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High molecular weight water-soluble chitosan acts as an accelerator of macrophages activation by recombinant interferon γ via a process involving L-arginine-dependent nitric oxide production

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Running title: NO production by water-soluble chitosan

SUMMARY

High molecular weight water-insoluble chitosan alone has been previously shown to exhibit *in vitro* stimulatory effect on macrophages nitric oxide (NO) production. However, high molecular weight water-soluble chitosan (WSC) had no effect on NO production by itself. When WSC was used in combination with recombinant interferon-γ (Rifn-γ), there was a marked cooperative induction of NO synthesis in a dose-dependent manner. The optimal effect of WSC on NO synthesis was shown at 24 h after treatment with rIFN-γ. The increased production of NO from rIFN-γ plus WSC-stimulated RAW 264.7 macrophages was decreased by the treatment with N^G monomethyl-L-arginine. The increase in NO synthesis was reflected, as an increased amounts of inducible NO synthase (iNOS) protein. Synergy between rIFN-γ and WSC was mainly dependent on WSC-induced nuclear factor-κB activation. The present results indicate that WSC may provide various activities such as anti-microbial, anti-tumoral, and anti-viral. In addition, since NO has emerged as an important intracellular and intercellular regulatory molecule having functions as diverse as vasodilation, neural communication, cell growth regulation and host defense, it is tempting to hypothesize that this WSC is involved in the local control of the various fundamental processes such as cardiagra, cardiac infarction, impotence etc.

Keywords: Water-soluble chitosan; Macrophages; Nitric oxide; Nuclear factor-κB; Host defense

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INTRODUCTION

Nitric oxide (NO) is a highly reactive molecule produced from a guanidino nitroten of L-arginine in a reaction catalyzed by a family of NO synthase (NOS) enzymes (Nathan, 1992). Authentic NO accounts for many of the biological properties of endothelium-derived relaxing factor, including inhibition of platelet aggregation, relaxation of vascular smooth muscle via the activation of soluble guanylate cyclase

(Nathan, 1992; Moncada, 1991). A second type of NOS family has been described in macrophages. This inducible isoform of NOS (iNOS) is not expressed under physiological conditions, but can be induced by cytokines and/or lipopolysaccharide (LPS) in many types of RAW 264.7 macrophages. The activity of NOS can be inhibited competitively by substrate analogs such as N^G-monomethyl-$_L$-arginine (N^GMMA). For the production of NO in macrophages, interferon-γ (IFN-γ) is required as a priming signal before those RAW 264.7 macrophages can subsequently be triggered by a second signal for example, LPS, phorbol ester, or tumor necrosis factor-α (TNF-α; Nathan, 1992; Jun *et al.*, 1994). Activated macrophages are a major source of cytokine and induction of cytokine gene expression by LPS occurs primarily at the level of transcription and involves the action of several transcription factors, including members of the nuclear factors-κB (NF-κB)/rel, C/EBP, Ets, and AP-1 protein families (Sweet and Hume, 1996). NF-κB/Rel bind to specific consensus DNA element present on the promoter of target genes initiates the transcription of various inflammatory cytokines, adhesion molecules and chemokines (Kuprash *et al.*, 1995; Galien *et al.*, 1996; Collart *et al.*, 1990). At least five different genes belong to the NF–κB family: NFKB1 (p105/50), NFKB2 (p100/p52), Rel A (p65), Rel B, and c-Rel. Most commonly, NF-κB dimers are composed of the Rel A (p65) and NFKB1 (p50) or NFKB2 (p52) subunits (Siebenlist *et al.*, 1994; Read *et al.*, 1994; Whitley *et al.*, 1994; Neish *et al.*, 1992; Hou *et al.*, 1994; Ledebur and Park, 1995). NF-κB normally residues in the cytoplasm, where it's retained by association with IκB protein (α, β, γ) that mask the nuclear localization signal (Baeuerle and Henkel, 1994). NF-κB function is regulated through rapid degradation of its inhibitory molecule IκB. Inflammatory stimuli, such as cytokines, initiate a signaling cascade that can lead to activation of recently identified IκB kinase (Mercurio *et al.*, Zandi *et al.*, 1997; DiDonato *et al.*, Regnier *et al.*, 1997).

