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Fucoidan Suppresses Prostaglandin E_2 Production and Akt Activation in Lipopolysaccharide-Stimulated Porcine Peripheral Blood Mononuclear Cells

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Abstract : Fucoidan, a cell wall polysaccharide found in the brown seaweed, is reported to have broad-spectrum biological activities. The objectives of this study were to examine the effect of fucoidan on prostaglandin E_2 (PGE_2) and cyclooxygenase-2 (COX-2) expression in lipopolysaccharide (LPS)-stimulated porcine peripheral blood mononuclear cells (PBMCs) and to determine whether these effects are involved in Akt activation. The levels of PGE_2 production in the culture supernatants from PBMCs were determined by the enzyme-linked immunosorbent assay (ELISA) kit and the levels of COX-2 mRNA were measured by real time polymerase chain reaction (RT-PCR). Akt activity was determined by Western blot analysis. Fucoidan in LPS-naïve PBMCs has no effect on PGE_2 production and COX-2 mRNA expression. Furthermore, fucoidan does not affect Akt activation in LPS- naïve PBMCs. However, PGE_2 production and COX-2 mRNA expression on PBMCs were remarkably enhanced by LPS stimulation. Akt activity was also increased by LPS. Increasing effects of PGE_2 production and COX-2 mRNA expression in PBMCs induced by LPS were suppressed by addition of fucoidan. In addition, fucoidan reduced an increase in Akt activity in LPS-stimulated PBMCs. These results suggested that fucoidan exerts potent anti-inflammatory properties by suppression of PGE_2 production, COX-2 mRNA expression and Akt activation in LPS-stimulated PBMCs.

Key words **:** fucoidan, Akt, anti-inflammation, PBMCs, PGE_2, COX-2, porcine.

Introduction

The inflammation process is a body's mechanism which induce inflammatory responses to stimulate the innate immunity against intruders (7). Cyclooxygenase-2 (COX-2) is important in the eicosanoid metabolism during inflammation. It is highly inducible in response to inflammatory stimuli resulting in enhanced prostaglandin (PG) release (31). PGE_2 is regarded as an important mediator in the processes of inflammation produced by COX-2 (34).

The Akt signal molecule is reported to mediate nuclear factor (NF)-κB activation via IkB kinase (IKK) activation. IKK activation is regulated by phosphorylation through various upstream kinases such as NF-κB-inducing kinase and Akt, which are involved in cellular signaling in response to pro-inflammatory stimuli (11). It has been reported that LPS-induced NF-κB activation is directly mediated as a main upstream molecule of NF-κB through the phosphorylation of Akt (6). Activation of Akt plays an important role in the expression of inducible nitric oxide synthase (iNOS) and COX-2 (8).

Fucoidan is a cell wall polysaccharide found in the extracellular matrix of brown algae and contains variable amounts of fucose, uronic acid, galactose, xylose, and sulfates (21). Fucoidan has diverse biological activities of potential medicinal value including inflammatory modulatory effect (5). Fucoidan also enhances both humoral and cell-mediated immune responses under in vitro and in vivo conditions (9). It was recently suggested that fucoidan has an anti-inflammatory effect by suppressing nitric oxide (NO) production through the down-regulation of iNOS gene expression in LPS- stimulated porcine PBMCs. In addition, these effects were accompanied by changes in AP-1 activation (25). However, it remains unclear whether fucoidan influences PGE_2 production and Akt activation.

The aim of the present study was to examine the effects of fucoidan on production of PGE_2 and expression of COX-2 in LPS-stimulated porcine PBMCs. In addition, it was investigated whether this effect is associated with a change of Akt activity or not.

Materials and Methods

Reagents

LPS from *Escherichia coli* 0127:B8 (Sigma-Aldrich, St. Louis, MO, USA) was diluted with phosphate buffered saline (PBS) to a final concentration of 100 μg/ml as stock solution and used with the concentration of 1 μg/ml. Fucoidan purified from *Focus vestculosus* was purchased from Sigma-Aldrich and passed through a 0.45 μm membrane filter (Millipore Corporation, Bedford, MA, USA). The primary antibodies for Akt (rabbit anti-rh Akt polyclonal antibody (IgG)) (Santa Cruz Biotechnology, Dallas, Texas, USA) and p-Akt (rabbit anti-rm pAkt polyclonal antibody (IgG)) (Cell Signaling

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Technology Inc., Danvers, MA, USA), Percoll®, RPMI 1640 medium (Sigma-Aldrich), and fetal bovine serum (FBS) (Gibco Company, Grand Island, NY, USA) were commercially purchased.

