Protective effect of porphyran isolated from discolored nori (*Porphyra yezoensis*) on lipopolysaccharide-induced endotoxin shock in mice

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Highlights

Porphyran from discolored nori (dc-porphyran) shows protective effect on LPS-induced endotoxin shock in mice.

F1, a fraction purified from dc-porphyran shows protective effect superior to dc-porphyran.

LPS-induced increases in serum levels of NO and TNF-α are suppressed by F1 treatment.

LPS-induced increase in liver MDA level is suppressed by F1 treatment.

LPS-induced increase in peritoneal exudate cell secretion of NO and TNF-α is suppressed by F1 treatment.

Abstract

Porphyran isolated from discolored nori (*Porphyra yezoensis*) (dc-porphyran) showed in vitro antioxidant and anti-inflammatory effects superior to those of porphyran isolated from normal nori. One fraction (F1) purified from dc-porphyran by DEAE-chromatography showed the highest inhibitory effect among all fractions. Therefore, we investigated the protective effects of dc-porphyran and F1 on LPS-induced endotoxin shock in mice. Intraperitoneal (i.p.) treatment with dc-porphyran or F1 (100 mg/kg) 60 min prior to i.p. injection of LPS (30 mg/kg) completely protected mice from LPS lethality. At 10 mg/kg concentration, F1 demonstrated significantly more protection than dc-porphyran. Intravenous (i.v.) challenge of LPS, even at 20 mg/kg, was more lethal than i.p. administration; i.v. injection of F1 (100 mg/kg) with LPS significantly improved the survival rate. However, i.v. dc-porphyran (100 mg/kg) produced an even lower survival rate than that of LPS alone. To further examine the mechanism of the

protective effects of dc-porphyran and F1 on LPS-induced endotoxin shock, we examined pro-inflammatory mediators such as NO and TNF-α in serum. F1 significantly reduced the levels of these markers. Additionally, F1 significantly decreased the malondialdehyde level in the liver, a marker of oxidative stress, while dc-porphyran had almost no effect. Furthermore, F1 significantly decreased the production of TNF-α and NO in peritoneal exudate cells harvested from LPS-challenged mice, while dc-porphyran treatment showed a lesser decrease. Our results suggest that porphyran isolated from discolored nori, especially F1, is capable of suppressing LPS-induced endotoxin shock in vivo.

Key words: *Porphyra yezoensis*, porphyran, LPS, endotoxin shock, anti-inflammatory activity

1. Introduction

Edible seaweeds are useful sources of natural polysaccharides, which have drawn great attention from diverse research fields in the development of new medicines, health foods, or

supplements [1, 2]. Most of the seaweed-derived polysaccharides can generally function as indigestible dietary fibers, which provide various beneficial effects on human health, such as lowering blood cholesterol level and blood pressure, and protective effect against infectious and inflammatory diseases [3]. Some polysaccharides are even known to act as potent immune modulators [4, 5].

Porphyra species are edible red algae cultivated and consumed in East and South-East Asian countries such as Japan, Korea, and China. Edible sheet-like dried seaweed called “nori,” prepared from *Porphyra* species, is traditionally used in Japanese cuisine such as sushi. Owing to the recent increase in the popularity of such Japanese dishes, the consumption of nori is now growing worldwide. Nori is known to contain some biologically functional components such as dietary fibers, taurine, polyunsaturated fatty acids, carotenoids, and mycosporine-like amino acids (porphyra-334), as well as minerals, vitamins, and a relatively high amount of proteins. Hence, nori is a health food and can contribute to human health as a functional food.

Porphyran is one of the main constituents of *Porphyra yezoensis*, and is related to

agarose, a linear sulfated polysaccharide consisting of D-galactose, 3, 6-anhydro-L-galactose, 6-*O*-methyl-D-galactose, and L-galactose-6-sulfate [6-8]. In addition to its health benefits as a dietary fiber in nori, previous studies have found that porphyran has diverse biological activities including antitumor, immuno-modulating, antioxidant, antihyperlipidemic, and hypercholesterolemic activities [9-13]. A previous study demonstrated that porphyran prepared from *P. yezoensis* inhibited nitric oxide (NO) production from lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophages in a concentration-dependent manner through the inhibition of NF-κB activation [14]. These results suggest that porphyran can exhibit anti-inflammatory activity. Furthermore, our recent study showed that discolored nori with no commercial value contains a much higher amount of porphyran than normal nori [15]. Chemical and biological analyses revealed that porphyrans obtained from discolored nori (dc-porphyran) have relatively lower molecular size than those from normal nori (n-porphyran), and dc-porphyran exhibits greater antioxidant activity than n-porphyran. Interestingly, dc-porphyran showed potent inhibitory effect on nitric oxide (NO) production in

LPS-stimulated RAW264.7 cells by preventing the expression of inducible NO synthase [15]. Furthermore, our recent study demonstrated that dc-porphyran was separated into four fractions (F1-F4) by DEAE-anion exchange chromatography, and one fraction (F1) showed the highest inhibitory effect on NO production from LPS-stimulated RAW264.7 cells. These findings suggest that dc-porphyran, especially F1, might be useful for the treatment of inflammatory diseases and endotoxin shock [15].

To evaluate this possibility, in this study, we examined the effect of dc-porphyran and F1 on LPS-induced endotoxin shock in mice.

2. Materials and methods

2.1. Materials

Dc-porphyran was prepared from discolored nori, and F1 was separated from dc-porphyran

by DEAE-chromatography as described previously [15]. LPS from *Escherichia coli* 0111: B4 (purified by phenol extraction) was purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). For experiments *in vivo*, dc-porphyran, F1, and LPS were dissolved in pyrogen-free saline. TNF-α capture antibody and anti-mouse TNF-α monoclonal antibody were purchased from Endogen, Inc. (Boston, MA, USA). Other chemicals were of the highest grade commercially available.