Chitosan, an non-acetylated or partially deacetylated chitin (a linear homopolymer of beta (1-4)-linked N-acetylglucosamine) has been proposed as biomaterial because of its apparent satisfactory biocompatibility. The mechanisms by which water-insoluble chitosan (This chitosan was solubilized in 1% acetic acid.) stimulates macrophages are not clear, but Peluso et al have previously reported that chitosan has on in vitro stimulatory effect on NO production (Peluso *et al.*, 1994). An easily water-soluble chitosan have a high reactivity than water insoluble chitosan (Kim *et al.*, 1998).

In the present study, we show that high molecular weight water-soluble chitosan (WSC) synergistically induces the NO production by RAW 264.7 macrophages when the cells are treated by recombinant IFN-γ (rIFN-γ), but WSC alone has no effect on the production of NO. In addition, to investigate the mechanism of WSC-induced NO production, we examined the transcription factor NF-κB activation and the ability of NF-κB inhibitor such as pyrrolidinedithiocarbamate (PDTC) to block WSC-induced effect. PDTC decreased NO production that had been induced by rIFN-γ plus WSC. These findings may explain that WSC influences on NO production via the NF-κB signaling pathway.

MATERIALS AND METHODS

Reagents

Murine rIFN-γ (1×10^6 U/ml) was purchased from Genzyme (Munchen, Germany). RPMI 1640, N-(1-naphtyl)-ethylenediamin edihydrochloride, LPS, sodium nitrite, and PDTC were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antisera to iNOS and NF-κB were obtained from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology, Inc. (CA), respectively. N^GMMA was purchased from Calbiochem (San Diego, CA). All reagents and media for tissue culture experiments were tested for their LPS content with use of a colorimetric Limulus amoebocyte lysate assay (detection limit, 10 pg/ml; Whittaker Bioproducts, Walkersville, MD). None these reagents contained endotoxins. Tissue culture plates of 96 wells and 100-mm diameter dishes were purchased from Nunc (Naperville, IL). High molecular weight water-soluble chitosan (average molecular weight 300,000 Da) was obtained from JA KWANG Co., Ltd. Korea.

Cell cultures

RAW 264.7, a macrophage-like cell line, were obtained from the American Type Culture Collection (ATCC), Rockville, MD. The RAW 264.7 macrophages were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2 and 95% air.

Preparation of WSC

Special care was taken to prevent endotoxin contamination of the WSC solution as follows. The WSC was dissolved in distilled water and then filtered through a 0.2 μm filter. The WSC stock solution (1mg/ml) was stored at -20°C. Immediately before use, the WSC was diluted in endotoxin free water.

Measurement of nitrite concentration

NO synthesis in cell cultures was measured by a microplate assay method, as previously described (Nathan, 1992). To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphtyl)-ethylenediamine dihydrochlori-de/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 8 μM of NO_2^-, this value was determined in each experiment and subtracted from the value obtained from the medium with RAW 264.7 macrophages.

Western blot analysis

Whole cell lysates were made by boiling RAW 264.7 macrophages in sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose paper. The membrane was then blocked with 10% skim milk in PBS-tween-20 for 1 h at room temperature and then incubated with anti-iNOS, TNF-α and NF-κB antibodies. After washing in PBS-tween-20 three times, the blot was incubated with secondary antibodies for 30 min and the antibody-specific proteins were visualized by the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp).

