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Effects of Fucoidan on Nitric Oxide Production and Activator Protein-1 Activation in Lipopolysaccharide-Stimulated Porcine Peripheral Blood Mononuclear Cells

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Abstract : Fucoidan which is sulfated polysaccharide extracted from brown seaweed has a wide variety of internal biological activities. The objectives of this study were to examine the effect of fucoidan on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated porcine peripheral blood mononuclear cells (PBMCs) and to investigate whether this effect is involved in the expression of inducible nitric oxide synthase (iNOS) and the activation of activator portein-1 (AP-1). The levels of NO production and AP-1 activity in the culture supernatants from porcine PBMCs were measured by the enzyme-linked immunosorbent assay and the levels of iNOS and AP-1 mRNA were determined by real time polymerase chain reaction. Fucoidan in LPS-naïve PBMCs has no effects on the production of NO and activity of AP-1. Expressions of iNOS and AP-1 mRNA in LPS-naïve PBMCs were also not affected by treatment of fucoidan. However, NO production, AP-1 activity and expressions of iNOS and AP-1 mRNA were dramatically increased in PBMCs stimulated with LPS. Enhancing effects of NO production and AP-1 activity in PBMCs induced by LPS were reduced by addition of fucoidan. Fucoidan also inhibited an increase in expressions of iNOS and AP-1 mRNA in LPS-stimulated PBMCs. These results suggested that fucoidan exerts anti-inflammatory effect by down-regulating production of NO via suppressing expression of iNOS and activity of AP-1 in LPS-stimulated porcine PBMCs.

Key words **:** fucoidan, AP-1, anti-inflammation, PBMCs, NO, iNOS, porcine.

Introduction

Fucoidan is sulfated polysaccharide extracted from brown algae and contains considerable amounts of L-fucose and sulfate (4,21). Fucoidan has received considerable attention as an anti-thrombotic property because its structural characteristics are similar to those of heparin (3). It has also been shown to have many important biological activities including antiviral (1), anti-adipogenic (35), anti-coagulant (14), anti-angiogenic (18), and anti-inflammatory effects (10,24).

Lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMCs) resulted in expression of mRNAs for many cytokines such as interleukin (IL)-1, IL-10, IL-12, tumor necrosis factor (TNF)-α, interferon (IFN)-γ (33) and inducible nitric oxide synthase (iNOS) (28). These cytokines increase inflammatory response. Nitric oxide (NO) is one of the important inflammatory mediators, reflecting the degree of inflammation like PGE_2 regulated by cyclooxygenase-2 (COX-2) and is produced from L-arginine by NOS (6). In particular, iNOS is produced mainly by immune cells in inflammation condition (27). A certain level of NO production by iNOS controls the replication of intracellular bacteria, parasites and tumor cells (12,25). However, excessive production of NO can damage host-cells (19). In addition, overproduction of NO can lead to infectious, ischemic, traumatic, and neurodegenerative diseases (5,23). For this reason, regulation of NO production plays a central role in a variety of diseases.

Activator protein-1 (AP-1) activity is activated by LPS-stimulation via phosphorylation of mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), c-jun N-terminal kinas (JNK) and p38 MAPK. This action induces expression of iNOS, COX-2 and a variety of interleukins (13,31).

In this study, the effects of fucoidan on production of NO and expression of iNOS in LPS-naïve and LPS-stimulated porcine PBMCs were investigated. In addition, it was determined whether this effect is associated with a change of AP-1 activity.

Materials and Methods

Chemicals and reagents

Fucoidan purified from *Focus vestculosus* was purchased from Sigma-Aldrich (St. Louis, MO, USA) and passed through a 0.45 μm membrane filter (Milipore Corporation, Bedford, MA, USA). LPS from *Escherichia coli* 0127:B8 (Sigma-Aldrich), Percoll® solution (specific gravity, 1.080; Sigma-Aldrich), RPMI 1640 medium (Sigma-Aldrich) and fetal bovine serum (FBS) (Gibco Company, Grand Island, NY, USA) were purchased commercially.

