Food Sci. Biotechnol. Vol. 17, No. 1, pp. 106 ~ 113 (2008)

Food Science and Biotechnology

β-Glucan Suppresses LPS-stimulated NO Production Through the Down-regulation of iNOS Expression and NFκB Transactivation in RAW 264.7 Macrophages

Jeong-Lye Yang, Ji-Hyun Jang[1], Vinodhkumar Radhakrishnan[1,2], Yang-Ha Kim[3], and Young-Sun Song[1]*

Busan Newport Import Food Inspection Office, Busan Regional Food & Drug Administration, Busan 618-410, Korea
[1]BK21 Center for Smart Food and Drugs, Biohealth Products Research Center, Inje University, Kimhae, Gyeongnam 621-749, Korea
[2]Department of Biochemistry, University of Madras, Guindy Campus, Chennai 600-025, India
[3]Department of Food and Nutrition, Ehwa Womans University, Seoul 120-750, Korea

Abstract The antioxidant and anti-inflammatory protective effects of β-glucan from barley on RAW 264.7 murine macrophage cells induced by lipopolysaccharide (LPS) were examined. The RAW 264.7 murine macrophages were preincubated with various concentrations (0-200 μg/mL) of β-glucan and stimulated with LPS to induce oxidative stress and inflammation. The β-glucan treatments were found to reduce thiobarbituric acid-reactive substance (TBARS) accumulation, and enhance glutathione levels and the activities of antioxidative enzymes, including superoxide dismutase (SOD), catalase, glutathione reductase, and glutathione peroxidase (GSH-px) in the LPS-stimulated macrophages as compared to the LPS-only treated cells. Nitric oxide (NO) production was significantly suppressed in a dose-dependent manner ($p<0.05$) with an IC_{50} of 104 μg/mL. Further treatment with β-glucan at 200 μg/mL suppressed NO production to 2% of the LPS-control, and suppressed the levels of inducible nitric oxide synthase (iNOS) protein and mRNA in a dose-dependent manner. The specific DNA binding activity of nuclear factor κB (NFκB) was significantly suppressed by β-glucan treatment with an IC_{50} of 220 μg/mL in a dose-dependent manner. Finally, barley β-glucan ameliorates NO production and iNOS expression through the down-regulation of NFκB activity, which may be mediated by attenuated oxidative stress in RAW 264.7 macrophages.

Keywords: barley β-glucan, oxidative stress, nuclear factor κB, inducible nitric oxide synthase expression, nitric oxide, macrophage

Introduction

Currently, β-glucan is accepted as one of the most powerful immune response modifiers (1). Several studies conducted in the past decade have shown that it inhibits tumor development (2,3), enhances defenses against bacterial, viral, fungal, and parasitic challenge (4,5), activates macrophages (6), and induces the production of cytokines (7), nitric oxide (NO), and arachidonic acid metabolites (8). Among the several mechanisms proposed for the protective effects of β-glucan, a major one is related to the antioxidant capacity of the molecule (9,10).

NO and inducible nitric oxide synthase (iNOS) are potent biological mediators produced by various mammalian cells and tissues (11,12). The production of NO is regulated by intracellular NOS, which exists in 3 isoforms. Among these isoforms, iNOS is regulated at the transcriptional level by the exposure of cells to other inflammatory stimuli such as reactive oxygen species (ROS) (13,14) and LPS or cytokines, including tumor necrosis factor α, and interferon-γ in macrophages (11,12). High NO levels along with decreased antioxidant enzyme activities, lead to peroxynitrite formation (15). Furthermore, it is known that NO interacts with thiol-containing glutathione (GSH). Subsequent depletion of GSH is shown to increase the sensitivity of cells to the toxic effects of NO (16).

*Corresponding author: Tel: +82-55-320-3235; Fax: +82-55-321-0691
E-mail: fdsnsong@inje.ac.kr
Received July 4, 2007; accepted August 6, 2007

Nuclear factor κB (NFκB), an inducible transcription factor, is activated in response to various extracellular stimuli, including cytokines, lipopolysaccharide (LPS), and oxidative stress (17-20). The promoter of murine macrophages encoding iNOS contains a consensus sequence for the binding of NFκB, which is necessary to confer inducibility of LPS (21). NFκB sites have been identified in the promoter region of the iNOS gene (22). Therefore, expression of iNOS is closely related to the transactivation of NFκB (23). Thus, the suppression of iNOS expression, NO production, and NFκB transactivation may be effective biomarkers for the attenuation of inflammatory processes and diseases (24).

Studies have shown that β-glucans can be isolated from almost every species of yeast. However, little information is available about the effects of β-glucan derived from barley on NFκB, a ROS-sensitive transcriptional factor that regulates iNOS expression and subsequent NO production in LPS-stimulated murine macrophages. These important aspects led us to investigate the effects of barley β-glucan on oxidative stress status, NO production, iNOS expression, and NFκB activation in RAW 264.7 macrophages stimulated by LPS.

Materials and Methods

Materials Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and TRIzol® reagent were obtained from Gibco BRL (Gibco BRL, Gaithersburg, MD, USA). LPS, dimethyl sulfoxide (DMSO), glutamine,

protease inhibitor cocktail, nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), phenylmethylsulfonyl fluoride (PMSF), SDS, Nonidet® P40 substitute (NP-40), and Griess reagent were obtained from Sigma Chemical (St. Louis, MO, USA). A Maloney murine leukemia virus (MMLV) first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA), NFκB-specific oligonucleotide and T4 polynucleotide kinase (Promega, Madison, WI, USA), and microspin G-25 columns (Amersham Bioscience, Piscataway, NJ, USA) were used for reverse transcription-polymerase chain reaction (RT-PCR) and electrophoretic mobility shift assay (EMSA). Bradford protein assay reagent and SDS-PAGE standards were acquired from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The anti-mouse iNOS antibody was from BD Transduction Laboratories (Lexington, KY, USA), and the anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Abcam (Cambridge, UK). Alkaline phosphatase conjugated goat anti-mouse immuno-globulin G (IgG) secondary antibody was acquired from Jackson ImmunoResearch (West Groove, PA, USA). The nitrocellulose membranes were from Schleicher and Schuell (Keene, NH, USA). The murine macrophage RAW 264.7 cell line was obtained from the American Type Culture Collection (TIB-71; Rockville, MD, USA). All other chemicals were of the highest commercial grade available.

