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ORIGINAL ARTICLE

Luteolin Partially Inhibits LFA-1 Expression in Neutrophils Through the ERK Pathway

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Abstract— Luteolin inhibits the adhesion of neutrophils to microvascular endothelial cells and plays an important anti-inflammatory role, owing to its mechanism of suppressing the expression of lymphocyte function-associated antigen-1 (LFA-1) in the neutrophils. Our study deals with the different signaling pathways participating in LFA-1 expression in neutrophils along with the regulation of luteolin in order to elucidate new anti-inflammatory targets of luteolin, thus providing a basis for clinical applications. In our study, neutrophils were separated using density gradient centrifugation and the cAMP levels were determined using ELISA. Additionally, phosphorylation levels of p38 mitogen-activated protein kinase (MAPK), extracellular regulated protein kinase (ERK), phosphatidylinositol-3-kinase (PI3K), and Janus kinase (JAK) were also detected by Western blotting. LFA-1 expression was estimated using flow cytometry. The results showed that inhibiting agents used against p38 MAPK, ERK, PI3K, and JAK could significantly inhibit LFA-1 expression on neutrophils ($p < 0.05$, $p < 0.01$). Luteolin also induced a noteworthy elevation of cAMP in neutrophil supernatants ($p < 0.01$). It could also significantly inhibit ERK phosphorylation ($p < 0.05$, $p < 0.01$), and had no obvious effect on p38 MAPK phosphorylation in neutrophils ($p > 0.05$). However, phosphorylation of PI3K and JAK was not detected in neutrophils. To conclude, the p38 MAPK, ERK, PI3K, and JAK pathways are involved in the regulation of LFA-1 expression in neutrophils, and luteolin partially inhibits LFA-1 expression by increasing cAMP levels and suppressing ERK phosphorylation.

KEY WORDS: ERK; p38 MAPK; cAMP; adhesion molecule; neutrophil; luteolin.

INTRODUCTION

Neutrophils form the largest proportion of the inflammatory cell community and play important roles in non-specific immune processes such as preventing the invasion of pathogens and removing foreign bodies and necrotic tissues. To exert anti-inflammatory effect, the neutrophils first have to adhere to vascular endothelial cells, penetrate the blood vessels, and finally reach the inflammatory site. However, increase in the expression of adhesion molecules on neutrophil surface is a prerequisite for the adhesion. Luteolin was found to be a specific phosphodiesterase (PDE) 4 inhibitor that we obtained from the plant Folium Perillae. Our early studies showed that luteolin could inhibit the adhesion between neutrophils and microvascular endothelial cells

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by inhibiting the PDE4 activity. Further research also indicated the mechanism to be related to suppressing the LFA-1 expression in neutrophils [1].

LFA-1 is an essential adhesion molecule expressed on the neutrophils and plays an important role in the occurrence and development of inflammation. LFA-1 is mostly located in cellular storage pool present during the resting state of cells. Under the effect of inflammatory factors, LFA-1 expression can increase 3–10 times in several minutes by its transposition and fusion with the membrane, while this affinity significantly improves through configuration changes [2, 3]. LFA-1 expression on neutrophils from peripheral as well as lung blood significantly rises in diseases such as acute pancreatitis, leukemia, or severe eclampsia, which further increases with the aggravation of inflammation [4–6]. Researches show that the expression of adhesion molecules in neutrophils is mainly regulated by signaling pathways of p38 mitogen-activated protein kinase (MAPK), extracellular regulated protein kinase (ERK), phosphatidylinositol-3-kinase (PI3K), and Janus kinase (JAK) [7–10], all of which, in turn, involve regulation by cAMP [11–18]. Therefore, we studied the role of these pathways in LFA-1 expression in the porcine neutrophils. Also, by controlling cAMP concentrations, we investigated the regulation of luteolin and the phosphorylation of signal proteins for revealing the new anti-inflammatory targets of luteolin.