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Isolation of porcine peripheral blood mononuclear cells (PBMCs)

Clinically healthy 6 month crossmixed pigs were used as blood donors in slaughterhouse (Farm story, Cheongju, Korea). Porcine PBMCs were isolated as described elsewhere (17). Heparinized porcine peripheral blood was drawn from the jugular vein, immediately diluted with an equal volume of phosphate-buffered saline (PBS) without calcium and magnesium, and overlaid 1:1 on a Percoll® solution. After centrifugation at 400×*g* for 45 min at room temperature, the cells at the interface between the plasma and Percoll® solution were harvested and treated with 0.83% NH_4Cl in a tris-base buffer (pH 7.2) for 5 min to lyse remaining erythrocytes. The resulting porcine PBMCs were washed three times with PBS. PBMCs were resuspended in RPMI 1640 medium with 2 mM L-glutamine, 5% heat-inactivated FBS, and 200 μg/ml of streptomycin.

Cell culture

The PBMCs seeded at a density of 2×10^6 cells/ml or 3×10^6 cells/ml in a twenty-four-multi well plate (Nunc company, Naperville, IL, USA) were incubated with fucoidan (0, 50, 100 or 200 μg/ml) in the presence or absence of LPS (1 μg/ml) for indicated times at 37°C in a 5% CO_2 humidified atmophere. The same amount of PBS was added to control cells without treatment of fucoidan. After an incubation, all culture supernatants were collected after centrifugation at 900×*g* for 10 min and stored at −70°C until used.

Nitric oxide (NO) assay

NO concentration in the culture medium was measured by a porcine NO enzyme-linked immunnosorbent assay (ELISA) kit (Mybiosource, San Diego, CA, USA) following the manufacturer's protocol. In brief, culture supernatants from PBMCs treated with fucoidan and/or LPS for 24 h were placed in 96-well plates with standard reagents. After 90 min of incubation, wells were washed three times with wash buffer and incubated with biotinylated porcine NO antibody liquid (dilution 1:100) for 1 h at 37°C. After three successive washes, they were incubated with enzyme-conjugate liquid (dilution 1: 100) for 30 min at 37°C. Then, five successive washes were performed and the reactions were blocked by adding stop solution reagent in each well. Optical density was determined using automated microplate reader at 450 nm.

Activator protein-1 (AP-1) transcription factor assay

AP-1 activity was determined using the porcine transcription factor/AP-1 ELISA kit (Mybiosource) following the manufacturer's protocol. The assay was similar tothat of NO ELISA kit except that culture supernatants from PBMCs treated with fucoidan and/or LPS for 1 h were used.

Real time polymerase chain reaction (RT-PCR)