2.2. Animal and treatment

Specific pathogen-free (SPF) male ddY mice (4 weeks old, 17.0-26.0 g) were obtained from Texam, Nagasaki, Japan. These mice were housed at a constant 24 ℃ under controlled conditions of 12 h light/dark cycles and provided with free access to laboratory-standard food CE-2 (Texam, Nagasaki, Japan) and water. All mice were treated according to the Guidelines of the Japanese Association for Laboratory Animal Science and the Guidelines for Animal

Experiments of Nagasaki University, Japan. Dc-porphyran or F1 (10 or 100 mg/kg) was administered intraperitoneally (i.p.) 60 min prior to LPS (30 mg/kg) i.p. injection, or intravenously (i.v.) together with LPS (20 mg/kg). Saline alone was used as control. Five mice were used for each test group (n=5). The mortality of mice was recorded 80 h after LPS injection in each test group. Blood samples were collected and the liver was excised after CO_2/O_2 euthanasia at 12 h after LPS injection.

2.3. Measurement of serum TNF-α levels

The level of tumor necrosis factor (TNF)-α in the serum of each treated mouse was measured by sandwich enzyme-linked immunosorbent assay (ELISA) with two antibodies to two different epitopes on the mouse TNF-α molecule by a method similar to that described in a previous study [14]. The TNF-α concentrations were estimated from reference to a standard curve for a serial two-fold dilution of murine recombinant TNF-α.

2.4. Nitrite assay for the estimation of nitric oxide (NO)

The level of nitrite, a stable reaction product of NO with O_2, was determined in the serum from each treated mouse using the Griess assay method as described in a previous study [16]. In brief, each serum sample was deproteinized by addition of sulfosalicylic acid and subsequent incubation for 30 min at 24°C After centrifugation (10,000 ×*g* for 15 min), the supernatant (100 µl) of each sample was mixed with 5% NH_4Cl buffer (150 µl) and 5% NaOH (30 µl). To convert nitrate to nitrite, a working solution (0.31 M phosphate buffer, pH 7.5, 0.1 mM FAD, 1 mM NADPH, and 10 U/ml nitrate reductase) was added to the reaction mixture and the reaction was allowed to proceed in the dark for 1 h. The final reaction mixture was added to an equal volume of Griess reagent (3 mM sulfanilic acid and 30 µM *N*-1-naphthyl-ethylenediamine dihydrochloride, and 25% glacial acetic acid) in 96-well plates and incubated for 10 min at 24°C. Nitrite levels were determined by measuring the absorbance at 540 nm using a MULTISCAN GO (Thermo Fisher Scientific Inc., MA, USA). A calibration curve was made

with known concentrations of $NaNO_2$ standard solution.

2.5. Measurement of lipid peroxidation

Malondialdehyde (MDA), an indicator of lipid peroxidation in liver homogenates, was determined by the thiobarbituric acid-reactive substance method [17]. The absorbance of the reaction product was detected at 532 nm, and the MDA levels were calculated as nmol/mg protein.

2.6. Preparation of peritoneal cells and macrophages

At 12 h after i.v. injection of LPS (20 mg/kg) together with dc-porphyran or F1 (100 mg/kg), peritoneal cells were harvested by peritoneal lavage with 10 ml cold Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10 U/ml heparin, which yielded 1-2 × 10^6 cells/mouse. After washing twice

with DMEM by centrifugation at 200 ×*g*, the cells were resuspended in DMEM supplemented with 10% FCS. The cells were placed in 96-well plates at a density of 8×10^4 cells/well, and were incubated for 24 h at 37°C. Then the medium was removed and NO and TNF-α levels in the medium were measured as described above. To obtain monolayers of peritoneal macrophages for the analysis of inducible NO synthase mRNA, 1×10^6 peritoneal cells harvested from mice after 12 h i.v. injection of LPS (20 mg/kg) together with dc-porphyran or F1 (100 mg/kg) were allowed to adhere to the 35 × 10 mm tissue culture dish by culturing them 2 h at 37°C in CO_2 incubator. Nonadherent cells were removed by gently washing three times with warm PBS. 3×10^5 macrophages were subjected to reverse transcription-polymerase chain reaction (RT-PCR) as described previously [14].

2.7. Statistical analysis

All the experiments were repeated at least three times. Data were expressed as means ± standard deviation (S.D.). Tested groups were compared with appropriate controls using Student's *t*-test.

Differences were considered significant at $P < 0.05$.

3. Results

3.1. Protective effects of dc-porphyran and F1 on LPS-induced mortality in mice

In our previous study, we found that dc-porphyran, especially F1, a fraction prepared from dc-porphyran, showed *in vitro* anti-inflammatory activity superior to that of porphyran prepared from normal nori [15]. Hence, in this study, we evaluated the ability of dc-porphyran and F1 to protect mice from LPS-induced mortality. When dc-porphyran or F1 (100 mg/kg) was i.p. administered 1 h before LPS (30 mg/kg) i.p. injection, LPS lethality was completely suppressed (Fig. 1A). At a lower dose (10 mg/kg), both porphyran samples showed a partial protective effect, and F1 was slightly more effective than dc-porphyran (Fig. 1A). Intravenously administered LPS (20 mg/kg) was considerably more lethal than i.p.

administered LPS, and caused 100% mortality within 48 h. Intravenous administration of F1 (100 mg/kg) together with LPS resulted in a significant increase in survival rate to 60% after 80 h, whereas dc-porphyran enhanced the toxic effect of LPS, and all the mice died within 24 h (Fig. 1B). At a lower dose of dc-porphyran and F1 (10 mg/kg), both porphyran samples showed similar protective effect on LPS-induced lethality, with 40% survival rate 80 h after LPS injection (Fig. 1B).