Porcine peripheral blood mononuclear cells (PBMCs)

All experimental procedures and animal use were approved by the ethics committee of the Chungbuk National University. Clinically healthy 6 months crossmixed pigs were used as blood donors in slaughterhouse (Donga food Co. Ltd., Cheongju, Korea). Peripheral blood drawn in heparinized tube from anterior vena cava, diluted with an equal volume of phosphate-buffered saline (PBS) without calcium and magnesium, and overlaid 1:1 on a Percoll® solution (Sigma-Aldrich; 1.080 gravity). 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 RBC lysis buffer (iNtRON biotechnology, Seongnam, Korea) for 5 min to lyse remaining erythrocytes. The resulting PBMCs were washed three times with PBS. PBMCs were resuspended in RPMI 1640 medium with 5% heat-inactivated FBS, 1% 100 U/ml penicillin and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 humidified atmosphere.

Cell culture

The PBMCs seeded at a density of 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 atmosphere. After an incubation, all culture supernatants were collected after centrifugation at 900 g for 15 min and stored at −70°C until used.

Prostaglandin E_2 (PGE_2) assay

The PGE_2 concentration in the culture medium was measured by a porcine PGE_2 enzyme-linked immunnosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, USA) following the manufacturer's protocol. In brief, culture supernatants from PBMCs treated with various concentration of fucoidan with or without LPS (1 μg/ml) for 24 h were placed in 96-well plates with standard reagents. Wells were incubated with PGE_2 conjugate liquid and monoclonal PGE_2 antibody liquid for 24 h at 4°C. After 24 h of incubation, wells were washed three times with wash buffer and incubated with substrate solution for 1 h at 37°C. Then, the reactions were blocked by adding stop solution reagent in each well. Optical density was determined using automated microplate reader at 405 nm.

Real time polymerase chain reaction (RT-PCR)

Porcine PBMCs (3×10^6 cells/ml) were incubated with fucoidan (0, 50, 100 or 200 μg/ml) in the presence or absence of LPS (1 μg/ml) for 6 h to measure the expression of COX-2 mRNA. Total RNA was extracted using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the method outlined in the protocol, and the concentration of total RNA was determined by measuring the absorbance at 260 nm. First strand complementary DNA (cDNA) was prepared by subjecting total RNA (1 mg) to reverse transcription using Moloney Murine Leukemia Virus RT (Invitrogen Co.) and random primers (9-mers; Takara Bio Inc, Otsu, Shiga, Japan). 2 μl of cDNA template was added to 10 μl of 2SYBR Premix Ex Taq (Takara Bio) and 10 pmol of each specific primer. Reactions were carried out for 40 cycles. At the end of the extension phase of each cycle, fluorescence intensity was measured. 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. The expression of COX-2 was quantified relative to that of 18S RNA. The oligonucleotides for COX-2 and 18S RNA gene, which were based on the cDNA sequence, were described below.

Cyclooxygenase-2
sense 5' - TCCTGCCCT TCTGGTAGA AA - 3'
antisense 5' - CTGAATCGAGGCAGTGTTGA - 3'

18S RNA
sense 5' - CTCAACACGGGAAACCTCAC - 3'
antisense 5' - CGCTCCACCAACTAAGAACG - 3'

Data for each sample were analyzed by comparing cycle threshold (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: $R = 2 - [\Delta CT \text{ sample} - \Delta CT \text{ control}]$.

