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Pseudo-allergic reaction caused by Qingkailing injection partially *via* the PI3K-Rac1 signaling pathway in RBL-2H3 cells

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Qingkailing injection (QKLI) is a kind of multi-component traditional Chinese medicine injection. It has been widely used in clinical practice, but in recent years, it has caused more and more adverse reactions, mainly manifested as pseudo-allergic symptoms. To explore the potential mechanism of the pseudo-allergic reaction by QKLI, basophilic leukemia cell line 2H3 (RBL-2H3) was chosen. The results showed that QKLI at doses of 5, 10 and 20 mL L⁻¹ activated phosphoinositide 3-kinase (PI3K) activity and also increased the levels of Ras-related C3 botulinum toxin substrate 1 (Rac1), p21 protein-activated kinase 1 (Pak1), LIM kinase (Limk1) and cofilin (an actin polymerization regulator) proteins. What's more, QKLI aggravated the depolymerization of F-actin. NSC23766, a Rac1 inhibitor, reversed the previous results in QKLI-treated RBL-2H3 cells. In addition, when the Rac1 gene was knocked down using lentiviral vector-loaded shRNA in RBL-2H3 cells, the PI3K activity and depolymerization of F-actin were downregulated, hinting that the pseudo-allergic reaction was significantly reduced. In general, the pseudo-allergic reaction induced by QKLI was likely to be based on PI3K-Rac1 signaling pathways partially.

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Introduction

An allergic reaction is a response to tissue damage or dysfunction that occurs when an immune organism is exposed to the same antigen again. The reaction is characterized by rapid onset, strong reaction and fast extinction.^{1,2} The current pathogenesis of allergic reactions mainly includes the following four pathways: (1) non-immune activation of the complement system, release of bioactive peptides, such as C_{3a}, C_{4a}, C_{5a} and other anaphylatoxins, these anaphylatoxins promote the release of allergic media;³ (2) direct stimulation of mast cells or basophils by drugs to release allergic mediators, such as certain non-steroidal anti-inflammatory drugs, opioids, compound 48/80, substance P, *etc.*;⁴ (3) change in the synthesis and release pathway of arachidonic acid metabolites;⁵ and (4) acute allergy pathway. A pseudo-allergic reaction⁶ is related to the bioactive activity such as degranulation and release of histamine from mast cells or basophils, all of which are an immediate allergic reaction, but obviously, a pseudo-allergic reaction has different mechanisms from an allergic reaction.⁷ Unlike an allergic reaction, a pseudo-allergic reaction is usually caused by the first dose. Pseudo-allergies do not cause

antigen-specific immune responses, but they do cause histamine release, activation of the complement system, and so on.⁸

Phosphoinositide 3-kinases (PI3Ks) is a family of lipid kinases that phosphorylate the 3'-OH group of the phosphoinositide inositol ring.⁹ In various signaling events, phosphorylated lipids are produced in the cell membrane and recruit and activate various signaling molecules. PI3Ks can be classified into three groups according to the structure and specific substrate (class I, class II and class III).¹⁰ PI3K consists of a regulatory subunit (P85) and a catalytic subunit (P110).^{11,12} When the corresponding ligand binds to the membrane receptor, P85 is activated to start recruiting P110, which is activated in the vicinity of the cell membrane, thereby catalyzing the production of phosphatidylinositol diphosphate (PIP₂) on the inner surface of the membrane to produce phosphatidylinositol triphosphate (PIP₃). As the second messenger, PIP₃ activates 3-phosphoinositide-resistant protein kinase-1 (PDK1). PDK1 can phosphorylate the T308 site of Akt.^{13,14} When Akt is activated, TSC2 is phosphorylated, whereby the inhibition of Rheb by the TSC1/2 complex is eliminated, and Rheb activates mTORC1. mTORC1 can phosphorylate the T389 site of ribosomal S6 kinase-1 (S6K1). S6K1 phosphorylates and activates rpS6, which increases the translation of proteins required for cell growth, which is involved in cell proliferation, leading to important biological events such as cell survival, cellular metabolism, and cytoskeletal reorganization.