Nuclear protein extraction

Preparation of crude nuclear extract was basically as described (Schoonbroodt *et al.*, 1997). Briefly, RAW 264.7 macrophages were washed in 1 ml of ice-cold PBS,

centrifuged at 1,000 × g for 5 min, resuspended in 400 μl of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, O.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 × g for 30 s. Pelleted nuclear proteins were gently resuspended in 50 μl of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15,000 × g for 5 min at 4°C. Aliquotes of the supernatant that contained nuclear proteins were frozen in liquid nitrogen and stored at -70°C.

Electrophoretic mobility shift assays (EMSA)

An established EMSA method, with slight modifications, was used (Camandola et al., 1996). Nuclear protein (10 μg) was incubated for 20 min at room temperature with 20 μg of bovine serum albumin, 2 μg of poly (dI-dC) from Pharmacia (Uppsala, Sweden), 2 μl of buffer C (20 mM HEPES/KOH, 20% glycerol, 100 mM KCl, 0.5 mM PMSF, pH 7.9), 4 μl of buffer F (20% ficoll-400, 100 mM HEPES/KOH, 300 mM KCl, 10 mM DTT, 0.5 mM PMSF, pH 7.9), and 20,000 cpm of a ^{32}P-labeled oligonucleotide in a final volume of 20 μl. DNA-protein complexes were resolved at 180 V for 4 h in a taurine-buffered, native 4 % polyacrylamide gel, dried, and visualized (with autography using a Fuji x-ray film).

Statistical analysis

Collected data were expressed as mean ± S.E. Statistical analysis was performed by the Student's *t*-test to express the difference between two groups.

RESULTS

Effect of WSC on rIFN-γ-induced NO production

Initially, we wished to determine whether RAW 264.7 macrophages could be stimulated by WSC, either alone or in combination with rIFN-γ, to induce NO production. RAW 264.7 macrophages were cultured either in medium alone or in medium that contained rIFN-γ (10 U/ml). Then, RAW 264.7 macrophages were stimulated with WSC at various times during 48 h culture, and NO release was measured by using the Griess method. As Table 1 shows, WSC alone did not induce NO synthesis, whereas WSC in combination with rIFN-γ synergistically increased NO synthesis in RAW 264.7 macrophages. The maximum cooperative effect of WSC for NO release was shown at 24 h after rIFN-γ treatment. The dose-dependent effects of WSC in the presence of rIFN-γ on NO synthesis were shown in Fig. 1. The synergistic effect always was maximal at 1g/ml of WSC.

Inhibition of WSC-induced NO production by N^GMMA

To determine if the signaling mechanism in WSC-induced NO production is involved in the $_L$-arginine-dependent pathway in RAW 264.7 macrophages, the cells were incubated for 6 h in the presence of rIFN-γ plus N^GMMA. The production of nitrite by rIFN-γ plus WSC in RAW 264.7 macrophages was progressively inhibited with increasing amount of N^GMMA. The WSC-induced accumulation of nitrite was significantly blocked by N^GMMA (1 mM, 10 mM) (Fig. 2).

Table 1. Time-Dependent Effect of WSC on rIFN-γ-Induced NO production in RAW 264.7 macrophages

Addition			
RIFN-γ (10 U/ml)	WSC (1 μg/ml)	LPS (10 μg/ml)	Nitrite concentration (μM)
−	−	−	<5
+	−	−	17±1.5
+	−	+	484±3.2*
−	+0 h	−	<5.0
+	+12 h	−	43.6±4.2*
+	+24 h	−	51.7±3.4*
+	+48 h	−	50.9±1.5
+	+24 h	+	55.1±3.2

RAW 264.7 macrophages (3×10^5 cells/well) were stimulated with WSC (1μg/ml) at various times after incubation. The amount of nitrite released by RAW 264.7 macrophages was measured after 48 h of the Griess method. Values are the mean ± S.E. of four independent experiments each run in duplicate. *P < 0.05, significantly different from the control.

