Original Article

Protective effects of luteolin against lipopolysaccharide-induced acute lung injury involves inhibition of MEK/ERK and PI3K/Akt pathways in neutrophils

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Aim: To investigate whether luteolin, the major polyphenolic components of Lonicera japonica, has beneficial effects against lipopolysaccharide (LPS)-induced acute lung injury (ALI) and to determine whether the protective mechanism involves anti-inflammatory effects on neutrophils.

Methods: ALI was induced with intratracheal instillation of LPS in mice. The level of ALI was determined by measuring the cell count and protein content in bronchoalveolar lavage (BAL) fluid. Neutrophils were stimulated with formyl-Met-Leu-Phe (fMLP) or LPS in vitro. Chemotaxis and superoxide anion generation were measured to evaluate neutrophil activation. The potential involvement of intracellular signaling molecules in regulating neutrophil activation was analyzed by using Western blot.

Results: LPS induced ALI in mice, as evidenced with leukocyte infiltration and protein leakage into the lungs. Luteolin attenuated LPS-induced leukocyte infiltration and protein extravasation. In cell studies, luteolin attenuated the fMLP-induced neutrophil chemotaxis and respiratory burst (IC50 0.2±0.1 µmol/L and 2.2±0.8 µmol/L, respectively), but had a negligible effect on superoxide anion generation during phorbol myristate acetate stimulation. Furthermore luteolin effectively blocked MAPK/ERK kinase 1/2 (MEK), extracellular signal-regulated kinase (ERK), and Akt phosphorylation in fMLP- and LPS-stimulated neutrophils.

Conclusion: These results indicate that luteolin has beneficial effects against LPS-induced ALI in mice, and the attenuation of neutrophil chemotaxis and respiratory burst by luteolin involves the blockade of MEK, ERK-, and Akt-related signaling cascades.

Keywords: acute lung injury; chemotaxis; luteolin; mitogen activated protein kinase; neutrophils; respiratory burst; PI3K/Akt

Introduction

ALI is a cause of acute respiratory failure that develops in patients from a variety of clinical disorders, including sepsis, pneumonia, aspiration of gastric and oropharyngeal contents, and major trauma. It is characterized by refractory hypoxemia, acute respiratory distress, and bilateral pulmonary infiltrates, and it is often associated with multiple organ failure in the later stage[1]. Clinically, ALI is a common complication of infection by Gram negative bacteria and an important cause of morbidity and mortality among humans[2]. LPS, an outer membrane component of Gram negative bacteria, can cause serious multiple organ dysfunctions, particularly within the respiratory system[3]. Therefore, LPS might be a determinant pathogenic molecule in the initiation and/or propagation of certain cases of ALI.

The histological changes of pulmonary microvascular permeability and neutrophil inflammatory responses are hallmarks of the early stage of ALI[4]. Accumulating evidence suggests that neutrophils play a critical role in ALI[4,5]. In general, neutrophils are the first line players of the
host defense system. Through chemotaxis, degranulation, respiratory burst, and phagocytosis, neutrophils migrate from the peripheral blood to the injured sites and ingest invasive pathogens. However, excessive neutrophilic activation releases toxic ROS, cytokines, and proteolytic enzymes, leading to tissue destruction[8-10]. The released products from activated neutrophils disrupt alveolar-capillary integrity, amplify inflammatory reactions, damage pulmonary cells, and contribute to lung injury[9]. Due to their mediating role, neutrophils are an important target for therapeutic intervention against ALI. Thus, the inhibition of excessive neutrophil activation by blocking chemotaxis and toxic mediator release may present a useful strategy for the treatment of various inflammatory diseases including ALI.