Porcine PBMCs (2×10^6 cells/ml) were incubated with fucoidan (0-200 μg/ml) in the presence or absence of LPS (1 μg/ml) for 6 h to measure the expression of iNOS mRNA and for 1 h to measure the expression of AP-1 mRNA. Total RNA was extracted using the Trizol reagent (Invitrogen Company, Carlsbad, CA, USA) according to the methods outlined in the protocol, and the concentration of total RNA was determined by investigating the absorbance at 260 nm. First-strand complementary DNA was prepared by subjecting total RNA (1 mg) to reverse transcription using Moloney Murine Leukemia Virus RT (Invitrogen Company) and random primers (9-mers; Takara Bio, Inc., Otsu, Shiga, Japan). To determine the conditions for logarithmic-phase PCR amplification of iNOS, AP-1 and cytochrome c oxidase subunit (1A), mRNA aliquots (1 mg) were amplified using 40 cycles. The 1A gene was PCR amplified to rule out the possibility of RNA degradation and was used to control for variations in mRNA concentration in the RT reaction. Complementary DNA was amplified in 20 ml PCR mixtures containing 1 unit Taq polymerase (iNtRON Biotechnology, Inc., Sungnam, Korea), 2 mM deoxyribonucleotide triphosphate and 10 pmol specific primers. The threshold fluorescence intensity for all samples was set manually. The reaction cycle at which the PCR products exceeded this threshold was identified as the threshold cycle (CT) of the exponential phase of PCR amplification. Oligonucleotides for iNOS were based on the complementary DNA sequence 5'-ACC TCA ACA AAG CTC TCA GC-3' (sense) and 5'-CGG GAA AAC TCC AAG ATG CT-3' (antisense). Oligonucleotides for AP-1 were based on the complementary DNA sequence 5'-GAT CCT GAA GCA GAG CAT GA-3' (sense) and 5'-ATA GGA ACT GGG TAG GGG TC-3' (antisense). The primer for the 1A gene was 5'-CAC CGT AGG AGG TCT AAC G-3' (sense) and 5'-GTA TCG TCG AGG TAT TCC G-3' (antisense). Data for each sample were analyzed by comparing CT values at constant fluorescence intensity. The amount of transcript was inversely related to the observed CT, and for every two-fold dilutions of the transcript, the CT was expected to increase by one increment. Relative expression (R) was calculated using the equation: $R = 2\text{-}[\Delta CT \text{ sample} - \Delta CT \text{ control}]$.

Statistical analyses

All statistical analyses were performed using GraphPad prism 6 software (GraphPad software, San Diego, CA, USA). One-way ANOVA was used to determine the statistical significance of the differences between control and treatment groups, followed by a Dunnett test. Comparisons of two groups were done using the *t* test. *P* values of less than 0.05 were considered to be statistically significant. Data are expressed as means and standard deviations (SD).

Results

Fucoidan does not increase NO production in porcine PBMCs

To examine the effect of fucoidan on NO production in LPS-naïve porcine PBMCs, the amount of NO in the culture supernatants from PBMCs treated with fucoidan for 24 h was measured. Production of NO was not increased by fucoidan (50 and 200 μg/ml) compared with untreated control (Fig 1).

Fucoidan decreases excessive NO production in LPS-stimulated PBMCs

To investigate the effect of fucoidan on NO production by LPS-stimulated PBMCs, the amount of NO in the culture

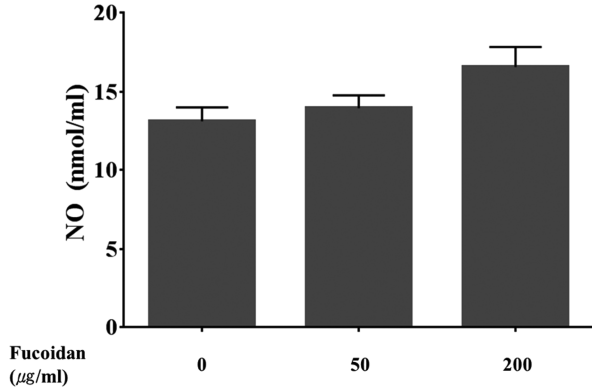

Fig 1. Effect of fucoidan on NO production in porcine PBMCs. The amount of NO in culture supernatants from PBMCs (3×10^6 cells/ml) treated with fucoidan (0-200 μg/ml) for 24 h was measured using ELISA assay. The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett test. Comparison of two groups was made by t-test.