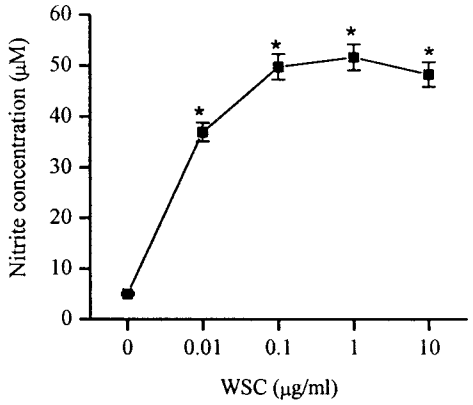

Fig. 1. Dose-dependent effect of WSC on NO synthesis in rIFN-γ-treated RAW 264.7 macrophages. RAW 264.7 macrophages (3×10^5 cells/well) were cultured with rIFN-γ (10 U/ml). The RAW 264.7 macrophages were then stimulated with various concentration of WSC for 6 h after incubation. After 24 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean ± S.E. of five independent experiments each run in duplicate. *P<0.01; significantly different from the control.

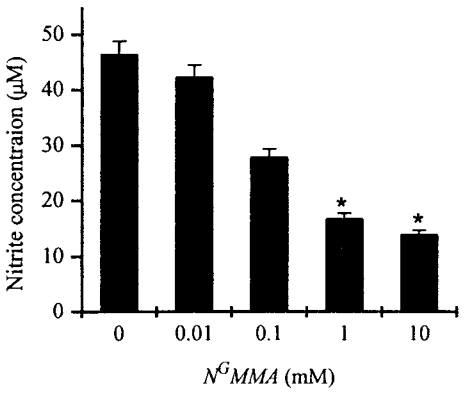

Fig. 2. Effect of NO synthase inhibitors on WSC-induced nitrite accumulation in the

cultured medium of RAW 264.7 macrophages. RAW 264.7 macrophages (3×10^5 cells/well) were incubated for 6 h with rIFN-γ plus various concentrations of N^GMMA. The RAW 264.7 macrophages were then treated with WSC (1 μg/ml) and cultured for 42 h. NO release was measured by the Griess method (nitirite). NO (nitrite) released into the medium is presented as the mean ± S.E. of three independent experiments each run in duplicated. *P<0.01; significantly different from the control.

Effect of WSC on rIFN-γ-induced iNOS expression

Data in Fig. 3 show the effects of rIFN-γ plus WSC treatments on the expression of iNOS protein in RAW 264.7 macrophages. We performed the Western blot analysis using anti-iNOS antibody. WSC alone partially increased on the expression of iNOS protein (data not shown), whereas WSC plus rIFN-γ or LPS plus rIFN-γ synergistically increased on the expression of iNOS protein in RAW 264.7 macrophages. WSC-induced expression of iNOS was decreased by N^GMMA (10 mM). Fig. 3B, the iNOS synthesis in Fig. 3A is normalized to control value using personal densitometer.

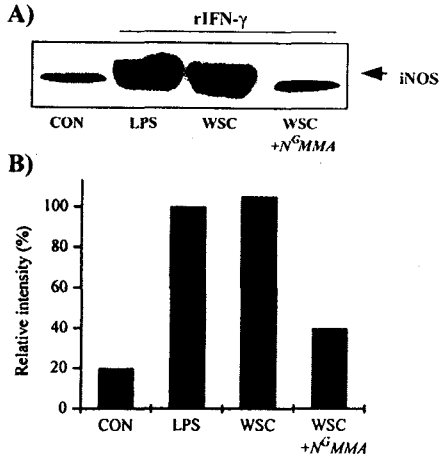

Fig. 3. Effect of WSC on the expression of iNOS by rIFN-γ plus WSC-induced RAW 264.7 macrophages. RAW 264.7 macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The RAW 264.7 macrophages were then stimulated WSC (1 μg/ml) or LPS (10 μg/ml) for 12 h. The protein extracts were prepared and samples were analyzed for iNOS expression by Western blotting as described in the method (A). iNOS levels were quantitated by densitometry (B).