MATERIALS AND METHODS

Chemicals

Anti-ERK (ab4819), anti-p38 (ab38283), and goat anti-rabbit IgG H&L (HRP) (ab205718) antibodies were purchased from Abcam; anti-JAK-1 (225499) was procured from Invitrogen; and anti-PI3K p85 (orb106105) and mouse anti-pig LFA-1-FITC (MCA2308F) were purchased from Biorbyt and AbD Serotec, respectively. Phosphatase inhibitor PhosSTOP and protease inhibitor complete tablets were bought from Roche. Rolipram and fMLP were bought from Sigma. The cAMP kit (P138911) and CL Luminol were procured from R&D systems and GE Healthcare, respectively. The developer and fixer used during Western blotting were purchased from Kodak. Leukocyte lysate was obtained from Shanghai Qiyi Biotech Co., Ltd., while rabbit anti-β-actin (Loading Control) was purchased from Beijing Boaosen Biotechnology Co., Ltd. The pre-stained colored protein molecular weight standard, SDS-PAGE protein loading buffer, and the Western blot primary and secondary antibody diluents were bought from Shanghai Biyuntian Biotechnology Co., Ltd. We purchased luteolin from the National Institute for the Control of Pharmaceutical and Biological Products. Dextran T-500 was obtained from Pharmacia, lymphocyte separation medium from Tianjin Haoyang Biological Product Technology Co., Ltd. All the reagents involved in our study were analytically pure.

Separation of Neutrophils

Fresh porcine blood was drawn in heparin-containing vacutainers/vials from the slaughterhouse. Highly purified neutrophils were obtained by Chen's method [19]. Purified neutrophils were adjusted to a concentration of 5×10^8 cells/ml in PBS for subsequent experiments.

Measurement of LFA-1 Expression on Neutrophils

Neutrophil suspension (5×10^7 cells/ml in reaction solution) and blockers of SB203580, U0126, LY294002, or AG490 were added into each of the respective wells of a 24-well cell culture plate. The minimum reactive concentrations of the blockers used in the test were half the inhibitory concentrations of the kinase, and reactive concentrations of SB203580 were 0.6, 3, and 5 μmol/l; U0126 were 60, 300, and 600 nmol/l; and for both LY294002 and AG490 were 1, 5, and 10 μmol/l. Plates were incubated for 30 min at 37 °C in a 5% CO_2 incubator. Formyl-MLP was added into each well to a final concentration of 100 nmol/l with an exception of the blank well. A total of 500 μl reaction mixture volume for each group was again placed into the incubator for 1 h. Subsequently, the contents of each well were transferred separately into centrifuge tubes and centrifuged for 5 min at 1000 ×g. The cells were then rinsed twice with PBS and suspended in 500 μl PBS. Subsequently, 10 μl FITC-LFA-1 was added to each of the samples, allowed to react for 30 min at room temperature in the dark, and then rinsed two times with PBS. In all the samples, we detected the positive rate of LFA-1 of 20,000 cells with a BD FACSCalibur (BD Biosciences, USA).

Detection of Different Concentrations of Luteolin on cAMP Levels and Protein Phosphorylation

Groups of blank control, control, luteolin (reactive concentrations were 1, 5, and 10 μmol/l), and rolipram were used for the assay. Neutrophils (5×10^7/ml in reaction solution) and compounds (or PBS in case of controls) were added to each group, and the cells were

preincubated for 30 min at 37 °C in a 5% CO_2 incubator. Formyl-MLP was added to each well to obtain a final concentration of 100 nmol/l except for the blank control. A total of 500 μl reaction volume for each group was again placed into the incubator to stimulate the culture for 1 min. The contents of each well were quickly cooled in ice water, and 150 μl of the cell suspensions were centrifuged at 4 °C, 2000 ×g for 8 min. The supernatants were used to detect cAMP using ELISA. Also, 100 μl cell lysates (containing a sufficient amount of protease and phosphatase inhibitors) and 25 μl loading buffer were added into the cell pellets for cell lysis. The phosphorylation states of p38 MAPK, ERK, PI3K, and JAK in neutrophils were detected by conventional Western blotting using β-actin as a positive control and using anti-p38, anti-ERK, anti-PI3K p85, and anti-JAK-1 as the antibody. The transferred protein bands were analyzed using GraphPad Prism 5 and the relative phosphorylation changes of proteins were represented as the ratio of target protein *vs.* β-actin expression.

