WI, USA) to a specific activity of  $>5 \times 10^7$  cpm/ $\mu$ g DNA. The radioactivity was determined in a Liquid Scintillation Analyzer (TRI-CARB 2900TR, Packard Instrument, Meriden, CT, USA).

## 2.9. Electrophoretic mobility shift assay (EMSA) (Chen and Wang, 1999)

Nuclear protein (5  $\mu$ g) was incubated for 20 min at 30 °C with 2  $\mu$ g salmon sperm DNA, 20 ng of labeled oligonucleotides and 14.5  $\mu$ L binding buffer (20 mM Hepes-KOH containing 0.2 mM EDTA, 100 mM KCl, 2 mM MgCl<sub>2</sub>, 20% glycerol, 0.2 mM DTT, and 0.5 mM PMSF, pH 7.9) in a final volume of 20  $\mu$ L. Protein-DNA complexes were separated from the free DNA probes by electrophoresis through 5% native polyacrylamide gels containing 0.5 $\times$  TBE (Amresco Inc., Solon, Ohio, USA). The gel was resolved at 4 °C for 2 h at 200 V, dried under vacuum on a Whatman 3MM paper (Schleicher and Schuell, Dassel, Germany), and

visualized with autoradiography using a Kodak Bio-Max X-ray film (Kodak, Rochester, NY).

## 2.10. Statistical analysis

The results are presented as the mean  $\pm$  SD. Data were analyzed by the statistical analysis system (SAS) program. Statistical comparisons were made using the Student's *t*-test. Differences of  $p < 0.05$  were considered statistically significant.

# 3. Results

## 3.1. Effects of the chitosanalytic products on NO production and iNOS expression

A low-molecular-weight chitosan (LMWC) with a molecular mass of 20.0 kDa and a chitooligosaccharide mixture (designated as oligomixture) containing sugars with a degree of polymerization (DP) of 1–6 were separated from the chitosan hydrolysate. The weight percentages of the chitooligosaccharides with DP 1–6 in oligomixture were: 36.8%, 9.5%, 2.7%, 2.1%, 16.3% and 32.6%, respectively (Wu and Tsai, 2004). All samples of the chitosan hydrolysate, LMWC and the oligomixture have been demonstrated *in vivo* to increase the levels of serum IgG and IgM antibodies and induce the proliferation of mouse lymphocyte (Wu and Tsai, 2004). These lyophilized chitosanalytic products (chitosan hydrolysate, LMWC and oligomixture) were used in this study to investigate their effects on the induction of NO production and iNOS expression in RAW 264.7 macrophages.

LPS, known as endotoxin, has strong activity on immune response and the endotoxin contamination in samples will totally revert the results. Therefore, the Pyrotell<sup>®</sup> Limulus Amebocyte Lysate kit with a detection limit of 0.25 EU/mL (1 EU approximately equal to 100 pg of LPS) was used to detect the presence of endotoxin in all tested samples in this study including chitosan hydrolysate, LMWC, oligomixture, and chitohexaose, *N*-acetyl-chito-hexaose, glucosamine, and *N*-acetyl-glucosamine. When the tested samples at concentration of 100  $\mu$ g/mL were added to the Amebocyte Lysate reagent, negative reaction (reagent still being solution-type, no any gel formed) was observed for all tested samples. In addition, the 3 samples of chitosan hydrolysate, LMWC and oligomixture alone did not induce NO production in RAW 264.7 macrophages (data not shown). All these results confirm that the 3 chitosanalytic products used in this study are endotoxin-free. When the hydrolysate or oligomixture in combination with IFN- $\gamma$  (10 ng/mL) synergistically increased the production of NO. The dose-dependent effects of hydrolysate and the oligomixture, in the presence of IFN- $\gamma$ , on NO production are shown in Fig. 1A. At 100  $\mu$ g/mL, the hydrolysate and oligomixture increased the NO production of RAW264.7 macrophages by 1.9 and 3.2 times, respectively. On the other hand, LMWC inhibited NO production. LPS, used as the positive control, strongly stimulated NO production in RAW264.7 macrophages, as shown in the upper-left