3.2. Effect of dc-porphyran and F1 on LPS-induced production of TNF-α and NO in mice

Since LPS-induced lethality is mediated by the production of inflammatory mediators such as TNF-α and NO, we examined the effect of dc-porphyran and F1 on LPS-induced production of these mediators in mice. Intravenous administration of LPS (20 mg/kg) induced an increase in serum nitrite levels (27 μM) estimated by the Griess assay. Simultaneous injection of F1 reduced the nitrite level significantly to 19 μM, while dc-porphyran was almost ineffective (Fig.

2A). An increase in the serum level of TNF-α (270 ng/ml) was also observed, and dc-porphyran or F1 (100 mg/kg) reduced the value to 200 or 80 ng/ml, respectively, suggesting that F1 more effectively reduces the TNF-α level in mice than does dc-porphyran (Fig. 2B).

3.3. Effect of dc-porphyran and F1 on LPS-induced oxidative stress in liver

It has been pointed out that excessive ROS production is related to the progression of sepsis or endotoxin shock [18, 19], and ROS-induced oxidative stress in multiple tissues contributes to high mortality rate [20, 21]. Hence, we examined the effect of dc-porphyran and F1 on lipid peroxidation level in the liver by measuring MDA, a marker of oxidative stress. MDA level in the liver homogenate from mice injected only with LPS was increased compared to the level in control mice. Pretreatment with F1 significantly reduced the MDA level, while dc-porphyran had almost no effect (Fig. 3).

3.4. Effect of dc-porphyran or F1 on NO and TNF-α producing activities of peritoneal exudate cells from LPS injected mice

To investigate the effect of i.v. dc-porphyran or F1 on immune-competent cells in mice, peritoneal cells were harvested from mice 12 h after i.v. injection of LPS (20 mg/kg) together with dc-porphyran or F1 (100 mg/kg). The cells were incubated in RPMI 1640 medium supplemented with 10% FCS at 37 ℃ for 24 h, and then NO and TNF-α levels in the culture supernatants were measured. As shown in Fig. 4A, NO levels in the culture medium of the peritoneal cells from LPS-injected mice were evidently higher than in those from control mice. Simultaneous injection of dc-porphyran or F1 with LPS resulted in the suppression of the NO levels, and F1 was significantly more effective than dc-porphyran. Consistent with these results, the iNOS mRNA levels in peritoneal macrophages were significantly reduced with F1 injection (Fig. 4B). Similar to NO results, an increase in TNF-α levels in the culture medium of peritoneal cells from LPS-injected mice was observed, and F1 exhibited more effective

suppressive effect on increased TNF-α level than did dc-porphyran (Fig. 4C).

4. Discussion

NO is a gaseous free radical, and plays various physiological roles depending on the sites at which it is generated [22-26]. NO is produced by NO synthase (NOS); endothelial NOS, (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) have been identified so far [27]. In both innate and adaptive immune systems, NO produced by macrophages through iNOS plays a role in anti-infectious immune responses as an important modulator [26]. However, the excess NO produced by iNOS in activated macrophages is involved in numerous severe inflammatory diseases including sepsis and arthritis [28, 29]. Since NO particularly induces impaired vascular reactivity and causes pathological changes [30], the selective inhibition of iNOS expression in macrophages can lead to a certain therapeutic effect for inflammatory diseases. Similar to our previous *in vitro* results on porphyran [14], it has been reported that fucoidan, a

sulfated polysaccharide isolated from brown algae, inhibits NO production and iNOS expression in LPS-activated macrophages [31]. The inhibitory effect of fucoidan on NO production and iNOS expression in other cell types is also reported [32, 33]. In contrast to these findings, it has been reported that fucoidan induces NO production in macrophages through a p38 MAP kinase and NF-κB activation mechanism [34]. Although the exact reason for this opposite result is still unclear, it may be attributable to the structural differences between the fucoidans used. In fact, it has been reported that biological activities of fucoidans isolated from nine different sources differed depending on the sources and compositions [35]. Related to these findings, our previous study on porphyran showed that the bioactivities of porphyrans isolated from different growth conditions of red alga nori (*Porphyra yezoensis*) were quite different [15]. Namely, a porphyran isolated from discolored nori inhibited NO production in LPS-stimulated RAW264.7 cells, whereas no significant effect was observed for porphyran isolated from normal nori. These findings suggest that the bioactivities of naturally occurring polysaccharides such as fucoidan and porphyran differ depending on the structural features,

which can even be altered by the growth conditions of algae. Our previous study demonstrated that F1, a fraction purified from porphyran isolated from discolored nori, showed the most potent inhibitory effect on NO production by LPS-stimulated RAW264.7 cells. Since F1 has the lowest molecular size among the four separated fractions, the molecular size is an important structural factor influencing the bioactivities. Our preliminary study showed that enzymatic digestion of porphyran resulted in a significant increase in the inhibitory effect on NO production in LPS-stimulated RAW264.7 cells (data not shown). Consistent with previous *in vitro* results [15], in the present study, porphyran isolated from discolored nori, especially F1, was found to be protective against LPS-induced endotoxin shock in mice (Fig. 1 A, B). Evidently superior protective effects of F1 against LPS lethality were observed in both i.p. and i.v. injection models compared to that of parental dc-porphyran. To our knowledge, this is the first report showing that porphyran is capable of exhibiting a protective effect on LPS lethality *in vivo*. After i.v. injection of LPS, NO level in the mouse serum increased significantly. Simultaneously injected F1 suppressed the LPS-induced increase in serum NO level, while the

effect of dc-porphyran was almost marginal (Fig. 2A). LPS-induced increase in TNF-α level in the serum was also significantly reduced by F1 (Fig. 2B). TNF-α is known as a major cytokine involved in endotoxin shock [36], and it induces several cytokines such as IL-1, IL-6, and IL-10, and activates inflammatory cells [37]. Therefore, compounds with the ability to suppress the production of NO and TNF-α can be promising candidates as therapeutic agents for inflammatory diseases and endotoxin shock. In addition, F1 also has ability to reduce the MDA level (Fig. 3). It indicates that F1 may have high potential as an effective therapeutic agent. It has been reported that naringin, a naturally occurring flavonoid with activity similar to F1, suppressed the LPS-induced production of NO and TNF-α, and reduced the LPS lethality *in vivo* [38]. Hence, suppression of NO and TNF-α production is a common mechanism of porphyran and naringin responsible for protection against endotoxin shock, although there is no structural similarity between porphyran and naringin.