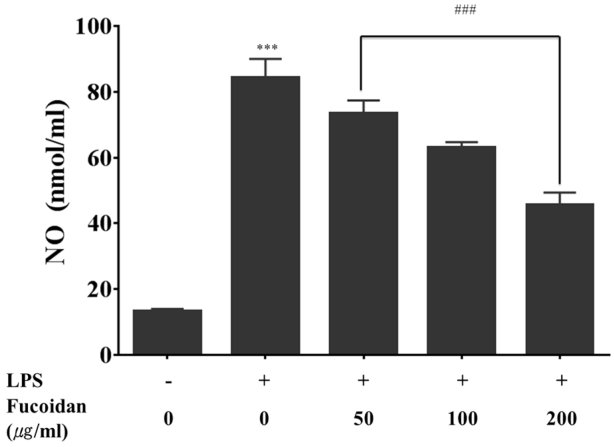

Fig 2. Effect of fucoidan on NO production in LPS-stimulated porcine PBMCs. Cells (3×10^6 cells/ml) were treated with LPS (1 μg/ml) and fucoidan (0-200 μg/ml) for 24 h. The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett test. Comparison of two groups was made by t-test. ***$p < 0.001$ *vs.* control. ###$p < 0.001$ *vs.* LPS.

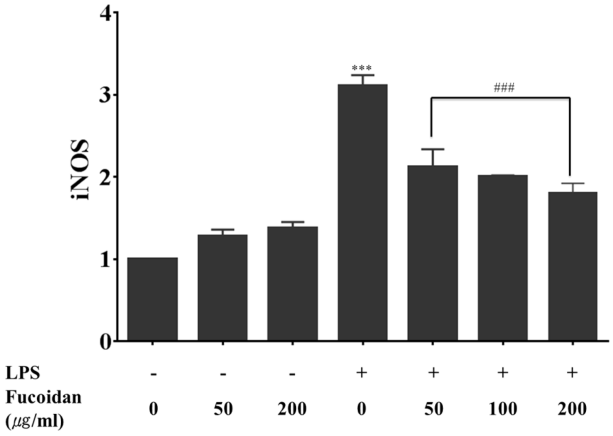

Fig 3. Effect of fucoidan on expression of iNOS mRNA in LPS-stimulated porcine PBMCs. Cells (2×10^6 cells/ml) were incubated with fucoidan (0-200 μg/ml) in the presence or absence of LPS (1 μg/ml) for 6 h. Then, expression of iNOS mRNA was examined by RT-PCR. The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett test. Comparison of two groups was made by t-test. ***$p < 0.001$ *vs.* control. ###$p < 0.001$ *vs.* LPS.

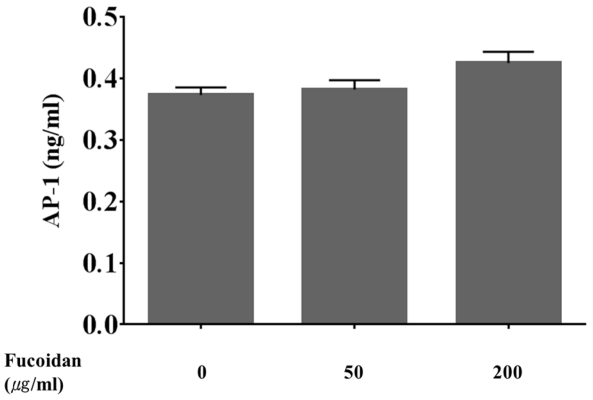

Fig 4. Effect of fucoidan on AP-1 activity in porcine PBMCs. AP-1 activity in the culture supernatant from PBMCs (3×10^6 cells/ml) treated with fucoidan (0-200 μg/ml) for 1 h was measured. The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett test.

supernatants from PBMCs treated with LPS (1 μg/ml) and fucoidan (0-200 μg/ml) for 24 h was measured. As shown in Fig 2, treatment of PBMCs with LPS remarkably increased the production of NO ($P < 0.001$) compared with untreated control. However, LPS-induced NO production was significantly ($P < 0.001$) decreased in dose-dependent manner by the addition of fuocidan (50, 100 and 200 μg/ml).

