Inhibitory effect of sulphated polysaccharide porphyran on nitric oxide production in lipopolysaccharide-stimulated RAW264.7 macrophages

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Porphyran, extracted from an edible red alga (*Porphyra yezoensis*), is a sulphated polysaccharide with a wide variety of biological activities including anti-tumour, antioxidant and immuno-modulating activities. In this study, we examined the effect of porphyran on nitric oxide (NO) production in mouse macrophage cell line RAW264.7 cells. Although no significant activity of porphyran to induce NO or tumour necrosis factor-α (TNF-α) production in RAW264.7 cells was observed at the concentration range tested (10–500 μg/ml), it was found for the first time that porphyran inhibited NO production and expression of inducible nitric oxide synthase (iNOS) in RAW264.7 cells stimulated with lipopolysaccharide (LPS). In the presence of 500 μg/ml porphyran, NO production and expression of iNOS in LPS-treated RAW264.7 cells were completely suppressed. On the other hand, porphyran showed only a marginal effect on the secretion of TNF-α from LPS-stimulated RAW264.7 cells. Electrophoretic mobility shift assay (EMSA) using infrared dye labelled oligonucleotide with nuclear factor-κB (NF-κB) consensus sequence suggested that porphyran inhibited the LPS-induced NF-κB activation. The LPS-inducible nuclear translocation of p65, and the phosphorylation and degradation of IκB-α were also inhibited by the pre-treatment with porphyran. Our results obtained in *in vitro* analysis suggest that porphyran suppresses NO production in LPS-stimulated macrophages by the blocking of NF-κB activation.

Keywords: iNOS/nitric oxide/NF-κB/porphyran/sulphated polysaccharide.

Abbreviations: DMEM, Dulbecco's modified Eagle's minimum essential medium; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; PBS, phosphate-buffered saline; RT-PCR, reserve transcription-polymerase chain reaction; TNF-α, tumour necrosis factor-α.

Many marine resources, especially marine algal polysaccharides such as alginate, fucoidan, carrageenan and agarose, have recently drawn a great attention from diverse research fields to develop new drugs and health foods or supplements. A number of studies have demonstrated that various polysaccharides from marine algae have various bioactivities including anti-tumour and immuno-modulatory activities. Among these polysaccharides, there have been extensive studies on the biological activities of fucoidan, a fucose-containing sulphated polysaccharide with particular focus on its immuno-modulatory actions. For instance, it has been reported that fucoidan induces nitric oxide (NO) production in RAW264.7 cells through p38 mitogen-activated protein kinase (MAP kinase) and nuclear factor-kappa B (NF-κB)-dependent signal transduction *via* macrophage scavenger receptors (1). However, another study found that blocking of macrophage scavenger receptors did not result in the inhibition of the fucoidan activity, suggesting that such receptor is not involved in fucoidan action (2). Recent studies have reported that fucoidan inhibits the release of NO from lipopolysaccharide (LPS)-stimulated RAW264.7 cells (3, 4). Thus, the precise action mechanism of fucoidan in terms of NO production in stimulated macrophages remains a matter of debate.

Porphyra species are the important edible red algae abundantly cultivated in East and Southeast Asia including Japan, and are commonly known as 'nori' and traditionally used to prepare sushi. Porphyran, one of the main constituents of *Porphyra yezoensis*, related to agarose, is a linear sulphated polysaccharide comprising the hot-water soluble portion of the cell wall and intracellular matrix (5–7). It is constituted of D-galactose, 3, 6-anhydro-L-galactose, 6-O-methyl-D-galactose and L-galactose-6-sulphate. In addition to the health benefit as a major dietary fiber in 'nori', previous studies have reported that porphyran also has diverse physiological activities including anti-tumour, immuno-modulating, antioxidant, anti-hyperlipidemic and hypercholesterolemic activities (8–12).

Although several studies on the bioactivities of porphyran have been conducted, it has not been studied yet whether porphyran affects the formation of NO in LPS-stimulated macrophages. Thus, in this study, we examined the effect of porphyran on NO production in LPS-stimulated RAW264.7 cells. Unexpectedly, we found that porphyran inhibited NO production and the expression of inducible nitric oxide synthase (iNOS) by blocking NF-κB activation in LPS-stimulated RAW264.7 cells.

Materials and Methods

Materials
LPS from *Escherichia coli* 0111: B4 (purified by phenol extraction), fucoidan (from *Fucus vesiculosus*) and custom oligonucleotides (iNOS and β-actin primers) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Alamar blue cell counting reagent was purchased from Invitrogen, Ltd. (Paisley PA4 9RF, UK). Sulphanilic acid, *N*-1-naphthyl-ethylenediamine dihydrochloride, glacial acetic acid and 5-methylphenazium-methyl sulphate (PMS) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NF-κB SN50 (cell-permeable inhibitor peptide) and NF-κB SN50M (cell-permeable inactive control peptide) were purchased from Calbiochem (La Jolla, CA, USA). PrimeScript® first strand cDNA synthesis kit for reserve transcription-polymerase chain reaction (RT-PCR) was purchased from TaKaRa Bio Inc. (Otsu, Shiga, Japan). GoTag Green Master Mix was purchased from Promega KK. (Tokyo, Japan). Rabbit anti-mouse iNOS antibody and goat anti-rabbit IgG-horseradish peroxidase conjugated secondary antibody were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit anti-mouse β-actin antibody was obtained from Abcam Inc. (Cambridge, USA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG and enhanced chemiluminescence western blotting detection reagents were obtained from Amersham Biosciences UK Ltd. (Buckinghamshire HP7 9NA, UK). BCA protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA, USA). Anti-IκB-α, anti-c-Rel (p65) and anti-phospho-IκB-α antibodies were obtained from Cell Signaling Thechnology, Inc. (Beverly, MA, USA). Sepasol-RNA I Super, protease inhibitor cocktail, nitro blue tetrazolium (NBT) and nicotinamide adenine dinucleotide (NADH) were obtained from Nacalai Tesque Co. (Kyoto, Japan). Tumour necrosis factor-α (TNF-α) capture antibody and anti-mouse TNF-α monoclonal antibody were purchased from Endogen, Inc. (Boston, MA, USA). NF-κB consensus IRDye® 700 infrared dye labelled oligonucleotides probe was purchased from LI-COR Bioscience (Lincoln, NE USA). Other chemicals were of the highest grade commercially available.