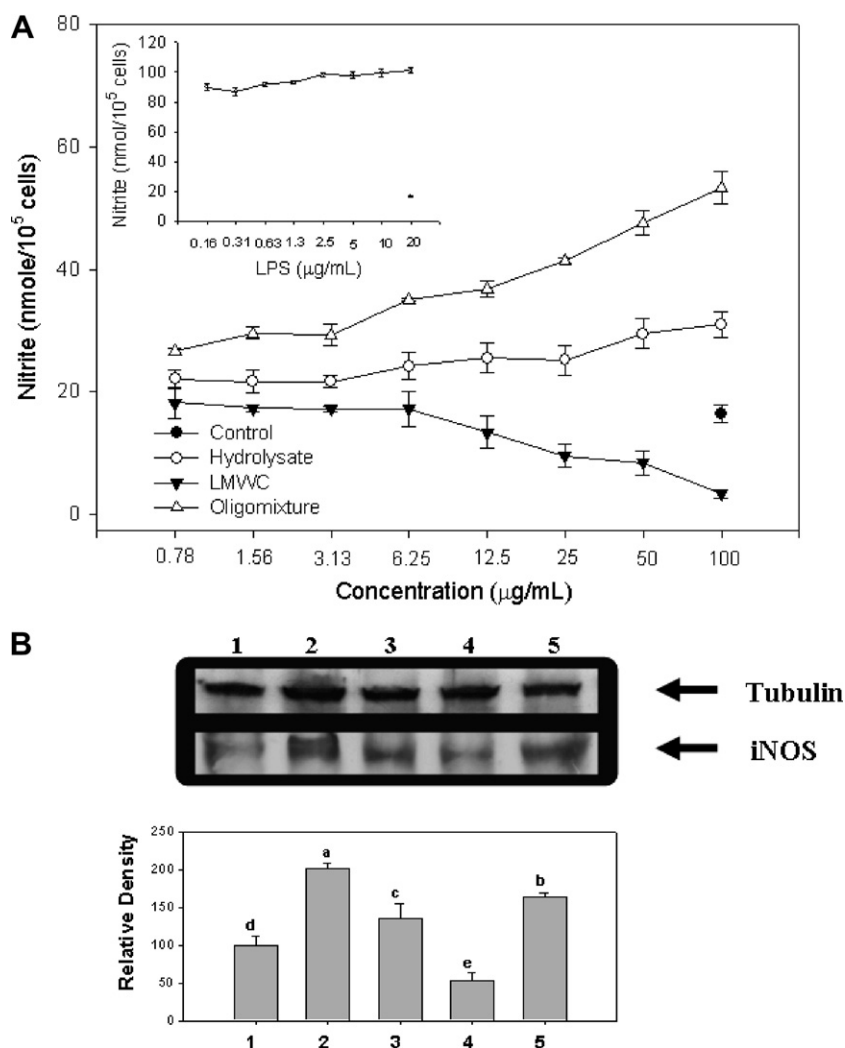


Fig. 1. (A) Dose-dependent effect of chitosan hydrolysate, low-molecular-weight chitosan (LMWVC), chitooligosaccharide mixture (oligomixture), and LPS on nitrite production in the IFN- $\gamma$ -stimulated RAW264.7 macrophages ( $5 \times 10^5$  cells/mL). Nitrite was measured by the Griess reagent method after incubation with the tested samples at 37 °C for 24 h. The nitrite concentrations were calculated by comparing with OD<sub>540</sub> of standard solutions of NaNO<sub>2</sub> prepared in culture medium. The data are the means  $\pm$  SD of tetraplicate samples. (B) The effect of chitosanalytic products on the expression of iNOS in IFN- $\gamma$ -stimulated RAW264.7 macrophages. Bar graph represent the densitometry of blot, and error bars show means  $\pm$  SD of triplicate samples ( $p < 0.05$ ). The relative density was normalized by the ratio of iNOS to tubulin. The RAW264.7 macrophages were incubated with the tested samples at 37 °C for 12 h, and the total cellular protein was then collected. The LPS concentration was 1  $\mu$ g/mL, and the sample concentration of chitosanalytic products was 100  $\mu$ g/mL. The expression of iNOS was determined by Western blotting as described in Section 2. Line 1, IFN- $\gamma$ ; Line 2, IFN- $\gamma$  + LPS; Line 3, IFN- $\gamma$  + chitosan hydrolysate; Line 4, IFN- $\gamma$  + LMWVC; Line 5, IFN- $\gamma$  + oligomixture.

corner in Fig. 1A. Fig. 1B shows the Western blots, illustrating the expression of the iNOS in the lysates of RAW 264.7 macrophages treated with IFN- $\gamma$  alone, or in combination with chitosanalytic samples or LPS. RAW 264.7 macrophages moderately expressed iNOS after 12 h incubation with IFN- $\gamma$  alone. However, IFN- $\gamma$  plus hydrolysate/oligomixture-treated RAW 264.7 macrophages significantly increased the expression of iNOS protein; especially the oligomixture-treated cells showed a higher level of iNOS than the hydrolysate-treated cells. On the other hand, LMWVC reverted the expression of iNOS in RAW264.7 macrophages (Fig. 1B). As expected, the NO production was greatly decreased when L-NMMA, the

NO synthase inhibitor, was added to RAW 264.7 macrophages incubated with IFN- $\gamma$  alone, IFN- $\gamma$  plus LPS, or IFN- $\gamma$  plus chitosanalytic samples (Fig. 2).

Since the oligomixture sample contained sugars with DP 1–6, the effects of the presence of the acetyl group and the DP size of the sugars on the enhancing NO production of RAW 264.7 macrophages were further investigated. As shown in Fig. 3, neither glucosamine nor *N*-acetyl glucosamine showed a stimulating effect on NO production in the IFN- $\gamma$ -treated RAW264.7 macrophages, whereas both *N*-acetyl chitohexaose and chitohexaose upregulated NO production. However, the enhancing effects of *N*-acetyl chitohexaose and chitohexaose on NO production were

