Research Article

Isoforskolin Inhibits Lipopolysaccharide Activation of the TLR4/MyD-88/NF-κB Pathway in Small Airway Epithelial Cells

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Abstract

Object: Isoforskolin (ISOF) is isolated from the plant *Coleus forskohlii*, native to Yunnan China, which has attracted much attention for its biological effects. It has been demonstrated to attenuate inflammation of rat ALI induced by Lipopolysaccharide (LPS). In this study, we hypothesize that ISOF inhibits a main pathway activated by LPS in human small airway epithelial cells (BEAS-2B): cascade of toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), and nuclear factor kappa-B (NF-κB) signaling.

Methods: BEAS-2B cells were pretreated with ISOF (1.0μM), forskolin (FSK, 1.0μM), roflumilast (RF, 1.0μM) and dexamethasone (DEX, 50μM) prior to LPS (2 μg/mL). Protein and mRNA levels of TLR4, MyD88, and NF-κB in BEAS-2B were determined using Western blot and Real-time quantitative PCR. Effects of ISOF and LPS on cell viability were tested by using MTT method.

Results: Our results showed that LPS augmented the protein and mRNA levels of TLR4, MyD88, and NF-κB in BEAS-2B cells. Following LPS treatment, protein and mRNA levels of TLR4, MyD88, NF-κB in BEAS-2B decreased significantly. Pretreatment with ISOF attenuated the effects of LPS. Moreover, ISOF (with dose < 5.0 μM) and LPS (with dose < 20 μg/mL) has no effect on cell viability of BEAS-2B at 6h.

Conclusions: These results have shown that ISOF inhibits TLR4/MyD88/NF-κB signal pathway, which is activated by LPS, through down-regulation of mRNA and protein levels of TLR4, MyD88 and NF-κB in BEAS-2B.

Keywords: *Coleus Forskohlii*; Dexamethasone; Forskolin; Inflammation; Roflumilast

Introduction

Acute Respiratory Distress Syndrome (ARDS) is a clinical syndrome of acute respiratory failure due to acute lung inflammation that often leads to multi-organ system failure and death [1]. Acute pulmonary infection culminating in ARDS is a critical complication in gram-negative bacterium induced sepsis [2]. Lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria, is one of the major causes of sepsis [3]. It has been shown that the inflammatory process evoked by LPS is mediated by the transmembrane receptor 4 (TLR4) [4,5]. Myeloid differentiating factor 88 (MyD88) is an adaptor of TLR. After the recognition of LPS by TLR4, a series of signaling cascades, including MyD88, are initiated. MyD88 then activates nuclear factor kappa B (NF-κB) family members and mitogen activated protein kinase [6]. NF-κB is an important transcription factor associated with inflammatory responses and is present in almost all type of cells [7]. NF-κB can control DNA transcription, regulate cytokine production and cell survival time, and is involved in the body's immune, inflammation, and stress physiological pathology [8]. Therefore, inhibition of NF-κB activity is an effective way to block inflammation [9]. NF-κB regulates the secretion of various inflammatory factors such as TNF-alpha, IL-6 and IL-1 beta and participates in the development and progression of ARDS cascade inflammation [10,11].

The plant *Coleus forskohlii Briq*, which is mainly distributed in India, China, Thailand, and Brazil, has been used to treat many diseases such as hypertension , obesity, glaucoma, asthma, bronchitis, convulsions and so on [12-15]. Isoforskolin (ISOF) is isolated from *Coleus forskohlii* native to Yunnan in China, which has attracted attention for its biological effect such as inhibition of airway remodeling and anti-inflammation [16-18]. Our previous research [19] showed that ISOF had an anti-inflammatory effect on acute lung injury of rats induced by LPS, but the mechanism is still unknown. Altogether, to elucidate the potential mechanism of ISOF anti-inflammation, we hypothesize that ISOF inhibits the TLR4/MyD88/NF-κB pathway which is activated by LPS.