Detection of Luteolin on cAMP Levels and Protein Phosphorylation at Different Time Points

Groups of blank control, control, luteolin, and rolipram were used. Neutrophils (5×10^7/ml in reaction solution) and compounds (or PBS in case of controls) were added to each group, and the cells were preincubated for 30 min at 37 °C in a 5% CO_2 incubator. Formyl-MLP was added to each well to obtain a final concentration of 100 nmol/l except for the blank control. A total of 500 μl reaction volume for each group was again placed into the incubator to stimulate the culture for 30 s, 1 min, 5 min, and 30 min. The contents of each well were quickly cooled in ice water, and 150 μl cell suspension was centrifuged at 4 °C, 2000 ×g for 8 min. The supernatants were used to detect cAMP level using ELISA. Also, 100 μl cell lysates (containing a sufficient amount of protease and phosphatase inhibitors) and 25 μl loading buffer were added into the cell pellets for cell lysis. The phosphorylation states of p38 MAPK, ERK, PI3K, and JAK in neutrophils were detected by conventional Western blotting using β-actin as a positive control and using anti-p38, anti-ERK, anti-PI3K p85, and anti-JAK-1 as the antibody. The transferred protein bands were analyzed using GraphPad Prism 5, and the relative phosphorylation changes of proteins were represented as the ratio of target protein *vs.* β-actin expression.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5. Statistical significance among different cell treatments was assessed by Student's *t* test. *P* value less than 0.05 was considered statistically significant. Data are expressed as mean ± SEM of "n" independent experiments performed in triplicate.

RESULTS

Effect of Blockers on LFA-1 Expression in Neutrophils

Formyl-MLP of the control significantly promoted the LFA-1 expression in neutrophils compared with that of blank control samples ($p < 0.01$). In comparison to control, 3 and 6 μM of SB203580, a p38MAPK blocker, significantly inhibited LFA-1 expression in neutrophils ($p < 0.01$) with the inhibition rates of 17.3% and 16.1%, respectively. Next, 60, 300, and 600 nM of U0126, an ERK blocker, was seen to inhibit LFA-1 expression in neutrophils ($p < 0.01$) with the inhibition rates of 15.2%, 17%, and 12.1%, respectively. Concentrations of 1, 5, and 10 μM of LY294002, a PI3K blocker, inhibited LFA-1 expression in neutrophils ($p < 0.05$, $p < 0.01$) with the inhibition rates of 15.2%, 25.8%, and 16.8%, respectively, while 1, 5, and 10 μM of AG490, a JAK blocker, significantly inhibited LFA-1 expression in neutrophils ($p < 0.01$) with the inhibition rates of 28.5%, 29.5%, and 35.6%, respectively (Fig. 1).

Effect of Different Concentrations of Luteolin on cAMP Levels and Protein Phosphorylation

Formyl-MLP of control group induced significantly decrease of the cAMP concentration in neutrophil supernatants compared with that of blank control ($p < 0.05$). When compared with control, 0.01, 0.1, and 0.5 μM of luteolin increased the cAMP concentration in neutrophil supernatants ($p < 0.01$). Rolipram also significantly raised cAMP concentration in neutrophil supernatants ($p < 0.05$), but was weaker than that induced by luteolin (Fig. 2).

Formyl-MLP of the control group did not promote p38 MAPK phosphorylation in neutrophils at 1 min compared with that of blank control ($p > 0.05$). In contrast to control, 0.01, 0.1, and 0.5 μM of luteolin concentrations had some promoting effect on p38 MAPK phosphorylation in neutrophils, but this effect was not a significant effect ($p > 0.05$). Rolipram also did not exert any significant effect on p38 MAPK phosphorylation in neutrophils ($p > 0.05$) (Fig. 3).