Preparation of porphyran
Porphyran was prepared from nori (*P. yezoensis*) as reported previously (*13*). In brief, dry sheets (3 g) of nori were homogenized with 500 ml of 85% ethanol and heated at 75°C with constant stirring for 1 h, and then filtered to remove 85% ethanol-soluble substances. This extraction was repeated again. The residue was washed with methanol and dried to obtain 2 g of partially decolourized powder. The decolourized powder was extracted with 1,000 ml of distilled water at 95°C with constant stirring for 1.5 h and centrifuged at 8 000g for 20 min, and then the supernatant was concentrated to 600 ml by using a rotary evaporator, and then digested with 50 units of nuclease P1 at 37°C for 24 h. After that, the mixture was heated for 5 min in the boiling water bath, and centrifuged to remove the precipitates. To the supernatant, sodium acetate and acetic acid were added to make a 0.5 M sodium acetate solution, pH 5.0, and then ethanol was added to the concentration of 42% (v/v). After 1 h, the precipitates in the solution were removed by centrifugation, and ethanol was added to the supernatant to the concentration of 60%. The precipitate was dissolved in distilled water, dialysed against distilled water and lyophilized to obtain 0.4 g of porphyran. Before use, porphyran solution was filtered through an endotoxin-removing filter (Zetapor Dispo filter) purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan).

Cell culture
RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured in CO$_2$ (5%) incubator at 37°C in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml), which was used as the growth medium throughout the experiments otherwise specified.

Cytotoxicity assay
Cytotoxicities of polysaccharide samples were measured by the Alamar blue assay as described previously (*14*). In brief, adherent RAW264.7 cells in 96-well plates (3×10^4 cells/well) were treated with varying concentrations of porphyran or fucoidan for 24 h in the growth medium, and then Alamar blue reagent was added to the cells at a final concentration of 10%. After 2 h incubation at 37°C, the absorbance of each well was measured at 570 and 600 nm using a multiwell scanning spectrophotometer (Thermo Electron Co., Yokohama, Japan).

Measurement of DNA fragmentation
Adherent RAW264.7 cells in 35 mm-diametre culture dishes (2×10^6 cells/dish) were treated with 500 μg/ml of porphyran or fucoidan in the growth medium at 37°C for 24 h. After removal of the medium, the cells were lysed in 300 μl of ice-cold lysis buffer containing 0.5% Triton X-100, 10 mM Tris-HCl pH 8.0 and 20 mM EDTA. The cell lysates were centrifuged for 20 min at 15,000g to separate DNA fragments (supernatant) from intact DNA (pellet). The DNA contents in supernatant and pellet fractions were measured using the diphenylamine reagent, and the extent of DNA fragmentation was estimated as described previously (*15*).

Nitrite assay for the estimation of NO
Nitrite, a stable reaction product of NO with O$_2$, in the supernatants from each treated RAW264.7 cells, was determined by the method based on Griess assay as described previously (*16*). In brief, adherent RAW264.7 cells in 96-well plates (3×10^4 cells/well) were treated with varying concentrations of porphyran (0−500 μg/ml) for 1 h in the growth medium, and then LPS was added at the final concentration of 2 ng/ml. After 18 h incubation at 37°C, Griess reagent (100 μl: 3 mM sulphanilic acid and 30 μM *N*-1-naphthyl-ethylenediamine dihydrochloride and 25% glacial acetic acid) was added to 50 μl of cultured supernatant. After 20 min incubation at room temperature, the optical density was measured at 540 nm using a multiwell scanning spectrophotometer (Thermo electron Co., Yokohama, Japan). Calibration curve was made with known concentration of NaNO$_2$ standard solution.

Isolation of RNA and RT-PCR for the detection of iNOS mRNA
Adherent RAW264.7 cells in 24-well plates (5×10^5 cells/well) were pre-treated with porphyran at the concentrations of 0, 250 and 500 μg/ml for 1 h in the growth medium, and then incubated with or without LPS at the final concentration of 2 ng/ml. After 4 h incubation, total RNA of each treated cells was isolated using Sepasol-RNA I Super. Total RNA (2.5 μg) was reverse transcribed with an oligo dT primer in a 10 μl using PrimeScript® first strand cDNA synthesis kit according to the manufacturer's instruction. PCR was performed with 1 cycle of 70 s at 95°C, 25 cycles of 55 s at 93°C, 45 s at 61°C, 40 s at 72°C and 1 cycle of 100 s at 72°C in a 25 μl reaction mixture containing 12.5 μl of GoTag Green Master Mix, 0.5 μl of forward and reverse iNOS primers (1 μM each) or β-actin primers (10 pM each), 0.5 μl of first strand cDNA and 11 μl nuclease-free water. The primer sequences were 5′-CAACCA GTATTATGGCTCCT-3′ (forward) and 5′-GTGACAGCCCGGT CTTTCCA-3′ (reverse) for mouse iNOS (*17*) and, 5′-GGAGAAGA TCTGGCACCACACC-3′ (forward) and 5′-CCTGCTTGCTGATC CACATCTGCTGG-3′ (reverse) for mouse β-actin (*18*). The β-actin primer was used as an internal control. Each PCR reaction (10 μl) product was run on 2% agarose gels containing 0.1 μg/ml ethidium bromide, and the amplified products (835 bp for iNOS and 840 bp for β-actin) were detected by a Light capture (ATTO Co., Tokyo, Japan).