Materials and Methods

Chemicals and kits

ISOF and FSK (purity 99%) was provided as white crystals by Kunming Beike Norton Pharmaceutical Co., Ltd. DEX was purchased from Yangtze River Pharmaceutical Company (Yangzhuo, Zhejian, China). LPS (Escherichia coli 055: B5), DMSO (0227) and roflumilast (SML1099) were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Sterile water for injection (H41024923) was purchased from Sinopharm Group Health and Pharmaceutical Co (Tianjing, China). Mouse TLR4 polyclonal antibody (ab13556), Rabbit MyD88 polyclonal antibody (ab2068), Rabbit NF-κB (P65) antibody (ab7970) and anti-beta actin antibody (ab49900) were purchased from abcam company (California, USA). Rabbit GAPDH monoclonal antibody (2118) was purchased from Cell-signaling Technology Inc (Massachusetts, USA). DMEM (high glucose) (NZM1284), Trypsin 0.25% Solution (SH30042.01), Penicillin/Streptomycin Solution (SV30010) and DPBS (NZM1284) were purchased from Hyclone (Louisiana, USA). Trizol (15596018) was purchased from Invitrogen (California, USA). PrimeScript™ RT reagent Kit with gDNA Eraser (AK3302) and SYBR Rremix Ex Taq™II (AK8901) were purchased from TakaRa Biotechnology Co., Ltd., (Shanghai, China). FBS (16010-159) was purchased from Gibico (California, USA).

BEAS-2B Culture and Treatment

BEAS-2B were obtained from ATCC (USA) and grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics in 37°C constant temperature incubator (5% CO_2 + 95% air). Tightly confluent monolayers of BEAS-2B from the 4th−15th passage were used in all experiments. Control cells were treated with vehicle control (DMSO volume concentration was 1/1000). LPS group was only given LPS (concentration). Treatment groups were given ISOF (1.0μM), FSK (1.0μM), RF (1.0μM), DEX (50μM) respectively for 0.5h, and then each group was treated with LPS (2μg/mL) for 6h. ISOF+RF group and FSK+RF group cells were given RF (0.5μM) for 0.5h and then ISOF (0.5μM) or FSK (0.5μM) for 0.5h followed by incubation with LPS (2μg/mL) for 6h. At the end of the experiment, cells of each group were collected for RT-PCR assay and Western blot analysis as described in the following sections. All experiments were performed in replicates (n = 3-6).

MTT Assay of Cell Viability

BEAS-2b cells were plated in 96-well flat-bottomed plates at a density of 2×10^4 cells/mL and 90μL/well. Cells were cultured in normal condition (37°C, 5% CO_2 + 95% air), and then were treated with LPS (0.25, 1.0, 5.0, 20 and 50μM) or ISOF (2.5, 5.0, 10, 20 and 50μM) for 6, 12 and 24h. After the treatments, 20 μL of MTT (5mg/mL) was added to each well and the plates were incubated for 4 hours at 37°C (5% CO_2 + 95% air). Then, 15μL DMSO was added to each well and incubated 10m. Cell viability was determined by measuring the ability of metabolically active cells to convert the yellow tetrazolium salt MTT into purple formazan crystals with a microplate reader at 490 nm. Results of three independent experiments (n = 3) were used for statistical analysis.

Total RNA extraction and Real-Time fluorescent quantitative PCR

Total RNA was extracted from BEAS-2B by using RNAiso Plus according to the manufacturer's instructions. Concentration and purity were determined by determining the absorbance at 260

and 280 nm. Approximately 1 µg of total RNA was used to synthesize cDNA (Prime Script® RT reagent kit and gDNA Eraser) according to the manufacturer's protocol. The primers of human TLR4, NF-κB and GAPDH were designed using the Primer Select software program and synthesized by TakaRa Biotechnology Co., Ltd. The sequences of primers human TLR4, NF-κB and GAPDH used in this study were TLR4 (forward, AGACCTGTCCCTGAACCCTAT, and reverse CGATGGACTTCTAAACCAGCCA), GAPDH (forward, GCACCGTCAAGGCTGAGAAC and reverse, TGGTGAAGACGCCAGTGGA), and NF-κB (forward, ATGTGGAGATCATTGAGCAGC, and reverse CCTGGTCCTGTGTAGCCATT) respectively. Target mRNA was quantified by using 7500 Real-time fluorescence quantitative PCR Detection System (ABI company, USA). The reactions were performed in a total volume of 20µL with 2µL of cDNA used as atemplate and 10µM of forward and reverse primers. The target sequence and GAPDH were amplified and this procedure was conducted in triplicate. The following thermal cycling conditions were used: 30s at 95 °C; 40 cycles of 95 °C for 5seconds; 40 cycles of 65 °C for 30seconds. The results are expressed as relative expression ratio.