Formyl-MLP of control significantly promoted ERK phosphorylation in neutrophils at 1 min compared with that of blank control ($p < 0.05$). Luteolin

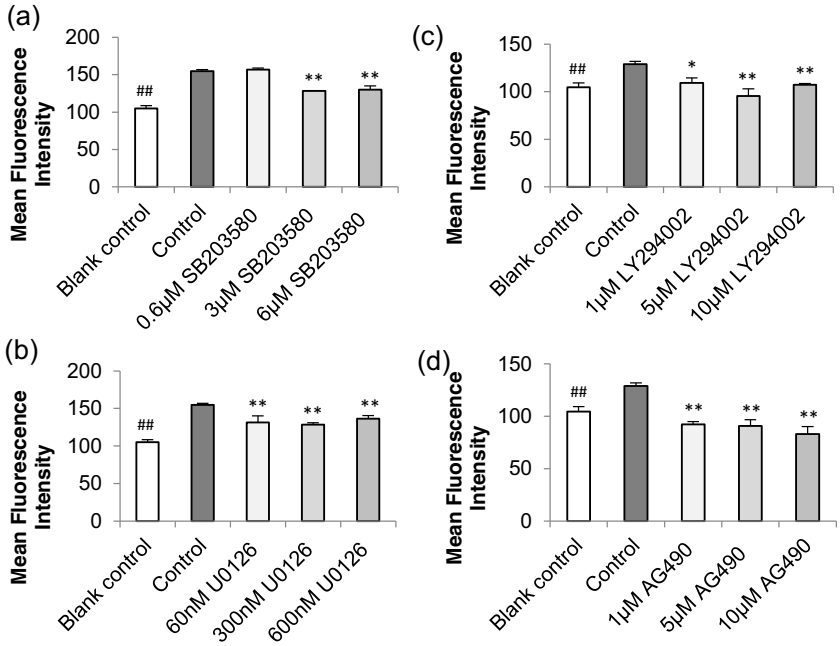

Fig. 1. Neutrophils were exposed to blockers for 30 min, and then stimulated with 100 nM fMLP for 1 h. LFA-1 expression on neutrophils was detected by flow cytometry. (a) SB203580, a p38MAPK blocker; (b) U0126, an ERK blocker; (c) LY294002, a PI3K blocker; (d) AG490, a JAK blocker. The experiment was performed three times with comparable results. ##$p < 0.01$ *versus* control group, *$p < 0.05$ and **$p < 0.01$ *versus* control group.

inhibited fMLP-induced ERK phosphorylation in a dose-dependent manner. In contrast with the control group, 0.1, and 0.5 μM luteolin concentrations inhibited ERK phosphorylation ($p < 0.05$), wherein the inhibition rates of 0.01, 0.1, and 0.5 μM luteolin on ERK phosphorylation were 25.0%, 53.90%, and 59.2%, respectively. Rolipram was also significantly

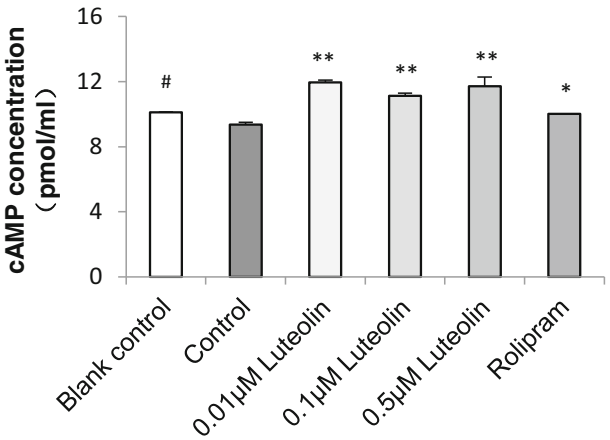

Fig. 2. Neutrophils were exposed to luteolin for 30 min and stimulated with 100 nM fMLP for 1 min. Cyclic-AMP concentrations in neutrophil supernatants were detected with the help of an ELISA kit. The experiment was performed three times with similar results. #$p < 0.05$ *versus* control group, *$p < 0.05$ and **$p < 0.01$ *versus* control group.