Effect of WSC on NO production though NF-κB activation

We also investigated that the effects of WSC on signal transduction pathway of NO production. NF-κB activation assessed by Western blot analysis. As shown Fig. 4A, stimulation of RAW 264.7 macrophages with LPS after the treatment of rIFN-γ resulted in increased protein synthesis of NF-κB (p65 and p50). WSC partially increased on protein synthesis of NF-κB by itself (data not shown). When WSC was used in combination with rIFN-γ, there was a marked cooperative activation of NF-κB. To confirm the effect of WSC on rIFN-γ-induced NF-κB activation in RAW 264.7 macrophages, EMSA were performed. RAW 264.7 macrophages were incubated with medium alone, rIFN-γ plus LPS or rIFN-γ- plus WSC and nuclear extracts were prepared and incubated with a ^{32}P-labeled DNA oligonucleotide containing the recognition site of NF-κB. Specific NF-κB binding activity was detected in the lanes and DNA binding activity was increased by rIFN-γ-plus WSC than medium alone (Fig. 4B).

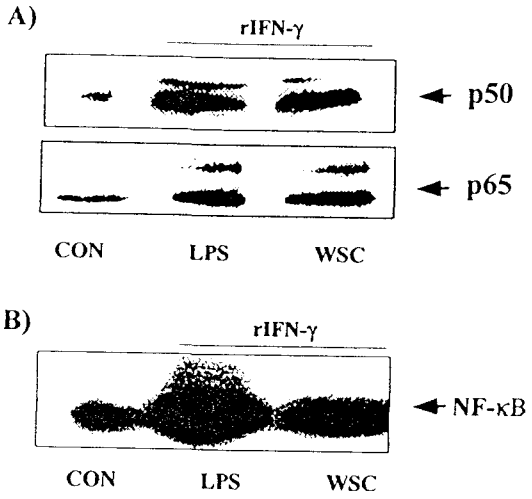

Fig. 4. Effect of WSC on the NF-κB activation by rIFN-γ plus WSC-induced RAW 264.7 macrophages. RAW 264.7 macrophages (5 × 10^6 cells /well) were incubated for 6 h with rIFN-γ (10 U/ml). The RAW 264.7 macrophages were then stimulated WSC (1 μg/ml) or LPS (10 μg/ml) for 1 h. Total protein extracts were prepared and samples were analyzed for NF-κB activation by Western blotting as described in the method (A). Nuclear extracts were prepared and samples were analyzed for NF-κB activation by EMSA as described in the method (B).

Inhibition of WSC-induced NO production by PDTC

As an approach to define the signaling mechanism of WSC on NO synthesis, we examined the influence of NF-κB inhibitor PDTC in rIFN-γ plus WSC or rIFN-γ plus LPS-treated RAW 264.7 macrophages. Adding PDTC to the rIFN-γ plus WSC or rIFN-γ plus LPS-treated RAW 264.7 macrophages decreased the synergistic effects of WSC on NO synthesis significantly (Table 2).

Table 2. Effect of PDTC on rIFN-γ plus WSC-induced NO production in RAW 264.7

Addition				
RIFN-γ (10 U/ml)	WSC (1 μg/ml)	LPS (10 μg/ml)	PDTC (100 μM)	Nitrite concentration (μM)
−	−	−	−	<5
+	−	+	−	48.43
+	+	−	−	43.64
+	−	+	+	11.22*
+	+	−	+	11.33*

macrophages

RAW 264.7 macrophages (3 × 10^5 cells/well) were stimulated with WSC. The amount of nitrite released by RAW 264.7 macrophages was measured after 48 h of the Griess method. Values are the mean ± S.E. of three independent experiments each run in duplicate. *$P < 0.01$, significantly different from the positive control.