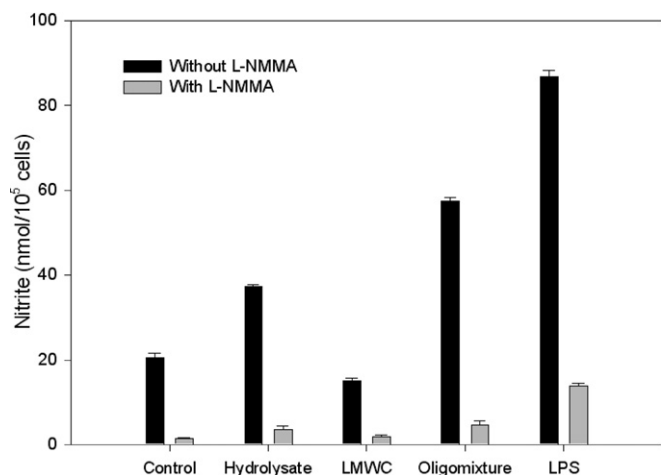


Fig. 2. The effect of the L-arginine analogues (L-NMMA) on the nitrite production in IFN- $\gamma$ -stimulated RAW264.7 macrophages ( $5 \times 10^5$  cells/mL). The RAW264.7 macrophages ( $5 \times 10^5$  cells/mL) were incubated with IFN- $\gamma$  alone (control) or IFN- $\gamma$  plus tested sample at 37 °C for 24 h in the presence ( $\square$ ) or absence ( $\blacksquare$ ) of 2 mM *N*<sup>G</sup>-monomethyl-L-arginine acetate salt (L-NMMA), and assayed for nitrite production. The LPS concentration was 1  $\mu$ g/mL, and the sample concentration of chitosanalytic products was 100  $\mu$ g/mL. The nitrite concentrations were calculated by comparing with OD<sub>540</sub> of standard solutions of NaNO<sub>2</sub> prepared in culture medium. The data are the means  $\pm$  SD of the tetraplicate samples.

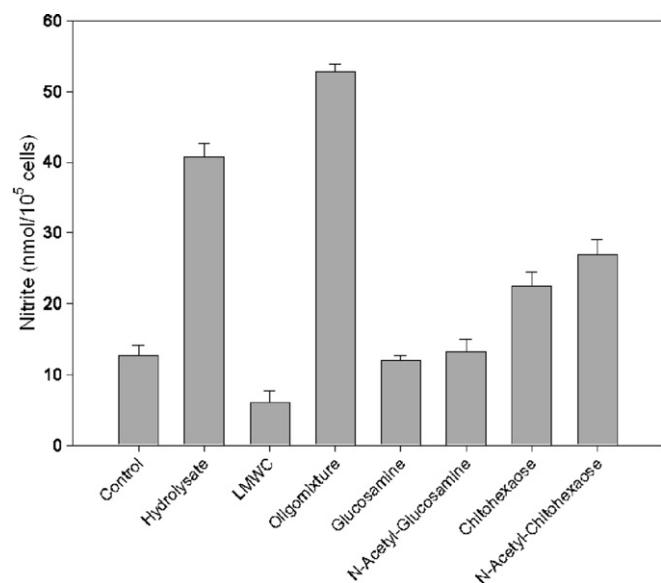


Fig. 3. Nitrite production in RAW 264.7 macrophages incubated with IFN- $\gamma$  alone (control) or IFN- $\gamma$  plus tested samples. The tested samples include chitosan hydrolysate, low-molecular-weight chitosan (LMWC), chitoooligosaccharide mixture (oligomixture), *N*-acetyl chitohexaose, chitohexaose, glucosamine, *N*-acetyl glucosamine, and LPS. The nitrite was measured by the Griess reagent method after incubation of the cells with the tested samples at 37 °C for 24 h. The LPS concentration was 1  $\mu$ g/mL, and the sample concentration of chitosanalytic products or sugars was 100  $\mu$ g/mL. The nitrite concentrations were calculated by comparison with OD<sub>540</sub> of standard solutions of NaNO<sub>2</sub> prepared in culture medium. The data are the means  $\pm$  SD of tetraplicate samples.

lower than those of the hydrolysate and oligomixture samples.

### 3.2. Inhibition of chitosanalytic sample-induced NO production by NF- $\kappa$ B inhibitors

It is known that iNOS expression is regulated by various transcription factors, especially by NF- $\kappa$ B (May and Ghosh, 1998; Kleinert et al., 2003). In order to know whether NF- $\kappa$ B is involved in IFN- $\gamma$  plus chitosanalytic sample-induced NO production in RAW264.7 macrophages, MG132, the NF- $\kappa$ B inhibitor was added into RAW264.7 macrophages incubated with alone, or in combination with LPS or chitosanalytic samples for 24 h. As shown in Fig. 4, little NO was detected when MG132 was added into RAW264.7 macrophages in each treatment, suggesting that the NF- $\kappa$ B plays a positive regulative role in the production of NO in RAW264.7 macrophages treated with IFN- $\gamma$  plus various chitosanalytic samples.

### 3.3. Effects of chitosanalytic products on iNOS synthesis through NF- $\kappa$ B activation

Inactive NF- $\kappa$ B in the cytosol is composed of protein dimers (p50, p65) and its inhibitor I $\kappa$ B. Upon activation the NF- $\kappa$ B in the cytosol releases its I $\kappa$ B, and the residual proteins (e.g. p50, p65) of NF- $\kappa$ B migrate into the nucleus to bind with target genes (Karin and Ben-Neriah, 2000). In order to determine if the chitosanalytic products can activate NF- $\kappa$ B to enhance iNOS production, the cytoplasmic proteins and nuclear proteins were isolated from the RAW264.7 macrophages treated with samples for 1 h, separated by SDS-PAGE, and analyzed by Western blot analysis. The data in Fig. 5A demonstrates that in the presence of IFN- $\gamma$ , LPS, hydrolysate, or oligomixture significantly