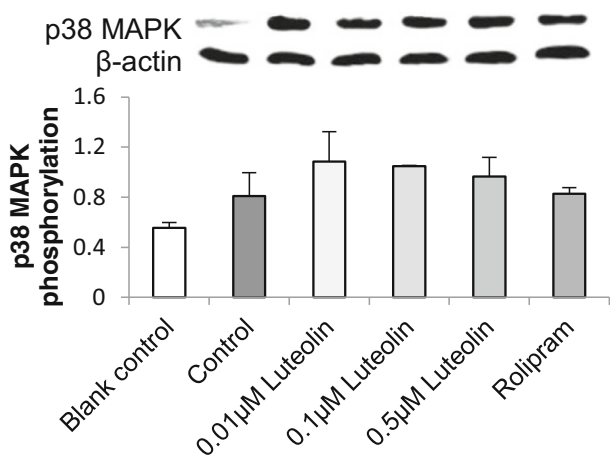

Fig. 3. Neutrophils were exposed to luteolin for 30 min, stimulated with 100 nM fMLP for 1 min, and p38 MAPK phosphorylation was detected by Western blotting subsequently. β-actin was used as a loading control. The relative phosphorylation of p38 MAPK was represented as the ratio of the target to β-actin band intensities. Representative figures (upper panel) and densitometric analysis (lower panel) of p38 MAPK phosphorylation have been shown here. The experiment was performed three times with similar results.

able to inhibit ERK phosphorylation ($p < 0.05$) with an inhibition rate of 65.8% (Fig. 4).

Effect of Luteolin on cAMP Levels and Protein Phosphorylation at Different Time Points

Formyl-MLP of control significantly leads to decrease in cAMP concentration in the neutrophil supernatants at different time points compared with that of blank control ($p < 0.01$). In comparison with control, 0.5 μM luteolin clearly increased the cAMP concentration in neutrophil supernatant at 30 s, 1 min, 5 min, and 30 min ($p < 0.01$). Rolipram also significantly raised cAMP concentration in neutrophil supernatants at 1 min, 5 min, and 30 min ($p < 0.05$, $p < 0.01$), but this effect was weaker than that seen in the case of luteolin (Fig. 5).

Formyl-MLP of control did not induce any significant change in p38 MAPK phosphorylation in neutrophils at 30 s, 1 min, 5 min, and 30 min compared with that of blank control ($p > 0.05$). Although 0.5 μM luteolin had a certain role in raising p38 MAPK phosphorylation in neutrophils in contrast with control, this alteration was not significant ($p > 0.05$). In addition, 1 μM rolipram significantly promoted p38 MAPK phosphorylation in neutrophils at 30 s and 5 min ($p < 0.05$, $p < 0.01$) (Fig. 6).

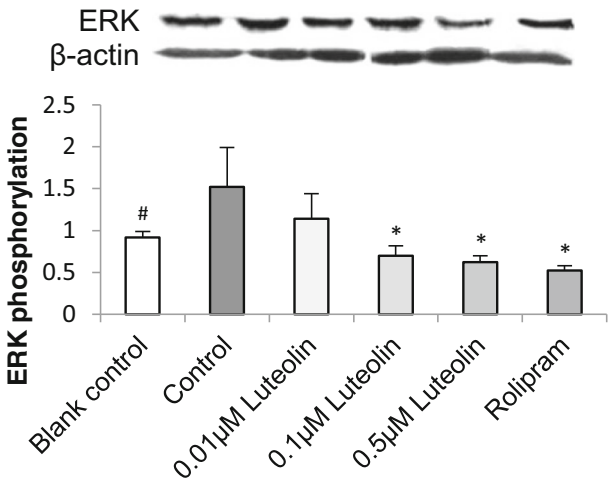

Fig. 4. Neutrophils were exposed to luteolin for 30 min and stimulated with 100 nM fMLP for 1 min. ERK phosphorylation was detected by Western blotting with β-actin as a loading control. The relative phosphorylation of ERK was represented as the ratio of the band intensities of target to β-actin. Representative figures (upper panel) and densitometric analysis (lower panel) of ERK phosphorylation are shown here. The experiment was performed three times with similar results. #$p < 0.05$ *versus* control group, *$p < 0.05$ *versus* control group.

