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Fucoidan Upregulates Chemotactic Activity of Porcine Peripheral Blood Polymorphonuclear Cells to Interleukin-8 by PI3K Activation

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Abstract : Fucoidan increases the chemotactic activity of peripheral blood polymorphonuclear cells (PMNs) through interleukin (IL)-8 produced by peripheral blood mononuclear cells (PBMCs). It has been demonstrated that fucoidan can regulate the chemotaxis of PMNs by activating F-actin polymerization. The objectives of this study are to investigate the direct effect of fucoidan on the chemotaxis of porcine PMNs and to examine whether this effect is associated with changes in phosphoinositide 3-kinase (PI3K) activity. The chemotactic activity of porcine PMNs was evaluated by modified Boyden chamber assay. Akt phosphorylation activity, a main downstream of PI3K, was measured by Western blotting assay. Fucoidan itself has no chemoattractant effect for PMNs. However, direct treatment of PMNs with fucoidan showed higher chemotactic activity to porcine recombinant (pr) IL-8 than that of PMNs without fucoidan. The increased chemotactic activity of fucoidan-treated PMNs to pr IL-8 was suppressed by treatment of wortmannin, an inhibitor of PI3K. Treatment of PMNs with fucoidan also increased Akt phosphorylation level. This increase was also suppressed by wortmannin. These results suggested that fucoidan can upregulate chemotactic activity of porcine PMNs to IL-8, which is associated with PI3K activation.

Key words **:** chemotaxis, fucoidan, PI3K, Akt, PMNs, porcine, IL-8.

Introduction

Neutrophils are highly specialized cells of the innate immune system that perform the first line of defense against bacterial and fungal infections (25). In host defense, neutrophil chemotaxis is crucial step in enabling neutrophils to migrate to sites of injury or infection, and refers to the sensing and crawling of neutrophils along a chemoattractant such as leukotriene B4 and interleukin (IL)-8 gradient (7). During chemotaxis, neutrophils display changes of cytoskeletal dynamics that accompanies F-actin polymerization, F-actin-enriched pseudopodia formation, and myosin II assembly in the leading edge (8,37).

Phosphoinositide 3-kinases (PI3K) are a family of enzymes linked to an incredibly diverse set of key cellular functions such as cell growth, proliferation, motility, differentiation, survival and intracellular trafficking (11,15,29). It was reported that phosphatidylinositol (3,4,5) triphosphate [PtdIns(3,4,5)P_3] produced by PI3K plays a central role in the cell polarization and migration during chemotactic movement (33,36). It also demonstrated that PI3K in macrophage and neutrophils is required for chemotaxis of chemoattractant-mediated respiratory burst, and activation of Akt, a serine/threonine protein kinase (13,22,34).

Fucoidan is sulfated polysaccharides found in the extracellular matrix of brown algae. Component of a fucoidan molecule includes D-galactose, D-mannose, D-xylose, L-rhamnose, D-glucuronic acid residues, acetyl groups and sulfates (24). Fucoidan has many potent biological activities including anti-inflammatory (12) and antioxidant effects (23). Fucoidan also has a variety of immunomodulatory effects. It enhances phagocytic capacity and oxidative burst activity of peripheral blood polymorphonuclear cells (PMNs), which is mainly mediated by tumor necrosis factor (TNF)-α released from fucoidan-treated peripheral blood mononuclear cells (PBMCs) (19). It induces activation and maturation of dendritic cells and can promote production of interferon-γ by CD4 and CD8 T cells (16). Moreover, it was recently suggested that fucoidan increases the chemotactic activity of canine PMNs via IL-8 produced by PBMCs (14). It was also demonstrated that fucoidan directly regulates the chemotaxis of canine PMNs by activating F-actin polymerization (18).