Fucoidan suppresses iNOS mRNA expression in LPS-stimulated PBMCs

We investigated the question of whether inhibition of NO production by fucoidan in LPS-stimulated PBMCs was involved in levels of iNOS mRNA, which were examined after treatment of fucoidan (0-200 μg/ml) with or without LPS (1 μg/ml) for 6 h. Expression of iNOS mRNA in PBMCs without LPS was not increased by treatment of fucoidan (50, 100 and 200 μg/ml) relative to untreated control cells. However, levels of iNOS mRNA expression were significantly ($P < 0.001$) increased by treatment of LPS. This increased levels of iNOS mRNA induced by LPS were significantly ($P < 0.001$) decreased in dose-dependent manner by the addition of fucoidan (50, 100 and 200 μg/ml) (Fig 3).

Fucoidan has no effect on AP-1 activity in porcine PBMCs

To investigate whether fucoidan affects AP-1 activity in porcine PBMCs, AP-1 activity was examined in the culture supernatant from PBMCs treated with fucoidan for 1 h. AP-1 activity was not increased by treatment of fucoidan (50 and 200 μg/ml) in LPS-naïve PBMCs (Fig 4).

Fucoidan suppresses AP-activation in LPS-stimulated PBMCs

To investigate whether fucoidan can modulate AP-1 path-

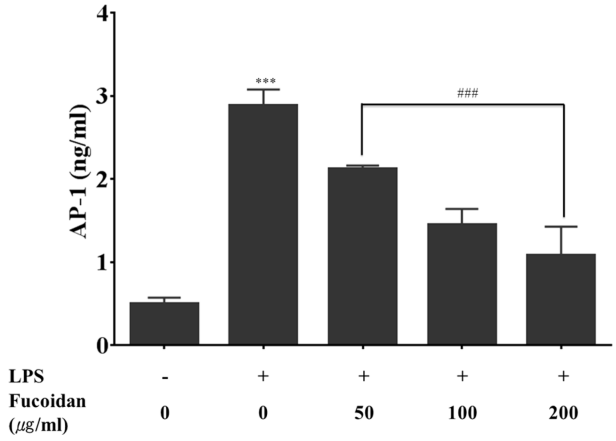

Fig 5. Effect of fucoidan on AP-1 activity in LPS-stimulated porcine PBMCs. AP-1 activity in culture supernatant from PBMCs (3×10^6 cells/ml) treated with fucoidan (0-200 μg/ml) with or without LPS (1 μg/ml) for 1 h was measured. The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett test. Comparison of two groups was made by t-test. ***$p < 0.001$ *vs.* control. ###$p < 0.001$ *vs.* LPS.

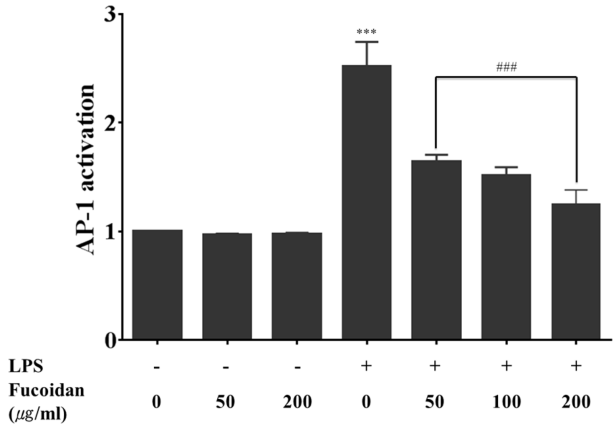

Fig 6. Effect of fucoidan on expression of AP-1 mRNA in LPS-stimulated porcine PBMCs. Cells (2×10^6 cells/ml) were incubated with fucoidan (0-200 μg/ml) in the presence or absence of LPS (1 μg/ml) for 1 h. Expression of AP-1 mRNA was investigated by RT-PCR. The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett test. Comparison of two groups was made by t-test. ***$p < 0.001$ *vs.* control group. ###$p < 0.001$ *vs.* LPS.

way in LPS-stimulated PBMCs, we measured AP-1 activity in culture supernatant from porcine PBMCs treated with fucoidan (0-200 μg/ml) with or without LPS (1 μg/ml) for 1 h. As shown in Fig 5, treatment of LPS alone markedly ($P < 0.001$) induced AP-1 activity when compared with that of unstimulated control. However, LPS-induced AP-1 activities in PBMCs were significantly ($P < 0.001$) decreased dose-dependently by treatment of fucoidan (50, 100 and 200 μg/ml).

