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Quercetin Reduces Chemotactic Activity of Porcine Peripheral Blood Polymorphonuclear Cells

Gyeong-Rok Hwa[1]
Changhwan Ahn[2]
Hakhyun Kim[1]
Byeong-Teck Kang[1]
Eui-Bae Jeung[1]
Mhan-Pyo Yang[1,*]

[1]Department of Veterinary Medicine, College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea
[2]Department of Veterinary Medicine, College of Veterinary Medicine, Jeju National University, Jeju Special Self Governing Province, Jeju 63243, Korea

*Correspondence: mpyang@chungbuk.ac.kr

ORCID
Gyeong-Rok Hwa: https://orcid.org/0000-0003-0725-2107
Changhwan Ahn: https://orcid.org/0000-0002-7176-7108
Hakhyun Kim: https://orcid.org/0000-0002-8882-2329
Byeong-Teck Kang: https://orcid.org/0000-0002-4471-4342
Eui-Bae Jeung: https://orcid.org/0000-0001-8936-916X
Mhan-Pyo Yang: https://orcid.org/0000-0002-8043-0152

Abstract Quercetin, a flavonoid found in fruits and vegetables, exhibits a strong anti-inflammatory activity. The objective of this study was to examine the effect of quercetin on chemotactic activity of peripheral blood polymorphonuclear cells (PMNs) to culture supernatant from peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharide (LPS). In addition, we determined whether this effect is related to interleukin (IL)-8 and changes in cytoskeleton. The chemotactic activity of PMNs was evaluated by a modified Boyden chamber assay. Total cellular filamentous (F)-actin levels were measured by method of fluorescence microscopy. The levels of IL-8 mRNA and protein were measured by real time polymerase reaction method and enzyme-linked immunosorbent assay, respectively. Quercetin (0-50 μM) itself has no chemoattractant effect for PMNs. The culture supernatant from PBMCs (2×10^6 cells/mL) treated with LPS (1 μg/mL) showed remarkable increase in chemotaxis of PMNs. However, this effect was reduced dose-dependently by treatment with quercetin. In addition, PBMCs treated with LPS revealed enhanced levels in IL-8 protein and mRNA. Co-treatment of LPS with quercetin (50 μM) in PBMCs decreased IL-8 production and expression. Treatment of quercetin (0-50 μM) on PMNs to rpIL-8 (10 nM) decreased dose-dependently the chemotactic activity of PMNs. Treatment of quercetin on PMNs to IL-8 also reduced their total cellular F-actin level. These results suggested that quercetin attenuates chemotactic activity of PMNs, which is mediated by down-regulation of IL-8 production from LPS-stimulated PBMCs and inhibition of F-actin polymerization in PMNs.

Key words quercetin, chemotactic activity, PMNs, IL-8, F-actin.

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Introduction

Neutrophils are the most abundant circulating blood leukocytes which provide the first-line defense against infection. However, neutrophils are involved in the pathology of various inflammatory conditions of host tissues. Upon infection or stimulation, local macrophages and other cells produce series of inflammatory mediators such as cytokines and chemokines that stimulate the adjacent microvessels and attract large numbers of neutrophils to migrate across the vascular wall and infiltrate into tissues (6). Neutrophil infiltration can eliminate pathogens via mechanisms such as the generation of reactive oxygen species (ROS) and the release of preformed oxidants and proteolytic enzymes from granules. The proteolytic enzymes released by degranulation may also cause the destruction of neighboring cells and the dissolution of tissue (16).

Lipopolysaccharide (LPS)-treated peripheral blood mononuclear cells (PBMCs) resulted in mRNAs expression for various cytokines such as interleukin (IL)-1, IL-10, IL-12, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, (34) and IL-8 (17). IL-8 is one of the major inflammatory mediators that recruit polymorphonuclear cells (PMNs) to inflamed sites (3). IL-8 plays a crucial role in various pathological conditions such as psoriasis, rheumatoid arthritis, occlusive vascular disease, septic shock (18) and inflammatory bowel disease (4). Accumulation of activated neutrophils and elevated IL-8 production are also observed in these diseases (32).