The objective of this study is to investigate whether fucoidan directly regulate the chemotactic activity of porcine PMNs and if so, whether this effect is associated with changes in PI3K activity

Material and Methods

Chemicals and reagents

Fucoidan purified from *Focus vesiculosus,* RPMI 1640 medium, and wortmannin, inhibitor of PI3K, were purchased commercially from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of fucoidan was prepared by dissolving it in phosphate buffered saline (PBS) to a final concentration of 20 mg/ml and passing it through a 0.45 μm

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membrane filter (Millipore Co., Bedford, MA, USA) before use. Porcine recombinant (pr) interleukin (IL) -8 (R&D systems Inc, Minneapolis, MN, USA), primary antibodies for Akt (rabbit anti-rh Akt polyclonal antibody (IgG)) (Santa Cruz Biotechnology, Dallas, Texas, USA) and primary antibodies for p-Akt (rabbit anti-rm pAkt polyclonal antibody (IgG)) (Cell Signaling Technology Inc., Danvers, MA, USA) were also purchased commercially.

PMNs isolation

Clinically healthy 6-month-old crossbred pigs in slaughterhouse (Donga food Co. Ltd., Cheongju, Korea) were used as blood donors. Heparinized porcine peripheral blood was drawn from the anterior vena cava, diluted with an equal volume of phosphate-buffered saline (PBS) without calcium and magnesium, and overlaid 1:1 on a solution (specific gravity, 1.080; Sigma-Aldrich Co.). After centrifugation at 400 g for 45 min at room temperature, the PMNs were obtained from the upper layer of sedimented erythrocytes after the removing the PBMCs layer. To purify the PMNs, the erythrocytes were allowed to settle for 60 min with 1.5% dextran (molecular weight, 200,000; Wako Ltd., Osaka, Japan) in PBS. The floating cells were then gently collected and pelleted by centrifugation at 900 g for 5 min. The residual erythrocytes were lysed by brief treatment with 0.83% NH_4Cl in a Tris-base buffer (pH 7.2) for 5 min. After that, PMNs were washed three times with PBS. The viability PMNs, as determined by trypan blue staining, always exceeded 98%. The resulting PMNs were resuspended in RPMI 1640 medium at 37°C under a 5% CO_2-humidified atmosphere.

Chemotaxis assay

The chemotactic activity of PMNs was determined as migration distance through millipore membrane filters by modified Boyden chamber assay, as previously described (32). Briefly, the chemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA) and FBS-free RPMI 1640 medium were pre-warmed for 2 h at 37°C. The lower chamber was filled with 200 μl of FBS-free RPMI 1640 medium containing pr IL-8 or fucoidan as the chemoattractant. A millipore membrane filters (120 μm thick and 3.0 μm pore size; millipore Corporation, Bedford, MA, USA) was placed on top of the well of the lower compartment. Then, 200 μl of PMNs suspension (2×10^6 cells/ml) containing fucoidan (100 and 200 μg/ml) or fucoidan (200 μg/ml) plus wortmannin (5 nM), an inhibitor of PI3K, in a minimal volume (< 1% of the medium) of dimethyl sulfoxide (DMSO) as the solvent was placed in the upper compartment. The same amount of DMSO as vehicle was added to the control well. DMSO was also added to the lower compartment to equalize the osmotic pressure with the compartment. The chambers were incubated for 40 min at 37°C in a 5% CO_2 humidified atmosphere. After incubation, the membrane filters were immediately removed, fixed in ethyl alcohol, dried, stained with hematoxylin, decolorized in ethyl alcohol, and mounted on a slide glass. The migrated distance of cells through the millipore membrane filter towards the other side was measured under a bright field microscopy at 400 × magnification. Five randomly selected fields per filter were examined in triplicate assay. The chemotactic responses of the input cells were evaluated as the absolute distance (μm/40 min) directionally migrated by the PMNs in response to chemoattractant.

Akt western blot analyses

Porcine PBMCs (2×10^6 cells/ml) were incubated with fucoidan (200 μg/ml) and/or wortmannin (5 nM) 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). A total 40 μg of cytosolic proteins was separated by 11% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 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 then blocked for 1 h with 5% skim milk (Difco™, Sparks, MD, USA) in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T). After blocking, the membranes were incubated with in primary antibodies Akt (Santa Cruz Biotechnology, Dallas, Texas, USA) and p-Akt (Cell Signaling Technology Inc., Danvers, MA, USA) for 60 min at room temperature (RT), followed the appropriate horseradish peroxidase-conjugated secondary antibodies (antirabbit, 1:2000, SantaCruz Biotechnology) in 3% skim milk containing PBS-T for 1 h. 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, Bethesda, 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 carried out by using GraphPad Prism 6 for windows (GraphPad Software Inc., San Diego, CA, USA). Results were compared by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test or two-way ANOVA with Bonferroni's *post hoc* test, as appropriate. P value of under 0.05 was considered statistically significant. Results are expressed as means ± standard deviation (± SD).