Formyl-MLP of control significantly induced ERK phosphorylation in neutrophils at 30 s, 1 min, and 5 min compared with that of blank control ($p < 0.01$). In contrast to control, 0.5 μM luteolin undoubtedly inhibited ERK phosphorylation at 1 min, 5 min, and 30 min ($p < 0.05$, $p < 0.01$), and the inhibition rates of 0.5 μM luteolin on ERK phosphorylation at 1 min, 5 min, and 30 min were 41.9%, 17.4%, and 10.6%, respectively. ERK phosphorylation was also significantly inhibited by 1 μM rolipram at 1 min, 5 min, and 30 min ($p < 0.05$, $p < 0.01$) (Fig. 7).

No target bands of PI3K and JAK were detected in porcine neutrophils.

DISCUSSION

P38 MAPK is a type of protein kinase that is distributed in the cytoplasm and plays a noteworthy role in the occurrence, development, and outcome of various disease conditions such as inflammation and tumorigenesis by regulating the phosphorylation levels of nuclear transcription factors. P38 MAPK can regulate the chemotaxis, infiltration, cell surface marker expression, degranulation, respiratory burst, and apoptosis in neutrophils [20–24]. ERK is a key protein that transfers information from the cell surface to the nucleus, and can possibly phosphorylate target proteins in the cytoplasm, playing a role in the biological regulatory processes such as cell morphology. ERK participates in the activation, migration, extracellular trap, phagocytosis, and apoptosis of neutrophils [25–29]. PI3K/Akt is one of the most important signal pathways in cells and plays a key role in promoting cell proliferation. In neutrophils, PI3K is involved in the regulation of cell survival and homeostasis, activation, migration, extracellular trap, phagocytosis, peroxide production, elastase release, cell proliferation, and apoptosis [30–37]. JAK participates in various regulatory reactions in the body, and is able to bind with the corresponding target gene promoter in the nucleus to control the expression of the target proteins by activating STAT. JAK can regulate the survival, homeostasis, and *in vitro* fungicidal effect of neutrophils [34, 38]. Formyl-MLP is a bacterial metabolite having the chemoattractant capacity and activates leukocytes. The results (Fig. 1) showed that fMLP could significantly promote LFA-1expression in neutrophils, while blockers of p38 MAPK, ERK, PI3K, and JAK could significantly inhibit LFA-1 expression induced by fMLP, suggesting that

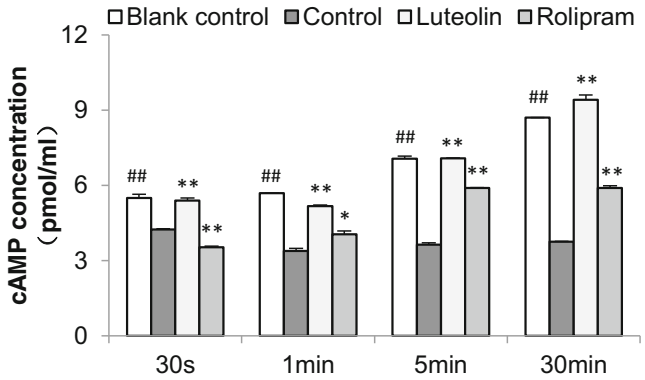

Fig. 5. Neutrophils were exposed to 0.5 μM luteolin for 30 min, and stimulated with 100 nM fMLP for 30 s, 1 min, 5 min, and 30 min. Cyclic-AMP concentration in neutrophil supernatants was detected by an ELISA kit. The experiment was performed three times with similar results. ##$p < 0.01$ *versus* control group, *$p < 0.05$ and **$p < 0.01$ *versus* control group.

pathways of p38 MAPK, ERK, PI3K, and JAK participate in the regulation of LFA-1 expression in neutrophils.