Western blot analysis

Analysis of iNOS was performed on whole-cell extracts. Adherent RAW264.7 cells in 35 mm-diametre culture dishes (2×10^6 cells/dish) were pre-treated with 0, 250 or 500 µg/ml porphyran for 1 h in the growth medium. After 5 h incubation with LPS (final 2 ng/ml), the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed with 100 µl extraction buffer (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1% CHAPS and 1% Triton X-100) containing 1% (v/v) protease inhibitor cocktail. After shaking for 30 min at 4°C, the extracts were obtained from the supernatant after centrifugation at 15,000g for 10 min, and the protein concentrations were measured with the BCA protein assay (Sigma) using bovine serum albumin as a standard. The extract was mixed with equal volume of $2 \times$ SDS-sample buffer and incubated at 100°C for 5 min. Samples containing 20 µg of protein were applied on 8% SDS-PAGE, and then electrically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 1% skim milk in TBS-0.1% Tween-20 (TBST). Immunostaining of the blot was performed with the specific antibody against mouse iNOS and β-actin. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody. The blots were detected using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). The analysis of IκB-α and phospho-IκB-α in cytosolic extracts and NF-κB p65 in nuclear extracts were performed, respectively. Adherent RAW264.7 cells (2×10^6 cells/dish) were incubated in serum-free DMEM for 2 h at 37°C, and then porphyran (0, 250 or 500 µg/ml) was added to the cells and incubated for 1 h. After 30 min incubation with LPS (final 2 ng/ml), the cells were washed three times with ice-cold PBS and incubated with 100 µl ice-cold cytosol extraction buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.2% Igepal CA-630, 1 mM dithiothreitol, 20 mM β-glycerophosphate, 1 mM sodium orthoranadate, 0.5 mM phenylmethysulphonyl fluoride, 1 µg/ml leupeptin and 1 µg/ml aprotinin) for 25 min on ice. The cytosolic extracs were collected after centrifugation at 7 000g for 5 min at 4°C. The nuclear pellets were re-suspended in 30 µl ice-cold nuclear extraction buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 0.45 M NaCl, 25% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethysulphonyl fluoride, 1 µg/ml leupeptin and 1 µg/ml aprotinin) and incubated on ice for 25 min, and the nuclear extracts were obtained after centrifugation at 15,000g for 10 min at 4°C. After measuring the protein concentrations, the extract was mixed with equal volume of $2\times$ SDS-sample buffer and incubated at 100°C for 5 min. Samples containing 20 µg of protein were applied on 12.5% SDS-PAGE, and then electrically transferred to a PVDF membrane. The western blot analyses of the cytosolic and nuclear extracts were conducted by the similar way as described above except using specific anti-NF-κB p65, anti-IκB-α and anti-phospho-IκB-α antibody.

Enzyme-linked immunosorbent assay

Adherent RAW264.7 cells in 24-well plates (5×10^5 cells/well) were treated with porphyran at the concentrations of 0, 250 and 500 µg/ml for 1 h at 37°C in the growth medium. After further 4 h incubation with LPS (final 2 ng/ml), the levels of TNF-α in culture supernatants of treated cells were measured by sandwich enzyme-linked immunosorbent assay (ELISA) with two antibodies to two different epitopes on TNF-α molecule by similar method as described previously (19). The TNF-α concentrations were estimated from a reference to a standard curve for serial two-fold dilution of murine recombinant TNF-α.

Electrophoretic mobility shift assay

Adherent RAW264.7 cells in 35 mm-diametre culture dishes (2×10^6 cells/dish) were incubated in serum-free DMEM for 2 h at 37°C, and then porphyran (0, 250 or 500 µg/ml) was added to the cells and incubated for 1 h. After further 30 min incubation with LPS (final 2 ng/ml), the nuclear proteins were extracted from the cells as described (20). Electrophoretic mobility shift assay (EMSA) were carried out with Odyssey® IRDye® 700 infrared dye labelled double-stranded oligonucleotides coupled with the EMSA buffer kit (LI-COR Bioscience, Lincoln, NE USA) according to manufacturer's instructions. Briefly, 5 µg of nuclear extract was incubated with 1 µl of IRDye® 700 infrared dye labelled double-stranded oligonucleotides, 2 µl of $10 \times$ binding buffer, 2.5 mM DTT, 0.25% Tween-20 and 1 µg of poly (dI-dC) in a total volume of 20 µl for 20 min at room temperature in the dark. Sample proteins were separated on a 4% polyacrylamide gel in $0.25 \times$ Tris-borate-EDTA running buffer for 60 min at 100 V. The gel was scanned by direct infrared fluorescence detection on the Odyssey® imaging system (LI-COR Bioscience, Lincoln, NE USA). NF-κB IRDye® 700 infrared dye labelled oligonucleotide sequences of the double-stranded DNA probes used were as follows: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′ (21, 22). The underlined nucleotides indicate the binding sites for NF-κB. The specificity of the binding was examined using competition experiments, where 100-fold excess of the unlabelled oligonucleotides were added to the reaction mixture before adding the infrared dye labelled oligonucleotide.

Superoxide radical-scavenging activity

The superoxide radical-scavenging activity of porphyran was assessed by the method described previously (23). For the assay, 0.5 µl of 30 mM NBT and 0.5 µl of 46.8 mM NADH in 16 mM Tris-HCl buffer (pH 8.0) were added to 100 µl of sample solution in 16 mM Tris-HCl buffer (pH 8.0) (final 10–1,000 µg/ml). The reaction was initiated by adding 0.5 µl of 6 mM 5-methylphenazium-methyl sulphate (PMS) to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance was measured at 560 nm by a spectrophotometer. Decrease in absorbance of the reaction mixture reflected increased superoxide radical-scavenging activity. The superoxide radical-scavenging activity was calculated using following equation:

$$O_2^- \text{ scavenging activity } (\%) = (1 - A\text{sample}/A\text{control}) \times 100$$

where A_{sample} is the absorbance in the presence of sample and $A_{control}$ is the absorbance of Tris-HCl buffer alone without sample.

Statistical analysis

All the experiments were repeated at least three times. Data were expressed as means $\pm$ SD and analysed using one-way analysis of variance (ANOVA) followed by a Dunnett's test to determine any significant differences. A value of $P < 0.05$ was considered statistically significant.