DISCUSSION

Water-insoluble chitosan has been simply shown to exhibit in vitro stimulatory effect on peritoneal macrophages NO production without combination with rIFN-γ (Peluso *et al.*, 1994). In this study, however, WSC alone did not induce NO synthesis. NO synthesis in RAW 264.7 macrophages by WSC can highly stimulated in combination with rIFN-γ. WSC had a maximal effect on NO synthesis at a concentration of 1 μg/ml in rIFN-γ-treated RAW 264.7 macrophages. The results of this study suggest that WSC may provide a second signal for synergistic induction of NO synthesis in RAW 264.7 macrophages. NO, the initial product of oxidation of $_L$-arginine, exhibits a multitude of biological actions (Garthwaite *et al.*, 1988; Peunova and Enikolopov, 1995). N^GMMA, an analog of $_L$-arginine, inhibited rIFN-γ plus WSC-induced NO production in RAW 264.7 macrophages. The strong inhibition of nitrite production by N^GMMA indicates that it is likely to depend upon NOS.

At present, the precise physiological significance of NO synthesis by WSC is unknown. However, during the last few years, NO as a potent macrophage-derived effector molecule against a variety of bacteria, parasites, and tumors has received increasing attention (Stuehr *et al.*, 1989; Nathan and Hibbs, 1991). More recent studies suggest that NO also have antiviral effects in both murine and human cells. Croen (1993) demonstrated that stimulation of a murine macrophages cell line with IFN-γ and LPS resulted in high level NO production and a 1000-fold inhibition of herpes simplex virus-1 replication. NO has been reported to play an important role for the mitogenic effect of angiogenic factor, for example, in the angiogenic process. Eroglu *et al.* (1999) showed that large tumor burden was associated with significantly increased levels of vascular endothelial growth factor and NO. Also, Klotz *et al.* (1999) reported that bladder carcinoma tissue had a high iNOS content; benign tissue did not. NO generation by iNOS also influences the cytotoxicity of macrophages and tumor- induced immunosu -ppression. NO production by WSC indicates that it may provide various activities such as anti-microbial, anti-tumoral, and anti-viral under specific conditions in vivo. In addition, since NO has emerged as an important intracellular and intercellular regulatory molecule having functions as diverse as vasodilation, neural communication, cell growth regulation and host defense (Nathan, 1992), it is tempting to hypothesize that this molecule is involved in the local control of the various fundamental processes. In most experimental model, macrophage activation is a two-step process (Kim and Moon, 1996; Russell *et al.*, 1977). After induction of iNOS by cytokine, the elaborated NO elicits direct effects. Probably, this response could appear to reflect a rapid but nonspecific type of immune activity, contrast with the slower but highly site-specific recognition actions. Signal transduction pathway of NO production has been previously reported that LPS stimulation of rIFN-γ-primed macrophages induces NF-κB activation (Kim *et al.*, 1995). NF-κB is now known to be ubiquitously expressed and to play a major role in controlling the expression of protein involved immune, inflammatory and acute phase responses (Baeuerle and Henkel, 1994; Sha, 1998). Expression of iNOS and TNF-α genes is dependent on the activation of a transcription factor, NF-κB. NF-κB is a central mediator of the immune response. The addition of NF-κB modulator PDTC inhibits the synergistic effect of WSC with rIFN-γ on NO synthesis. The present results demonstrated that the capacity of WSC to

increase NO synthesis from rIFN-γ-primed RAW 264.7 macrophages was the result of WSC-induced NF-κB activation.

In conclusion, our results demonstrate that WSC acts as an accelerator of RAW 264.7 macrophages activation by rIFN-γ via a process involving L-arginine-dependent NO production. Then, RAW 264.7 macrophages were stimulated with rIFN-γ plus WSC, the NF-κB activation significantly increased. These results suggest that WSC influences on NO production via the NF-κB signaling pathway and is important in the development of macrophages activation.

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