Cyclic-AMP regulated by chemokines can control the expression of adhesion molecules [39]. It can efficiently regulate fMLP-induced Ca^{2+} influx in neutrophils and can control fMLP-induced anions by activating the cAMP/PKA pathway while inhibiting the ERK pathway [40]. Cyclic-AMP also regulates LFA-1 expression in T cells *via* the cAMP/Rap1 pathway [41]. In addition, splitomicin and tetrandrine can inhibit fMLP-induced Mac-1 expression to reduce degree of inflammation by increasing cAMP level in neutrophils [40, 42].The results (Fig. 2 and Fig. 5) indicated that fMLP could significantly reduce cAMP concentration in neutrophil culture media, whereas luteolin could clearly increase cAMP concentrations in fMLP-induced neutrophil culture media; therefore, the mechanism was probably related to the inhibitory effect of luteolin on cAMP-PDE activity [1].

Formyl-MLP can induce p38 MAPK activity in neutrophils. Secondary metabolites of marine *Pseudomonas* (N11) could inhibit fMLP-induced phosphorylation of p38 MAPK and JNK in human neutrophils; however, this inhibition was not observed for ERK and AKT [43]. The fMLP also activates the NADPH oxidase by stimulating the increase in p38 MAPK activity in HL60 cells [44]. Interestingly, MSP68 short peptide is capable of inhibiting fMLP-induced p38 MAPK activity in human neutrophils [45]. Studies show that luteolin is involved in the regulation of the p38 MAPK pathway. Luteolin generally inhibits the p38 MAPK activity in normal body cells and plays an anti-inflammatory role [46–49], while promoting p38 MAPK activity in abnormal tumor tissues by exerting anti-tumor effect [50, 51]. There are four subtypes of p38 MAPK that can be activated by different mediators and produce different biological effects. Moreover, different subtypes have variable response to the same stimulus,

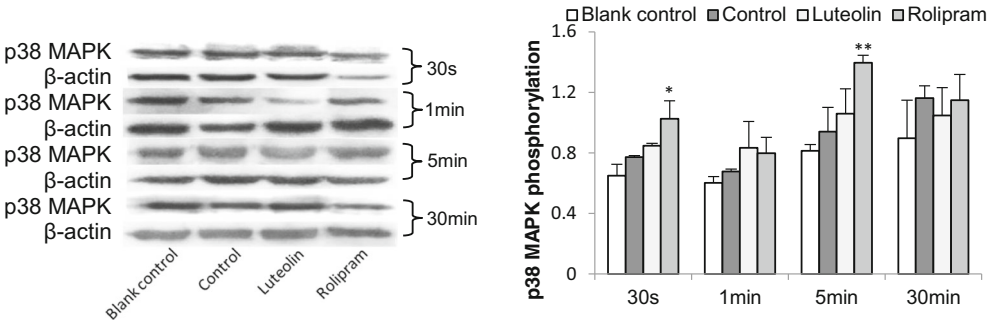

Fig. 6. Neutrophils were exposed to 0.5 μM luteolin for 30 min and stimulated with 100 nM fMLP for 30 s, 1 min, 5 min, and 30 min. The p38 MAPK phosphorylation was detected by Western blotting with β-actin as a loading control. The relative phosphorylation of p38 MAPK was represented as the ratio of band intensities of the target and β-actin. Representative figures (left panel) and densitometric analysis (right panel) of p38 MAPK phosphorylation are shown here. The experiment was carried out three times with similar results. *$p < 0.05$ and **$p < 0.01$ *versus* control group.