Cell culture and treatment The murine macrophage RAW 264.7 cell line was obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. Cells in 10 mm dishes (5×10^6 cells/dish) or 24-well plates (4×10^5 cells/well) were pre-incubated with and without indicated concentrations of barley β-glucan (50, 100, and 200 μg/mL) for 2 hr, and then incubated with LPS (1 μg/mL) for 20 hr at 37°C in a humidified atmosphere containing 5% CO_2. The cells that were untreated provided the negative control without LPS (untreated), while the cells treated with LPS alone provided the positive control (control).

Cell viability Cell viability was assessed by measuring the uptake of neutral red supravital dye by the viable cells according to the procedure of Fautz *et al.* (25). After culturing the cells as described previously, the medium was removed and replaced with 0.5 mL of fresh medium containing 1.14 mmol/L of neutral red. After incubation for 3 hr, the medium was removed and the cells were washed twice with phosphate buffered saline solution (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation for 15 min at room temperature in the presence of 1 mL of cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L dithiothreitol (DTT), and Triton X-100 (1%, v/v)] containing acetic acid (1%, v/v) and ethanol (50%, v/v). To measure the dye taken up, the cell lysis products were centrifuged and the absorbance of the supernatant was measured spectrophotometrically at 540 nm.

Nitrite assay The nitrite that accumulated in the culture medium was an indicator of NO production and was measured according to the Griess reaction (26). One-hundred μL of each medium supernatant was mixed with 50 μL of sulphanilamide (1% in 5% phosphoric acid) and 50 μL of naphthylenediamine dihydrochloride (0.1%), and then incubated at room temperature for 10 min. The absorbance at 550 nm was measured against a $NaNO_2$ serial dilution standard curve, and then nitrite production was determined.

Lipid peroxidation Lipid peroxidation was measured by thiobarbituric acid reactive substance (TBARS) production, as described by Fraga *et al.* (27). The cells (4×10^5 cells/well) in 24-well plates were first incubated with and without the indicated concentrations of β-glucan for 2 hr, and then incubated with LPS (1 μg/mL) for 20 hr. Two-hundred μL of each medium supernatant was mixed with 400 μL of TBARS solution and then boiled at 95°C for 30 min. The absorbance at 532 nm was measured with a 1,1,3,3-tetraethoxypropane serial dilution standard curve, and the TBARS values were expressed as nmols of malondialdehyde equivalents.

GSH concentration GSH was measured using the enzymatic recycling procedure by Tietze (28), in which GSH is sequentially oxidized by 5,5"-dithiobis (2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase.

Antioxidant enzyme assays The cell suspensions were sonicated 3 times for 5 sec on ice, and then the cell sonicates were centrifuged at 10,000×g for 20 min at 4°C. The cell supernatants were used to test for the antioxidant enzyme activities. The protein concentration was measured using the Bradford assay (29) with bovine serum albumin as the standard. SOD activity was determined by monitoring the auto-oxidation of pyrogallol according to the method of Marklund and Marklund (30). One unit of SOD activity was defined as the amount of enzyme that inhibited the oxidation rate of 6 mM pyrogallol by 50%. Catalase activity was measured according to the method of Aebi (31) by following the decrease in absorbance of H_2O_2. The decrease in absorbance at 240 nm was measured for 2 min. Standards containing 0, 0.2, 0.5, 1, and 2 mM of H_2O_2 were used to construct a standard curve. GSH-peroxidase (GSH-px) activity was assayed by the method of Lawrence and Burk (32). One unit of GSH-px was defined as the amount of enzyme that oxidized 1 nmol of NADPH consumed per min. GSH reductase activity was measured by following the oxidation of NADPH according to the method of Inger and Bengt (33). One unit of GSH reductase was defined as the amount of enzyme that catalyzed the reduction of 1 nmol of NADPH per min.

Western blot analysis Western blot analysis was performed by the method of Katsuyama *et al.* (34) with slight modifications. The cells were washed twice with PBS, scraped into 0.5 mL of ice-cold lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholase, 1 mM PMSF] for 15 min, and then disrupted with a Handy Sonic Disrupter (Sonopuls UW 2070; Bandelin electronic, Berlin, Germany). The lysis buffer containing the disrupted cells was centrifuged at 13,000×g and 4°C for 20 min. The protein content of the lysate supernatants was determined with the

Bio-Rad protein assay reagent (Bio-Rad Laboratories). The protein samples (60 μg) from each lysate were separated on a 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membranes (Schlecher-Schuell). The membranes were blocked for 1 hr at room temperature with 5% nonfat dry milk in a buffer that contained 10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, and 0.05% Tween 20. The reactions were then incubated at 4°C overnight with a 1 : 10,000 dilution of rabbit anti-mouse iNOS antibody and a 1 : 1,000 dilution of rabbit anti-mouse GAPDH antibody in blocking buffer. After the membranes were washed, they were further incubated with a 1 : 10,000 dilution of alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody for 1 hr at room temperature. The blots were finally developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitroblue tetrazolium (NBT) color developing solution. The data were quantified using the Gel Doc EQ System (Bio-Rad Laboratories). All signals were normalized to the protein levels of the housekeeping gene, GAPDH, and expressed as a ratio.