Peritoneal exudate cells (PEC) harvested from mice intravenously injected with LPS showed increased generation of NO and TNF-α compared to PEC of control mice without LPS

challenge, suggesting that intravenously injected LPS eventually stimulated PEC. Simultaneous injection of F1 with LPS resulted in significant suppression of the activities of the PEC (Fig. 4A, B, C). These results suggest that porphyran, especially F1, is capable of suppressing the LPS-induced stimulation of immune-competent cells responsible for the generation of NO and TNF-α *in vivo*.

Regarding the action mechanism of porphyran, our previous *in vitro* study demonstrated that porphyran inhibited LPS-induced NF-κB activation in RAW264.7 cells through the suppression of phosphorylation and degradation of IκB-α, and subsequent nuclear translocation of the p65 large subunit [14]. These findings suggest that the inhibition of NF-κB activation may be a cellular level action mechanism of porphyran to inhibit iNOS expression in LPS-stimulated macrophages.

The activation status of various transcription factors is influenced by the intracellular redox condition [39]. It is known that activation of NF-κB is induced by various agents including reactive oxygen species [40], and redox regulation is involved in the activation of

NF-κB [41, 42]. Antioxidant reagents such as pyrrolidine dithiocarbamate and *N*-acetylcysteine inhibit the activation of NF-κB [43, 44], and these reagents also suppress the production of NO and TNF-α [43, 45]. Recently, it has been suggested that porphyran and fucoidan act as potential antioxidants or radical scavengers [46, 47]. In fact, our previous study demonstrated that a porphyran sample showed potent scavenging activity against superoxide anion and hydroxyl radical [15]. Furthermore, our previous fluorescent analysis with a ROS-specific probe revealed that porphyran reduced elevated levels of intracellular ROS in LPS-stimulated RAW264.7 cells, although it was slightly less effective than *N*-acetylcysteine [15]. Therefore, it is considered that dc-porphyran and F1 inhibited NF-κB activation through an antioxidant property, which may lead to a decrease in TNF-α and NO levels in the serum and eventually to an increase in survival rate of LPS-injected mice.

5. Conclusion

We found that porphyran isolated from discolored waste nori (dc-porphyran) showed a protective effect on LPS-induced lethality in mice. F1, a fraction further purified from dc-porphyran, showed significantly superior protective effect to parental dc-porphyran. NO and TNF-a levels in the serum of the mouse injected with LPS were significantly increased compared to control levels, and F1 reduced the increased levels of these mediators. The LPS-induced increase in the activities of peritoneal exudate cells (PEC) to produce NO and TNF-a was also suppressed by F1 treatment. The LPS-induced increase in MDA level in the liver was reduced by F1 treatment. These results suggest that a specific fraction of porphyran (F1) can be a promising candidate as an effective therapeutic agent for inflammatory diseases.

Acknowledgements

This work was supported in part by a Grant-in-Aid (15K07580) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a fund from

Nagasaki University Major Research Project (Research Initiative for Adaptation to Future Ocean Change), and a project fund (C36290) to D. Kim from the Korea Basic Science Institute (KBSI). This work was also supported by a Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fellows to M. Ueno.

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Figure legends

Fig. 1. Protective effect of dc-porphyran and F1 on LPS-induced lethality in mice. (A) Mice were intraperitoneally injected with dc-porphyran (□, ■) or F1 (△, ▲) at 10 (□, △) or 100 mg/kg (■, ▲） 1 h before intraperitoneal injection of LPS (30 mg/kg). Survival of each group (n=5) was observed 80 h after LPS injection. (B) Mice were intravenously injected with LPS (20 mg/kg) together with dc-porphyran (□, ■) or F1 (△, ▲) at 10 (□, △) or 100 mg/kg (■, ▲） simultaneously. Survival of each group (n=5) was observed 80 h after LPS injection. ◇, control; ○, LPS alone.

Fig. 2. Effects of dc-porphyran and F1 on LPS-induced increase in serum levels of NO and TNF-α. Dc-porphyran or F1 (100 mg/kg) was intravenously injected simultaneously with LPS (20 mg/kg) and the serum was prepared from mouse blood at 12 h after LPS injection. Control mice were injected with saline alone. The serum nitrite and TNF-α levels of each group (n=3) were determined by Griess assay and ELISA, respectively. Each column represents the mean of triplicate measurements. Each bar represents standard deviation. Asterisks denote significant

differences between the experimental groups. ($p < 0.05$).

Fig. 3. Effects of dc-porphyran and F1 on MDS level in liver. Dc-porphyran or F1 (100 mg/kg) was intravenously injected simultaneously with LPS (20 mg/kg). After 12 h, the MDA level of each group (n=3) in liver was determined. Each column represents the mean of triplicate measurements. Each bar represents standard deviation. Asterisks denote significant differences between the experimental groups. ($p < 0.05$).

Fig. 4. Effects of dc-porphyran and F1 on the activities of peritoneal exudate cells to produce NO and TNF-α. Peritoneal exudate cells were harvested from mice after 12 h intravenous injection of LPS (20 mg/kg) and dc-porphyran or F1 (100 mg/kg). The cells were washed twice with PBS by centrifugation and resuspended in DMEM containing 10% FCS, and cultured for 24 h at 37°C in 96-well plates (8×10^4 cells/well). (A) NO level in the culture medium was measured as described in the text. (B) For the analysis of inducible NO synthase mRNA, 3 ×

10^5 peritoneal macrophages were isolated from mice after 12 h intravenous injection of LPS (20 mg/kg) together with dc-porphyran or F1 (100 mg/kg) as described in the text. The cells were subjected to reverse transcription-polymerase chain reaction. (C) TNF-α level in the culture medium was measured by ELISA as described in the text. Each column represents the mean of triplicate measurements. Each bar represents standard deviation. Asterisks denote significant differences between the experimental groups. ($p < 0.05$).