Results

Cytotoxicities of porphyran and fucoidan on RAW264.7 cells

Since it has been reported that porphyran inhibits cell proliferation and induces apoptosis in AGS human gastric cancer cells (9), we first examined the cytotoxicity of porphyran on RAW264.7 cells. After 24 h incubation with varying concentrations of porphyran, the viabilities of the cells were determined by the Alamar blue assay. As shown in Fig. 1A, porphyran showed no cytotoxic effect on RAW264.7 cells up to 500 µg/ml. In contrast to the lack of significant cytotoxic effect of porphyran to RAW264.7 cells, fucoidan, a sulphated polysaccharide, showed cytotoxicity to RAW264.7 cells in a concentration-dependent manner at the same concentration range tested. DNA fragmentation (Fig. 1B) and morphological changes (Fig. 1C) were also induced in the cells treated with 500 µg/ml fucoidan, suggesting that the cytotoxicity of fucoidan was accompanied by induction of apoptosis, while no such significant apoptosis-related changes were observed in porphyran-treated RAW264.7 cells (Fig. 1). Therefore, it seems likely that porphyran and fucoidan are quite different in terms of cytotoxicity to RAW264.7 cells. Hence, it is considered that cellular toxicity of porphyran on RAW264.7 may not be a main reason for any observed changes in NO production or related events in the following studies.
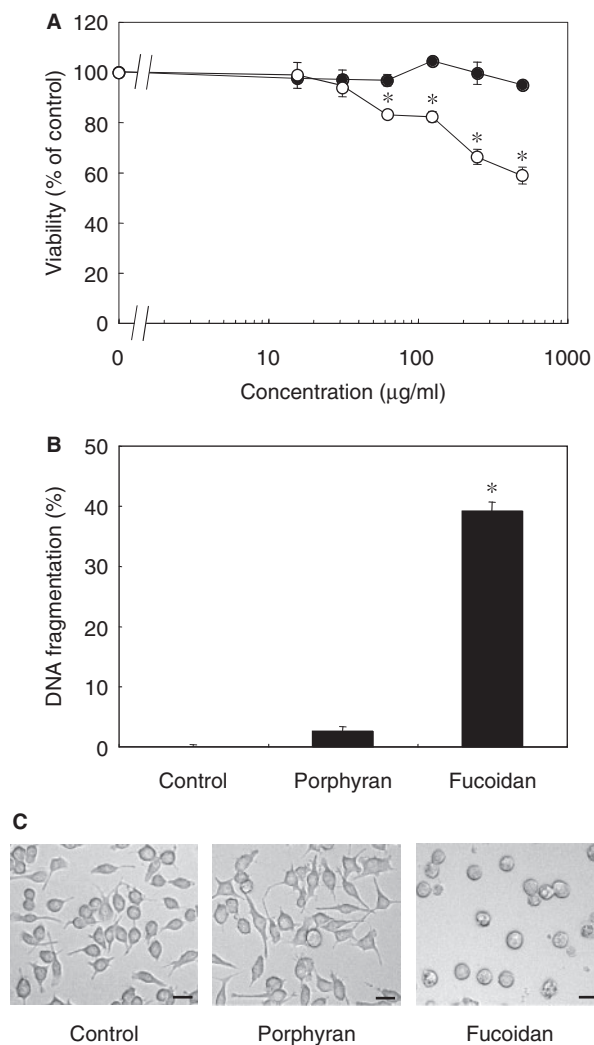
Fig. 1 Cytotoxic effects of porphyran and fucoidan on RAW264.7 cells. (A) Adherent cells (3×10^4 cells/well in 96-well plates) in the growth medium were treated with varying concentrations of porphyran (filled circle) or fucoidan (open circle) at 37°C. After 24 h, the cell viabilities were estimated by Alamar blue assay as described under 'Materials and Methods' section. Data represent means ± SD of triplicate measurements. Asterisks indicate significant differences between with and without fucoidan ($P < 0.05$). (B) Adherent RAW264.7 cells in 35-mm diametre culture dishes (2×10^6 cells/dish) were treated with 500 μg/ml of fucoidan or porphyran in the growth medium at 37°C. After 24 h, DNA fragmentations of the treated cells were examined by diphenylamine assay as described under 'Materials and Methods' section. Asterisk indicates significant difference between with and without fucoidan ($P < 0.05$). (C) Adherent RAW264.7 cells in 35-mm diametre culture dishes equipped with quartz glass were treated with 500 μg/ml of fucoidan or porphyran in the growth medium at 37°C. After 24 h, the cells were observed under phase contrast microscope (BIOREVO BZ9000, KEYENCE Co., Osaka, Japan). The bar indicates 20 μm.

Effect of porphyran on NO production in LPS-treated RAW264.7 cells

Because of the extremely short half-life of NO, we measured nitrite as a stable biomarker of NO production in LPS-stimulated RAW264.7 cells by Griess assay. Our preliminary study demonstrated that LPS potently induced NO production in RAW264.7 cells, even at a very low concentration (1 ng/ml). Time-course analysis showed that NO production was
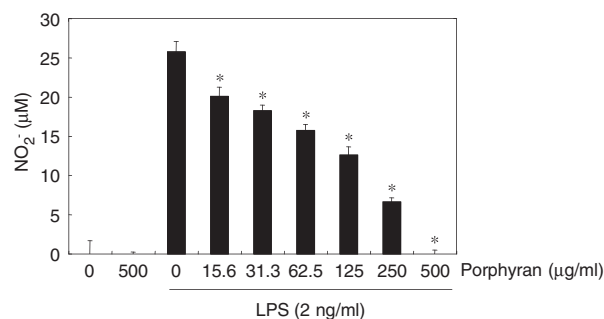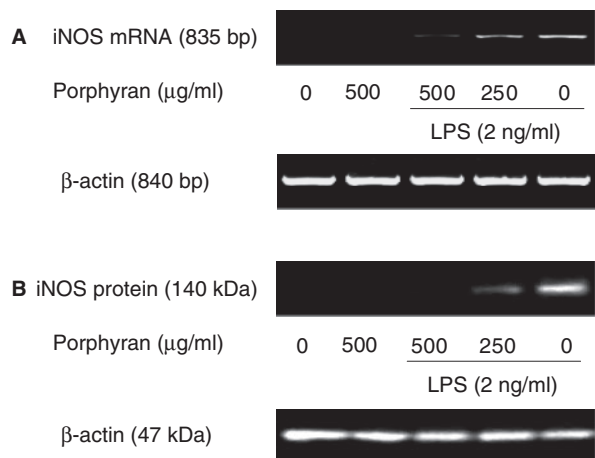