RNA preparation and iNOS mRNA analysis by the RT-PCR Total RNA was isolated using Trizol-reagent (Sigma Chemicals) according to the method of Chomczynski and Sacchi (35). Five μg of total RNA was used to produce the first strand of cDNA using the MMLV First Strand cDNA synthesis kit (Invitrogen). PCR (GeneAmp PCR System 2400; Perkin Elmer Life Sciences, Wellesley, MA, USA) was carried out in 50 μL of reaction mixture containing the first strand cDNA, 10× PCR buffer, 2.5 mM deoxy-ribonucleoside triphosphate (dNTP), 20 pM of each primer, and *Taq*. DNA polymerase (Perkin Elmer Life Sciences). The PCR primer sequences for iNOS and GAPDH were as follows: the primers for iNOS were 5'-GCC TTC AAC ACC AAG GTT GTC TGC A-3' (sense) and 5'-TCA TTG TAC TCT GAG GGC TGA CAC A-3' (anti-sense), and the primers for GAPDH were 5'-CAA TGC CAA GTA TGA TGA CAT-3' (sense) and 5'-CCT GTT ATT ATG GGG GTC TG-3' (anti-sense). The expected sizes of the PCR products were 920-bp for iNOS and 375-bp for GAPDH. The amplification profile consisted of an initial denaturation at 94°C for 1 min followed by denaturation at 94°C for 2.5 min (for iNOS and GAPDH); annealing at 59°C for 2 min (iNOS) and at 49°C for 2 min (GAPDH); and extension at 72°C for 2 min (for iNOS and GAPDH). Twenty-seven cycles for iNOS and GAPDH resulted in the best amplification profiles to recognize the differences among samples. The expression of the housekeeping gene, GAPDH, served as the control. The PCR products specific for each cDNA were analyzed by electrophoresis on 2% agarose gels containing ethidium bromide (0.5 μg/mL) at 50 V for 70 min, and were visualized with an UV trans-illuminator. The data were quantified using the Gel Doc EQ System (Bio-Rad Laboratories). All signals were normalized to the mRNA levels of the housekeeping gene, GAPDH, and expressed as ratios.

Electrophoretic mobility shift assay (EMSA) The nuclear protein was extracted using the slightly modified method of Dignam *et al.* (36). The cells in 100 mm dishes were lysed with buffer containing 0.6% Igepal, 0.15 M NaCl, 10 mM Tris (pH 7.9), 1 mM ethylenediamine tetraacetic acid (EDTA), and 0.1% protease inhibitor cocktail. They were then vortexed, kept on ice for 5 min, and centrifuged at 500×g for 5 min at 4°C. The pelleted nuclei were resuspended in 50 μL of extraction buffer [10 mM Hepes (pH 7.9), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM EDTA, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 1 mM DTT, and 0.33% protease inhibitor cocktail]. Following gentle mixing and then keeping on ice for 20 min, the samples were centrifuged at 500×g for 5 min at 4°C. The supernatant fraction was transferred to new tubes and stored at −70°C. The protein concentration was determined by the Bradford assay (29). For the EMSA, NFκB-specific oligonucleotide was end-labeled with [γ-^{32}P]-ATP using T_4 polynucleotide kinase (Promega), and then purified using microspin G-25 columns (Amersham Bioscience). An EMSA was performed according to the instruction manual of Promega. Five mg of nuclear protein, 2 μL of 5× binding buffer, 1 μL of ^{32}P-labeled NFκB, and 1 μL of 10× loading buffer were incubated for 30 min at room temperature. The DNA-protein complexes were separated from the unbound DNA probe by electrophoresis through a 4% polyacrylamide gel, using 0.5× Tris-borate EDTA buffer as the running buffer. The gels were exposed to a phosphor screen (Packard Instrument Company, Inc., Meriden, CT, USA) for 2 hr at −20°C, and the bands were quantitated by a phosphor image analyzer (Amersham Bioscience).

Statistical analysis All data are expressed as means ± SD. The statistical analyses were done on the SPSS program (SPSS 10.0; SPSS Institute, Chicago, IL, USA). One-way ANOVA with Duncan's multiple range tests (37) were used to examine the differences between groups. p-Values of <0.05 were considered significant, if not otherwise stated.

Results and Discussion

NO production In this study, we investigated the suppressive effect of barley β-glucan on NO production and intracellular oxidative stress, and how barley β-glucan regulates NFκB activation and iNOS expression as possible mechanisms of NO suppression, in an LPS-stimulated macrophage cell line (38,39). Barley β-glucan treatment was found to suppress NO production in a dose-dependent manner ($p<0.05$), with an IC_{50} of 104 mg/mL (Fig. 1). The treatment of barley β-glucan at 200 mg/mL suppressed NO production to 1% of the LPS-treated control. Cell viability was >95% at the administered concentrations as assessed by the neutral red assay, which supports the idea that the suppressive effect of β-glucan on NO production were not due to cell death (data not shown).

Few studies have reported that the β-glucan from yeast and mushrooms can increase NO production in primary macrophages, Kupffer cells, and RAW cells without LPS stimulation (6,8,40,41). Contradictory results were obtained by several researchers, who reported that β-glucan from yeast did not affect NO production in RAW cells (42,43) However, an increase of NO production at a high concentration of β-glucan (1,000 mg/mL) was previously

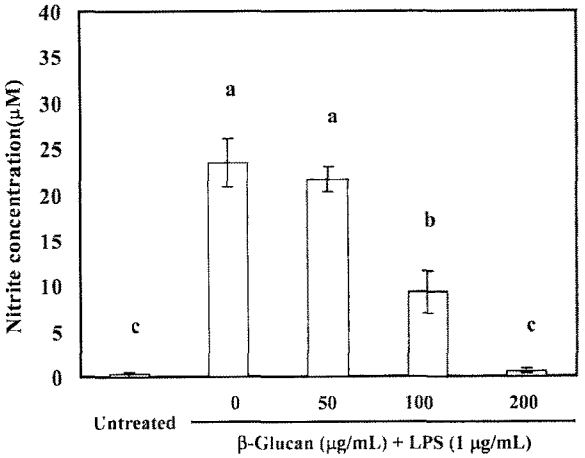

Fig. 1. Effects of β-glucan on NO production in LPS-stimulated RAW 264.7 macrophages. Data represent the means±SD of triplicate experiments. A value sharing the same superscript is not significantly different at $p<0.05$.