Chemotaxis of neutrophils is the process of directed migration by cells in gradients of chemoattractants such as n-formyl-methionine-leucine-phenylalanine (fMLP), complement-derived C5a, leukotriene (LT) B4 and IL-8 (21). During inflammation, chemoattractants induce rapid changes in neutrophil shape, causing cell polarization and allowing the cell to orient and migrate up a gradient of attractant to the site of inflammation (9,29). The migratory potential of neutrophils depends on rearrangement of the actin cytoskeleton to form a pseudopod toward the signal and the pseudopod contains highly dynamic actin filaments (8). Actin is a major cytoplasmic component of the cytoskeleton of neutrophils and exists in two main states: a globular monomeric form (G-actin) and a filamentous helical polymer (F-actin) (14). Change in motility is associated with actin polymerization as evidenced by an increase in F-actin or a decrease in G-actin (19).

Quercetin is a dietary flavonoid, which widely exists in red onions, grapes, apples, broccoli and particularly at high concentrations in capers (7). The use of quercetin is widely associated with a great number of beneficial effects, including antioxidant and anti-inflammatory activities (11). It was recently suggested that quercetin could suppress LPS-induced TNF-α and IL-8 productions in macrophages and lung A549 cells (36). Quercetin also inhibits mRNA levels of cytokines in LPS-induced colloid cells, such as tumor necrosis factor (TNF)-α and IL-1α. In addition, quercetin inhibits IL-6 production by LPS-stimulated neutrophils and this inhibitory effect may eliminate the accumulation of neutrophils into the inflammation sites through controlling neutrophil activation and neutrophil adhesion to endothelial cells (25). These observations suggest that quercetin has significant potential to prevent inflammation. Therefore, the objective of the study was to examine the effects of quercetin on chemotactic activity of PMNs to culture supernatant from LPS-stimulated PBMCs. In addition, it was determined whether this effect is associated with production of IL-8 and changes in cytoskeleton.

Materials and Methods

Chemicals and reagents

Quercetin, was purchased commercially from Sigma-Aldrich (St. Louis, MO, USA). The stock solution (0.5 mM) was prepared by dissolving quercetin in dimethyl sulfoxide (DMSO; Sigma-Aldrich), passing it through a 0.2 μm membrane filter (Millipore Co., Bedford, MA, USA) and stored at 4°C atmosphere. LPS from Escherichia coli 0127:B8, Percoll® solution, RPMI 1640 medium (Sigma-Aldrich), Acti-stain™ 488 phalloidin (Cytoskeleton, Denver, CO, USA), recombinant porcine (rp) IL-8 (R&D Systems Inc., Minneapolis, MN, USA) were purchased commercially.

Porcine peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) isolation

Clinically healthy 6-month-old pigs in slaughterhouse (Donga food Co. Ltd., Cheongju, Chungbuk, Republic of Korea) were used as blood donors. Peripheral blood drawn in heparinized tube was overlaid 1:1 on Percoll® solution (specific gravity, 1.080). The PBMCs at the interface between the plasma and Percoll® solution following centrifugation at 400 *g* for 40 min at room temperature were obtained and added with RBC lysis buffer (iNtRON biotechnology, Seongnam, Gyeonggi, Republic of Korea) for 5 min to lyse remaining erythrocytes. The PMNs were harvested from the upper layer of sedimented erythrocytes following despumating the PBMCs layer. For purification of the PMNs, the erythrocyte layer was treated with 1.5% dextran (molecular weight, 200,000; Wako Ltd., Osaka, Japan) in phosphate buffered saline (PBS) for 70 min. The floating cells were carefully harvested

and centrifused at 900 *g* for 5 min. The collected PBMCs and PMNs were washed two times with PBS. The viability of PBMCs and PMNs, as determined using trypan blue dye exclusion, always exceeded 97%. All cells were resuspended in RPMI 1640 medium with 5% heat-inactivated fetal bovine serum (FBS) (Gibco Company, Grand Island, NY, USA), 1% 100 U/mL penicillin and 100 μg/mL of streptomycin (Gibco Company) at 37°C in a 5% CO_2-humidified atmosphere.

Culture supernatant from PBMCs

The PBMCs seeded at a density of 2×10^6 cells/mL in a twenty-four-well plate (Nunc company, Naperville, IL, USA) were cultured with either LPS (1 μg/mL) or quercetin (0-50 μM) for 3 h at 37°C in a 5% CO_2 atmosphere. Control cells received the same amount of RPMI 1640 medium. After an incubation, culture supernatants were collected after centrifugation at 900 *g* for 15 min and stored at – 80°C until use.