Fig. 2 Effects of porphyran on NO production in LPS-treated RAW264.7 cells. Adherent cells (3×10^4 cells/well in 96-well plates) were pre-incubated with various concentrations of porphyran (0−500 μg/ml) in DMEM supplemented with 10% FBS at 37°C for 1 h, followed by the addition of LPS (final 2 ng/ml). After 18 h incubation, the NO levels in the supernatants of the treated cells were estimated as described under 'Materials and Methods' section. Data represent means ± SD of triplicate measurements. Asterisks indicate significant differences between with and without porphyran ($P < 0.05$).

initiated at 6 h and reached a stable and maximum level at 18 h, and the level continued to 24 h after stimulation with LPS (data not shown). Thus, in subsequent experiments, we evaluated nitrite concentration at 18 h. As shown in Fig. 2, nitrite level was almost undetectable in RAW264.7 cells treated with 500 μg/ml porphyran alone, and porphyran did not induce significant levels of NO at any other concentrations (1−1 000 μg/ml) tested in RAW264.7 cells (data not shown).

To examine whether porphyran affects LPS-induced NO production in RAW264.7 cells, cells were pre-incubated with porphyran (0−500 μg/ml) for 1 h in the growth medium, followed by stimulation with LPS (2 ng/ml). After 18 h incubation, the nitrite levels of the supernatants of the treated cells were measured by Griess assay. As shown in Fig. 2, porphyran inhibited NO production in LPS-stimulated RAW264.7 cells in a concentration-dependent manner, with 500 μg/ml porphyran completely blocking the LPS-inducible NO production. Considering that 500 μg/ml porphyran alone did not affect NO production and was not cytotoxic to RAW264.7 cells (Fig. 1), it seems obvious that inhibition of LPS-induced NO production in RAW264.7 cells by porphyran is unrelated to its any cytotoxic effects.

Effect of porphyran on expression of iNOS in LPS-treated RAW264.7 cells

To study whether the inhibition of NO production by porphyran is due to the inhibition of expression of inducible NO synthase (iNOS), which catalyses the generation of NO from L-arginine, RT-PCR and immunoblot analysis were performed to detect the levels of iNOS mRNA and protein expression, respectively. As shown in Fig. 3, treatment of cells with 2 ng/ml LPS resulted in increased expression of both iNOS mRNA and protein, while iNOS expression was not detected in RAW264.7 cells treated with control normal medium or 500 μg/ml porphyran alone. Pre-treatment of cells with porphyran, especially at

A iNOS mRNA (835 bp)

Porphyran (µg/ml)   0   500   500   250   0

LPS (2 ng/ml)

β-actin (840 bp)

B iNOS protein (140 kDa)

Porphyran (µg/ml)   0   500   500   250   0

LPS (2 ng/ml)

β-actin (47 kDa)

Fig. 3 Effects of porphyran on LPS-induced iNOS mRNA and iNOS protein levels in RAW264.7 cells. (A) Adherent cells (5×10^5 cells/well in 24-well plates) were pre-incubated with various concentrations of porphyran (0, 250 or 500 µg/ml) in DMEM supplemented with 10% FBS at 37°C for 1 h, followed by the addition of LPS (final 2 ng/ml). After 4 h incubation, the total RNA of each treated cells was subjected to RT-PCR analysis as described under 'Materials and Methods' section. (B) Adherent cells in 35 mm-diametre culture dishes (2×10^6 cells/dish) were pre-incubated with various concentrations of porphyran (0, 250 or 500 µg/ml) in DMEM supplemented with 10% FBS at 37°C for 1 h followed by the addition of LPS (final 2 ng/ml). After 5 h incubation, the whole-cell lysates were analysed by western blot analysis as described under 'Materials and Methods' section.

500 µg/ml, inhibited iNOS expression by nearly 100% at the transcription and translation levels in LPS-stimulated RAW264.7 cells.
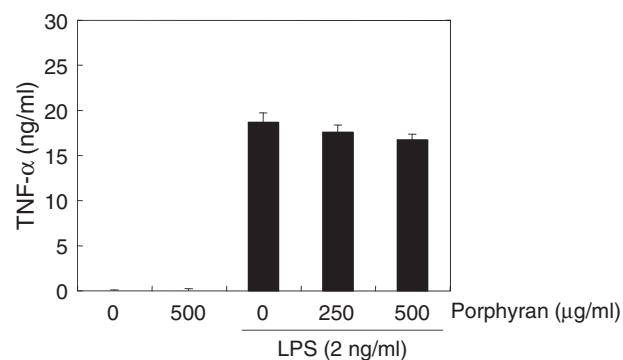
Effect of porphyran on TNF-α secretion in LPS-treated RAW264.7 cells

LPS activates various intracellular signalling cascades leading to the secretion of various cytokines in addition to the induction of NO production *via* iNOS expression. In fact, increased level of TNF-α was detected in LPS-treated RAW264.7 cells (Fig. 4). We next investigated whether porphyran can also affect the secretion of TNF-α from LPS-stimulated RAW264.7 cells. As shown in Fig. 4, porphyran showed only a marginal inhibitory effect on the TNF-α production in LPS-treated RAW264.7 cells even at 500 µg/ml, at which NO production was completely inhibited, though.

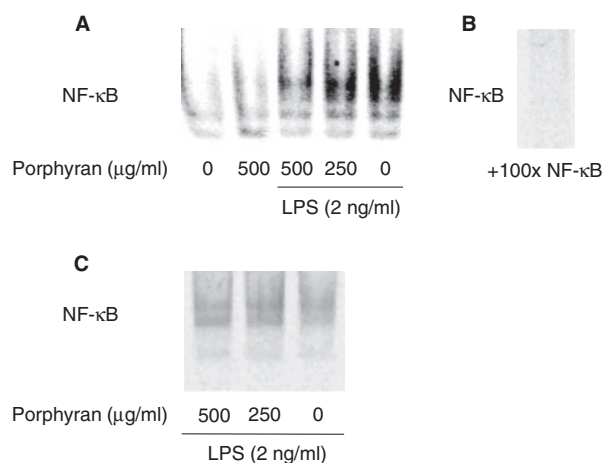### Effect of porphyran on LPS-induced NF-κB activation in RAW264.7 cells