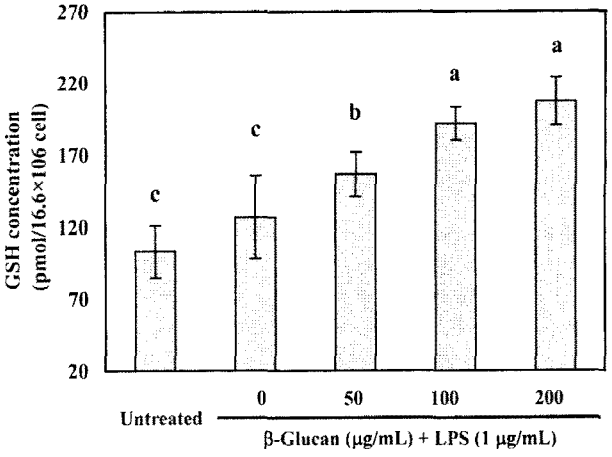

Fig. 3. Effects of β-glucan on GSH concentration in LPS-stimulated RAW 264.7 macrophages. Data represent the means ±SD of triplicate experiments. A value sharing the same superscript is not significantly different at $p<0.05$.

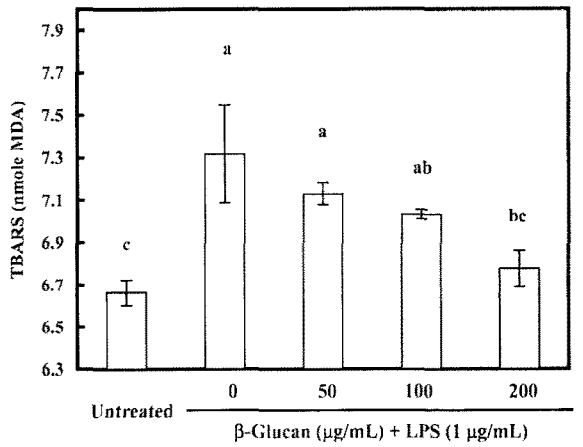

Fig. 2. Effects of β-glucan on TBARS generation in LPS-stimulated RAW 264.7 macrophages. Data represent the means± SD of triplicate experiments. A value sharing the same superscript is not significantly different at $p<0.05$.

reported (44). In our study, the barley β-glucan treatment at the level of 200 mg/mL without LPS stimulation did not support these findings (data not shown). These differences might be ascribed to the concentration and incubation time of the β-glucan.

Status of oxidative stress and antioxidative enzyme activities To assess the defensive function of barley β-glucan against oxidative stress, we investigated markers of oxidative stress status such as TBARS and GSH levels, and antioxidative enzyme activities. As shown in Fig. 2, treatment with LPS elevated the TBARS level, indicative of lipid peroxidation. Pre-incubation with barley β-glucan significantly decreased TBARS ($p<0.05$) in a dose-dependent manner, suggesting that lipid peroxidation was attenuated in the LPS-stimulated macrophages by the barley β-glucan treatment. Exposing the RAW 264.7 cells to LPS induced a significant increase in the GSH level. GSH levels were further enhanced in the LPS-treated RAW 264.7 cells pre-incubated with barley β-glucan as compared to the LPS-only treated cells (Fig. 3). The effects of barley β-glucan on the specific activities of antioxidant enzymes in the LPS-stimulated cells are shown in Table 1. Exposing the cells to LPS affected the enzyme activities; while exposure of the cells to barley β-glucan at 50, 100, or 200 mg/mL concentrations before LPS treatment significantly elevated all the enzyme activities except GSH-px, as compared to those of the LPS-stimulated control ($p<0.05$). β-Glucan pretreatment without LPS stimulation did not affect enzyme activity.

Lipid peroxidation, mediated by free oxygen radicals, is believed to be an important cause of damage and destruction in cell membranes, since the polyunsaturated fatty acids of cellular membranes are degraded by this process with consequent disruption of membrane integrity (45). Membrane peroxidation can lead to changes in membrane fluidity and permeability, and also to enhanced rates of protein degradation, and these will eventually lead to cell lysis (46). Our results demonstrate that barley β-glucan significantly inhibited MDA elevation, an end product of lipid peroxidation. Glutathione is an important constituent of intracellular protective mechanisms against several noxious stimuli, including oxidative stress, while reduced GSH is the main component of the endogenous non-protein sulfhydryl pool that scavenges free radicals in the cytoplasm (47). Because of their exposed sulfhydryl groups, non-protein sulfhydryls bind to a variety of electrophilic radicals and metabolites that may be damaging to cells (48). It has been proposed that antioxidants, which maintain the concentration of reduced GSH, may restore cellular defense mechanisms and block lipid peroxidation, and thus protect against intracellular oxidative stress. Depletion of GSH by oxi-LDL treatment, and recovery from the oxidative injury by the pre-incubation of aged garlic extract possessing antioxidative activity, was reported in cultured endothelial cells (49). However, our finding of GSH elevation by LPS alone was comparable to that of Yen and Lai (50), who treated RAW 264.7 cells with peroxynitrite, a powerful oxidant formed by the reaction of NO and superoxide. Iqbal *et al.* (15) also reported that LPS

Table 1. Effects of β-glucan on antioxidative enzyme activities in LPS-stimulated RAW 264.7 macrophages[1)]

	Untreated	β-Glucan (μg/mL) + LPS (1 μg/mL)			
		0	50	100	200
Catalase (μmol/mg protein/min)	530±53[b2)]	248±61[d]	363±50[c]	545±76[b]	770±65[a]
SOD (unit/mg protein)	13±1.5[e]	23±2.3[d]	28±2.7[c]	31±0.9[b]	34±3.2[a]
GSH-px (unit/mg protein)	4.7±0.2[b]	5.4±0.6[a]	5.5±0.4[a]	5.2±0.3[ab]	4.7±0.3[b]
GSH-reductase (unit/mg protein)	159±7[a]	97±9[cd]	112±13[c]	119±16[bc]	133±9[b]

[1)]LPS, Lipopolysaccharide; SOD, superoxide dismutase; GSH-px, glutathione peroxidase; GSH-reductase, glutathione reductase.
[2)]Data represent the means±SD of triplicate experiments. A value sharing the same superscript is not significantly different at $p<0.05$.

injection in rats increased GSH levels when compared to baseline levels, and then returned to baseline after 24 hr from the LPS injection. These observations might be justified by the finding that GSH synthesis was induced by a nano-molar concentration of NO, due to enhanced expression of the rate-limiting enzyme for GSH synthesis, glutamylcysteine synthetase (51).