Fig. 1A

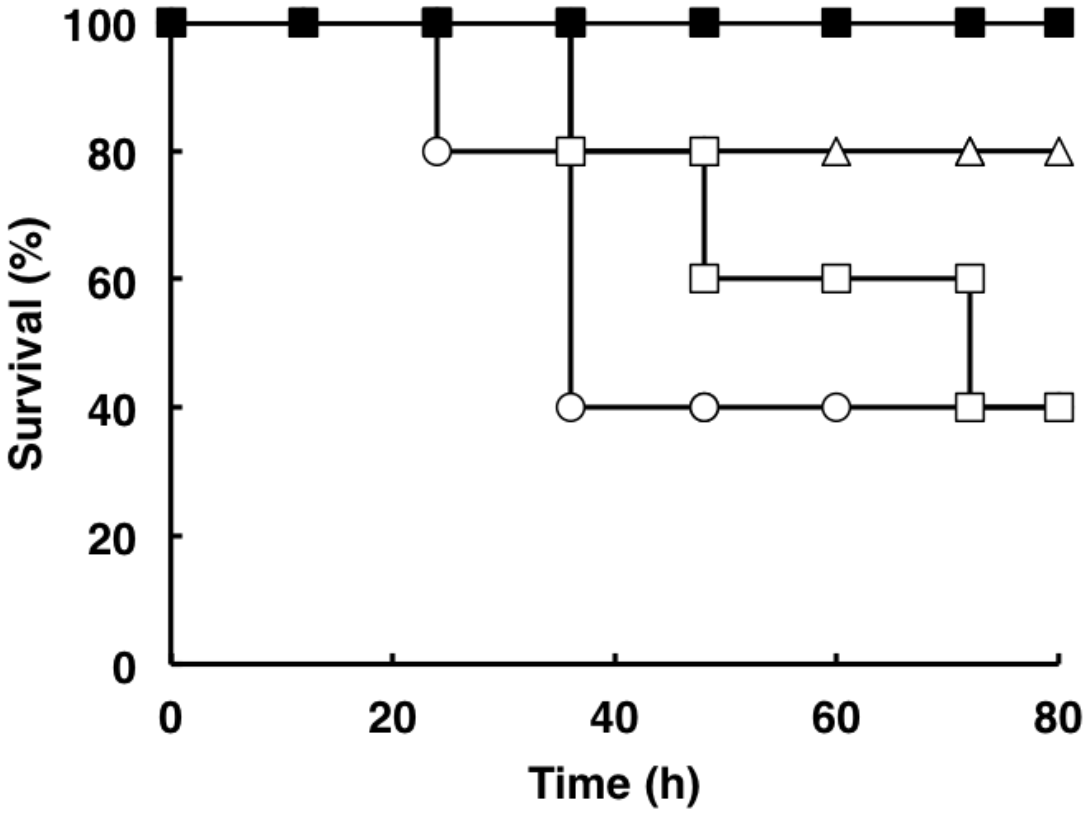


Fig. 1B

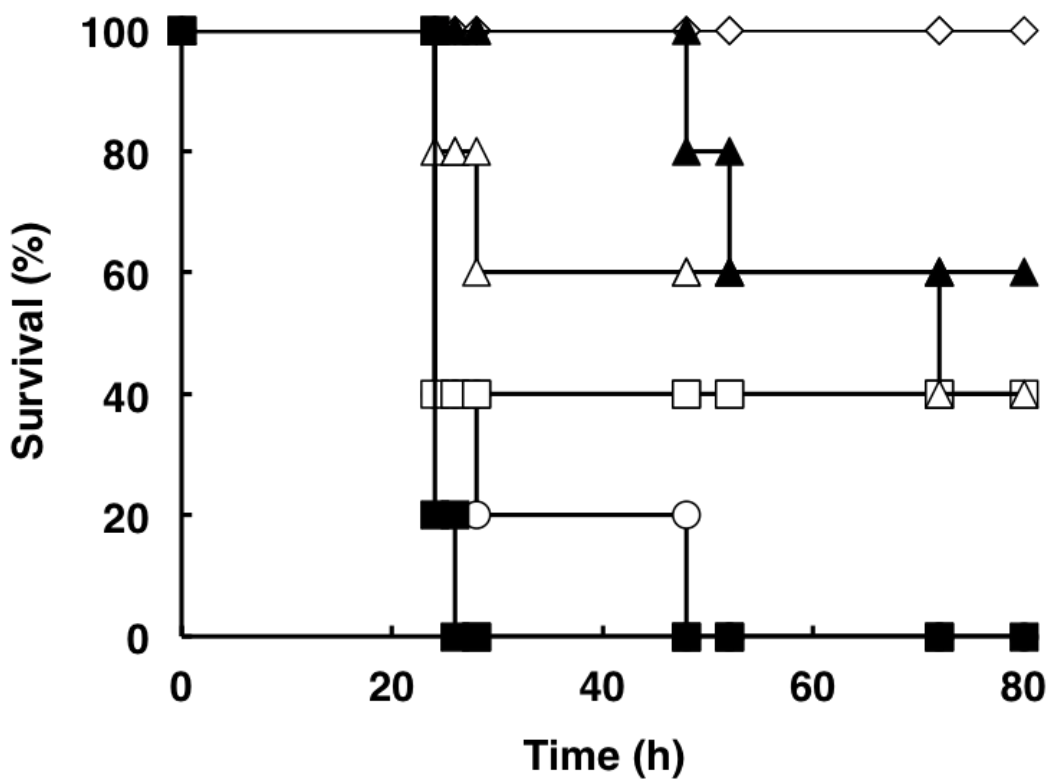


Fig. 2A

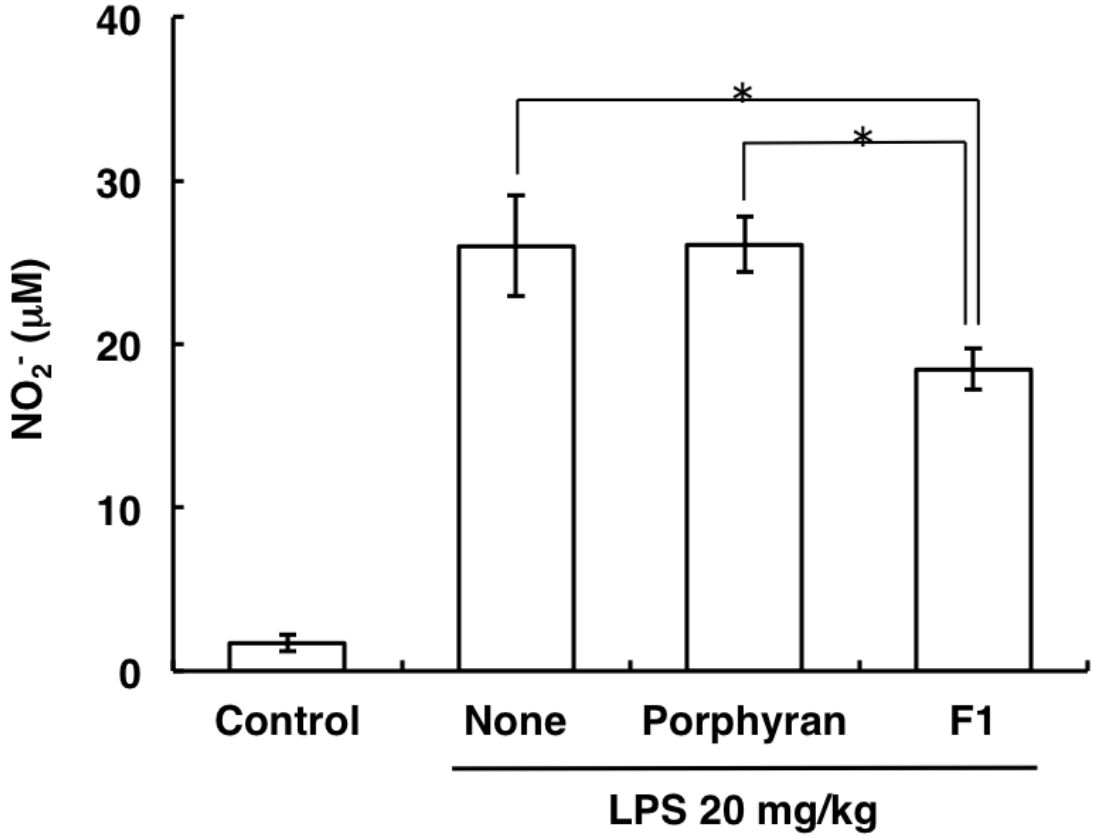


Fig. 2B