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Qingkailing injection (QKLI), a traditional Chinese medicine (TCM), is widely used in clinical practice,^{15,16} especially in upper respiratory inflammation and stroke.¹⁷ QKLI was specified as a “must-have” medicine for TCM emergency clinics in 1992.¹⁸ QKLI is processed from cholic acid, mother-of-pearl (powder), hyodeoxycholic acid, wolfberry, buffalo horn (powder), radix isatidis, baicalin, and honeysuckle. The auxiliary materials are disodium edetate, sodium thiosulfate and glycerin. However, in recent years, the pseudo-allergic reaction caused by QKLI has been frequently reported. According to the results of our previous experiment, QKLI at a dose of 5 mL kg⁻¹ could increase vascular permeability in mice, which was one of the main performance of the pseudo-allergic reaction, and treatment with 7.5 mL kg⁻¹ and 15 mL kg⁻¹ QKLI aggravated vascular permeability. QKLI at doses of 5, 7.5 and 15 mL kg⁻¹ reduced mean arterial blood pressure in a dose-dependent manner in rats.¹⁹ In the present study, we chose the rat basophil leukemia RBL-2H3 cells and explored the role of the PI3K-Rac1 signaling pathway during the process of the pseudo-allergic reaction induced by QKLI.

Materials and methods

Drugs and reagents

QKLI (batch number: 15070852) was purchased from HuaDong Medicine Co., Ltd; C48/80 (cat number: C2313) and NSC23766 (Rac1 inhibitor) (cat number: SML0952) were purchased from Sigma-Aldrich; DMEM was purchased from Hangzhou Genom Co., Ltd; trypsin was purchased from Gibco; fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd; PI3K assay kit (batch number: 33-016) was purchased from Millipore; BCA kit (batch number: P0010S), rabbit polyclonal antibodies for LIM domain kinase 1 (LIMK1), Pdc protein (Cdc42/Rac)-activated kinase 1 (PAK1), cofilin and Rac1 were all purchased from Cell Signaling Technology Co., Ltd; and GAPDH was purchased from Abcam.

Cell culture

The rat basophil leukemia cell line RBL-2H3 was purchased from the Cell Bank of China Science. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 0.1% antibiotics under a humidified atmosphere with 5% CO₂ at 37 °C. QKLI was diluted with the incubating solution and the final concentration was 5, 10, and 20 mL L⁻¹. The positive drug C48/80 was prepared using the incubation solution, and the final concentration was 10 µg mL⁻¹. The Rac1 inhibitor NSC23766 was prepared using the incubation solution, and the final concentration was 50 µM.

PI3 kinase assay

Cells were collected after treating with QKLI. Then, the PI3K activity was detected using the homogeneous time-resolved fluorescence (HTRF) assay (PI3K assay kit; Millipore) as described in the literature.²⁰ The HTRF ratio was determined

by using a microplate reader (SpectraMax M4, Molecular Devices).

Western blot analysis

Cells treated with test compounds were harvested and lysed in RIPA lysis buffer (Beyotime). Cell lysates were separated by SDS-PAGE, and transferred onto PVDF transfer membranes (Millipore, USA). Membranes were blocked and then incubated overnight at 4 °C with primary antibodies: GAPDH, LIMK1, PAK1, cofilin and Rac1 were at a 1 : 1000 dilution in the blocking solution. After washing three times in TBST for each 10 min, the membranes were incubated with secondary horseradish peroxidase-conjugated goat anti-mouse/rabbit immunoglobulin G. Then, the proteins were detected using an enhanced chemiluminescence detection kit. The images were obtained using a Mini-PROTEAN gel imaging system (Bio-Rad, USA).

Quantitative detection of F-actin

Cells were treated with culture medium containing the appropriate drug or an equal amount of negative control (incubation buffer) for 1 h; then, the culture medium was removed and washed three times with PBS. Cells were fixed with 4% paraformaldehyde at room temperature for 10 min, washed with PBS for 30 s, permeabilized with 0.5% Triton X-100 for 5 min and washed again with PBS for 30 s. Subsequently, 200 µL of 100 nM rhodamine-phalloidin (Cytoskeleton, Denver, CO, USA) were added, and the samples were incubated in the dark at room temperature for 30 min, washed three times with PBS and photographed under a confocal laser microscope. Alternatively, 300 µL of methanol were added and the samples were incubated in the dark for 2 h. The supernatant was transferred to a black plate with a transparent bottom and the fluorescence was detected at a wavelength of 535/590 nm.