The earlier evidence on the effect of LPS on antioxidative enzyme activity is not consistent. Ben-Shaul *et al.* (52) reported that LPS injection in rats increased SOD and catalase, but did not change GSH-px activity in the heart. Iqbal *et al.* (15) found that GSH-px activity was unaltered in LPS injected rat hearts. However, Watson *et al.* (53) found that the activities of GSH-px and SOD were decreased after LPS injection in rat liver. Our study found that the activity of catalase and GSH-reductase were slightly decreased by LPS treatment. However, the preincubation of barley β-glucan with the LPS-stimulated cells further enhanced the antioxidative enzyme systems, including catalase, SOD, and GSH-reductase, that ameliorate oxidative stress. When NO is produced in a large amount, it reacts rapidly with superoxide to form peroxynitrite, a powerful oxidant that damages many biological molecules, including DNA and proteins, and thus, leads to the amplification of inflammation and tissue injury (50,52). The elevated antioxidative enzyme activities found in our present experiment, such as those of catalase and SOD, may imply the adaptive process of cells exposed to increasing intracellular oxidative stress by LPS, or the antioxidant properties of β-glucan itself against oxidative stress (9,10,41,54). From these results, it is certain that barley β-glucan has a defensive mechanism against oxidative stress caused by LPS, through the elevation of GSH and antioxidative enzyme activity and the suppression of TBARS production.

Effects of barley β-glucan on iNOS protein and mRNA expressions and NFκB activity We investigated iNOS protein and mRNA expression in order to elucidate the mechanism of inhibition by the barley β-glucan. Although hardly detected in the unstimulated RAW 264.7 cells by Western blot analysis, iNOS protein was sufficiently expressed after stimulation with LPS (1 μg/mL) for 20 hr, and the presence of barley β-glucan in LPS (1 μg/mL) stimulated the cell cultures to have markedly decreased iNOS expression in a dose-dependent manner (Fig. 4). This result indicates that the decreased activity of iNOS by barley β-glucan could have resulted from the inhibition of iNOS protein expression. RT-PCR was performed to examine whether the inhibition of NO production by

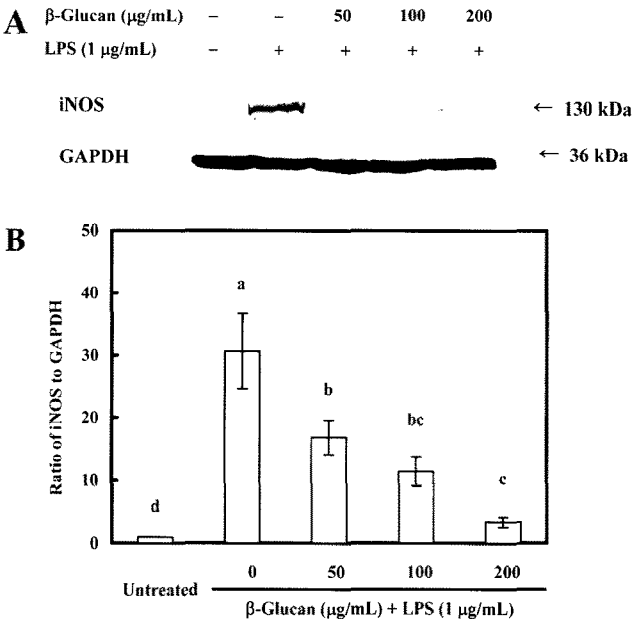

Fig. 4. Effects of β-glucan on iNOS protein expression in LPS-stimulated RAW 264.7 macrophages. (A) Levels of iNOS protein were measured by Western blot analysis using a monoclonal antibody for murine iNOS. The blot was rehybridized with antibody against GAPDH to verify equal loading of the protein in each lane. GAPDH was used as an internal control. (B) All signals were normalized to protein levels of the housekeeping gene, GAPDH, and expressed as a ratio. Data represent the means ±SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p<0.05$.

barley β-glucan was involved in iNOS mRNA expression. The LPS-stimulated macrophages had induced expression of iNOS mRNA, but not that of GAPDH. The pretreatment of barley β-glucan with LPS suppressed the expression of iNOS mRNA in a dose-dependent manner ($p<0.05$). However, the barley β-glucan did not affect the expression of the housekeeping gene GAPDH (Fig. 5). We also performed EMSA to examine whether the barley β-glucan interfered with the binding of NFκB to DNA; NFκB is a transcriptional factor regulating several genes, including iNOS. The specific DNA binding of NFκB, using EMSA, showed that LPS treatment of the RAW 264.7 cells enhanced NFκB activation (Fig. 6A). Pre-incubation of the cells in the presence of barley β-glucan at 50-200 μg/mL had an inhibitory effect on NFκB activity, with an IC_{50} of 220 μg/mL in a dose-dependent manner (Fig. 6B). We had