Chemotaxis assay

The chemotaxis of PMNs was measured as migration distance through millipore membrane filters using modified Boyden chamber method, as previously described (33). The chemotaxis chamber (Neuro Probe, Gaitherburg, MD, USA) and medium (FBS-free) were pre-warmed for 1 h at 37°C. The lower chamber was filled with 200 μL of culture supernatant or rpIL-8. A millipore filters (3.0 μm pore size and 120 μm thick; Millipore Corporation, Bedford, MA, USA) was layed on top of the well of the lower compartment. Then, 200 μL of PMNs suspension (1×10^6 cells/mL) was placed in the upper compartment. In case of rpIL-8 in the lower chamber, PMNs were pre-treated with quercetin for 30 min. The chambers were incubated for 40 min at 37°C in a 5% CO_2 atmosphere. The membrane filters after incubation were immediately removed, fixed in ethyl alcohol, stained with hematoxylin, and mounted on a slide glass. The migration distance of cells through millipore filter was observed under a microscopy at 400 × magnification. Randomly 5 selected fields per filter were observed in triplication. The chemotaxis of the input cells was measured as the absolute distance (μm/40 min) directionally migrated by the PMNs to chemoattractant.

Measurement of IL-8 in culture supernatant

The culture supernatant from PBMCs treated with quercetin and/or LPS was collected after 3 h incubation. The IL-8 levels in the culture supernatant were measured by the direct sandwich ELISA in the porcine IL-8 kit (R&D systems Inc., Minneapolis, MN, USA). All samples, controls and standard were tested in triplicate. The optical density was determined at 450 nm. The levels of IL-8 were quantified from standard curves generated by purified porcine IL-8 tested at 8 titration points.

Real time-polymerase chain reaction (RT-PCR)

Total RNA was extracted by the Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) and determination of concentration of total RNA was performed by measuring the absorbance at 260 nm. First strand cDNA was made by reverse transcription of total RNA (1 μg) by moloney murine leukemia virus reverse transcriptase (Invitrogen Co) and primers (9-mers; Takara Bio Inc., Otsu, Shiga, Japan). To test the conditions for logarithmic phase PCR amplification of the target mRNA, aliquot (1 μg) was amplified using different cycle numbers. The PCR product of Sus scrofa 18S ribosomal RNA (RN18S, 2302-bp) expression was used as a control for the variation of mRNA concentrations in the RT reactions. A linear relation between number of amplification cycles and PCR product band visibility was observed for the target mRNAs. The target genes and the 18S gene were quantified using 30 and 28 cycles, respectively. cDNA was amplified in a 20 μL PCR containing 1 U Taq polymerase (iNtRON Bio Inc., Seongnam, Kyunggi, Korea), 2 mM dNTPs, and 10 pmol specific primers. PCR conditions were denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The oligonucleotides for the IL-8 were based on the deposited cDNA sequence: 5′-CTG CTT TCT GCA GCT CTC T-3′ (sense) and 5′-GCA GAC CTC TTT TCC ATT G-3′ (antisense). The primers for the 18S gene were: 5′-CGC GGT TCT ATT TTG TTG G-3′ (sense) and 5′-AGT CGG CAT CGT TTA TGG T-3′ (anti-sense). The PCR products (8 μL) were loaded on 2.3% agarose gel, stained with ethidium bromide following electrophoresis. Intensity of the PCR bands was directly scanned from the agarose gel and analyzed by a molecular analysis program version 4.5.1 (Gel Doc 1000, Bio-Rad, Hercules, CA, USA).

Determination of total cellular F-actin content

PMNs were placed in 24-well plates (1×10^6 cells/mL) and incubated with quercetin for 30 min, followed by incubation with rpIL-8 for 5 min at 37°C in a 5% CO_2 atmosphere. Afterward, the reaction was stopped with 2% paraformaldehyde. The cells were then wash with PBS containing 1% Triton X-100 and resuspended for 1 min, and washed three times with PBS solution, and stained in the dark for 20 min at 37°C with 100 nM of Acti-stain™ 488 Phalloidin. After staining, the cells were washed two times with PBS. Microscopic analysis of fluorescent images was performed using an Olympus CKX3 inverted microscope (Olympus, Tokyo, Japan). The

F-actin content of the cells was determined by quantitating the pixel fluorescence intensity using the ImageJ software (ImageJ 1.46, National Institute of Health (NIH), Bethesda, MD, USA) (13). Randomly 5 selected fields per slide were examined in triplicate assay.