Fucoidan down-regulates AP-1 mRNA expression in LPS-stimulated PBMCs

In order to examine the effect of fucoidan on expression of AP-1 mRNA in LPS-stimulated PBMCs, we investigated AP-1 mRNA levels after treatment of fucoidan (0-200 μg/ml) with or without LPS (1 μg/ml) for 1 h. Expression of AP-1 mRNA in LPS-naïve PBMCs was not induced by treatment of fucoidan (50 and 200 μg/ml) relative to untreated control cells. However, levels of iNOS mRNA was significantly ($P < 0.001$) increased by treatment of LPS. This increased levels of iNOS mRNA induced by LPS was significantly ($P < 0.001$) reduced in dose-dependent manner by the addition of fucoidan (50, 100 and 200 μg/ml) (Fig 6).

Discussion

LPS that binds the toll-like receptor (TLR) 4 complex is well known as endotoxin. LPS promotes the secretion of pro-inflammatory cytokines and induces the inflammation condition by inducing NO (11,33). In this study, we used LPS to examine the effect of fucoidan on the inflammatory responses in porcine PBMCs.

We examined whether fucoidan has effects on production of NO and expression of iNOS in porcine PBMCs. In the present study, fucoidan in LPS-naïve PBMCs did not show any effect on NO production and expression of iNOS mRNA. It has been known that fuocidan has no effect on NO production and expression of iNOS in LPS-naïve cells such as BV2 microglia cells (29) and primary microglia cells (9). These findings suggested that fucoidan does not induce NO production and expression of iNOS in LPS-naïve PBMCs.

As expected, LPS-stimulated porcine PBMCs revealed the overproduction of NO and high expression of iNOS mRNA in comparsion with LPS- naïve PBMCs. However, treatment of fucoidan in LPS-stimulated PBMCs decreased the over-production of NO and the expression of iNOS mRNA. LPS-induced excessive production of NO and expression of iNOS have been reported to be suppressed by a variety of biological materials in murine macrophages (7,8,15,26). It is also possible that cell cytotoxicity and cell death induced by fucoidan attribute to the decrease of NO production. However, fucoidan treatment in the presence of LPS did not reduce cell viability (29), even at a concentration of 300 μg/ml of fucoidan (34), suggesting that suppression of NO production was not due to cytotoxicity and death of porcine PBMCs by fucoidan. Therefore, these results suggested that fucoidan can suppress excessive production of NO with expression of iNOS mRNA in LPS-stimulated porcine PBMCs.

Expression of iNOS is regulated by transcription factors including NF-κB and AP-1 (20). NF-κB is activated by phosphorylation of inhibitory kappa B and translocated into nucleus to activate gene, when inflammation is induced by LPS (22,30). AP-1 regulated by phosphorylation of MAPKs can also modulate gene transcription in various biological processes including inflammation and immune responses (20, 32). However, the effect of fucoidan on AP-1 activity, especially in porcine PBMCs, was not examined. In the present study, we found that treatment of fucoidan has no effects on both activity of AP-1 and expression of AP-1 mRNA in LPS-naïve PBMCs. Chlorogenic acid in LPS-naïve RAW 264.7 cells (31) and clarithromycin in LPS-naïve human monocyte (16) have also been reported to show no effect on AP-1. It was, therefore, thought that fucoidan has no effects on activ-

ity of AP-1 and expression of AP-1 mRNA in LPS-naïve PBMCs.