Western blot analyses

Porcine PBMCs (3×10^6 cells/ml) were incubated with fucoidan (0, 100 or 200 μg/ml) in the presence or absence of LPS (1 μg/ml) to measure the Akt activation. Cellular protein was extracted using RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM EDTA and proteinase inhibitors). Cytosolic protein (40 mg per lane) was separated on 11% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride transfer membrane (Perkin Elmer Co., Wellesley, MA, USA) in a TransBlot Cell (TE-22 Hoefer Inc., Sanfrancisco, CA, USA) according to the manufacturer's protocol. The membranes were blocked for 60 min with 5% skim milk (Difco™, Sparks, MD, USA) in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T), then incubated in primary antibodies Akt (Santa Cruz Biotechnology) and p-Akt (Cell Signaling Technology Inc.) for 60 min at room temperature (RT). After washing in buffer, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, 1:2000, Santa Cruz Biotechnology) for 1 h at RT. After washing, the blots were developed by incubation in enhanced chemiluminescent reagent (Amersham Biosciences, Little Chalfont, UK) and detected by ChemiDoc equipment GenGnome 5 (Syngene, Cambridge, UK). Signal specificity was confirmed by blotting in the absence of primary antibody and bands were standardized to Akt immunoreactive

bands visualized in the same membrane after stripping. Density measurements for each band were performed with NIH Image J software (NIH, Rockville, MD, USA). Background samples from an area near each lane were subtracted from each band to obtain mean band density.

Statistical analyses

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

Results

Fucoidan has no effect on PGE_2 production in porcine PBMCs

To assess the effect of fucoidan on PGE_2 production, the amount of PGE_2 in the culture supernatants from PBMCs treated with fucoidan for 24 h was measured. PGE_2 from PBMCs was not produced by treatment of fucoidan (0-200 μg/ml) (Fig 1).

Fucoidan suppresses overproduction of PGE_2 in LPS-stimulated porcine PBMCs

To examine the effect of fucoidan on PGE_2 production by LPS-stimulated PBMCs, the amount of PGE_2 in the culture supernatants from PBMCs treated with LPS (1 μg/ml) and/or fucoidan (0-200 μg/ml) for 24 h was measured. As shown in Fig 2, treatment of PBMCs with LPS dramatically increased the production of PGE_2 ($P < 0.001$) when compared with PBMCs without LPS. However, Production of PGE_2 induced by by LPS was significantly ($P < 0.01$-0.001) decreased by the addition of fuocidan (50-200 μg/ml).

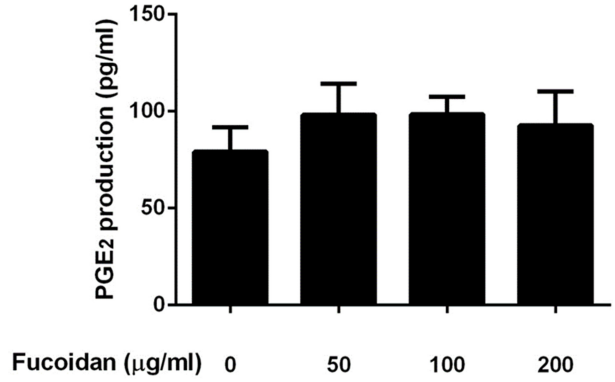

Fig 1. Effect of fucoidan on PGE_2 production in porcine PBMCs. Cells (3×10^6 cells/ml) were treated with fucoidan (0-200 μg/ml) for 24 h. The amount of PGE_2 in culture supernatants from PBMCs was measured using ELISA kit. The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett test. PGE_2, prostaglandin E_2; PBMCs, peripheral blood mononuclear cells.

Fucoidan has no effect on COX-2 mRNA expression in porcine PBMCs

To investigate the question of whether fucoidan affects COX-2 mRNA expression in porcine PBMCs, levels of COX-2 mRNA were examined 6 h after treatment of fucoidan (0-200 μg/ml). Expression of COX-2 mRNA in PBMCs without LPS was not changed by treatment of fucoidan (0-200 μg/ml) (Fig 3).