Statistical analyses

All statistics were determined by GraphPad prism 6 software (GraphPad software, San Diego, CA, USA). Comparisons of 2 groups were done using the Student's t-test. One-way analysis of variance (ANOVA) was used to test differences between the treatment and control groups, followed by Dunnett's post hoc test. p value of <0.05 was considered to statistical significance. Data were expressed as means ± standard deviations (SD).

Results

Quercetin has no chemoattractant activity for PMNs

To examine whether quercetin has any chemoattractant activity for PMNs, the distance of migration of PMNs in response to either rpIL-8 or quercetin in the lower chamber was measured (Fig. 1). The chemotactic activity of PMNs showed significant increase ($p < 0.001$) by rpIL-8 (10 nM) as compared with control. However, the presence of quercetin in the lower chamber revealed no effects on chemotaxis of PMNs when compared with that of the controls.

Quercetin reduces chemotaxis of PMNs by culture supernatant from PBMCs with LPS

To examine the effect of culture supernatant from PBMCs with either LPS (1 μg/mL) or quercetin (10-50 μM) on chemotaxis of PMNs, the distance of migration of PMNs to culture supernatant was measured. The culture supernatant from LPS-treated PBMCs significantly increased ($p < 0.001$) the migrated distance of PMNs relative to untreated control. However, this effect was significantly reduced ($p < 0.001$) dose-dependently by treatment with quercetin (Fig. 2).

Quercetin decreases IL-8 production and mRNA expression in LPS-treated PBMCs

To examine the effect of quercetin on IL-8 production by LPS-treated PBMCs, the amount of IL-8 in the culture supernatant from PBMCs treated with quercetin (50 μM) and/or LPS (1 μg/mL) for 3 h was measured. Treatment of PBMCs with LPS significantly increased ($p < 0.001$) the production of

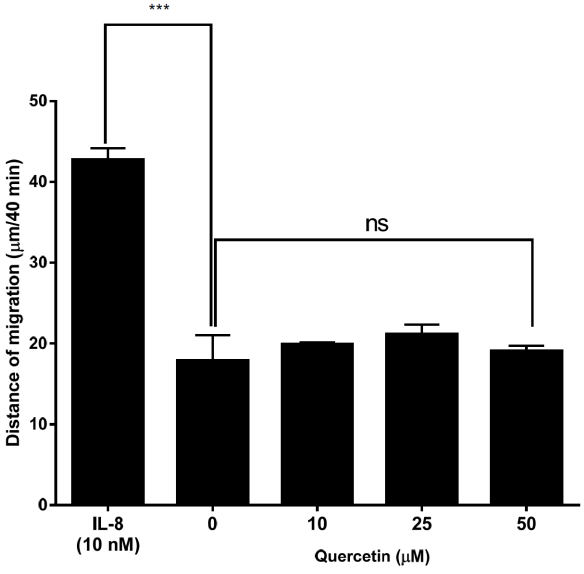

Fig. 1. Chemoattractant effect of quercetin on chemotaxis of PMNs. Freshly isolated PMNs (1×10^6 cells/mL) were placed in the upper chamber and rpIL-8 (10 nM) or quercetin (0-50 μM) was added to the lower chamber. After 40 min incubation, the distance migrated by the cells through the filter was measured. Data represent the means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's post hoc test. Comparison of two groups was made by Student's *t*-test. ***$p < 0.001$ vs. untreated PMNs. ns, not significant. IL-8, interleukin-8.