Immunofluorescence

The cells were inoculated into the laser confocal culture dish at a concentration of 1 × 10⁵ cells per mL on the previous day. After 24 hours, the culture medium was discarded and the cells were washed with PBS 3 times. The corresponding drug or negative control (incubation solution) was given, and the solution was discarded after 1 h of placement in the incubator. The solution was rinsed with PBS at room temperature 3 times. The cells were fixed with 4% paraformaldehyde at room temperature for 10 min, and washed with PBS for 30 s. 0.5% Triton X-100 was permeated for 5 min and was washed with PBS for 30 s. 100 nM ghost pen cyclic peptide was added and incubated at room temperature without light for 30 min. 100 nM DAPI was added to stain for 30 s, and then washed with PBS for 3 times. Fluorescence was observed using a Zeiss confocal microscope.

Lentiviral vector-loaded shRNA to interfere with Rac1 expression

Lentiviral vector-loaded shRNA was used to interfere with Rac1 expression. Briefly, a lentivirus expression plasmid containing

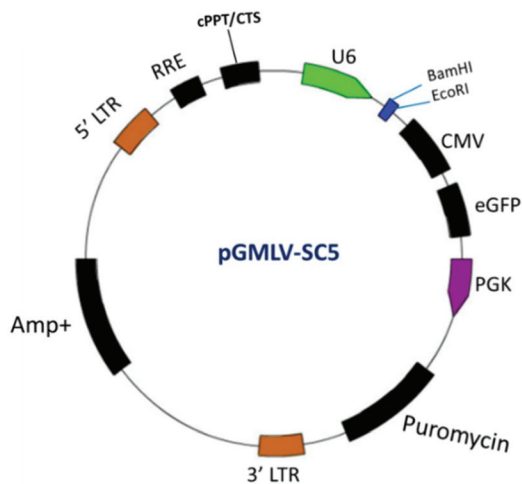


Fig. 1 The vector map of pGMLV-SC5 RNAi.

small interference RNA targeting Rac1 was constructed by GeneChem (Shanghai, China) and was used to infect the RBL-2H3 cells in the presence of $6 \mu\text{g mL}^{-1}$ polybrene. As shown in Fig. 1, the vector was linearized through the restriction endonucleases BamH I (GGATCC) and EcoR I (GAATTC), which was connected to the target RNAi sequence and constructed as the lentiviral vector pgmLv-sc5 RNAi with the target RNAi sequence. Cells were then selected by puromycin, and the knockdown of Rac1 was confirmed by real-time PCR and western blot.

Statistical analysis

The data are expressed as mean $\pm$ SD and were analyzed using SPSS for Windows Version 13.0. Comparisons of the data from different groups were performed using a one-way ANOVA test. A P value less than 0.05 was considered statistically significant.

Results

QKLI increased the level of PI3K in RBL-2H3 cells

HRTF assay was applied to detect the PI3K activity in RBL-2H3 cells and the higher the HRTF ratio, the lower the kinase activity, and *vice versa*. As shown in Fig. 2, compared with the negative control group, the fluorescence rates of QKLI at different doses of 5, 10 and 20 mL L^{-1} were decreased ($P < 0.01$), indicating that the P13 kinase activity was increased in a dose-dependent manner. When the cells were co-treated with 5, 10 and 20 mL L^{-1} QKLI and a Rac1 inhibitor, NSC23766, the fluorescence rates were increased, indicating that its kinase activity decreased significantly ($P < 0.01$).