Results

Fucoidan has no chemoattractant activity for porcine PMNs

To examine whether fucoidan has any chemoattractant activity for PMNs, the migrated distances of PMNs in response to either fucoidan or pr IL-8 in the lower chamber were measured. Pr IL-8 significantly increased ($p < 0.001$) the chemotactic activity of PMNs at concentrations of 10 nM compared with the vehicle-treated control (Fig 1). However, the presence of fucoidan (100 and 200 μg/ml) in the lower

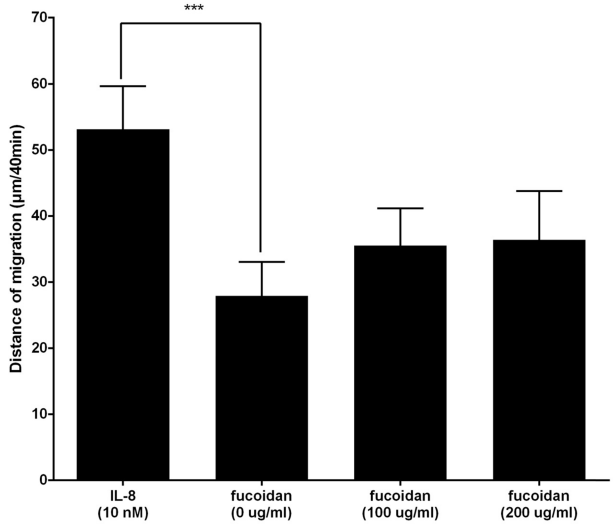

Fig 1. Chemoattractant effect of fucoidan on porcine PMN chemotaxis. Freshly isolated PMNs (2×10^6 cells/ml) were placed in the upper chamber and pr IL-8 (10 nM) or fucoidan (100 or 200 μg/ml) was added to the lower chamber. After 40 min incubation, the distance migrated by the cells through the filter was measured. Data represent the means ± SDs (n = 3). ***P < 0.01 vs. fucoidan-untreated PMNs (one-way ANOVA followed by Tukey's test).

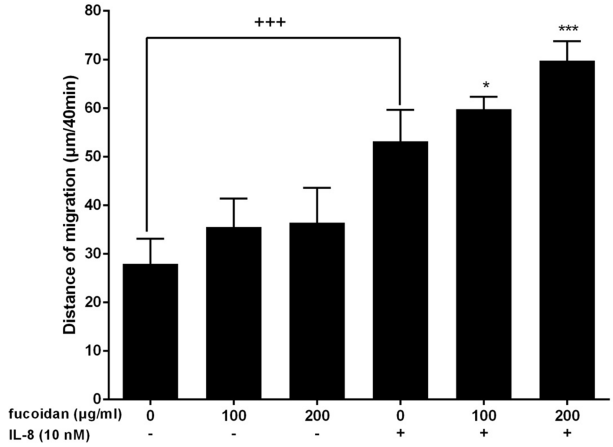

Fig 2. Chemotactic activity of porcine PMNs treated with fucoidan. The chemotactic activity of PMNs (2×10^6 cells/ml) treated with fucoidan (100 or 200 μg/ml) in the upper chamber was measured in the presence or absence of pr IL-8 (10 nM) in the lower chamber after 40 min. Data represent the means ± SDs (n = 3). +++p < 0.001 vs. fucoidan-untreated PMNs in the absence of IL-8 (one-way ANOVA followed by Tukey's test); *p < 0.05, ***p < 0.001 vs. fucoidan-untreated PMNs in the presence of IL-8 (two-sample *t*-test).

chamber showed no effects on chemotaxis of PMNs as compared with that of the controls.