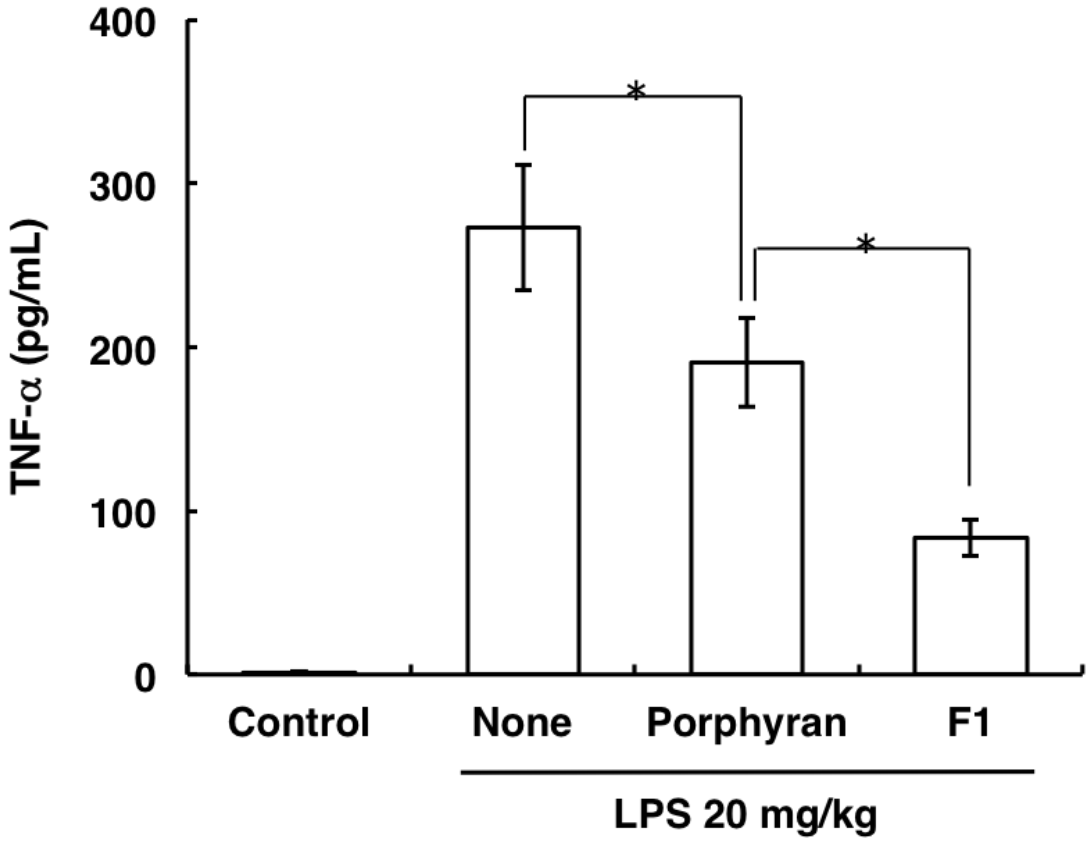

400
300
200
100
0
TNF-α (pg/mL)
*
*
Control
None
Porphyran
F1
LPS 20 mg/kg

Fig. 3

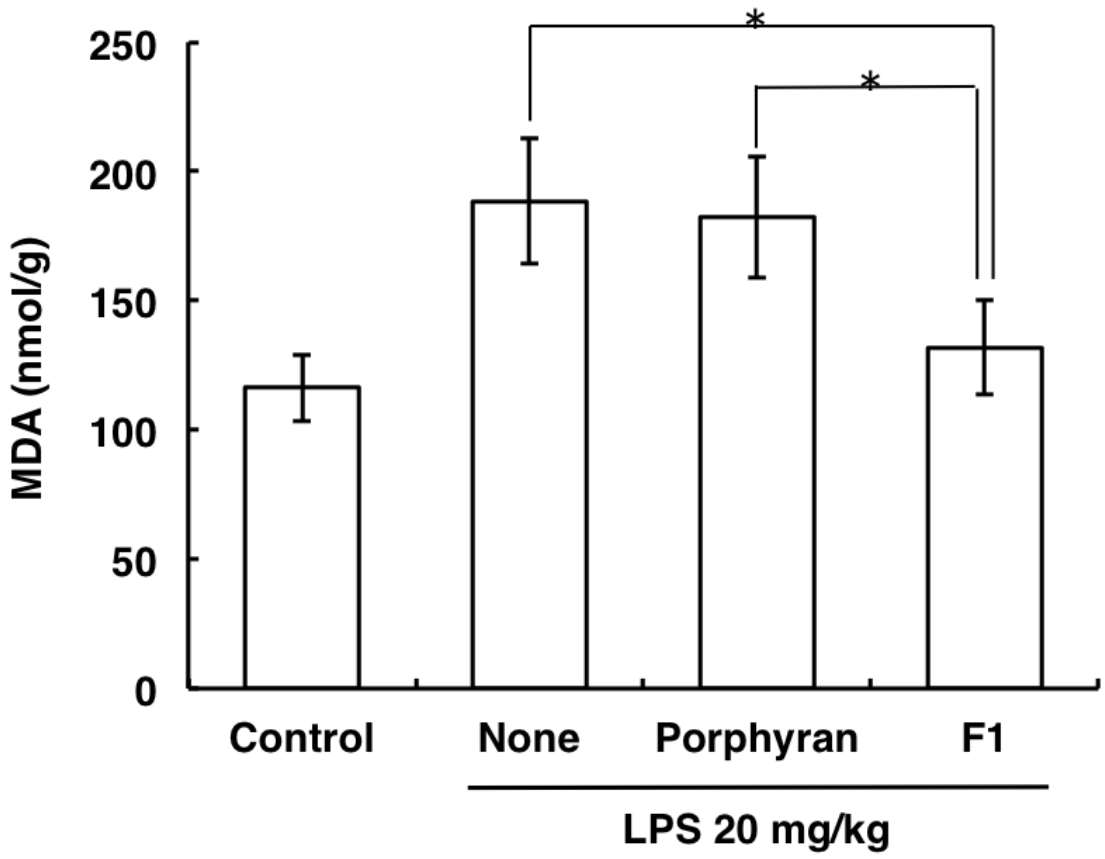

MDA (nmol/g)
250
200
150