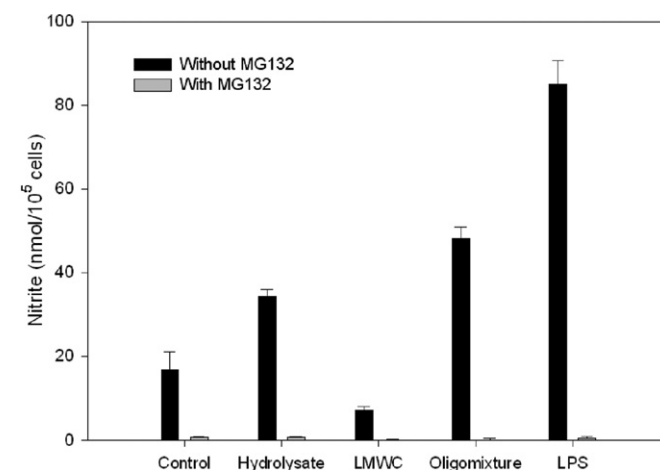


Fig. 4. Effect of a NF- $\kappa$ B inhibitor (MG132) on the nitrite production in IFN- $\gamma$ -stimulated RAW264.7 macrophages. The RAW264.7 macrophages ( $5 \times 10^5$  cells/mL) were incubated with IFN- $\gamma$  alone (control) or IFN- $\gamma$  plus tested sample at 37 °C for 24 h in the presence ( $\square$ ) or absence ( $\blacksquare$ ) of 10  $\mu$ M of MG132, and assayed for nitrite production. The LPS concentration was 1  $\mu$ g/mL, and the sample concentration of the chitosanalytic products was 100  $\mu$ g/mL. The nitrite concentrations were calculated by comparison with OD<sub>540</sub> of standard solutions of NaNO<sub>2</sub> prepared in culture medium. The data are the means  $\pm$  SD of tetraplicate samples.

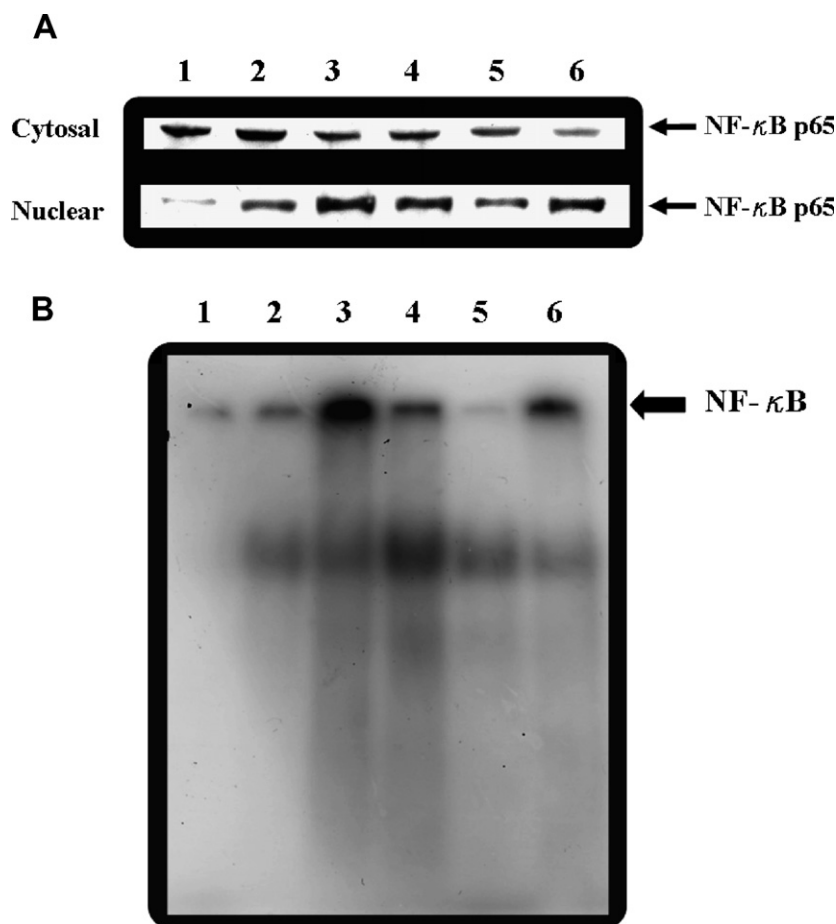


Fig. 5. The effect of chitosanolytic products on the activation of NF- $\kappa$ B in RAW264.7 macrophages. The RAW264.7 macrophages were incubated alone (negative control), or with IFN- $\gamma$ , or IFN- $\gamma$  plus the tested sample at 37 °C for 1 h, and their cytoplasmic and nuclear proteins were collected separately. The LPS concentration was 1  $\mu$ g/mL, and the sample concentration of chitosanolytic products was 100  $\mu$ g/mL. (A) The cytoplasmic and nuclear proteins were prepared and analyzed for NF- $\kappa$ B p65 level by Western blotting as described in Section 2. (B) Nuclear proteins were analyzed for NF- $\kappa$ B binding activity by electrophoretic mobility shift assay (EMSA) as described in Section 2. Line 1, negative control; Line 2, IFN- $\gamma$ ; Line 3, IFN- $\gamma$  + LPS; Line 4, IFN- $\gamma$  + chitosan hydrolysate; Line 5, IFN- $\gamma$  + LMWC; Line 6, IFN- $\gamma$  + oligomixture.

increases the amounts of NF- $\kappa$ B p65 protein in the nuclear extracts of RAW264.7 macrophages; while LMWC attenuates the nuclear p65 protein level. To further confirm that the hydrolysate- or oligomixture-activated NF- $\kappa$ B, like LPS-activated NF- $\kappa$ B, can actually bind with DNA, EMSA was carried out with nuclear protein extract of RAW 264.7 macrophages incubated with IFN- $\gamma$  alone, or IFN- $\gamma$  plus the tested samples for 1 h. As shown in Fig. 5B, in the presence of IFN- $\gamma$ , LPS strongly enhanced the DNA-binding activity of NF- $\kappa$ B. Both hydrolysate and oligomixture also significantly enhanced NF- $\kappa$ B binding activity, whereas LMWC significantly attenuated the IFN- $\gamma$ -induced activation of NF- $\kappa$ B in RAW264.7 macrophages.