NF-κB is an essential transcription factor for the induction of several inflammatory mediators including iNOS (24, 25). Therefore, an EMSA on the nuclear extracts from LPS-stimulated RAW264.7 cells was performed using IRDye® 700 infrared dye labelled oligonucleotide with NF-κB consensus sequence to examine whether porphyran-caused inhibition of iNOS induction is due to the suppression of NF-κB activation. As shown in Fig. 5A, increase in band thickness of the slow migrating nucleoprotein complex was observed after the LPS treatment for 30 min, suggesting that NF-κB was activated by LPS.
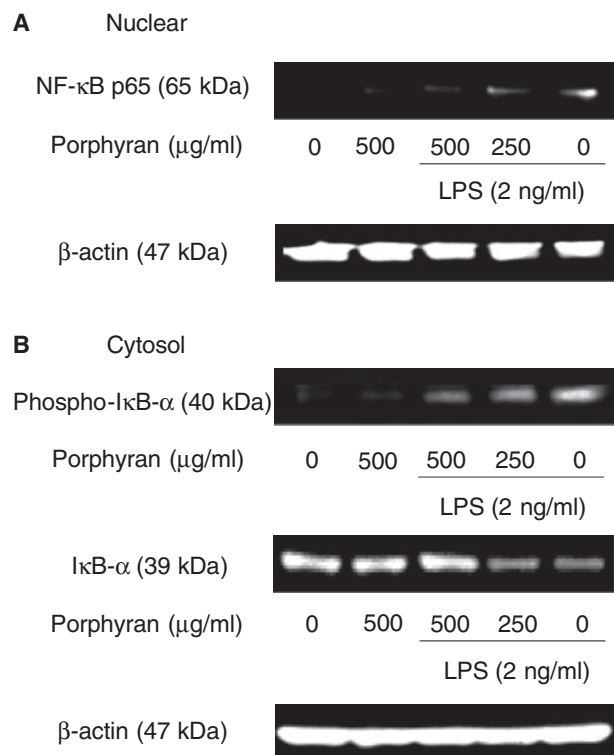

Fig. 4 Effect of porphyran on LPS-induced TNF-α secretion from RAW264.7 cells. Adherent cells (5×10^5 cells/well in 24-well plates) were pre-incubated with various concentrations of porphyran (0, 250 or 500 µg/ml) in DMEM supplemented with 10% FBS at 37°C for 1 h, followed by the addition of LPS (final 2 ng/ml). After 4 h incubation, the TNF-α levels in the culture medium of the treated cells were measured by ELISA as described under 'Materials and Methods' section. Data represent means ± SD of triplicate measurements.

A

NF-κB

Porphyran (µg/ml)   0   500   500   250   0

LPS (2 ng/ml)

B

NF-κB

+100x NF-κB

C

NF-κB

Porphyran (µg/ml)   500   250   0

LPS (2 ng/ml)

Fig. 5 Effects of porphyran on LPS-induced NF-κB activation in RAW264.7 cells. (A) *In vivo* effect of porphyran on DNA binding of NF-κB. Adherent cells in 35 mm-diametre culture dish (2×10^6 cells/dish) were incubated in serum-free DMEM at 37°C for 2 h, and then the cells were incubated with various concentrations of porphyran (0, 250 or 500 µg/ml) in serum-free DMEM at 37°C for 1 h, followed by the addition of LPS (final 2 ng/ml). After 30 min incubation, gel shift assay of NF-κB was performed with nuclear extracts prepared from the treated cells as described under 'Materials and Methods' section. (B) EMSA was performed on the nuclear extract prepared from LPS-stimulated RAW264.7 cells in the presence of 100-fold excess unlabelled oligonucleotides with the same NF-κB consensus sequence. (C) *In vitro* effect of porphyran on DNA binding of NF-κB. Porphyran (0, 250 or 500 µg/ml) was added to the assay mixture containing the nuclear extracts prepared from the LPS-stimulated RAW264.7 cells and infrared dye labelled oligonucleotides with NF-κB consensus sequence, and incubated for 20 min, and then subjected to EMSA.

Pre-treatment with porphyran (250 or 500 µg/ml) resulted in the inhibition of the LPS-inducible NF-κB DNA binding in a concentration-dependent manner. The addition of 100-fold excess of the unlabelled oligonucleotides with same sequences to the nuclear extract resulted in almost complete abolishment of the binding activity, which confirms that the binding is specific to

A   Nuclear

NF-κB p65 (65 kDa)

Porphyran (μg/ml)	0	500	500	250	0

LPS (2 ng/ml)

β-actin (47 kDa)

B   Cytosol

Phospho-IκB-α (40 kDa)

Porphyran (μg/ml)	0	500	500	250	0

LPS (2 ng/ml)

IκB-α (39 kDa)

Porphyran (μg/ml)	0	500	500	250	0

LPS (2 ng/ml)

β-actin (47 kDa)

Fig. 6 Effects of porphyran on the nuclear translocation of NF-κB p65 and phosphorylation and degradation of IκB-α in LPS-treated RAW264.7 cells. (A) Western blot analysis was conducted on NF-κB p65 in the nuclear extracts prepared from RAW264.7 cells treated with porphyran and subsequent stimulation with LPS as described under 'Materials and Methods' section. (B) Western blot analyses were conducted on the IκB-α and phosphorylated IκB-α in the cytosolic extracts prepared from RAW264.7 cells treated with porphyran and subsequent stimulation with LPS as described under 'Materials and Methods' section.

NF-κB (Fig. 5B). To examine whether porphyran directly inhibits NF-κB binding to DNA, the nuclear extracts prepared from the LPS-treated RAW264.7 cells were treated with porphyran and labelled NF-κB oligonucleotides. The *in vitro* exposure of the LPS-activated nuclear extracts to porphyran caused no decrease in the NF-κB DNA binding (Fig. 5C). Hence, it seems likely that porphyran itself does not inhibit NF-κB DNA binding activity.