QKLI increased Rac1, PAK1, LIMK1 and cofilin in RBL-2H3 cells

As shown in Fig. 3, compared with the negative control group, the expression level of the Rac1 protein was increased in QKLI-treated RBL-2H3 cells ($P < 0.01$), and the expression levels of

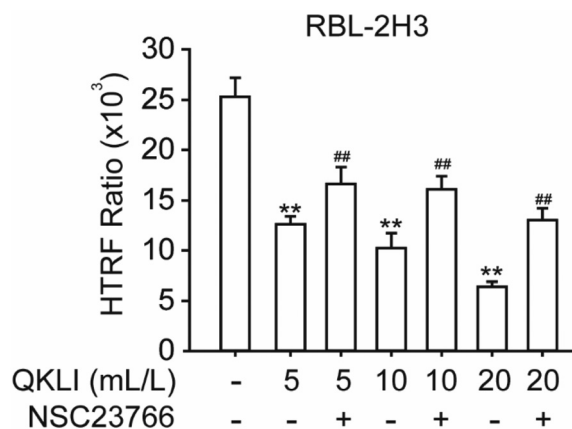


Fig. 2 The effect of QKLI on the PI3K kinase activity in RBL-2H3 cells. The cells were treated with 0, 5, 10 and 20 mL L^{-1} QKLI with or without the Rac1 inhibitor NSC23766, and then the PI3K activity was detected by the HTRF assay. Each study was performed three times and the error bars represent the SD around the mean. ** $P < 0.01$ compared with the control group, ## $P < 0.01$ compared with the corresponding QKLI group (without NSC23766).

its downstream proteins including PAK1, LIMK1 and cofilin in RBL-2H3 cells were also increased ($P < 0.01$), showing a dose-dependent relationship. After co-treating with NSC23766 and QKLI, the expression of Rac1, PAK1, LIMK1 and cofilin proteins was reduced compared with the QKLI group ($P < 0.01$).

QKLI promoted the depolymerization of F-actin in RBL-2H3 cells

A cytoskeletal polymer controls the morphology and kinetics characteristics of eukaryotic cells, including three main forms: actin filament (F-actin), microtubule and intermediate filament; they are assembled into the network structure to resist deformation of cells, but in response to external stress to reassemble, they play a very important function in maintaining cell integrity. The aggregation and depolymerization of F-actin and microtubules are the direct factors of the changes in cell morphology. Meanwhile, molecular motors play an important role in the assembly process of various components of cells.

As shown in Fig. 4, in the negative control group, the cell morphology was spindle-shaped without obvious morphological changes. After treatment with QKLI, F-actin was induced to depolymerize, and the cytoskeletal rearrangement was promoted. The depolymerization state of F-actin in QKLI-treated RBL-2H3 cells was obvious, and the degree of depolymerization was increased in a dose-dependent manner. In addition, the depolymerization of intracellular F-actin in co-treated with QKLI and the Rac1 inhibitor group was significantly reduced and lower than that in the QKLI group.

The effect of gene silencing detected by real-time PCR and western blotting

Using a slow viral vector system, the Rac1 gene expressing siRNA interference recombinant virus was successfully