#### 3.4. Effects of polymyxin B on chitosanolytic product- or LPS-induced NO production

Polymyxin B has been known to inhibit macrophage activation by LPS through binding of lipid A in LPS

(Morrison and Jacobs, 1976). In order to understand whether both oligomixture and LPS are sharing similar structures and accordingly, they may pass through similar pathways to regulate NO production in RAW264.7 macrophages, the macrophages were pretreated with polymyxin B (10<sup>3</sup> U/mL) for 1 h and then stimulated with IFN- $\gamma$  alone or IFN- $\gamma$  plus samples for 24 h. The data in Fig. 6 shows that polymyxin B does not revert the stimulation effect of IFN- $\gamma$  on NO production in RAW264.7 macrophages. However, polymyxin B significantly decreases the NO production by 59.4%, 60.1% and 86%, respectively, in RAW264.7 macrophages incubated with IFN- $\gamma$  plus hydrolysate, IFN- $\gamma$  plus oligomixture, or IFN- $\gamma$  plus LPS.

#### 3.5. Effect of anti-CD14, anti-TLR4, and anti-CR3 antibody treatments on chitosanolytic product-induced NO production

CD-14 and TLR4 are the macrophage receptors for LPS (Triantafyllou and Triantafyllou, 2002), and the complement

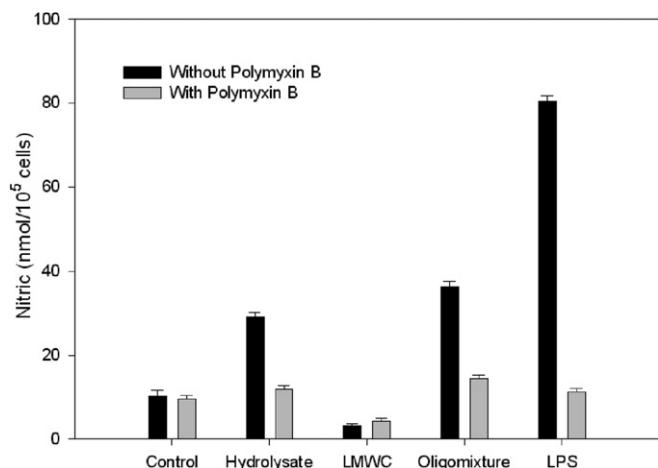


Fig. 6. The effect of polymyxin B on nitrite production induced by IFN- $\gamma$  alone or IFN- $\gamma$  plus tested sample in RAW264.7 macrophages. RAW264.7 macrophages ( $5 \times 10^5$  cells/mL) were incubated at 37 °C for 1 h in the presence ( $\square$ ) or absence ( $\blacksquare$ ) of  $10^3$  U polymyxin B. The macrophages were then stimulated with IFN- $\gamma$  alone (control) or IFN- $\gamma$  plus tested sample samples, incubated for 24 h, and assayed for nitrite production. The LPS concentration was 1  $\mu$ g/mL, and the sample concentration of chitosanolytic products was 100  $\mu$ g/mL. The nitrite concentrations were calculated by compared with OD<sub>540</sub> of standard solutions of NaNO<sub>2</sub> prepared in culture medium. The data are the means  $\pm$  SD of tetraplicate samples.

receptor CR3 is identified as the leukocyte membrane receptor for *N*-acetyl glucosamine and  $\beta$ -glucans (Thorton et al., 1996). To further investigate whether the oligomix-

ture binds similar receptors in RAW 267.4 macrophages such as LPS to regulate NO production, we performed antagonistic studies in IFN- $\gamma$  plus tested sample-induced NO production by using anti-CD14, anti-TLR4, and anti-CR3 antibodies. The treatment of RAW264.7 macrophages with anti-CD14, anti-TLR4, and anti-CR3 antibodies significantly decreased IFN- $\gamma$  plus oligomixture-induced NO production (Fig. 7D). Similarly, anti-CD14, anti-TLR4, and anti-CR3 antibodies also significantly decreased the NO production induced by LPS (Fig. 7A). Both anti-CD14 and anti-TLR antibodies did not affect the NO level of RAW 264.7 macrophages incubated with IFN- $\gamma$  plus LMWC. However, anti-CR3 antibody could revert the inhibition effect on NO production by LMWC, which resulted in higher NO synthesis in RAW 267.4 macrophages (Fig. 7C). Both anti-CD14 and anti-TLR4 antibodies significantly decreased IFN- $\gamma$  plus hydrolysate-induced NO production (Fig. 7B). This suggests that oligomixture induces NO production in IFN- $\gamma$ -stimulated RAW264.7 macrophages through binding with the receptors of CD14, TLR4, and CR3, which is similar with LPS. LMWC may bind with CR3 receptor to inhibit the NO production in RAW 264.7 macrophages.