The process of the activation of NF-κB proceeds through phosphorylation and degradation of the IκB-α inhibitory subunit and subsequent translocation of p65/p50 complex into the nucleus (26). Thus, the effect of porphyran on nuclear translocation of p65 was also examined by immunblotting. As shown in Fig. 6A, the nuclear levels of the p65 protein increased in LPS-treated RAW264.7 cells, and the LPS-induced nuclear translocation of p65 was blocked by porphyran in a concentration-dependent manner. In addition, immunoblot analysis using specific antibodies indicated that both the phosphorylation and degradation of IκB-α were also inhibited by a pre-treatment with porphyran (Fig. 6B). These results suggest that porphyran blocks the nuclear translocation of NF-κB through the inhibition of the phosphorylation and subsequent degradation of IκB-α. Specific NF-κB
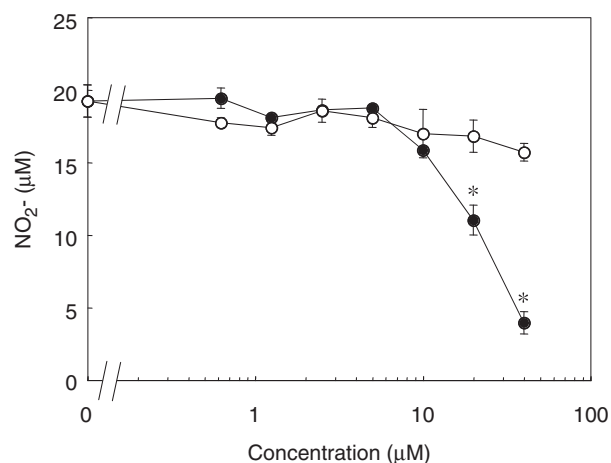
Fig. 7 Effects of specific NF-κB inhibitor peptide (SN50) and its inactive control peptide (SN50M) on NO production in LPS-treated RAW264.7 cells. Adherent cells (3×10^4 cells/well in 96-well plates) were pre-incubated with various concentrations of SN50 (filled circle) or SN50M (open circle) in DMEM supplemented with 10% FBS at 37°C for 2 h, followed by the addition of LPS (final 2 ng/ml). After 18 h incubation, the NO level in the supernatants of the treated cells were estimated as described under 'Materials and Methods' section. Data represent means ± SD of triplicate measurements. Asterisks indicate significant differences between with and without SN50 ($P < 0.05$).

inhibitor peptide (SN50) blocked LPS-induced NO production in a concentration-dependent manner, while inactive control peptide SN50M had no effect (Fig. 7). These results indicate that NF-κB activation is required for LPS-induced NO production, and may support the idea that inhibition of NF-κB activation by porphyran is its main action mechanism to inhibit NO production in LPS-stimulated RAW264.7 cells.
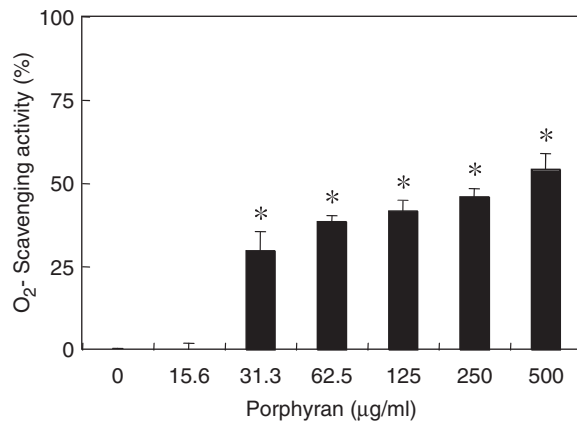
Superoxide radical-scavenging activity of porphyran
It has been reported that sulphated polysaccharides from marine algae including fucoidan and porphyran have antioxidant activities (27–32), and naturally occurring antioxidant compounds such as β-carotene have suppressive effect on NF-κB activation in macrophages stimulated with LPS (33). Thus, there is a possibility that antioxidant property of porphyran might be partly responsible for the inhibitory effects on the inflammatory response of LPS-stimulated RAW264.7 cells. To study this point, the antioxidant activity of porphyran was assessed by the surperoxide radical-scavenging activity. As shown in Fig. 8, porphyran significantly scavenged surperoxide radical in a concentration-dependent manner. Porphyran at final concentration of 500 μg/ml scavenged surperoxide anion radical by >50%.

Discussion

Porphyran is a linear sulphated polysaccharide composed of galactose and 3, 6-anhydrogalactose, which are partially substituted with galactose-6-sulphate and 6-O-methyl-galactose. In the present study, we found that porphyran inhibited the production of NO through the inhibition of iNOS expression in

Fig. 8 Scavenging effects of porphyran on superoxide anion radical.
Scavenging activity of porphyran towards superoxide anion radical
was measured as described under 'Materials and Methods' section.
Experiments were performed in triplicate, and values shown are the
means ± SD. Asterisks indicate significant differences between with
and without porphyran ($P < 0.05$).

LPS-stimulated RAW264.7 cells. RT-PCR analysis
revealed that iNOS mRNA level was significantly
reduced by porphyran in LPS-treated RAW264.7
cells. Since β-actin mRNA levels remained unchanged,
the possibility of the non-specific actions of porphyran
on gene expression may be excluded. Thus, the inhi-
bition of NO production is due to the inhibition of
iNOS mRNA transcription. These results differ from
those obtained using murine peritoneal macrophages,
which demonstrated that porphyran fraction prepared
from *P. yezoensis* induced NO and TNF-α production
(*34*, *35*). Although the exact reason for these differ-
ences is uncertain now, it may be due to the different
cell types used in the studies, as well as differences in
sources and preparation procedures of porphyrans
used. In fact, it has been known that porphyran
has a quite complicated and heterogeneous structure
depending on the source of the porphyran, and the
even cultivation manner or harvest time also influences
the structure of porphyran derived from same algal
species (*36*, *37*). Although further studies are required
for the clarification of the mechanism of porphyran to
inhibit NO production in LPS-stimulated macro-
phages as well as for its structure-activity relationship,
it seems that our results can exclude the possibility that
porphyran directly interferes with interaction between
NF-κB protein and DNA, since no significant inhibi-
tory effect of porphyran on NF-κB DNA binding was
observed in *in vitro* EMSA (Fig. 5C).