The data of the present study showed that treatment of LPS increases both AP-1 activity and expression of AP-1 mRNA in PBMCs. These increases were reduced by addition of fucoidan in PBMCs stimulated with LPS. In addition, fucoidan has been found to suppress AP-1 activity in LPS-induced BV2 microglia cells and RAW 264.7 cells (29,34). These findings indicated that fucoidan can also suppress AP-1 activity in LPS-stimulated porcine PBMCs. Since AP-1 modulates NO production by regulating expression of iNOS, the effects of fucoidan on NO production and iNOS expression would be exerted by down-regulation of AP-1 activity in porcine PBMCs stimulated with LPS. NO production and iNOS expression can be also modulated by upstream pathway including myeloid differentiation factor 88 (MyD88) via regulation of AP-1 and NF-κB (7,8). However, the effects of fucoidan in porcine PBMCs on upstream pathway were not examined. If fucoidan has any effect on upstream pathway, it can modulate NF-κB pathway. If so, it is possible that action pathway is more responsive to fucoidan. In contrast, if fucoidan has no effect on upstream pathway, it directly modulates AP-1 activity. If so, NF-κB blocking materials which regulate NF-κB may create a synergy effect with fucoidan. Therefore, investigations of the effect of fucoidan on upstream pathway need to explain precise mechanism.

Pigs have many inflammatory diseases such as inflammatory bowel disease, atrophic rhinitis and arthritis, which reduce growth rate (2). In the present study, we showed that fucoidan decreases excessive production of NO with reduction of iNOS expression by regulating activity of AP-1 in LPS-stimulated porcine PBMCs. These results suggested that fucoidan has anti-inflammatory effect in inflammatory condition of PBMCs. Therefore, fucoidan may have effect on porcine inflammatory diseases and may be used for feed additives.

In conclusion, the data supported our hypothesis that the effect of fucoidan on NO production is related to the level of AP-1 activation induced by LPS stimulation. The results of this study suggested that fucoidan can suppress NO production through the down-regulation of iNOS gene expression in LPS-stimulated porcine PBMCs. These effects were accompanied by changes in AP-1 activation.

Acknowledgments

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LPS로 자극한 돼지 말초혈액 단핵구세포의 Nitric Oxide (NO) 생산 및 Activator Protein-1 (AP-1) 활성화에 있어 Fucoidan의 효과

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요 약 : Fucoidan은 갈조류로부터 추출되는 황산다당류로 다양한 생리학적 활성을 갖고 있다. 본 연구의 목적은 LPS로 자극한 돼지 말초혈액 단핵구세포(PBMCs)의 NO 생산에 있어 fucoidan의 효과를 검토하고, 이러한 효과가 iNOS의 발현과 AP-1의 활성화와 관련이 있는지를 조사하는데 있다. LPS 무처치 돼지 PBMCs에서 fucoidand의 처리는 NO 생산과 AP-1활성에 대해 효과를 보이지 않았다. 또한 iNOS와 AP-1의 mRNA 발현도 fucoidan 처치에 의해 영향을 받지 않았다. 그러나 LPS로 자극한 PBMCs에서는 NO 생산과 AP-1의 활성 그리고 iNOS와 AP-1의 mRNA 발현이 현저하게 증가하였다. 이와 같은 LPS에 의한 돼지 PBMCs의 NO 생산과 AP-1 활성증가는 fucoidan 첨가에 의해 감소되었다. 또한 fucoidan은 LPS에 의한 iNOS와 AP-1의 mRNA 발현증가도 억제시켰다. 이상의 결과는 fucoidan이 LPS 자극 돼지 PBMCs에서 iNOS 발현과 AP-1 활성의 억제와 함께 NO 생산을 하향 조절함으로써 항염증효과를 나타내는 것으로 사료되었다.

주요어 : fucoidan, AP-1, NO, iNOS, 항염증, 말초혈액단핵구세포, 돼지