100
50
0
*
*
Control
None
Porphyran
F1
LPS 20 mg/kg

Fig. 4A

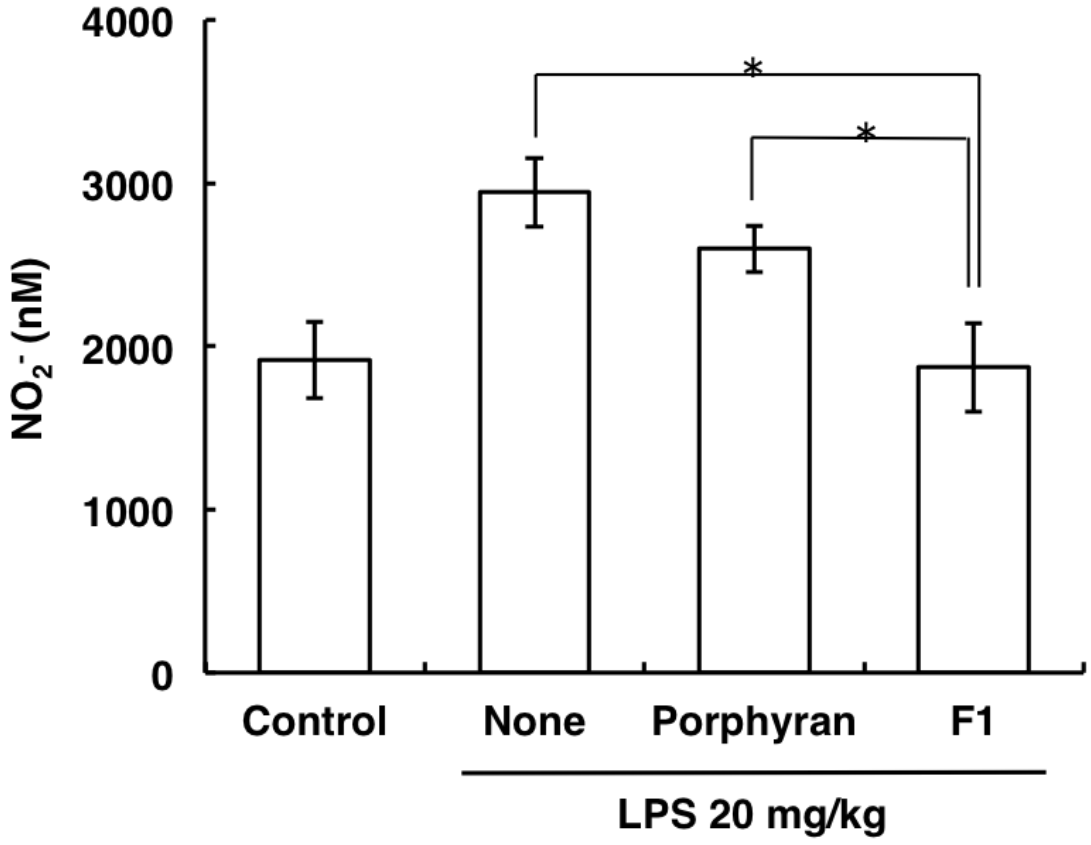

4000
3000
2000
1000
0
NO_2^- (nM)
*
*
Control
None
Porphyran
F1
LPS 20 mg/kg