#### 4. Discussion

Some contrary results were found regarding the effect of chitosan on the NO synthesis in macrophages (Jeong et al.,

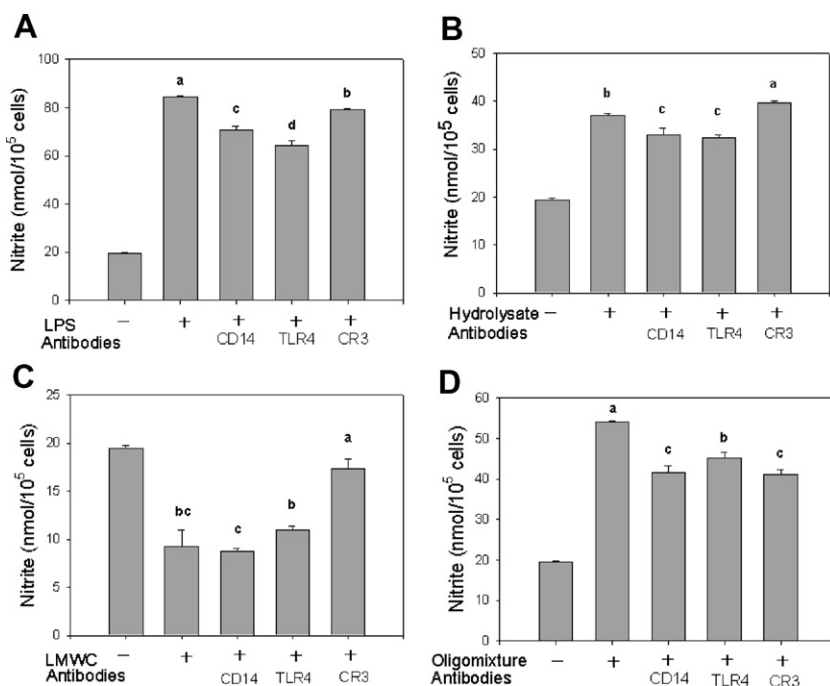


Fig. 7. The effects of anti-CD14, anti-TLR4 and anti-CR3 antibodies treatments on the nitrite production induced by IFN- $\gamma$  alone or IFN- $\gamma$  plus tested sample in RAW264.7 macrophages. RAW264.7 macrophages ( $5 \times 10^5$  cells/mL) were incubated at 37 °C for 1 h in the presence or absence of 5  $\mu$ g/mL antibodies. The macrophages were then stimulated with 10 ng/mL IFN- $\gamma$  plus (A) 1  $\mu$ g/mL LPS, (B) 100  $\mu$ g/mL hydrolysate, (C) 100  $\mu$ g/mL LMWC, and (D) 100  $\mu$ g/mL oligomixture, incubated for 24 h, and assayed for nitrite production. The nitrite concentrations were calculated by comparison with OD<sub>540</sub> of standard solutions of NaNO<sub>2</sub> prepared in culture medium. The data are the means  $\pm$  SD of tetraplicate samples. Values with different superscripts are significantly different at  $p < 0.05$ .

2000; Hwang et al., 2000). Since LPS strongly induces NO production in macrophages (Schmidt et al., 1992) and a tiny amount of LPS contamination in samples may totally revert the results, all samples used in this study, including the hexamer standards of chitohexaose and *N*-acetyl chitohexaose, the chitosan hydrolysate and its two components of LMWC and a chitooligosaccharide mixture (oligomixture) were examined as endotoxin-free before use. The oligomixture in the hydrolysate was proven to significantly enhance the NO production in IFN- $\gamma$ -primed RAW264.7 macrophages, whereas LMWC inhibited the NO production (Fig. 1A). This is similar to some reports that also mentioned that chitosan oligomers in combination with IFN- $\gamma$  synergistically enhance the NO production of macrophages (Seo et al., 2000; Yu et al., 2004). In this study we further found that the size of chitooligosaccharide significantly affects its stimulating effect on NO production; it was demonstrated that both *N*-acetyl chitohexaose and chitohexaose significantly enhanced NO production, whereas *N*-acetyl glucosamine and glucosamine did not enhance NO production (Fig. 3).

NO is a highly reactive substance produced originally from the L-arginine-NO pathway catalyzed by a family of NO synthase (NOS). The inducible NOS (iNOS) has been described in macrophages and can be induced by many cytokines and bacterial products (Schmidt et al., 1992; Otterlei et al., 1994), among which LPS is a strong inducer of iNOS (Schmidt et al., 1992). Therefore, LPS was used in this study as the positive control. Using a typical Western blotting assay for the cellular iNOS content in macrophages (Fig. 1B) and through inhibition of NO production by the iNOS inhibitor, L-NMMA (Fig. 2), the oligomixture in this study was proven to induce the iNOS synthesis, and accordingly it enhances NO production.

The expression of iNOS is regulated by various transcription factors, including NF- $\kappa$ B, IRF-1, STAT-1 $\alpha$ , and AP-1 etc (Kleinert et al., 2003). The NF- $\kappa$ B had been shown to be the crucial regulator in the NO production in macrophages incubated with IFN- $\gamma$  plus chitosan (Jeong et al., 2000). In this study we found that the oligomixture-induced NO production was almost totally inhibited by MG-132, an inhibitor of NF- $\kappa$ B (Grisham et al., 1999) (Fig. 4). The co-treatment of IFN- $\gamma$  plus the oligomixture caused a marked activation of NF- $\kappa$ B, which can migrate into the nucleus (Fig. 5A) and strongly bind with the DNA segment (Fig. 5B). All of these results demonstrated that in the presence of IFN- $\gamma$ , NF- $\kappa$ B in RAW 264.7 macrophages is positively regulated by the oligomixture for iNOS expression; and as such the NO production is increased.