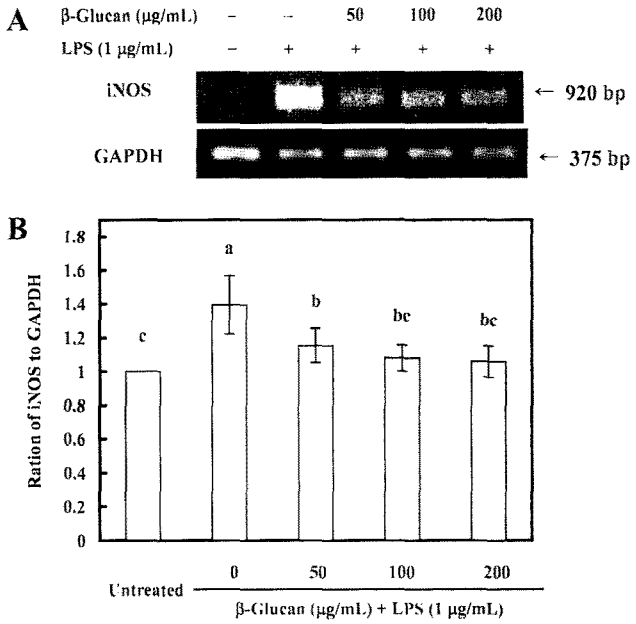

Fig. 5. Effects of β-glucan on iNOS mRNA expression in LPS-stimulated RAW 264.7 macrophages. (A) Levels of iNOS mRNA were determined by RT-PCR analysis. GAPDH was used as an internal control. (B) All signals were normalized to mRNA levels of the housekeeping gene, GAPDH, and expressed as a ratio. Data represent the means±SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p<0.05$.

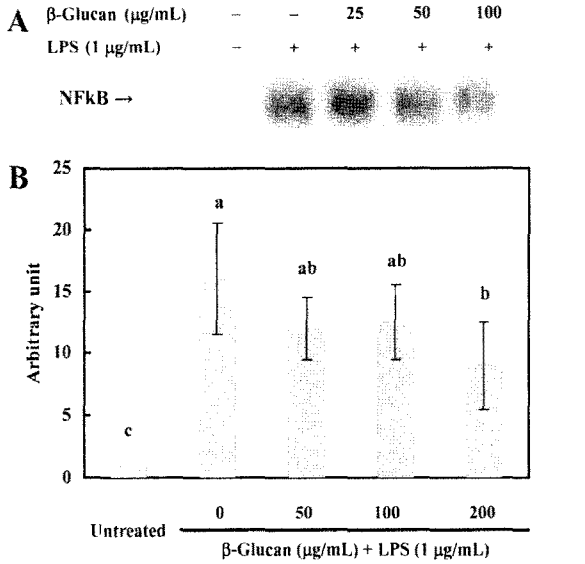

Fig. 6. Effects of β-glucan on NFκB activity in LPS-stimulated RAW 264.7 macrophages. (A) DNA binding activity of NFκB was performed by EMSA. (B) Values are expressed as the relative intensity of radioactivity. Data represent the means±SD of triplicate experiments. A value sharing the same superscript is not significantly different at $p<0.05$.

used 5 mg of nuclear protein that was extracted from the macrophages treated with different levels of barley β-glucan and LPS. Thus, the decreased level of NFκB activity was not due to cell death. β-Glucan inhibited the accumulation of nitrite, and this decrease in NO production was consistent with the inhibition of LPS-induced iNOS gene expression, as analyzed by Western blot and RT-PCR analysis, respectively. The finding that LPS-stimulated iNOS protein and mRNA expression were suppressed by barley β-glucan further supports the hypothesis that barley β-glucan's inhibition of LPS-stimulated NO production may be mediated through the down-regulation of NFκB, since the activation of NFκB is critical for the induction of iNOS gene expression in macrophages stimulated with LPS (22). It is known that the promoter region of the iNOS gene contains several consensus sequences for the binding of NFκB (17). Based on the observations made in this study, the inhibitory effect of barley β-glucan on NFκB activation induced by LPS in RAW 264.7 cells, can be explained by the ameliorated intracellular oxidative stress in the signaling pathway of LPS to NFκB activation. Indeed, the suppressive effect of barley β-glucan on NFκB activation coincides with the ameliorated NO, TBARS, elevated GSH generation, and enhanced antioxidative enzyme activities. Our data support the view that antioxidants are cell specific in their ability to inhibit NFκB, and clearly suggest that β-glucan acts in the RAW 264.7 murine macrophage cell line on the signal transduction pathway relating to LPS stimulation and NFκB activation. β-Glucan ameliorated intracellular oxidative stress by delaying the consumption of cellular GSH and by enhancing antioxidative enzyme activities, which resulted in the inhibition of NFκB activation, iNOS protein and mRNA expression, and NO production by LPS. NO production was attenuated through decreases in iNOS protein and mRNA expression as well as NFκB activity by the barley β-glucan, which might have attributed to the attenuation of intracellular oxidative stress in reverse by ameliorating peroxynitrite formation (55). These biological actions of β-glucan are possible through the C-type-lectin-like receptor, Dectin-1, a receptor for β-glucan on murine macrophages (56). In conclusion, the results suggest that barley β-glucan may attenuate inflammatory diseases by suppressing NO production through the inhibition of iNOS protein expression and iNOS mRNA transcription, to an extent that is associated with the inhibition of NFκB transactivation, which may be solely mediated by the antioxidative and anti-inflammatory properties of barley β-glucan.

Acknowledgments

This study was supported by a Korean Research Foundation Grant # (KRF-2002-075-E00002).

References

1. Bittencourt VC, Figueiredo RT, da Silva RB, Mourao-Sa DS, Fernandez PL, Sassaki GL, Mulloy B, Bozza MT, Barreto-Bergter E. An α-glucan of *Pseudallescheria boydii* is involved in fungal phagocytosis and Toll-like receptor activation. J. Biol. Chem. 281: 22614-22623 (2006)
2. Hetland G, Sandven P. β-1,3-Glucan reduces growth of *Mycobacterium tuberculosis* in macrophage cultures. FEMS Immunol. Med. Mic. 33: 41-45 (2002)
3. Guzel S, Sunamak O, As A, Celik V, Ferahman M, Nuri MM, Gazioglu E, Atukeren P, Mutlu O. Effects of hyperbaric oxygen and Pgg-glucan on ischemic colon anastomosis. World J. Gastroentero.