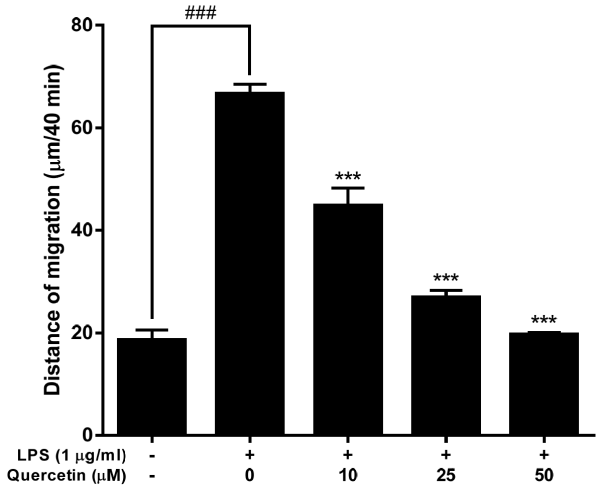

Fig. 2. Chemotaxis of PMNs in response to culture supernatant from LPS (1 μg/mL)-stimulated PBMCs treated with quercetin (0-50 μM). The culture supernatant from LPS-stimulated PBMCs treated with or without quercetin for 3 h were placed in the lower chamber. Freshly isolated PMNs (1×10^6 cells/mL) were placed in the upper chamber. After 40 min incubation, the distance migrated by the cells through the filter was measured. Data represent the means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's post hoc test. Comparison of two groups was made by Student's *t*-test. ###$p < 0.001$ vs. control. ***$p < 0.001$ vs. culture supernatant from LPS-stimulated PBMCs without quercetin. LPS, lipopolysaccharide.

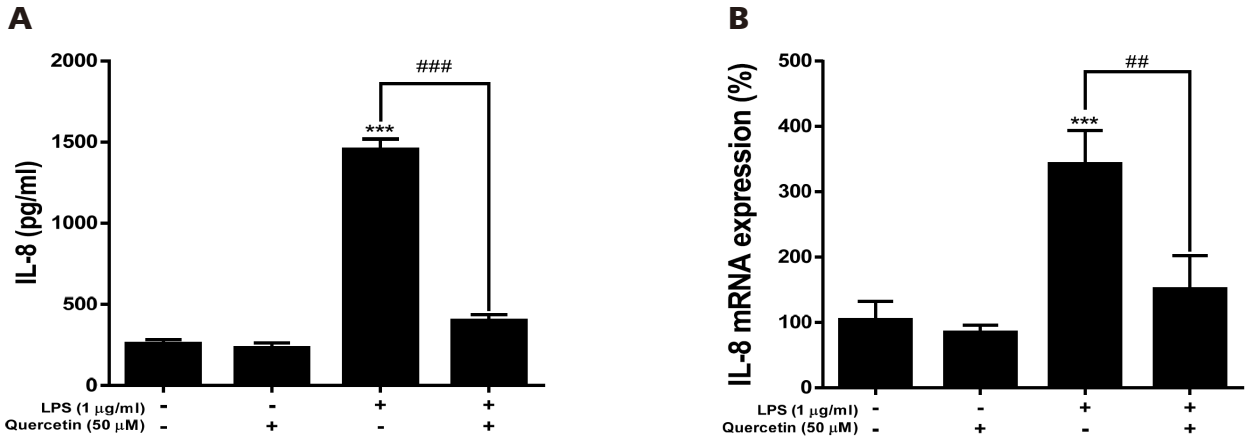

Fig. 3. Effect of quercetin on IL-8 production (A) and mRNA expression (B) in LPS-stimulated PBMCs. LPS-stimulated PBMCs (2×10^6 cells/mL) were incubated with or without quercetin (50 μM) for 3 h. The concentration of IL-8 in the culture supernatant from PBMCs was measured by ELISA (A). IL-8 mRNA expression in PBMCs was measured by real time-PCR analysis. Signals were quantified by a digital analysis program (B). One-way ANOVA was used for statistical analysis, followed by a Dunnett's post hoc test. Comparison of two groups was made by Student's *t*-test. Data represent the means ± SD (n = 3). ***$p < 0.001$ vs. control. $^{##}p < 0.01$ and $^{###}p < 0.001$ vs. LPS-treated PBMCs without quercetin.

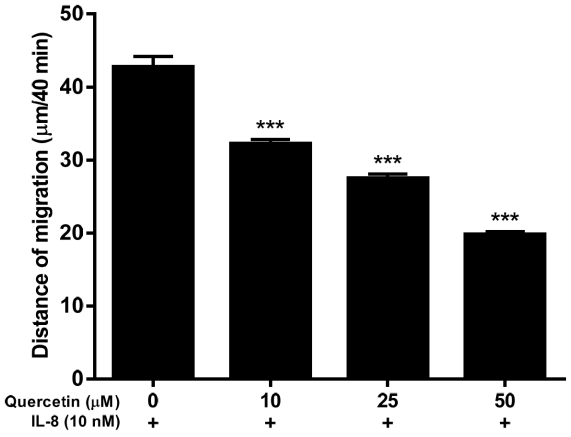

Fig. 4. Effect of quercetin on chemotaxis of PMNs to rpIL-8 (10 nM). PMNs were treated with quercetin for 30 min before the modified Boyden chamber assay. The chemotactic activity of PMNs (1×10^6 cells/mL) treated with quercetin in the upper chamber was measured in the presence of rpIL-8 (10 nM) in the lower chamber after 40 min. Data represent the means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's post hoc test. Comparison of two groups was made by Student's *t*-test. ***$p < 0.001$ vs. quercetin-untreated PMNs in the presence of IL-8.