In Chinese medicine, honeysuckle (Lonicera japonica) has been widely used for the treatment of upper respiratory tract infections. The beneficial effects of Lonicera japonica are attributable to its anti-inflammatory properties[10]. Among the bioactive components of Lonicera japonica, luteolin (3',4',5,7-tetrahydroxyflavone) is an active polyphenolic compound possessing anti-inflammatory activity and is widely distributed in the plant kingdom[11, 12]. Recent studies have demonstrated beneficial actions of luteolin through its anti-inflammatory activity. The research of modern scientists has shed new light on honeysuckle in the treatment of SARS and H1N1 influenza[13-15]. Luteolin protects mice against LPS-induced toxicity, alleviates bronchoconstriction and airway hyperreactivity in ovalbumin-sensitized mice, and decreases Chlamydia pneumoniae infection-induced inflammatory reactions[16-18]. In vitro, luteolin inhibits NO and pro-inflammatory cytokine expression in primary bone marrow-derived macrophages, gingival fibroblasts, alveolar macrophages, mast cells, and RAW 264.7 cells[19-22]. Studies also show that luteolin has a modulatory effect on neutrophils[16, 23, 24]. These observations imply that luteolin may be useful for mitigating inflammation. Given that ALI is characterized by a diffuse inflammatory process, including neutrophil accumulation/activation, increased vascular permeability, and parenchymal injury, the present study was designed to determine whether luteolin could ameliorate ALI induced by LPS in mice in an attempt to clarify the underlying molecular basis with a focus on neutrophil activation.

Materials and methods

Materials
HBSS and calcein-AM were obtained from Invitrogen. Mouse polyclonal antibodies against Akt, p38 MAPK, ERK, and MEK were obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against phospho-MEK, ERK, p38 MAPK, and Akt were purchased from Cell Signaling Technology. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Liu’s stain kit was purchased from Tonyar Biotechnology (Taiwan). The FluoroBlok™ Insert System was obtained from BD Biosciences. Other chemicals were purchased from Sigma-Aldrich. The final volume of DMSO in the reaction mixture was <0.5%.

Murine model of ALI
Male ICR mice (20–25 g) were obtained from BioLASCO (Taiwan). To determine the preventive effect of luteolin on LPS-induced ALI, mice were divided into five groups (n=6/group). Various doses of luteolin (18, 35, and 70 µmol/kg) or normal saline were intraperitoneally administered 0.5 h before a single intratracheal injection of LPS (100 µg/50 µL)[25]. To determine the therapeutic effect of luteolin on LPS-induced ALI, mice were divided into three groups (n=6/group). Luteolin (70 µmol/kg) or normal saline was intraperitoneally administered 5 min after intratracheal LPS administration. Animals that received normal saline (50 µL) pretreatment and challenge were referred to as the control group. Six hours after saline or LPS administration, mice were anesthetized with sodium pentobarbital (60 mg/kg). The lungs were lavaged in situ via tracheal cannula with five 1-mL aliquots of sterile saline, and the BAL was centrifuged. Total white blood cells and protein content were determined by counting the cells and using Bio-Rad protein assay reagents in the pellet and supernatant, respectively. All experiments were approved by the Institutional Animal Ethics Committee and conducted in accordance with the principles and guidelines of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isolation of neutrophils
The blood of Sprague-Dawley rats was collected from the abdominal aorta. Neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Paque, and hypotonic lysis of erythrocytes[26]. Purified neutrophils, containing >95% viable cells, were resuspended in HBSS containing 10 mmol/L HEPES, pH 7.4, and 4 mmol/L NaHCO₃ and kept in an ice bath before use.

Chemotaxis assay
The chemotaxis assay was performed using FluoroBlok Insert Systems according to the manufacturer’s instructions[25]. Briefly, neutrophils (1×10⁶ cells/mL) were incubated with 5 µmol/L calcine-AM for 15 min at 37 °C. After being washed, the cells were resuspended in HBSS containing 1% bovine serum albumin. Calcine-labeled cells were placed in FluoroBlok Inserts in the presence or absence of 0.1 µmol/L fMLP. Chemotaxis was measured by detecting the fluorescence of cells that had migrated through the pores (3 µm) to the lower chamber. Fluorescence changes, termed RFUs, were monitored at 535 nm with excitation at 488 nm using a fluorescence microplate reader.