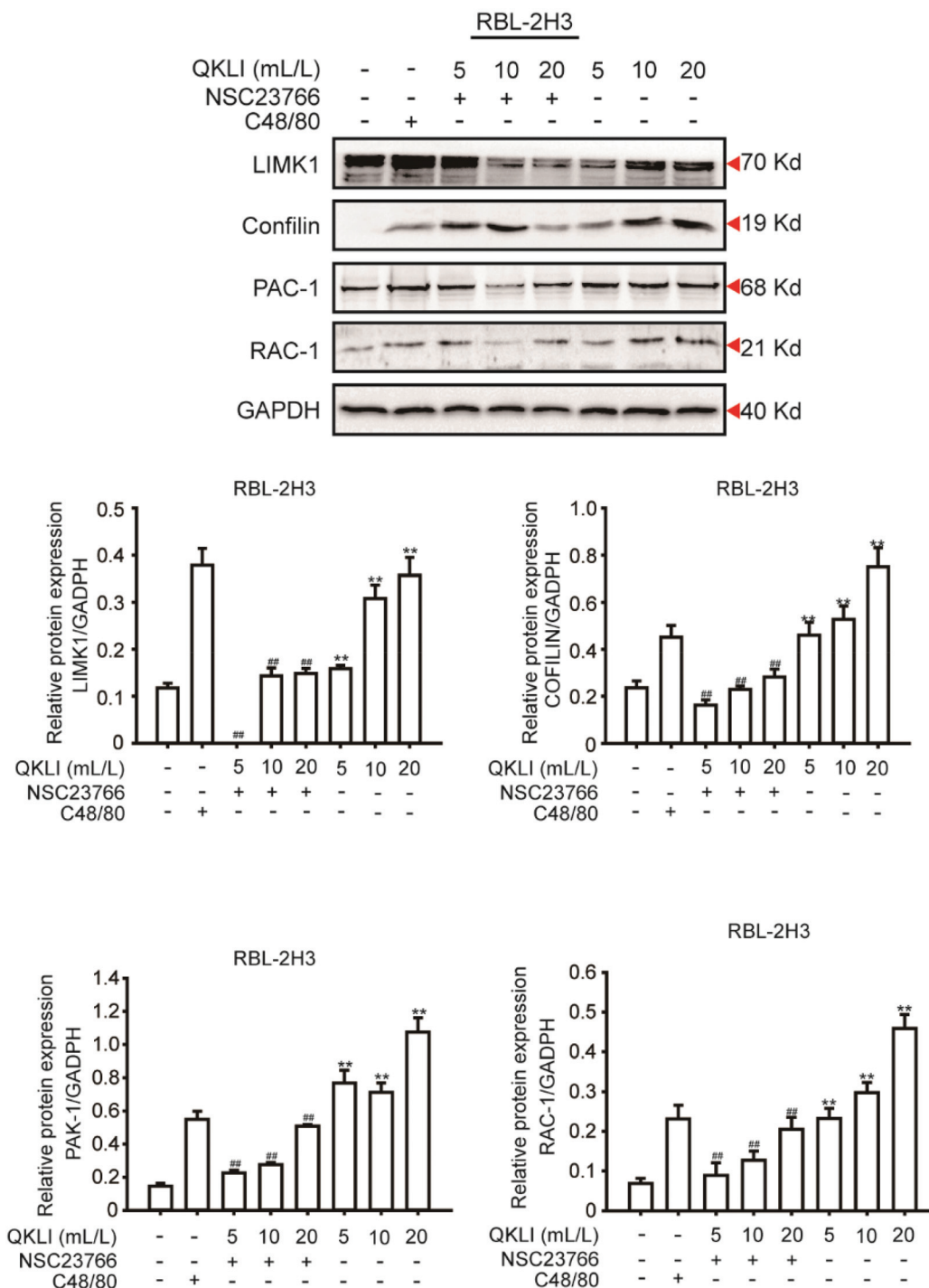


Fig. 3 The effect of QKL on Rac1, PAK1, LIM1 and cofilin proteins in RBL-2H3 cells. The cells were treated with 0, 5, 10 and 20 mL L⁻¹ QKLI with or without the Rac1 inhibitor NSC23766; then, Rac1, PAK1, LIM1 and cofilin proteins were detected by western blotting. Relative band intensities were used in order to quantify protein expression levels. ***P* < 0.01 compared with the control group, ##*P* < 0.01 compared with the corresponding QKLI group (without NSC23766).

obtained and a cell model of Rac1 gene defects RBL-2H3 Rac1-sh2 was constructed. The gene silencing effect in RBL-2H3 cells was verified by qPCR. The results showed that the Rac1 gene expression in the negative control group did not produce any change, and the slow virus vector carrying pieces of RNA

interference in the experiments for a specific fragment was different in gene expression quantity as shown in Fig. 5. Meanwhile, western blotting was performed with an anti-Rac1 antibody, and the results are shown in Fig. 5. Compared with the control group transfected with an empty vector, the