It is well known that LPS can bind to the surface receptors of CD14 (Wright et al., 1990), or TLR4 (Qureshi et al., 1999; Hoshino et al., 1999) in macrophages, activate the signal transduction system such as mitogen-activated protein kinases (MAPKs) pathway (Yang et al., 2000; Luo et al., 2003), and finally, that I $\kappa$ B is released from NF- $\kappa$ B, resulting in the activation of the NF- $\kappa$ B and iNOS

expression (Chen and Wang, 1999). The CR3 is the receptor for *N*-acetyl glucosamine and  $\beta$ -glucan (Thorton et al., 1996). Muzzarilli (1997) mentioned that the structure of chitosan is similar with the saccharide portion of lipid A in LPS, thus it can activate macrophages. Polymyxin B is an inhibitor of LPS by specific binding to lipid A. The fact that polymyxin B significantly reduces the IFN- $\gamma$  plus oligomixture induced NO production in RAW264.7 macrophages (Fig. 6) shows that the oligomixture, sharing similar monomers of *N*-acetyl glucosamine and glucosamine as chitosan, may have a similar structure as the lipid A in LPS and accordingly, it may similarly bind to the surface receptors of CD14 or TLR4 to activate the signal transduction system. By assessing the inhibition of anti-CD14, anti-TLR4 and anti-CR3 antibodies on NO production, the oligomixture was proven to bind with the receptors of CD14, TLR4 and CR3 in RAW 264.7 macrophages to activate the signal transduction cascade. Similarly in this study, LPS was also shown to bind with 3 receptors to initiate the activation of signal transduction (Fig. 7). Although LMWC shares the same structures of monomer as glucosamine and *N*-acetyl glucosamine as oligomixture, its bigger size may cause that it can only bind with the CR3 receptor (Fig. 7). Due to binding with different receptors on RAW 264.7 macrophages the oligomixture and LMWC have different effects on the signal transduction proteins in MAPKs (authors not published data). Finally, these two samples showed the opposite effects on the iNOS expression and NO production (Fig. 1).

In summary, this study demonstrated that in the presence of IFN- $\gamma$ , the oligomixture isolated from chitosan hydrolysate significantly stimulates RAW264.7 macrophages to secrete NO through the induction of iNOS expression. Based on our finding, the most likely mechanism that can account for this biological effect for the oligomixture involves binding of the oligomixture with the CD14, TLR4 and CR3 receptors, and then activating the NF- $\kappa$ B transcription factor in the RAW 264.7 macrophages.

## Acknowledgements

The financial supports from the National Science Council of Taiwan, ROC (NSC 93-2313-B-019-003, NSC 93-2120-M-019-001) and from the Center for Marine Bioscience and Biotechnology, NTOU (95529001D8) are gratefully acknowledged.

## References

- Chen, C.C., Wang, J.K., 1999. p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 cells. *Mol. Pharmacol.* 55, 481–488.
- Ding, A.H., Nathan, C.F., Stuehr, D.J., 1988. Release of reactive nitrogen intermediated and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141, 2407–2412.