Fucoidan suppresses expression of COX-2 mRNA in LPS-stimulated porcine PBMCs

We further examined the question of whether the inhibition of PGE_2 production by fucoidan in LPS-stimulated

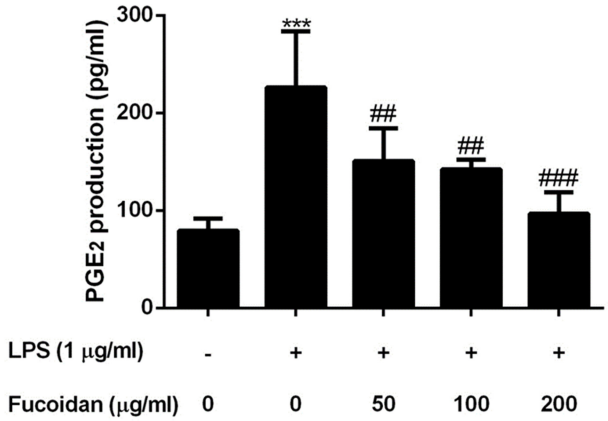

Fig 2. Effect of fucoidan on PGE_2 production in LPS-stimulated porcine PBMCs. Cells (3×10^6 cells/ml) were treated with fucoidan (0-200 μg/ml) and LPS (1 μg/ml) for 24 h. The amount of PGE_2 in culture supernatant from PBMCs treated with fucoidan and LPS was measured using ELISA kit. The data represent 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. ***$p < 0.001$ indicates *vs* control. ##$p < 0.01$ *vs* LPS. ###$p < 0.001$ *vs* LPS. PGE_2, prostaglandin E_2; LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells.

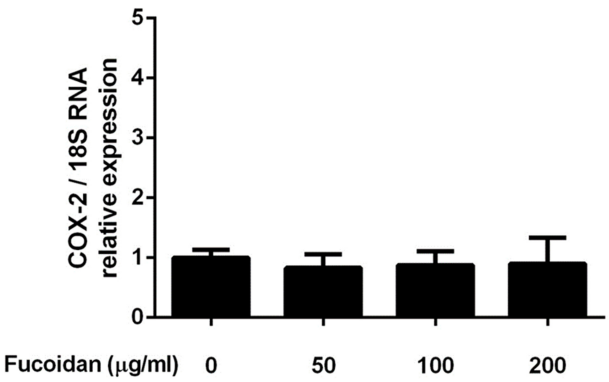

Fig 3. Effect of fucoidan on expression of COX-2 mRNA in porcine PBMCs. Cells (3×10^6 cells/ml) were incubated with fucoidan (0-200 μg/ml)) for 6 h. Then, expression of COX-2 was examined by RT-PCR. The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett test. COX-2, cyclooxygenase-2; PBMCs, peripheral blood mononuclear cells.

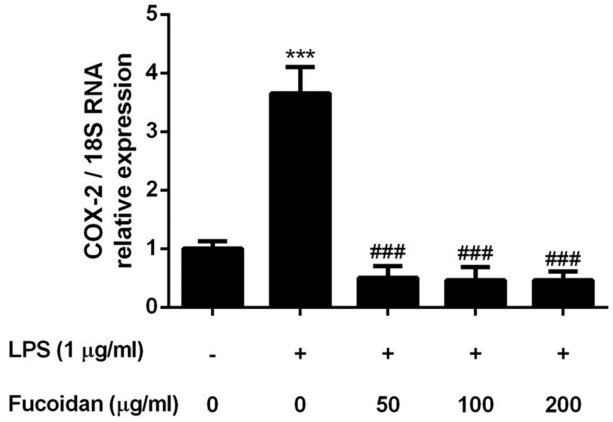

Fig 4. Effect of fucoidan on expression of COX-2 mRNA in LPS-stimulated porcine PBMCs. Cells (3×10^6 cells/ml) were incubated with fucoidan (0-200 μg/ml) in the presence of LPS (1 μg/ml) for 6 h. Then, expression of COX-2 was examined by RT-PCR. The data represent means ± SD (n = 3). One-way ANOVA was 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. COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells.