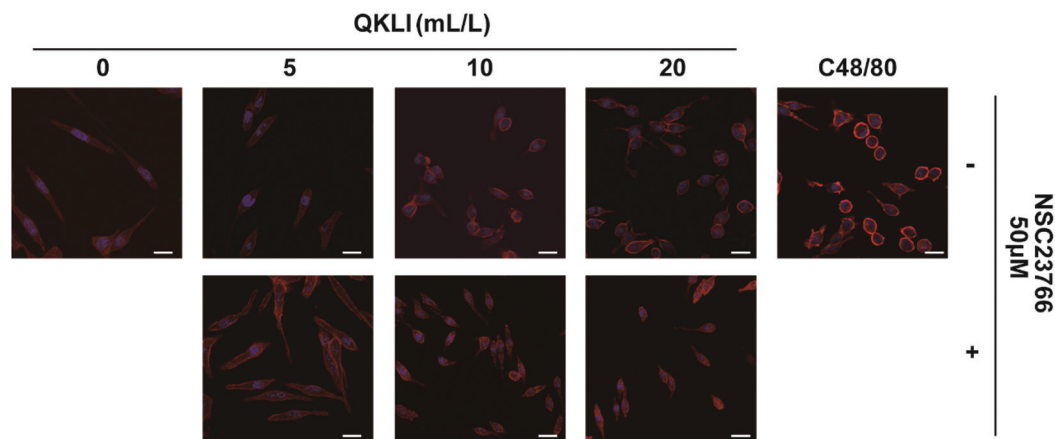


Fig. 4 The effect of QKLI on the depolymerization of F-actin in RBL-2H3 cells. The cells were treated with 0, 5, 10 and 20 mL L⁻¹ QKLI with or without the Rac1 inhibitor NSC23766, and then the cells were stained with rhodamine phalloidin and DAPI and photographed under a confocal laser microscope. Scale bar = 100 μm.

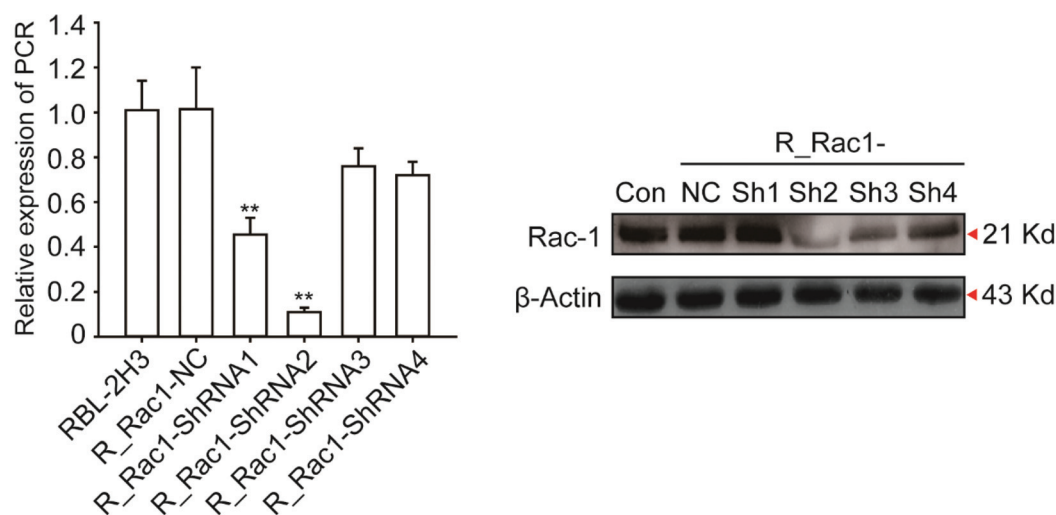


Fig. 5 Relative expression of the Rac1 gene and protein was detected by real-time PCR and western blotting. The experiments were performed three times independently, and the error bars represent the SD around the mean. ** $P < 0.01$ compared with the RBL-2H3 group.

expression of Rac1 in cells was significantly inhibited after transfection with a plasmid containing Rac1 sh RNA. The results indicated that the stable transfection cell lines of RBL-2H3 with the Rac1 gene defect were successfully obtained.