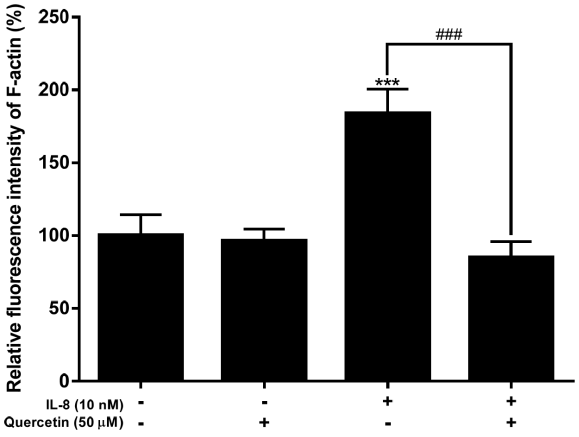

Fig. 5. Effect of quercetin treatment on total F-actin levels in PMNs. Isolated PMNs (1×10^6 cells/mL) were cultured with quercetin (50 μM) for 30 min before treatment with rpIL-8 (10 nM). Cells were then stained with FITC-labeled phalloidin. F-actin polymerization of cells was determined by immunofluorescence (n = 3 slides, 5 random fields per slide). Data represent the means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's post hoc test. Comparison of two groups was made by Student's *t*-test. ***$p < 0.001$ vs. control. $^{###}p < 0.001$ vs. IL-8-treated PMNs without quercetin.

IL-8 compared with untreated control. However, LPS-induced IL-8 production showed significant decrease ($p < 0.001$) by the addition of quercetin (Fig. 3A).

The expression of IL-8 mRNA in PBMCs in response to quercetin and/or LPS was also examined. IL-8 mRNA expression in PBMCs was significantly increased ($p < 0.001$) by LPS compared with untreated control. However, IL-8 mRNA expression in LPS-treated PBMCs was significantly decreased ($p < 0.01$) by treatment with quercetin (Fig. 3B).

Quercetin also decreases chemotaxis of PMNs to rpIL-8

The effect of quercetin on chemotactic activity of PMNs to rpIL-8 was evaluated. The chemotactic activity of PMNs in response to rpIL-8 (10nM) was significantly decreased ($p < 0.001$) dose-dependently by quercetin (Fig. 4).

Quercetin decreases total F-actin level of PMNs increased by rpIL-8

To examine the effect of quercetin on actin polymerization, the total cellular F-actin levels in PMNs with rpIL-8 (10 nM) and/or quercetin (50 μM) were measured. PMNs treated with rpIL-8 showed a significant increase ($p < 0.001$) in total cellular F-actin level relative to controls. This increase was significantly reduced ($p < 0.001$) by treatment with quercetin (Fig. 5).

Discussion

LPS, which is a major component of outer membrane of Gram-negative bacteria, is well known as endotoxin (5). It promotes host cells to produce various proinflammatory cytokines and induces inflammation. In this study, we used LPS to investigate the effect of quercetin on the inflammatory responses of porcine PBMCs. It was examined whether quercetin has direct effect on chemotactic activity of PMNs. Recombinant porcine IL-8 (10 nM) as a chemoattractant showed an enhancement in chemotaxis of PMNs. However, the data revealed that quercetin on chemotactic activity of PMNs as a chemoattractant has no effects. It was, therefore, thought that quercetin itself is not directly chemoattractant for PMNs.

Then, next experiment was conducted to investigate the effect of quercetin on chemotaxis of PMNs in inflammatory conditions induced by LPS. Our results showed that the chemotactic activity of PMNs was remarkably enhanced by culture supernatant from PBMCs with LPS. It has been known that upon stimulation with LPS, PBMCs secretes comparable amounts of proinflammatory cytokines such as IL-8 and TNF-α over time (12,15). A major neutrophil chemoattractant from LPS-activated human monocytes was IL-8 (23). Human monocytes, when activated with LPS, exhibited rapid expression of IL-8 mRNA and the expression of mRNA followed by cytokine synthesis (1). Therefore, it was assumed that enhanced chemotactic activity of PMNs by culture supernatant from LPS-treated PBMCs may be due to IL-8.