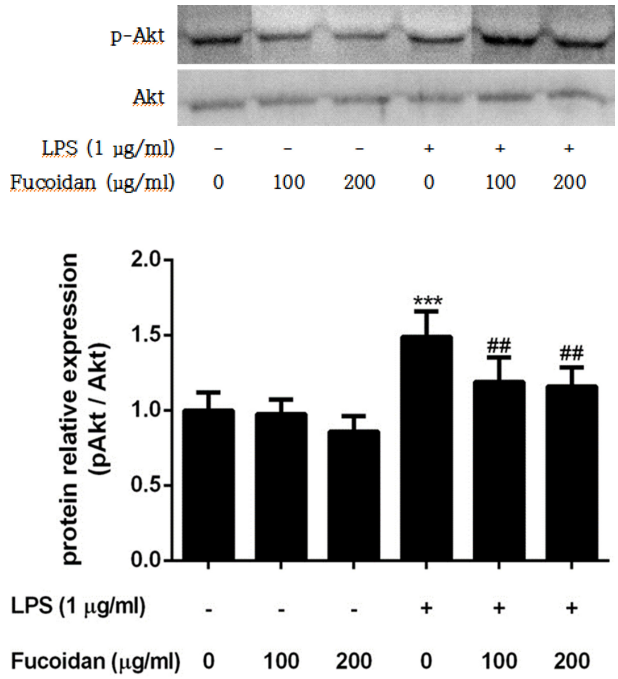

Fig 5. Effect of fucoidan on Akt activity in LPS-stimulated porcine PBMCs. Cells (3×10^6 cells/ml) were treated with fucoidan (0-200 μg/ml) for 2 h. The cells were also treated with LPS (1 μg/ml) for final 1 h. Total protein was subjected to 11% SDS-PAGE, followed by Western blotting using anti-Akt and anti-pAkt antibodies. 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. ##$p < 0.01$ *vs* LPS. LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells.

PBMCs is associated with levels of COX-2 mRNA, which were examined after treatment of fucoidan (0-200 μg/ml) and/or LPS (1 μg/ml) for 6 h. Levels of COX-2 mRNA expression were significantly ($P < 0.001$) increased by treatment of LPS relative to untreated control cells. This increased levels of COX-2 mRNA induced by LPS were significantly ($P < 0.001$) decreased by treatment of fucoidan (50-200 μg/ml) (Fig 4).

Fucoidan down-regulates phosphorylation of Akt in LPS-stimulated porcine PBMCs

In order to investigate the effect of fucoidan on activity of Akt in LPS-stimulated PBMCs, we examined Akt activation in PBMCs after treatment of fucoidan (100-200 μg/ml) for 2 h with or without LPS (1 μg/ml) for final 1 h. As shown in Fig 5, Akt phosphorylation in LPS-naïve PBMCs was not induced by treatment of fucoidan (100-200 μg/ml) relative to untreated control cells. However, phosphorylation of Akt was significantly ($P < 0.001$) increased by treatment of LPS. This increased levels of Akt phosphorylation was significantly ($P < 0.01$) reduced by pretreatment of fucoidan (100-200 μg/ml).

Discussion

LPS is the major constituent of the outer membrane of gram-negative bacteria which can stimulate diverse inflammatory reactions, inducing the release of related pro-inflammatory mediator such as PGE2 and inflammatory cytokines including interleukin (IL)-1, IL-10 and tumor necrosis factor (TNF)-α (1,4,30). In the present study, we used LPS originated from E. *coli* to examine the inflammatory responses in porcine PBMCs.

PGE_2 is a widely known inflammatory mediator derived from arachidonic acid through the action of cyclooxygenases (23). PGE_2 induced by COX-2 is potent inflammatory mediator that stimulate tumor growth and metasis by stimulating angiogenesis, cell proliferation, and invasion (20). For this reason, the suppression of PGE_2 production by the inhibition of COX-2 expression can be a very important therapeutic approach in the development of anti-inflammatory agents. In the present study, we investigated whether fucoidan has effects on production of PGE2 and expression of COX-2 in porcine PBMCs. Fucoidan in LPS-naïve PBMCs has no effect on PGE2 production and expression of COX-2 mRNA. It was demonstrated that fucoidan does not increase PGE2 production and expression of COX-2 in LPS-naïve cells such as BV2 microglia cells (24). In addition, it has been reported that PGE2 production and expression of COX-2 were not affected by various biological materials such as saponins in BV2 microglial cells (12) and 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone in RAW 264.7 macrophage (15). Therefore, it is suggested that fucoidan has no effect on PGE2 production and expression of COX-2 in LPS-naïve PBMCs.