Western Blot Analysis

The TLR4, MyD88, NF-κB protein levels in lysed cells were examined by Western blot analysis. Protein concentrations were determined by using BCA Protein Assay Kit (Beyotime Biotechnology, Haimen, Jiangsu, China). Total protein (20 µg) was fractionated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 10% milk solution at room temperature for 2h and incubated overnight at 4°C with the primary antibodies against TLR4 (1:500), MyD88 (1:700), NF-κB (1:700) and GAPDH (1:10000). After three washes, membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG antibody for 1.5 h at room temperature. After four washes, the protein was visualized with enhanced chemilumin essence kit (Sigma, USA). The density values of bands were quantified by densitometric analysis of scanned images (Scion Image 4.03). The relative protein ratio was calculated by determining the integrated intensity of the bands of each treated group as a ratio of the control condition.

Calculations and Statistical Analysis

Data were expressed as means ± SEM. Statistical analysis was performed using statistical software Sigma Stat 3.5. Graphs were generated using software Sigma plot 10. Comparisons were made using one-way ANOVA analysis. Skanlt Software for Multiskan GO was used to calculate the value of Real-time fluorescence PCR. $p < 0.05$ was considered statistically significant.

Results

ISOF does not decrease cell viability

BEAS-2B cells were incubated with ISOF (2.5–50

µM) for 6, 12, or 24h. Survival of cells was greater than 95% in all treatment groups (Figure 1). Moreover, BEAS-2B cells incubated with LPS (2 µg/mL) and ISOF (1-3 µM) for 6h showed no decrease in cell viability (Figure 2).
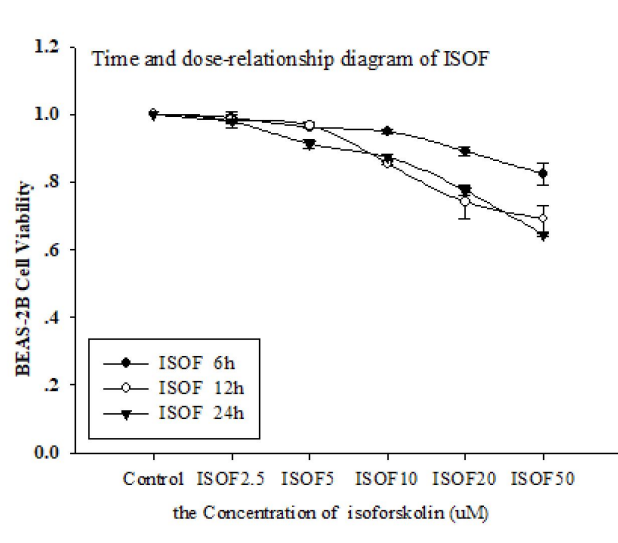

Figure 1: Time and concentration-dependent effects of ISOF on BEAS-2B cell viability; BEAS-2B cells were treated with ISOF (2.5, 5.0, 10, 20 and 50 µM) for 6, 12 and 24 hours respectively. Cell viability was determined via MTT assay and is expressed as the ratio of the cell viability of control. One-way ANOVA on Rank followed by SNK test. Data are means ± SEM; n = 4 independent experiments with independent culture.
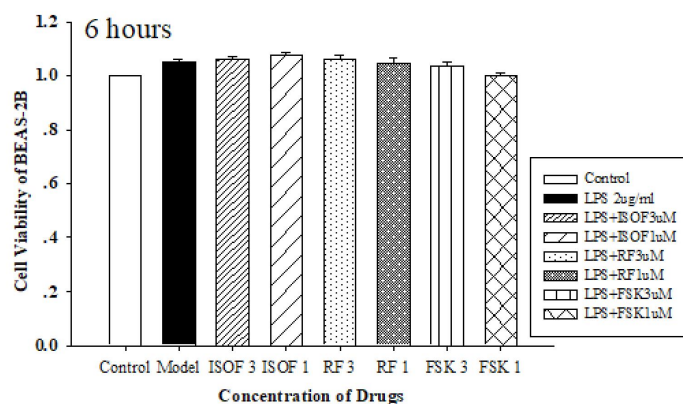

Figure 2: Effects of ISOF and LPS on BEAS-2B cell viability; the ratio of cell viability compared with control in normal BEAS-2B with ISOF and positive control drugs, RF and FSK (1 and 3µM), pretreatment for 0.5 hours prior to LPS. One-way ANOVA on Rank followed by SNK test. Data are means ± SEM; n = 4 independent experiments with independent culture.