Fig. 4B

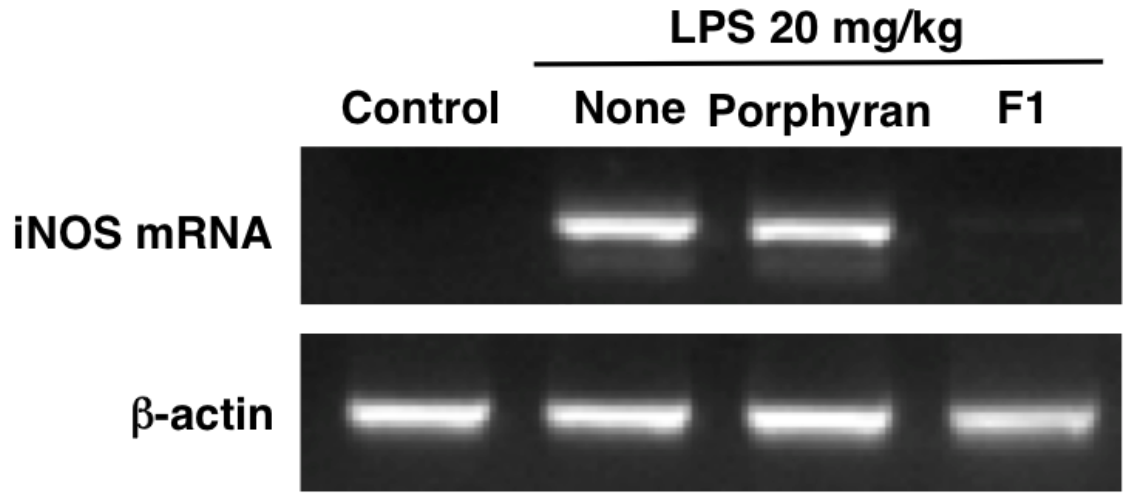
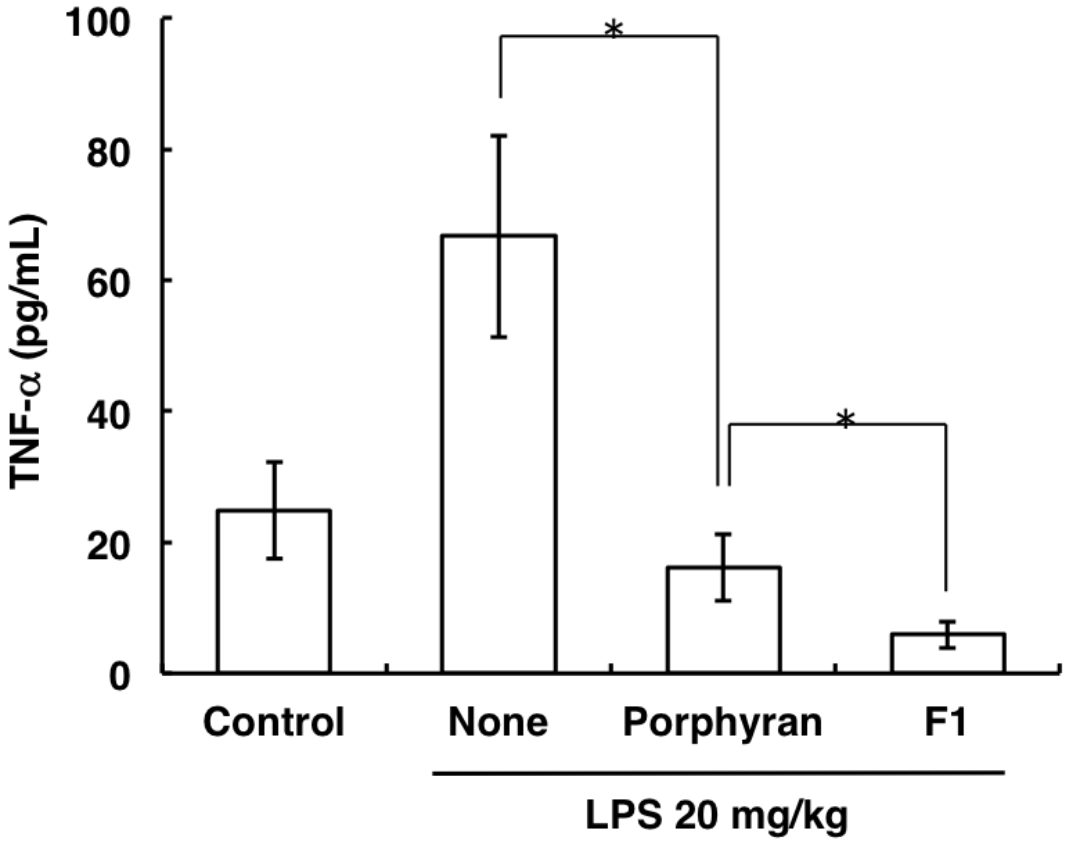


Fig. 4C


100
80
60
40
20
0
TNF-α (pg/mL)
*
*
Control
None
Porphyran
F1
LPS 20 mg/kg