LPS-stimulated porcine PBMCs revealed the excessive production of PGE2 and increased COX-2 mRNA expression in comparsion with LPS-naïve PBMCs. However, treatment of fucoidan in LPS-stimulated PBMCs suppressed overproduction of PGE2 and COX-2 mRNA expression. It is reported that overproduction of PGE2 and COX-2 expression stimulated by LPS were suppressed by a variety of bio-

logical materials such as geniposide in macrophages (29), chebulagic acid in RAW 264.7 macrophages (27), alantolactone in RAW 264.7 cells (4) and casticin in mouse macrophages (18). These results suggest that fucoidan can also suppress overproduction of PGE2 with COX-2 mRNA expression in LPS-stimulated porcine PBMCs.

Expression of COX-2 gene is largely mediated by NF-κB transcription factor (33). It has been well demonstrated that NF-κB activation is associated with the phosphorylation, ubiquitination, and subsequent degradation of IκBα (13). It was studied that effect of fucoidan on NF-κB is related to regulation of signal pathway. Fucoidan could regulate the inflammation response through inhibition of NF-κB activation in LPS-induced BV2 microglia cells (24). A previous study found that LPS could activate Akt signaling to turn on the transcription factors of NF-κB and express many inflammatory cytokines and COX-2 expression (19,35). In this study, we examined whether fucoidan has effects on Akt activity in porcine PBMCs. It was found that fucoidan has no effect on Akt activation in LPS-naïve PBMCs. Fuocidan in LPS-naïve BV2 microglia cells has also been reported to show no effect on Akt activation (24). Therefore, it is suggested that fucoidan has no effect on Akt activation in LPS-naïve PBMCs.

Treatment of LPS alone increased Akt activity in PBMCs. LPS has been shown to activate the Akt signaling pathway in several cell types such as murine adrenocortical cells (22), murine mesangial cells (28) and adrenal zona glomerulosa (10). However, these increases were suppressed by addition of fucoidan in PBMCs. Also, fucoidan reduced Akt activity in LPS-induced BV2 microglia cells (24). In addition, phlorofucofuroeckol A inhibit Akt activity induced by LPS in RAW 264.7 cells (14). Thus, these results suggested that fucoidan can suppress Akt activity in LPS-stimulated porcine PBMCs. Based on these findings, the effects of fucoidan on PGE2 production and COX-2 expression would be exerted by down-regulation of Akt activity in porcine PBMCs.

Steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to regulate symptoms of inflammation such as rheumatoid arthritis and atopic dermatitis (16). The levels of COX-2, PGE2, iNOS and proinflammatory cytokines could be suppressed by NSAIDs and steroids (26). However, it has been studied that long-term use of NSAIDs causes increased gastrointestinal toxicity and blood pressure, and increases vascular thrombosis and the incidence of heart failure (17,32). Also, long-term steroid use suppresses the immune system and results in more opportunities for pathogen infection (3). In this study, we showed that fucoidan suppresses overproduction of PGE2 with reduction of COX-2 expression by mediating activity of Akt in LPS-stimulated porcine PBMCs. In addition, it was recently suggested that fucoidan also exerts anti-inflammatory effect by down regulating production of NO via suppressing iNOS and activity of AP-1 in LPS- stimulated porcine PBMCs (25). These findings indicate that fucoidan has anti-inflammatory effect in inflammatory state of porcine PBMCs. Pigs have many inflammatory diseases including inflammatory bowel disease, arthritis and atrophic rhinitis (2). Based on our findings, fucoidan shows great potential as therapeutic agent and may be used in the future to treat porcine inflammation-associated diseases.

In conclusion, fucoidan can attenuate the LPS-induced overproduction of PGE2 and expression of COX-2 in porcine PBMCs. Furthermore, we showed that anti-inflammatory activity of fucoidan is achieved in part by inhibition of Akt activation. Taken together, fucoidan may be used as an anti-inflammatory therapeutic agent.

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