ISOF attenuates effects of LPS on mRNA levels of TLR4 and NF-κB in BEAS-2B cells

Levels of NF-κB and TLR4 mRNA in BEAS-2B were elevated after treatment with LPS. Pretreatment with ISOF significantly reduced mRNA levels of NF-κB and TLR4 of BEAS-2B. These results are consistent with our positive control drugs- FSK (1.0 μM), RF (1.0 μM), DEX (50 μM) (Figures 3,4).
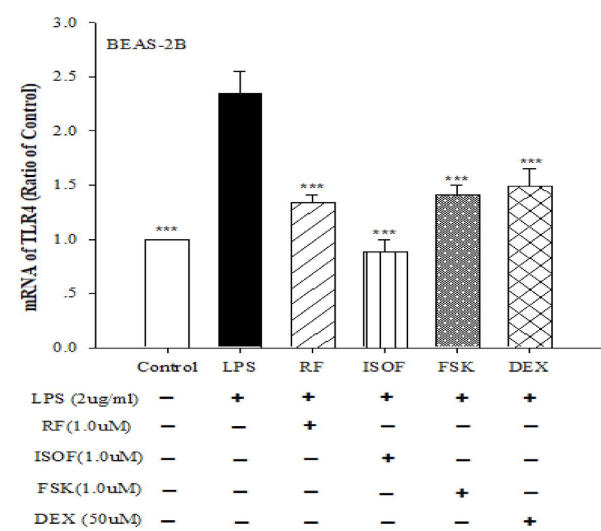

Figure 3: Effect of ISOF on mRNA expression of TLR4 in BEAS-2B. Ratio of control was calculated by determining cycle time. Control group, BEAS-2B was only given solvent (DMSO volume concentration was 1/1000) without LPS. BEAS-2B cells were pretreated with DMSO, ISOF (1.0μM), FSK (1.0μM), RF (1.0μM), DEX (50μM) respectively for 0.5 h. Each group was then treated with LPS (2μg/ml) for 6h.One-way ANOVA on Rank followed by SNK test, *P <0.05, **P< 0.01, compared to control. Data are presented as means ± SEM; n= 5 independent experiments with independent culture.

Figure 4: Effect of ISOF on mRNA expression of NF-κB in BEAS-2B. Ratio of control was calculated by determining cycle time. Control group, BEAS-2B was only given solvent (DMSO volume concentration was 1/1000) without LPS. BEAS-2B cells were pretreated with DMSO, ISOF (1.0μM), FSK (1.0μM), RF (1.0μM), DEX (50μM) respectively for 0.5 h. Each group was then treated with LPS (2μg/ml) for 6h.One-way ANOVA on Rank followed by SNK test, *P < 0.05, **P < 0.01, compared to control. Data are presented as means ± SEM; n= 5 independent experiments with independent culture.
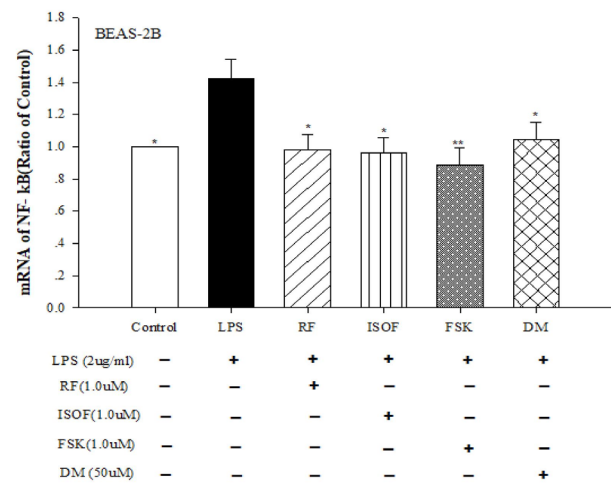
ISOF attenuates the effects of LPS on protein levels of TLR4, MyD88 and NF-κB in BEAS-2B cells