The PI3K activity was downregulated in RBL-2H3 Rac1-sh2 cells after treatment with QKLI

As shown in Fig. 6, there was no significant difference in the PI3K activity between the blank control and the Rac1-sh2 group in RBL-2H3 cells. The fluorescence rates of QKLI at doses of 10 mL L⁻¹ decreased compared with the blank group in RBL-2H3 cells ($P < 0.05$), suggesting that the PI3K activity was upregulated. After the Rac1 gene was silenced, the fluorescence rates of QKLI at doses of 10 mL L⁻¹ was increased compared with the QKLI group in RBL-2H3 cells, indicating

that the PI3K activity was significantly reduced in RBL-2H3 Rac1-sh2 cells, which suggested that the PI3K activity was regulated by the Rac1 gene in the process of the pseudo-allergic reaction induced by QKLI.

Effect of QKLI on the depolymerization of F-actin in RBL-2H3 Rac1-sh2 cells

As shown in Fig. 7, the performance of F-actin was normal in the blank control and the Rac1 shRNA2 group. After QKLI treatment, F-actin was induced to depolymerize, and cytoskeletal rearrangement was promoted, and F-actin in RBL-2H3 Rac1-sh2 also appeared in the depolymerization state, but the degree and concentration of depolymerization showed in a decrease, revealing that the silence of the Rac1 gene could reduce cytoskeletal fluidity in QKLI-treated RBL-2H3 cells and

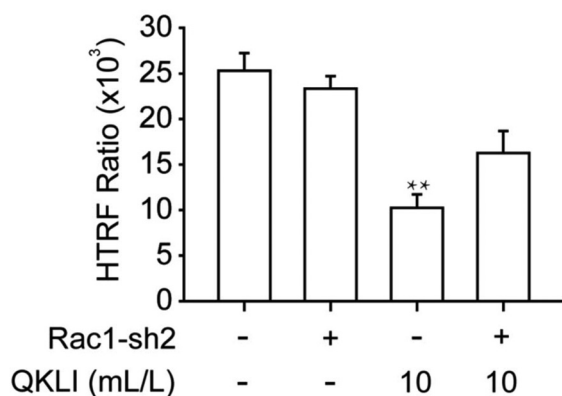


Fig. 6 The effect of Rac1 gene knockdown on the PI3K kinase activity in QKLI-treated RBL-2H3 cells. After the Rac1 gene was silenced, the cells were treated with 10 mL L⁻¹ QKLI, and then the PI3K activity was detected by the HTRF assay. The experiments were performed three times independently, and the error bars represent the SD around the mean. ***P* < 0.01 compared with the blank control group.

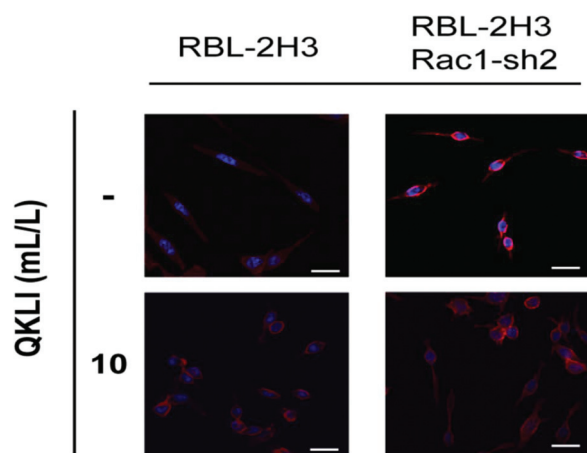


Fig. 7 The effect of Rac1 gene knockdown on the F-actin activity in QKLI-treated RBL-2H3 cells. After the Rac1 gene was silenced, the cells were treated with 10 mL L⁻¹ QKLI, and then the cells were stained with rhodamine phalloidin and DAPI and photographed under a confocal laser microscope. Scale bar = 100 μm.

the pseudo-allergic reaction induced by QKLI might be partly through the PI3K_Rac1 pathway.

Discussion

We hypothesized that the mechanism of the QKLI-induced pseudo-allergic reaction was a non-specific factor that was independent of specific receptors. The external environment changed the signal, which was transmitted from the outside of the cell to the cell through a signal transduction pathway, and finally caused the dense particles in the cell to migrate to the inner side of the cell membrane. Then, the membrane was fused, and the dense particle contents were effluxed. The

accumulation of lipid rafts on the surface of the cell membrane led to changes in its fluidity, which further caused changes in the cytoskeleton, which was the basis for degranulation of mast cells.