12: 1421-1425 (2006)
4. Nakagawa Y, Ohno N, Murai T. Suppression by *Candida albicans* β-glucan of cytokine release from activated human monocytes and from T cells in the presence of monocytes. J. Infect. Dis. 187: 710-713 (2003).
5. Sener G, Toklu H, Ercan F, Erkanli G. Protective effect of beta-glucan against oxidative organ injury in a rat model of sepsis. Int. Immunopharmacol. 5: 1387-1396 (2005)
6. Ha CH, Lim KH, Jang SH, Yun CW, Paik HD, Kim SW, Kang CW, Chang HI. Immune-enhancing alkali-soluble glucans produced by wild-type and mutant *Saccharomyces cerevisiae*. J. Microbiol. Biotechn. 16: 576-583 (2006)
7. Zekovic DB, Kwiatkowski S, Vrvic MM, Jakovljevic D, Moran CA. Natural and modified (1→3)-beta-D-glucans in health promotion and disease alleviation. Crit. Rev. Biotechnol. 25: 205-230 (2005)
8. Ljungman AG, Leanderson P, Tagesson C. (1→3)-Beta-D-glucan stimulates nitric oxide generation and cytokine mRNA expression in macrophages. Environ. Toxicol. Pharm. 5: 273-281 (1998)
9. Krizkova L, Durackova Z, Sandula J, Slamenova D, Sasinkova V, Sivonova M, Krajcovic J. Fungal β-(1-3)-D-glucan derivatives exhibit high antioxidative and antimutagenic activity *in vitro*. Anticancer Res. 23: 2751-2756 (2003)
10. Babincova M, Bacova Z, Machova E, Kogan G. Antioxidant properties of carboxymethyl glucan: Comparative analysis. J. Med. Food 5: 79-83 (2002)
11. Shen SC, Lee WR, Lin HY, Huang HC, Ko CH, Yang LL, Chen YC. *In vitro* and *in vivo* inhibitory activities of rutin, wogonin, and quercetin on lipopolysaccharide-induced nitric oxide and prostaglandin E (2) production. Eur. J. Pharmacol. 446: 187-194 (2002)
12. Stolz D. Peroxisomal localization of inducible nitric oxide synthase in hepatocytes. Hepatology 36: 81-93 (2002)
13. Park JY, Park CM, Kin JJ, Noh KH, Cho CW, Song YS. The protective effect of chlorophyll a against oxidative stress and inflammatory processes in LPS-stimulated macrophages. Food Sci. Biotechnol. 16: 205-211 (2007)
14. Cho HY, Park JY, Kim JK, Noh KH, Moon GS, Kin JI, Song YS. Quercetin ameliorates NO production via down-regulation of iNOS expression, NFκB activation, and oxidative stress in LPS-stimulated macrophage. Food Sci. Biotechnol. 14: 200-206 (2005)
15. Iqbal M, Cohen RI, Marzouk K, Liu SF. Time course of nitric oxide, peroxynitrite, and antioxidants in the endotoxemic heart. Crit. Care Med. 30: 1291-1296 (2002)
16. Berendji D, Kolb-Bachofen V, Meyer KL, Kroncke KD. Influence of nitric oxide on the intracellular reduced glutathione pool: Different cellular capacities and strategies to encounter nitric oxide-mediated stress. Free Radical Bio. Med. 27: 773-780 (1999)
17. Li N, Karin M. Is NF-kappa B the sensor of oxidative stress? FASEB J. 13: 1137-1143 (1999)
18. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. J. Biol. Chem. 273: 13245-13254 (1998)
19. Bonizzi G, Piette J, Schoonbroodt S, Greimers R, Havard L, Merville MP, Bours V. Reactive oxygen intermediate-dependent NF-kappaB activation by interleukin-1β requires 5-lipoxygenase or NADPH oxidase activity. Mol. Cell Biol. 19: 1950-1960 (1999)
20. Choi SY, Hwang JH, Ko HC, Park SY, Kim GO, Kim SY, Chang IS, Kwon HM, Kim SJ. Inhibitory action of *Tsunokaori Tangor* peel on the lipopolysaccharide-induced inflammatory response in RAW 264.7 macrophage cells. Food Sci. Biotechnol. 15: 270-276 (2006)
21. Nunokawa Y, Oikawa S, Tanaka S. Human inducible nitric oxide synthase gene is transcriptionally regulated by nuclear factor-kappaB dependent mechanism. Biochem. Bioph. Res. Co. 223: 347-352 (1996)
22. Lee AK, Sung SH, Kim YC, Kim SG. Inhibition of lipopoly-saccharide-inducible nitric oxide synthase, TNF- and COX-2 expression by suchinone effects on I-B phosphorylation, C/EBP and AP-1 activation. Brit. J. Pharmacol. 139: 11-20 (2003)
23. Lo AH, Liang YC, Lin-Shiau SY, Ho CT, Lin JK. Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-κB in mouse macrophages. Carcinogenesis 23: 983-991 (2002)
24. Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochem. Pharmacol. 58: 759-765 (1999)
25. Fautz R, Husein B, Hechenberger C. Application of the neutral red assay (NR assay) to monolayer cultures of primary hepatocytes: Rapid colorimetric viability determination for the unscheduled DNA synthesis test (UDS). Mutat. Res. 253: 173-179 (1991)
26. D'Agostino P, Ferlazzo V, Milano S, La Rosa M, Di Bella G, Caruso R, Barbera C, Grimaudo S, Tolomeo M, Feo S, Cillari E. Anti-inflammatory effects of chemically modified tetracyclines by the inhibition of nitric oxide and interleukin-12 synthesis in J774 cell line. Int. Immunopharmacol. 1: 1765-1776 (2001)
27. Fraga CG, Leibovita RM, Roeder RG. Lipid peroxidation measured as thiobarbituric-reactive substances in tissue slices: Characterization and comparision with homogenates and microsomes. Free Radical Bio. Med. 4: 155-161 (1988)
28. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidaized glutathione: Applications to mammalian blood and other tissue. Anal. Biochem. 27: 502-522 (1969)
29. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Ann. Biochem. 72: 248-254 (1976)
30. Marklund S, Marklund G. Involvement of the superoxide anion radical in antioxidant of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47: 469-474 (1974)
31. Aebi H. Catalase *in vitro*. Method Enzymol. 105: 121-126 (1984)
32. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem. Bioph. Res. Co. 71: 952-958 (1976)
33. Inger C, Bengt M. Methods in Enzymology. Academic Press Inc., Orlando, FL, USA. pp. 484-490 (1985)
34. Katsuyama K, Shichiri M, Marumo F, Hirata Y. NO inhibits cytokine-induced iNOS expression and NF-κB activation by interfering with phosphorylation and degradation of IκB-α. Arterioscl. Throm. Vas. Biol. 18: 1795-1802 (1998)
35. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinum thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159 (1987)
36. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract isolated from mammalian nuclei. Nucleic Acids Res. 11: 1475-1489 (1983)
37. Duncan DB. Multiple range tests for correlated and heteroscedastic means. Biometrics 13:164-176 (1957)
38. Ippouchi K, Itou H, Azuma K, Higashio H. Effect of naturally occurring organosulfur compounds on nitric oxide production in lipopolysaccharide-activated macrophages. Life Sci. 71: 411-419 (2002)
39. Wang J, Mazza G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-gamma-activated RAW264.7 macrophages. J. Agr. Food Chem. 50: 850-857 (2002)
40. Han MK, Lee JW, Jeong H, Kim YS, Ra SJ, Yoon KH. Nitric oxide, TNF-α, and TGF-β formation of rat kupffer cell activated by the β-glucan from *Ganoderma lucidum*. Korean J. Appl. Microbiol. Biotechnol. 57: 28-34 (1999)
41. Lee JW, Baek SJ, Bang KW, Kang SW, Kang SM, Kim BY, Ha IS. Biological activities of polysaccharide extracted from the fruit body and cultured mycelia *Phellinus linteus* IY001. Korean J. Food Sci. Technol. 32: 726-736 (2000)
42. Park JH, Kang MS, Kim HI, Chung BH, Lee KH, Moon WK. Study on immuno-stimulating of β-glucan isolated from the cell wall of yeast mutant *Saccharomyces cerevisiae* IS2. Korean J. Food Sci. Technol. 35: 488-492 (2003)
43. Kim HN, Lee JN, Kim GE, Lee YMA, Kim CW, Sohn JW. Comparative study of immune-enhancing activity of crude and mannoprotein-free yeast-glucan preparations. J. Microbiol. Biotechn. 9: 826-831 (1999)