- Feng, J., Zhao, L., Yu, Q., 2004. Receptor-mediated stimulatory effect of oligochitosan in macrophages. *Biochem. Biophys. Res. Commun.* 317, 414–420.
- Green, S.J., Nacy, C.A., Meltzer, M.S., 1991. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to *Leishmania* and other intracellular pathogens. *J. Leukocyte Biol.* 50, 93–103.
- Grisham, M.B., Palombella, V.J., Elliott, P.J., Conner, E.M., Brand, S., Wong, H.L., Pien, C., 1999. Inhibition of NF- $\kappa$ B activation in vitro and in vivo: role of 26S proteasome. *Meth. Enzymol.* 300, 345–363.
- Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., Akira, S., 1999. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol.* 162, 3749–3752.
- Hwang, S.M., Chen, C.Y., Chen, S.S., Chen, J.C., 2000. Chitinous materials inhibit nitric oxide production by activated RAW 264.7 macrophages. *Biochem. Biophys. Res. Commun.* 271, 229–233.
- Jeong, H.J., Koo, H.N., Oh, E.Y., Chae, H.J., Kim, H.R., Suh, S.B., Kim, C.H., Cho, K.H., Park, B.R., Park, S.T., Lee, Y.M., Kim, H.M., 2000. Nitric oxide production by high molecular weight water-soluble chitosan via nuclear factor- $\kappa$ B activation. *Int. J. Immunopharmacol.* 22, 923–933.
- Karin, M., Ben-Neriah, Y., 2000. Phosphorylation meets ubiquitination: the control of NF- $\kappa$ B activity. *Annu. Rev. Immunol.* 18, 621–663.
- Karupiah, G., Xie, Q.W., Buller, R.M.L., Nathan, C., Duarte, C., MacMicking, J.D., 1993. Inhibition of viral replication by interferon- $\gamma$ -induced nitric oxide synthase. *Science* 261, 1445–1448.
- Kleinert, H., Schwarz, P.M., Förstermann, U., 2003. Regulation of the expression of inducible nitric oxide synthase. *Biol. Chem.* 384, 1314–1364.
- Kobayashi, M., Watanabe, T., Suzuki, S., Suzuki, M., 1990. Effect of *N*-acetylchitohexaose against *Candida albicans* infection of tumor-bearing mice. *Microbiol. Immunol.* 34, 413–426.
- Kolios, G., Valatas, V., Ward, S.G., 2004. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* 113, 427–437.
- Lehoux, J.G., Grondin, F., 1993. Some effects of chitosan on liver function in the rat. *Endocrinology* 132, 1078–1084.
- Luo, S.F., Wang, C.C., Chien, C.S., Hsiao, L.D., Yang, C.M., 2003. Induction of cyclooxygenase-2 by lipopolysaccharide in canine tracheal smooth muscle cells: involvement of p42/p44 and p38 mitogen-activated protein kinases and nuclear factor-kappa B pathways. *Cell. Signal.* 15, 497–509.
- May, M.J., Ghosh, S., 1998. Signal transduction through NF- $\kappa$ B. *Immunol. Today* 19, 80–88.
- Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43, 109–142.
- Mori, T., Irie, Y., Nishimura, S.I., Tokura, S., Matsuura, M., Okumura, M., Kadosawa, T., Fujinaga, T., 1998. Endothelial cell responses to chitin and its derivatives. *J. Biomed. Mater. Res.* 43, 469–472.
- Morrison, D.C., Jacobs, D.M., 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* 13, 813–818.
- Muzzarilli, R.A.A., 1997. Human enzymatic activities related to the therapeutic administration of chitin derivatives. *Cell. Mol. Life Sci.* 53, 131–140.
- Naseem, K.M., 2005. The role of nitric oxide in cardiovascular diseases. *Mol. Aspects Med.* 26, 33–65.
- Nathan, C., 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* 6, 3051–3064.
- Nishimura, K., Nishimura, S., Seo, H., Nishi, N., Tokura, S., Azuma, I., 1987. Effect of multiporous microspheres derived from chitin and partially deacetylated chitin on the activation of mouse peritoneal macrophages. *Vaccine* 5, 136–140.
- Otterlei, M., Varum, K.M., Ryan, L., Espevik, T., 1994. Characterization of binding and TNF- $\alpha$ -inducing ability of chitosans on monocytes: the involvement of CD14. *Vaccine* 12, 825–832.
- Park, D.W., Kim, J.R., Kim, S.Y., Sonn, J.K., Bang, O.S., Kang, S.S., Kim, J.H., Baek, S.H., 2003. Akt as a mediator of secretory phospholipase A2 receptor-involved inducible nitric oxide synthase expression. *J. Immunol.* 170, 2093–2099.
- Petillo, G., Petillo, O., Ranieri, M., Santin, M., Ambrosio, L., Calabro, D., Avallone, B., Balsamo, G., 1994. Chitosan-mediated stimulation of macrophage function. *Biomaterials* 15, 1215–1220.
- Qureshi, S.T., Lariviere, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P., Malo, D., 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med.* 189, 615–625.
- Schmidt, H.H.H.W., Timothy, D., Warner, T.D., Nakane, M., Forstemann, U., Murad, F., 1992. Regulation and subcellular location of nitric oxide synthases in RAW264.7 macrophages. *Mol. Pharmacol.* 41, 615–624.
- Seo, W.G., Pae, H.O., Kim, N.Y., Oh, G.S., Park, I.S., Kim, Y.H., Kim, Y.M., Lee, Y.H., Jun, C.D., Chung, H.T., 2000. Synergistic cooperation between water-soluble chitosan oligomers and interferon-gamma for induction of nitric oxide synthesis and tumoricidal activity in murine peritoneal macrophages. *Cancer Lett.* 159, 189–195.
- Shibata, Y., Metzger, W.J., Myrvik, Q.N., 1997. Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. *J. Immunol.* 159, 2462–2467.
- Sugano, M., Watanabe, S., Kishi, A., Izume, M., Ohtakara, A., 1988. Hypocholesterolemic action of chitosans with different viscosity in rats. *Lipids* 23, 187–191.
- Suzuki, K., Tokoro, A., Okawa, Y., Suzuki, S., Suzuki, M., 1986. Effect of *N*-acetylchito-oligosaccharides on activation of phagocytes. *Microbiol. Immunol.* 30, 777–787.
- Thorton, B.P., Vetvicka, V., Pitman, M., Goldman, R.C., Ross, G.D., 1996. Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). *J. Immunol.* 156, 1235–1246.
- Tōei, K., Kohora, T., 1976. A conductometric method for colloid titrations. *Anal. Chem. Acta* 83, 59–65.
- Tokoro, A., Kobayashi, M., Tatewaki, N., Suzuki, K., Okawa, Y., Mikami, T., Suzuki, S., Suzuki, M., 1989. Protective effect of *N*-acetylchitohexaose on *Listeria monocytogenes* infection in mice. *Microbiol. Immunol.* 3, 357–367.
- Triantafyllou, M., Triantafyllou, K., 2002. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. *Trends Immunol.* 23, 301–304.
- Tsai, G.J., Su, W.H., 1999. Antibacterial activity of shrimp chitosan against *Escherichia coli*. *J. Food Prot.* 62, 239–243.
- Ueno, H., Yamada, H., Tanaka, I., Kaba, N., Matsuura, M., Okumura, M., Kadosawa, T., Fujinaga, T., 1999. Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs. *Biomaterials* 20, 1407–1414.
- Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C., 1990. CD14, a receptor for complexes of lipopolysaccharides (LPS) and LPS binding protein. *Science* 249, 1431–1433.
- Wu, G.J., Tsai, G.J., 2004. Cellulase degradation of shrimp chitosan for the preparation of a water-soluble hydrolysate with immunoactivity. *Fisheries Sci.* 70, 1113–1120.
- Xie, Q.W., Cho, H.J., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Troso, A., Nathan, C., 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 256, 225–228.
- Yang, H., Young, D.W., Gusovsky, F., Chow, J.C., 2000. Cellular events mediated by lipopolysaccharide-stimulated Toll-like receptor 4. *J. Biol. Chem.* 275, 20861–20866.
- Yu, Z., Zhao, L., Ke, H., 2004. Potential role of nuclear factor-kappaB in the induction of nitric oxide and tumor necrosis factor-alpha by oligochitosan in macrophages. *Int. Immunopharmacol.* 4, 193–200.