PI3K regulates numerous cellular biological processes. These include apoptosis, transcription, translation, metabolism, angiogenesis, and regulation of the cell cycle. PI3K is an upstream molecule of the Akt/mTOR pathway,²¹ and its aberrant activation can cause a series of reactions, including cell growth, proliferation and metastasis, transformation of epithelial cells into leaf cells, and angiogenesis. The activity of PI3K is mainly regulated by Rac1 GTPase.^{22,23} PAK1 is an effector protein that regulates cytoskeletal remodeling by Rac1, and its activity is also regulated by PI3K-Akt signaling.^{24,25} PAK1 associates Rac with cellular activity through phosphorylation of the myosin light chain. Phosphorylation of PAK1 simultaneously activates the LIM enzyme, which is the same target as human fitinin phosphorylation. Cofilin stimulates the depolymerization of F-actin while altering the cell structure, while LIM-activated human actin phosphorylation inhibits its activity.²⁶

In clinical medication, we found that the clinically recommended dose of QKLI of 20–40 mL 60 kg⁻¹ led to a severe pseudo-allergic reaction in patients. In order to enable cells to better simulate such a pseudo-allergic reaction to study its mechanism, QKLI at doses of 5, 10 and 20 mL L⁻¹ were applied in this experiment. The RBL-2H3 cell²⁷ was a cell model for screening antiallergic drugs *in vitro*. In the present study, F-actin produced depolymerization under the action of different concentrations of QKLI in RBL-2H3 cells, and the intracellular PI3K kinase activity and the levels of Rac1, PAK1, LIMK1 and cofilin proteins were increased to some extent. The degree of F-actin depolymerization and the levels of PI3K-Rac1 pathway proteins were upregulated in a dose-dependent way. In order to verify whether QKLI induced an allergic reaction through the PI3K-Rac1 signaling pathway, the Rac1 inhibitor NSC23766 was chosen, which could block the PI3K-Rac1 signaling pathway. The results showed that the activity of Rac1 was inhibited by the addition of the Rac1 inhibitor, and its upstream regulator PI3K activity was lowered. The expression of its downstream proteins PAK1, LIMK1 and cofilin protein was also decreased, and the degree of F-actin depolymerization was weakened.

The small molecule GTPase Rac is a member of the Rho small G protein superfamily and has a regulatory role in a variety of cellular biological activities, including cell growth, cytoskeletal remodeling, cell migration, and infiltration, which can cause the decomposition of cellular stress fibers. Rac includes 3 members, Rac1, Rac2, and Rac3. Rac1 is expressed in a variety of tissues, Rac2 is expressed only in cells of the blood system, and Rac3 is mainly expressed in cells of the nervous system.^{28,29} Studies have suggested that the high expression of Rac1 could disrupt the cadherin-dependent cell junction and alter the cell's morphological phenotype by regulating the polymerization and reorganization of F-actin, enhancing the cell migration ability.^{30,31} Therefore, the use of siRNA

to construct cells deficient in the Rac1 gene can more directly verify the role of the PI3K-Rac1 signaling pathway in an allergic reaction. Our results showed that the PI3K activity was decreased after treatment with QKLI in RBL-2H3 Rac1-sh2 cells. In addition, the degree of F-actin depolymerization also was decreased in RBL-2H3 Rac1-sh2 cells.

Conclusions

Under our experimental conditions, QKLI could stimulate the degranulation of mast cells, thereby inducing an allergic reaction. The QKLI-induced allergic reaction in RBL-2H3 cells was likely to be based on changes in cell membrane fluidity, which further induced changes in the cytoskeleton through the PI3K-Rac1 cell motor signal transduction pathway, and knock-down of Rac1 alleviated the PI3K activity and cytoskeletal fluidity. Therefore, it was believed that the QKLI-induced allergic reaction may be partly dependent on the PI3K-Rac1 signaling pathway.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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