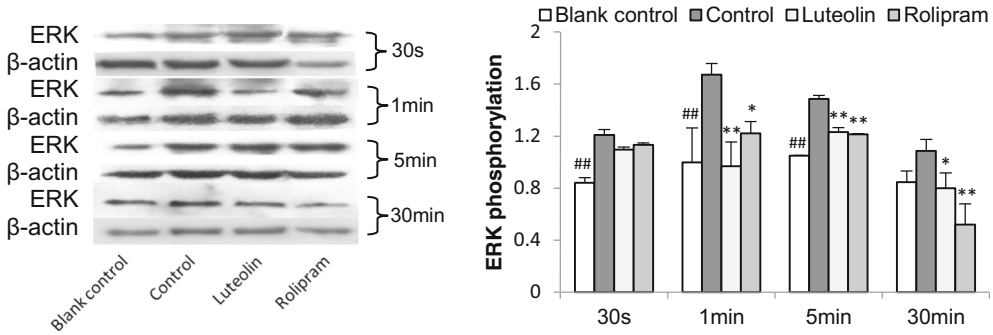

Fig. 7. Neutrophils were exposed to 0.5 μM luteolin for 30 min and were then stimulated with 100 nM fMLP for 30 s, 1 min, 5 min, and 30 min. The ERK phosphorylation was detected by Western blotting and β-actin was used as a loading control. The relative phosphorylation of ERK was represented as the ratio of the target band to β-actin band intensities. Representative figures (left panel) and densitometric analysis (right panel) of ERK phosphorylation are shown here. The experiment was carried out three times to get similar results. ##$p < 0.01$ *versus* control group, *$p < 0.05$, and **$p < 0.01$ *versus* control group.

which constitutes a complex and varied network of signal transduction pathways. Results (Fig. 3 and Fig. 6) showed that fMLP only had a limiting inducting effect on p38 MAPK phosphorylation in porcine neutrophils, which was not significant. Luteolin further had a slightly increasing effect on p38 MAPK phosphorylation induced by fMLP with no statistical significance.

Formyl-MLP can also promote ERK activity in inflammatory cells such as neutrophils. ERK is able to promote cell migration during the directional migration of human neutrophils to fMLP [52]. Also, fMLP-induced phosphorylation of PAK1/2 and ERK/JNK may promote transendothelial migration and chemotaxis of human neutrophils [53]. The fMLP also promotes BV-2 cell migration of mouse microglia cell line by activating ERK signaling pathway [54]. Luteolin has different regulatory effects on ERK pathway in different tissues. It is known to inhibit the movement, migration, and proliferation along with inducing apoptosis of lung adenocarcinoma cells through activation of MEK-ERK pathway [55]. Luteolin also induces apoptosis of human breast cancer cells by activating ERK and p38 MAPK-mediated nuclear translocation of apoptosis-inducing factor [51]. In addition, luteolin inhibits breast cancer cell invasion by suppressing the ERK/AP-1 and ERK/NF-κB signal cascades [56]. It has been shown that luteolin protects the brain from damage by persistent middle cerebral artery occlusion, inducing neurite outgrowth, and increased the cellular antioxidant defenses associated with activation of ERK signaling pathway [57, 58]. In another study, luteolin improved myocardial contractile function after cardiac ischemia/reperfusion injury and protected the cells from cardiotoxicity induced by doxorubicin. This role had a hidden relationship with inhibition of ERK pathway [59, 60]. Luteolin has a positive effect on LPS-induced acute lung injury in mice, and delayed neutrophil chemotaxis and respiratory burst, which was associated with blocking the MEK-, ERK-, and Akt-related signaling cascades [61]. The results (Fig. 4 and Fig. 7) showed that fMLP had a significant enhancing effect on ERK phosphorylation in porcine neutrophils, whereas luteolin had a dose- and time-dependent inhibitory effect on fMLP-induced ERK phosphorylation.

PI3K and JAK are also important signal proteins involved in the expression of adhesion molecules in neutrophils [8, 10, 62]. However, the target bands of these two proteins were not detected after many experiments, which may be related to their low expression in porcine neutrophils and needs further study.

Taken together, our results demonstrate that pathways of p38 MAPK, ERK, PI3K, and JAK are involved in the regulation of LFA-1 expression in neutrophils. Moreover, luteolin inhibits LFA-1 expression at least partly by increasing cAMP concentration and suppressing ERK phosphorylation, which will help us to elucidate the new anti-inflammatory target of luteolin, thereby laying a strong foundation in clinical settings.

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