44. Park HJ, Kim YB, Kang TS, Jung IS, Kim KY, Jeong HS. Immunomodulatory activities of oat bran extracts with different extraction conditions. Korean J. Food Sci. Technol. 37: 103-107 (2005)
45. Gitto E, Karbownik M, Reiter RJ, Tan D, Cuzzocrea S, Chiurazzi P, Cordaro S, Corona G, Trimarchi G, Barberi I. Effects of melatonin treatment in septic newborns. Pediatr. Res. 50: 756-760 (2001)
46. Garcia JJ, Reiter RJ, Guerrero JM, Escames G, Yu BP, Oh CS, Munoz-Hoyos A. Melatonin prevents changes in microsomal membrane fluidity during induced lipid peroxidation. FEBS Lett. 408: 297-300 (1997)
47. Ross D. Glutathione, free radicals, and chemotherapeutic agents. Mechanisms of free-radical induced toxicity and glutathione-dependent protection. Pharmacol. Therapeut. 37: 231-249 (1988)
48. Szabo S, Nagy L, Plebani M. Glutathione, protein sulfhydryls, and cysteine proteases in gastric mucosal injury and protection. Clin. Chim. Acta 206: 95-105 (1992)
49. Ide N, Lau BHS. Garlic compounds minimize intracellular oxidative stress and inhibit nuclear factor-kappa B nuclear factor- activation. J. Nutr. 131: 1020S-1026S (2001)
50. Yen GC, Lai HH. Inhibitory effects of isoflavones on nitric oxide-or peroxynitrite-mediated DNA damage in RAW 264.7 cells and phiX174 DNA. Food Chem. Toxicol. 40: 1433-1440 (2002)
51. Moellering D, Mc Andrew J, Patel RP, Forman HJ, Mulcahy RT, Jo H, Darley-Usmar VM. The induction of GSH synthesis by nanomolar concentrations of NO in endothelial cells: A role for gamma-glutamylcysteine synthetase and gamma-glutamyl transpeptidase. FEBS Lett. 448: 292-296 (1999)
52. Ben-Shaul V, Lomnitski L, Nyska A, Zurovsky Y, Bergman M, Grossman S. The effect of natural antioxidants, NAO, and apocynin, on oxidative stress in the rat heart following LPS challenge. Toxicol Lett. 123: 1-10 (2001)
53. Watson AM, Warren G, Howard G, Shedlofsky SI, Blouin RA. Activities of conjugating and antioxidant enzymes following endotoxin exposure. J. Biochem. Mol. Toxic. 13: 63-69 (1993)
54. Sener G, Sert G, Ozer Sehirli A, Arbak S, Uslu B, Gedik N, Ayanoglu-Dulger G. Pressure ulcer-induced oxidative organ injury is ameliorated by beta-glucan treatment in rats. Int. Immunopharmacol. 6: 724-732 (2006)
55. Sener G, Eksioglu-Demiralp E, Cetiner M, Ercan F, Yegen BC. Beta-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects. Eur. J. Pharmacol. 542: 170-178 (2006)
56. Willment JA, Marshall AS, Reid DM, Williams DL, Wong SY, Gordon S, Brown GD. The human beta-glucan receptor is widely expressed and functionally equivalent to murine dectin-1 on primary cells. Eur. J. Immunol. 35: 1539-1547 (2005)