NO is a gaseous free radical involved in various
physiological processes such as vasodilation, smooth
muscle regulation, neurotransmission and apoptosis
(*38–41*). NO also plays a role in anti-infectious
immune responses as an important modulator in
both innate and adaptative immunity (*42*). On the
other hand, the excess amount of NO produced by
iNOS in activated macrophages contributes to numer-
ous severe inflammatory diseases including sepsis and
arthritis (*43*, *44*). Since NO particularly induces the
impaired vascular reactivity and causes pathological
changes (*45*), the selective inhibition of iNOS

expression in inflammatory cells (*e.g.* macrophages)
may provide an important therapeutic strategy for
inflammatory diseases.

Similar to our present results on porphyran, it has
recently been reported that fucoidan, a sulphated
polysaccharide derived from brown algae, inhibits
NO production and the expression of iNOS in
LPS-activated macrophages (*3*). The inhibitory effect
of fucoidan on NO production and iNOS expression in
other cell types are also reported (*4*, *46*). Fucoidan
shows various biological activities. Particularly, its
anti-inflammatory and anti-complement actions have
drawn significant attention (*47*, *48*). The suppressive
effects on iNOS expression in activated macrophages
might partly explain its anti-inflammatory actions.
In contrast, it was reported that fucoidan induces
NO production from macrophages *via* a p38 MAP
kinase and NF-κB-dependent mechanism (*1*). Since
fucoidan has highly complicated structure similar
to porphyran, it can vary depending on the algal
sources. Comparative study on the anti-inflammatory,
anti-angiogenic, anticoagulant and anti-adhesive
activities of fucoidans prepared from nine species of
brown algae showed that the source and composition
of fucoidan likely affect the biological activity to
a certain extent (*49*). Further studies are necessary to
clarify the structure-activity relationship of sulphated
polysaccharides fucoidan and porphyran especially
their effects on NO production in activated
macrophages.

LPS can induce the secretion of inflammatory
cytokines such as TNF-α and IL-6 in addition to
iNOS expression in macrophages. In fact, increased
level of TNF-α was detected in the medium of
LPS-treated RAW264.7 cells. It is of interest to point
out that porphyran had only a marginal inhibitory
effect on the level of TNF-α. This observation suggests
that porphyran may not interrupt the extracellular
interaction between LPS and the specific receptors
but affect the intracellular signalling pathways such
as the activation process of transcription factor. The
promoter region of iNOS gene has several homologous
consensus sequences for binding of the transcription
factors such as NF-κB, c-jun/c-Fos heterodimers
known as AP-1, and CCAAT box enhancer binding
protein (C/EBP) (*50*, *51*). In this study, EMSA using
oligonucleotides probe corresponding to the consensus
binding site for NF-κB was carried out, and we found
that LPS-induced NF-κB activation was inhibited by
porphyran. It is well known that NF-κB exists in the
cytosol as an inactive trimeric complex in which the
p50/p65 protein dimer is associated with IκB known
as an inhibitory subunit (*52*). When the cells are stimu-
lated with extracellular stimulus, phosphorylation and
subsequent degradation of IκB occurs. Such changes
lead to dissociation of IκB from the complex and to
produce activated NF-κB, which translocates into
nucleus and activates the target gene expression.
The immunoblot analysis using the specific antibodies
demonstrated that the LPS-induced phosphorylation
and degradation of IκB-α, which is prerequisite for
p65 activation, was suppressed in the porphyran-
pretreated cells. In addition, the translocation of p65

into nuclear in response to LPS was also inhibited by porphyran. Thus, these results suggest that the inhibition of NF-κB activation through the inhibition of the phosphrylation and degradation of IκB-α may be an action mechanism of porphyran to inhibit iNOS expression in LPS-stimulated RAW264.7 cells.

The activation status of various transcription factors are influenced by the intracellular redox condition and controlled by the generation of ROS (50). Recently, it has been suggested that porphyran or fucoidan acts as a potential antioxidant or radical scavenger (30–32). In fact, our porphyran sample showed superoxide radical-scavenging activity. Regarding naturally occurring antioxidants, which have inhibitory activities on inflammatory responses of macrophages, it has recently been reported that lycopene, a major carotenoid in tomatoes, inhibited LPS-induced production of NO through the suppression of NF-κB activation in RAW264.7 cells, but had no effect on TNF-α (53). Similarity between porphyran and lycopene in terms of the effect on LPS-stimulated RAW264.7 cells suggests that the antioxidant property may be one of the important factors responsible for the anti-inflammatory activities towards LPS-stimulated macrophages. In addition to lycopene, diosgenin, a steroid saponin found in several plants, which has antioxidant activity, has also been reported to inhibit iNOS expression in LPS/interferon γ-activated murine macrophages without affecting the secretion of TNF-α (54). Since diosgenin inhibited LPS/interferon γ-induced NF-κB and AP-1 activation, the inhibition of iNOS expression by diosgenin was considered at the transcriptional level. As another example, prodigiosin isolated from marine bacteria *Hahella chejuensis* has also been reported to suppress LPS-induced NO production by inhibiting p38 MAPK, JNK and NF-κB activation in mouse peritoneal macrophages, but had no effect on the production of cytokines including TNF-α (55). Although porphyran, lycopene, diosgenin and prodigiosin are chemically unrelated compounds, they may have a similar action mechanism on activated macrophages. Namely, these compounds inhibit NO production through the inhibition of NF-κB activation without affecting the production of TNF-α, suggesting that NF-κB separately regulates iNOS expression and secretion of TNF-α. Other several natural compounds with antioxidant or free radical-scavenging activities have been reported to inhibit NO production by activated macrophages through the inhibition of the NF-κB activation (3, 56–58). Thus, it seems likely that the specific inhibition of NO production via suppression of NF-κB activation may be a common action mechanism of anti-inflammatory agents with antioxidant activity. Further studies are required to clarify this point as well as exact action mechanism of porphyran on the inhibition of NO production in activated macrophages.

In conclusion, our *in vitro* studies demonstrated that porphyran inhibited NO production in macrophages activated by LPS through the inhibition of NF-κB activation. This inhibition may explain some of the anti-inflammatory effects of porphyran.

Conflict of interest
None declared.

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