As shown in (Figures 5-7), protein levels of NF-κB, TLR4 and MyD88 in BEAS-2B cells were elevated after treatment with LPS. Pretreatment with ISOF prior to LPS significantly reduced protein levels of NF-κB, TLR4, MyD88 BEAS-2B. These results were consistent with our positive control drugs-FSK (1.0 μM), RF (1.0 μM), DEX (50 μM) .In the present study, our results showed that the LPS treatment increased the protein levels of TLR4, MyD88 and NF-κB in BEAS-2B cells. This result was similar to our previous research that LPS treatment increased the protein levels of TLR4, MyD88 and NF-κB in macrophages and ALI model of animal and further demonstrated that LPS signaled via TLR4 and MyD88 activated NF-κB in an inflammatory reaction. In addition, our research illustrated protein levels of NF-κB could be apparently raised in BEAS-2B stimulated by LPS.
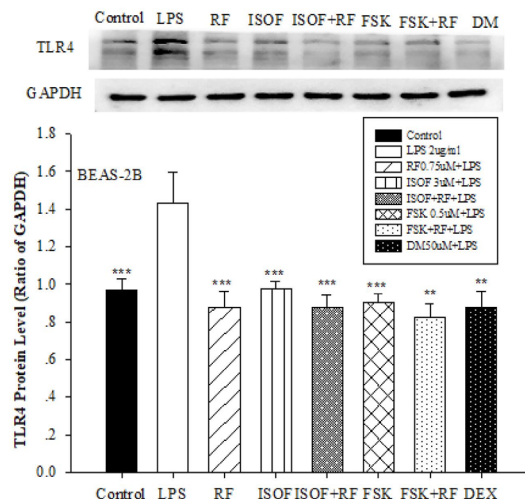

Figure 5: Effect of ISOF on protein level of TLR4 in BEAS-2B cells. Representative immunoblot and quantification of TLR4 protein expression in BEAS-2B. Cells treated with ISOF and other control drugs. Protein ratio of control was calculated by determining band intensity as a ratio of control. Control group, BEAS-2B cells were only given solvent (DMSO volume concentration was 1/1000) without LPS. BEAS-2B cells were pretreated with DMSO, ISOF (1.0μM), FSK (1.0μM), RF (1.0μM), DEX (50μM) respectively for 0.5h. Each group was then treated with LPS (2μg/ml) for 6h. One-way ANOVA on Rank followed by SNK test, *P< 0.05, **P< 0.01, compared to control. Data are presented as means ± SEM; n = 5 independent experiments with independent culture.
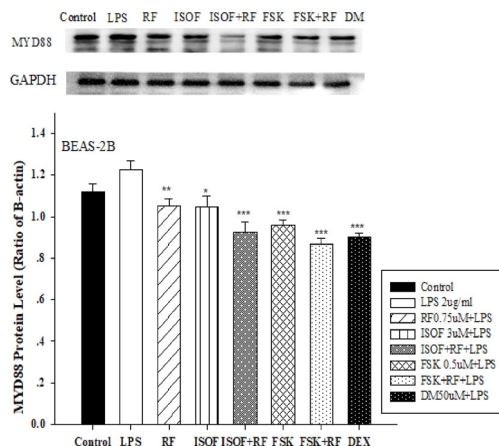