Measurement of superoxide anion generation
The generation of superoxide anion in neutrophils was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome c[25]. Briefly, the assay mixture contained a neutrophil suspension (2×10⁶ cells) and 40 µmol/L of ferricytochrome c in a final volume of 1.5 mL. Absorbance changes in the reduction of ferricytochrome c were monitored at 550 nm using a microplate reader.
Western blot analysis of protein phosphorylation

Neutrophils (2×10^7 cells/mL) were incubated with different concentrations of luteolin for 10 min before stimulation. The reactions were terminated with a 5 fold volume of cold HBSS. Cellular proteins were extracted with Laemmli sample buffer[25]. Proteins (60 µg per lane) were resolved by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dried milk and probed with anti-phospho-p38 MAPK, anti-phospho-Akt, anti-phospho-ERK, or anti-phospho-MEK antibodies. The blots were then stripped and reprobed with anti-p38 MAPK, anti-ERK, anti-Akt or anti-MEK antibodies to standardize protein loading. The membranes were then incubated with horseradish-peroxidase-conjugated secondary antibody. Detection was performed with the enhanced chemiluminescence reagent. The intensities of the protein bands were determined with a computer image analysis system (Multi Gauge; Fujifilm Corporation).

Statistical analysis

Statistical analyses were performed using ANOVA followed by the Bonferroni t test for multigroup comparisons; P<0.05 was considered significant for all tests. The curve estimation regression analysis with a logarithmic model (Excel) was used to calculate IC_{50} values.

Results

The beneficial effect of luteolin on LPS-induced ALI in mice

To demonstrate an effect of luteolin on ALI, a LPS-induced ALI mouse model was used. The central features of the pathogenesis of ALI are enhancement of vascular leakage and leukocyte infiltration in the lung[26–29]. Intratracheal administration of LPS induced an increase in pulmonary permeability. An increase in leukocyte infiltration (Figure 1A, P<0.01) and protein leakage (Figure 1B, P<0.01) in the lung were also detected in LPS-treated mice. The LPS-induced leukocyte infiltration and protein leakage were attenuated by pretreatment with luteolin in a concentration-dependent manner, and statistically significant differences were observed starting at a dose of 35 µmol/kg (Figure 1). Since luteolin shows preventive effects against LPS-induced ALI in mice, we wondered whether luteolin possesses a therapeutic effect. We found that posttreatment with luteolin also attenuated LPS-induced leukocyte infiltration (Figure 2A) and protein leakage (Figure 2B) in the lung. These results suggest that luteolin treatment can protect against LPS-induced ALI in mice, likely through the attenuation of LPS-induced vascular permeability changes and the reduction of leukocyte infiltration in the lung.

Effects of luteolin on neutrophil chemotaxis and the respiratory burst

The rapid accumulation and activation of neutrophils in the lung is a recognizable event and plays a critical role in the pathogenesis of ALI[30]. To obtain further insight into the protective effects of luteolin against ALI, a mechanistic study was conducted using a cultured neutrophil model. fMLP caused an increase in neutrophil chemotaxis in vitro (P<0.01). This response was attenuated by luteolin in a concentration-dependent manner with an IC_{50} value of 0.2±0.1 µmol/L (Figure 3A). During the assay, cell viability was >95% in the treatment with testing different concentrations of luteolin (assessed by lactate dehydrogenase efflux, data not shown).
ROS released from activated neutrophils play an important role in the initiation/propagation of microvascular permeability change and inflammation in LPS-induced ALI[30]. Stimulation of neutrophils with 1 μmol/L fMLP and 10 nmol/L PMA evoked superoxide anion generation. Luteolin had an inhibitory effect against fMLP-induced superoxide anion generation, with an IC$_{50}$ value of 2.2±0.8 μmol/L. However, no effect on PMA-induced superoxide anion generation was observed (Figure 3B). The results showed that luteolin was capable of inhibiting chemoattractant-induced neutrophil chemotaxis and the respiratory burst.