Direct treatment of fucoidan increases the chemotactic activity of porcine PMNs

Direct treatment effect of fucoidan on chemotactic activity of PMNs to IL-8 was evaluated. In the absence of pr IL-8 in the lower chamber, the migrated distances of PMNs treated

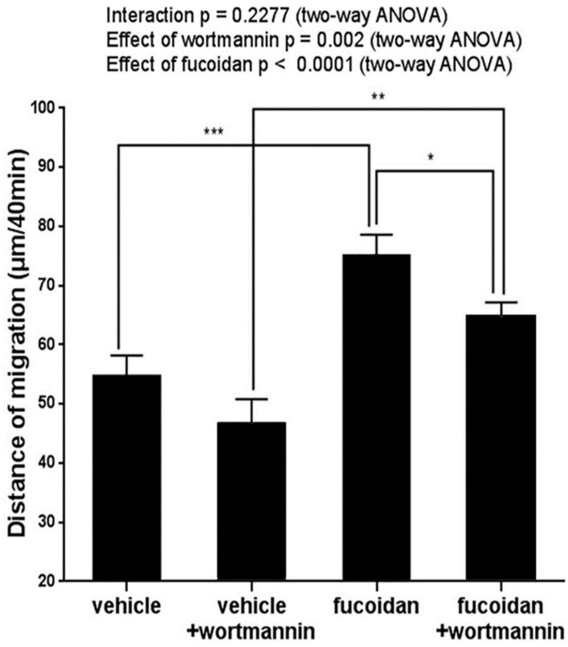

Fig 3. Effect of wortmannin, an inhibitor of PI3K, on the chemotactic activity of fucoidan-treated porcine PMNs. The chemotactic activity of isolated PMNs (2×10^6 cells/ml) treated with fucoidan (200 μg/ml) and/or wortmannin (5 nM) in the upper was measured after 40 min in the presence of pr IL-8 (10 nM) in the lower chamber. Two-way ANOVA was used for statistical analysis (fucoidan treatment in the presence of wortmannin). Data represent the means ± SDs (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (two-sample *t*-test).

with fucoidan (100 and 200 μg/ml) were not significantly different from that of vehicle-treated controls (Fig 2). However, the chemotactic activity of PMNs was significantly increased (p < 0.001) by the presence of IL-8 (10 nM) in the lower chamber. In the presence of pr IL-8, treatment with fucoidan (100 and 200 μg/ml) significantly increased (p < 0.05 to 0.001) the chemotactic activity of PMNs compared with that of the fucoidan-untreated PMNs (no fucoidan in the upper chamber).

Increased chemotactic activity of fucoidan-treated porcine PMNs in response to pr IL-8 is associated with PI3K activation

To investigate whether the ability of fucoidan to increase the chemotaxis of porcine PMNs in response to pr IL-8 (10 nM) is related to PI3K activation, the chemotactic activity of PMNs was examined by addition of wortmannin (5 nM), an inhibitor of PI3K. PMNs were exposed to fucoidan and/or wortmannin in the upper chamber for 40 min. As shown in Fig 3, in the naïve PMNs without fucoidan, there was no significant difference in the chemotactic activity of PMNs by treatment of wortmannin. However, the chemotactic activity of PMNs was significantly increased (p < 0.001) by treatment with fucoidan (200 μg/ml) when compared with naïve PMNs. This increase in chemotactic activity of PMNs by fucoidan was significantly reduced (p < 0.05) by wortmannin (5 nM). Moreover, when compared with treatment of wortmannin alone, the chemotactic