Figure 6: Effect of ISOF on protein level of MyD88 in BEAS-2B cells. Representative immunoblot and quantification of MyD88 protein expression in BEAS-2B. Cells treated with ISOF and other control drugs. Protein ratio of control was calculated by determining band intensity as a ratio of control. Control group, BEAS-2B cells were only given solvent (DMSO volume concentration was 1/1000) without LPS.BEAS-2B cells were pretreated with DMSO, ISOF (1.0μM), FSK (1.0μM), RF (1.0μM), DEX (50μM) respectively for 0.5h. Each group was then treated with LPS (2μg/ml) for 6h. One-way ANOVA on Rank followed by SNK test, *P< 0.05, **P< 0.01, compared to control. Data are presented as means ± SEM; n = 5 independent experiments with independent culture.
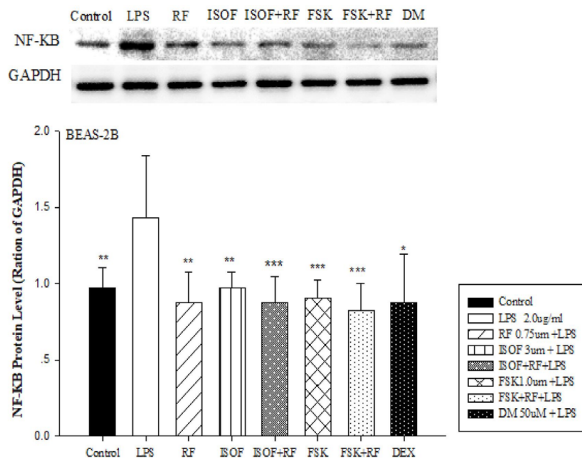
Figure 7: Effect of ISOF on protein level of NF-κB in BEAS-2B cells. Representative immunoblot and quantification of NF-κB protein expression in BEAS-2B. Cells treated with ISOF and other control drugs. Protein ratio of control was calculated by determining band intensity as a ratio of control. Control group, BEAS-2B cells were only given solvent (DMSO volume concentration was 1/1000) without LPS. BEAS-2B cells were pretreated with DMSO, ISOF (1.0μM), FSK (1.0μM), RF (1.0μM), DEX (50μM) respectively for 0.5h. Each group was then treated with LPS (2μg/ml) for 6h. One-way ANOVA on Rank followed by SNK test, *P< 0.05, **P < 0.01, compared to control. Data are presented as means ± SEM; n = 5 independent experiments with independent culture.

Discussion

ISOF was isolated from *Coleus forskohlii* native to Yunnan in China. The present study expands the knowledge regarding the role and biological effect of ISOF in inflammation induced by LPS. Our results showed that ISOF was able to attenuate the effects of LPS on protein levels of TLR4, MyD88 and NF-κB in BEAS-2B. Moreover, ISOF could reduce the mRNA levels of TLR4 and NF-κB in BEAS-2B induced by LPS. Our results indicate that ISOF inhibits the TLR4/MyD-88/NF-κB pathway, which activated by LPS. These results are similar with our another research result, which ISOF has anti-inflammation effect in human peripheral blood mononuclear leukocytes induced by LPS through down regulation protein levels of TLR4, MyD88, NF-kB, TNF-α and IL-1β [20].

As the receptor of LPS, TLR4 plays a principal role in the recognition of Gram- negative bacteria. MyD88 is an adaptor molecule that responds to the interaction of TLR4 with LPS [21]. The degree of NF-κB activation was reported to increase in patients with sepsis or acute lung injury [22,23], and the nuclear accumulation of NF-κB p65 was observed in alveolar macrophages from patients with acute lung injury caused by severe infection, in contrast to the alveolar macrophages from control patients [22,24]. The increased nuclear levels of NF-κB p65 were also confirmed in ML from patients with sepsis [23,25]. In addition, it appears that acute lung injury patients who do not survive exert greater and more prolonged NF-κB activation compared to patients who do survive [23,25]. These results suggest that NF-κB activation is central to the development of pulmonary inflammation and acute lung injury. Our work shows that ISOF could counteract the effect of LPS on increasing protein levels of NF-κB p65 in BEAS-2B cells.

Acute Lung injury and Acute Respiratory Distress Syndrome (ALI/ARDS) are serious clinical disorders of the lung. The mortality rates resulting from ALI/ARDS in Intensive Care Units (ICU) remain high at 30% to 40% [26]. The pathogenesis of ALI/ARDS is complex and involves multiple signal transduction processes and multiple cells [27]. The most common pathological condition of ARDS is sepsis. LPS released during sepsis is the major stimulus for the release of inflammatory mediators [28]. Administration of LPS to experimental cells causes the pathological condition of ongoing sepsis and concomitant ALI/ARDS-like systemic inflammatory reaction, hence we selected LPS to stimulate BEAS-2B which are involved in the occurrence and development of ALI / ARDS induced by sepsis [29-31].

Conclusion

In summary, the results of this study revealed that ISOF inhibited pathway of TLR4/ MyD-88/NF-κB, which activated by LPS. These findings indicate that ISOF could be a potential candidate for the treatment of acute lung injury.

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