The migrated distance of PMNs increased by culture supernatant from LPS-treated PBMCs was reduced by treatment of quercetin. This indicates that the effect of chemotactic cytokine, probably IL-8, in culture supernatant from LPS-treated PBMCs might be diminished by quercetin. So, we next investigated the IL-8 amount of culture supernatant from PBMC with LPS and/or quercetin. We found that IL-8 levels of LPS- and quercetin- treated PBMCs culture supernatants were lower than that of culture supernatant from LPS-treated PBMCs. These results indicated that quercetin downregulates PBMCs to produce IL-8, which increases chemotactic activity of PMNs. Moreover, quercetin also controled IL-8 mRNA expression in LPS-treated PBMCs. These findings suggest that quercetin attenuates the increased chemotaxis of PMNs by culture supernatant from LPS-treated PBMCs, which is mediated via the inhibition of IL-8 production. IL-8 is also produced in response to stimulation by TNF-α. And TNF-α-induced IL-8 production correlated with activation of phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) and NF-κB pathways (28). Previous study showed that quercetin inhibits LPS-treated NF-κB activation in RAW 264.7 macrophage (10) and inhibits TNF-α gene expression in normal PBMCs via modulation of the NF-κB system (27). NF-κB was also implicated in the pathogenesis of inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis, atherosclerosis, systemic lupus erythematosus, type I diabetes, and asthma (26). This observation suggested that quercetin reduces the chemotactic activity of PMN by inhibiting the NF-κB pathway, which is associated with the production of IL-8.

We examined whether quercetin is able to control chemotactic activity of PMNs induced by recombinant porcine IL-8. Quercetin also decreased chemotactic activity of PMNs to rpIL-8. However, recent evidence has demonstrated that quercetin does not alter surface expression of receptors for chemoattractant in PMNs. This inhibitory effect may not be explained by diminishing the expression of chemotactic receptors on the surface of PMNs (35). A possibility is suggested that decreased chemotactic activity mediated by quercetin might be associated with actin polymerization. Since these results link actin dynamics and quercetin-mediated chemotaxis in PMNs, we investigated the effect of quercetin on F-actin polymerization of PMNs. The increased F-actin levels induced by rpIL-8 were also inhibited by the addition of quercetin. These findings suggest that the effect of quercetin on the migration of PMNs in response to IL-8 is also associated with F-actin polymerization. It has been demonstrated that quercetin inhibits PI3K signaling pathway (20), which has been known as a modulator of chemotaxis of neutrophils, controlling cell polarization and intracellular co-localization of F-action to leading edges (31). These are suggested that PI3K signaling pathway can be a target of quercetin to inhibit neutrophil migration. Based on these observations, the effect of quercetin on chemotaxis of PMNs to IL-8 might be associated with decreasing F-actin polymerization through PI3K inhibition.

Indoor husbandry environments of animals are contaminated with various endotoxins. Endotoxin exposure is also

associated with various inflammatory diseases in animals (30). Especially, pigs have many inflammatory diseases including inflammatory bowel disease, arthritis and atrophic rhinitis (2). And livestock are also exposed to various stressors including diet, temperature, weaning and infection. Such stressors often affect homeostasis of animals by inducing systemic or local inflammatory statues (24). A recent our study has been demonstrated that quercetin inhibits neutrophil extracellular trap (NET) formation by suppressing production of TNF-α from LPS-stimulated PBMCs (22). In the present study, we showed that quercetin decreases not only excessive production of IL-8 in LPS-treated PBMCs but also chemotaxis of PMNs to IL-8 by affecting F-actin polymerization. These results suggested that quercetin has anti-inflammatory capacity in both PBMCs and PMNs.

Conclusions

The data indicated that quercetin attenuates chemotactic activity of PMNs to culture supernatant from LPS-stimulated porcine PBMCs. These findings suggest that quercetin has an anti-inflammatory capacity via the down-regulation of IL-8 production in LPS-treated PBMCs, and the inhibition of F-actin polymerization in PMNs.

Conflicts of Interest

The authors have no conflicting interests.

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