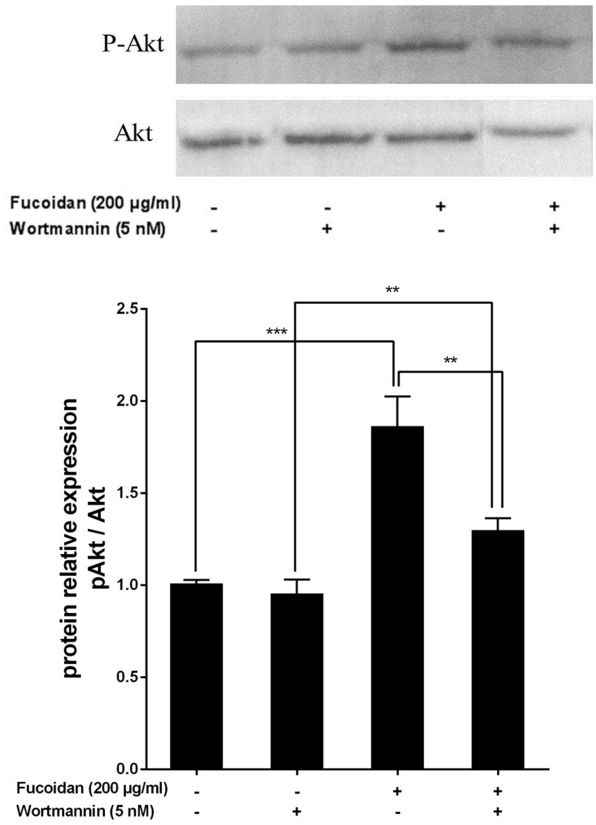

Fig 4. Effect of wortmannin on Akt phosphorylation levels in fucoidan-treated PMNs. Isolated PMNs (2×10^6 cells/ml) were treated with fucoidan (200 μg/ml) and/or wortmannin (5 nM) for 1h. Total protein (40 μg) was subjected to 11% SDS-PAGE, followed by Western blot analysis. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. Data represent the means ± SDs (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$ (two-sample *t*-test).

activity of PMNs treated with fucoidan plus wortmannin also showed a significant increase ($p < 0.01$).

Chemotactic activity of fucoidan-treated PMNs is associated with increased Akt phosphorylation

To investigate whether the effect of fucoidan on chemotactic activity of PMNs is associated with Akt phosphorylation, a downstream of PI3K, Akt phosphorylation levels in PMNs treated with fucoidan (200 μg/ml) and/or wortmannin (5 nM), an inhibitor of PI3K, were measured. Treatment with wortmannin had no effect on Akt phosphorylation levels in the naïve porcine PMNs (Fig 4). However, there was a significant increase ($p < 0.001$) in phosphorylation levels of fucoidan-treted PMNs compared with untreated cells. Then, increased Akt phosphorylation in fucoidan-treated PMNs was significantly ($p < 0.01$) reduced by the addition of wortmannin (5 nM). Wortmannin-treated PMNs showed significantly ($p < 0.01$) increased Akt phosphorylation levels by treatment with fucoidan.

Discussion

The effect of fucoidan on cell migration has been investigated in several types of cells. In fucoidan-treated bladder cancer cells or human lung cancer cells, cell growth and migration were inhibited (6,21), whereas in osteoblasts, chemotaxis and actin polymerization were increased by fucoidan treatment (17). In this study, we examined whether treatment with fucoidan directly increases the chemotactic activity of porcine PMNs. The results showed that fucoidan increases chemotactic activiy of PMNs in response to pr IL-8. Furthermore, we observed that treatment of wortmannin, PI3K inhibitor, in fucoidan-treated porcine PMNs reduces chemotactic activity and Akt phosphorylation levels, a main downstream of PI3K. These results suggest that increase in chemotactic activity of PMNs to IL-8 by fucoidan is associated with PI3K activation.

Cell migration along gradient of a chemoattractive substance was known as chemotaxis. The initial step of chemotaxis is to sense the chemoattractant and to transduce the signal that triggers cell polarization, which involves morphological changes and the asymmetrical redistribution of multiple proteins and lipids. Cell polarization enables the cell to maintain leading edge that protrudes and the rear edge that retracts. These pathways interact through the actin cytoskeleton (4).

PI3K is a key modulator of neutrophil chemotaxis, controlling cell polarization and the intracellular co-localization of F-action to the leading edges (30). All four class I PI3Ks (p110α, p110β, p110δ, and p110γ) are expressed in neutrophils (35). PI3Kγ, the dominant class I PI3K in neutrophil, catalyzes the 3'-phosphorylation of phosphatidylinositol 4,5-biphosphate [PtdIns(4,5)P2] to generate PtdIns(3,4,5)P3 (26). Local production and degradation of PtdIns(3,4,5)P3 at the plasma membrane result in a net accumulation of PtdIns (3,4,5)P3 at the leading edge and leads ultimately to actin polymerization, formation of pseudopodia and directional cell movement (20). It is shown that recruitment of neutrophil to sites of inflammation was significantly reduced in PI3Kγ knockout mice (22,31). Additionally neutrophils lacking PI3Kγ exhibit strong defects in adhesion (10). Therefore, we used wortmannin, PI3K inhibitor, to elucidate whether fucoidan increases chemotactic activity of PMNs via PI3K activation. The results of this study showed that the migrated distance of PMNs is reduced by the addition of wortmannin. Because Akt phosphorylation acts as downstream of PI3K, the effect of fucoidan on Akt phosphorylation was also investigated. It was observed that Akt phosphorylation levels are increased when PMNs are treated with fucoidan. The increased phosphorylation level induced by treatment of fucoidan was also suppressed by the addition of wortmannin. These findings suggest that the effect of fucoidan on the chemotactic activity of PMNs in response to pr IL-8 is associated with PI3K activation. Previous study showed that fucoidan also stimulates total cellular F-actin polymerization. The increased chemotactic activity of fucoidan-treated PMNs to IL-8 was suppressed by cytochalasin D, an inhibitor of F-actin polymerization (18). Based on these results, the increasing effect of fucoidan on chemotactic activity of PMNs to IL-8 may be associated with increasing F-actin polymerization through PI3K activation.

It is not known whether fucoidan can directly affect the

neutrophil cytoskeleton. It has been reported that fucoidan itself does not induce the migration of PMNs (14). However, in the presence of IL-8, treatment with fuocoidan increased the chemotactic activity of canine PMNs (18). In this study, fucoidan increased Akt phosphorylation levels of porcine PMNs. However, the migrated distances of PMNs were not affected by treatment with fucoidan in the absence of pr IL-8. Thus, fucoidan could not induce neutrophil migration without chemoattractant, despite increased PI3K activity, which is closely associated with chemotaxis. PtdIns(3,4,5)P3 produced by PI3K strongly influences cell motility via the regulation of the cytoskeleton. PtdIns(3,4,5)P3 is accumulated at sites of F-actin polymerization, causing transient pseudopod extension (31). In the absence of chemoattractant, cells randomly extend their pseudopodia, probably as self-organizing structures (5,28). However, for chemotaxis, pseudopodia formation must be biased to at the front of the cell and suppressed at the back (38). In the absence of IL-8, fucoidan presumably stimulates the intrinsic organization of the neutrophil cytoskeleton by PI3K activation. With this results, pseudopodia may be also randomly extended.

F-actin polymerization during chemoattractant-stimulated lamella extension in the human neutrophil is occurred by two distinct pathways (7). One pathway is dependent on PI3K activation. Downstream of this pathway is dependent on protein kinase C (PKC) and Akt. This pathway regulates the formation of 70% to 80% of the F-actin in the lamella region. The other pathway is dependent on the activation of Rho GTPases, rho-associated coiled-coil-containing protein kinase 1 (ROCK), Src family tyrosine kinases, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This pathway is modulated by cAMP. It is known that GTPases activation is dependent on PI3K activation (9). In addition, it was demonstrated that fucoidan increases oxidative burst activity related with NADPH oxidase (19). Therefore, stimulatory effects of fucoidan by PI3K activation may be associated with two distinct pathways that control F-actin polymerization. Furthermore, this effect may also be related with the oxidative burst of PMNs.

Although neutrophils constitute the first line of defense against infection, they have been considered one of the major contributors to host damage in inflammatory states (1). In addition, impaired neutrophil migration has been reported in sepsis, leukemia, and AIDs in humans and in diabetes in rats (2,27). It is, therefore, suggested that the direct stimulatory effect of fucoidan on porcine PMNs may be applicable for development of therapeutic interventions in porcine inflammatory disease including mastitis, ulcerative colitis, arthritis (3).

The overall results of this study support that fucoidan directly increases the chemotactic activity of porcine PMNs in respone to pr IL-8 by increasing Akt phosphorylation levels. This suggests that fucoidan may increase chemotaxis of porcine PMNs by upregulating PI3K activation.

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