Effects of luteolin on fMLP-stimulated p38 MAPK phosphorylation
p38 MAPK plays an important role in LPS-induced ALI[31] and in fMLP-induced neutrophil chemotaxis and the respiratory burst[32, 33]. To examine the effect of luteolin on p38 MAPK, the phosphorylation of p38 MAPK in fMLP-stimulated neutrophils was analyzed by Western blotting. fMLP stimulation induced an increase in p38 MAPK phosphorylation (1.9±0.2 fold, P<0.01). The inhibitory effect of luteolin on fMLP-increased p38 MAPK phosphorylation was observed only at concentrations of luteolin up to 30 μmol/L (61% inhibition) (Figure 4). The IC$_{50}$ values were 100- and 10-fold higher than those required for chemotaxis and for the respiratory burst, respectively.

Effects of luteolin on fMLP-stimulated ERK pathway
The ERK pathway participates in chemoattractant-induced neutrophil chemotaxis and the respiratory burst[32, 33] and in LPS-induced ALI[31]. Stimulation of neutrophils with fMLP resulted in an increase in the phosphorylation of ERK (P<0.01). Luteolin reduced the fMLP-induced ERK phosphorylation in a concentration-dependent manner, with an IC$_{50}$ value of 5.4±2.2 μmol/L (Figure 5). Activation of ERK is mediated through its phosphorylation by MEK in response to various stimuli[34]. Therefore, we next examined the effect of luteolin on the phosphorylation of MEK. As shown in Figure 6, MEK phosphorylation was weak in vehicle-treated cells, whereas a visible band was detected in response to fMLP stimulation (P<0.01). Luteolin decreased the fMLP-induced MEK phosphorylation in a concentration-dependent manner, with an IC$_{50}$ value of 0.1±0.1 μmol/L. These results suggest that the MEK- and ERK-related pathways play key roles in luteolin-mediated attenuation of fMLP-induced neutrophilic responses.

Effects of luteolin on fMLP-stimulated Akt phosphorylation
Protein kinase B/Akt has been implicated in LPS-induced ALI[9] and plays a critical role in neutrophil chemotaxis and the
respiratory burst\[^{[39]}\]. fMLP stimulation of neutrophils led to an increase in Akt phosphorylation \((P<0.01)\). Luteolin attenuated the Akt phosphorylation in a concentration-dependent manner, with an IC\(_{50}\) value of 1.8±1.0 µmol/L (Figure 7). These results suggest that the Akt-related pathways might actively participate in luteolin-mediated attenuation of fMLP-induced neutrophilic responses.

**Effects of luteolin on LPS-stimulated MEK, ERK, and Akt phosphorylation**

In parallel with LPS-induced injury, the effects of luteolin were also investigated in LPS-stimulated neutrophils. Like fMLP, LPS stimulation induced elevated phosphorylation of MEK \((4.0±0.7 \text{ fold}, P<0.01)\), ERK \((7.1±1.5 \text{ fold}, P<0.01)\), and Akt \((3.5±1.0 \text{ fold}, P<0.01)\). The increased phosphorylation was significantly attenuated by luteolin (Figure 8).

**Discussion**

An essential feature of experimental ALI is the presence of profound vascular leakage with movement of fluid and macromolecules into the interstitium and air space, events that directly contribute to the severe physiological derangements characteristic of this disorder. The impairment of pulmonary vascular barrier function enhances the transendothelial diapedesis of leukocytes into lung parenchymal tissues, further contributing to vascular and alveolar dysfunction in ALI\[^{[9]}\]. In the present study, we found that pretreatment or posttreatment with luteolin inhibited the leukocyte influx and pulmonary permeability of LPS-induced ALI in mice, as evidenced by the decrease in leukocytes and protein content in the BAL fluid. This animal study suggests that luteolin has a beneficial effect against experimental ALI induced by LPS. Furthermore, the attenuation of peripheral leukocyte infiltration indicates the potential involvement of an immunosuppressive mechanism in luteolin-mediated
neutrophil infiltration and activation.

NADPH oxidase contains several subunits that include both membrane-bound cytochrome b\textsubscript{558} (p22\textsuperscript{phox} and gp91\textsuperscript{phox}) and cytosolic subunits (p47\textsuperscript{phox}, p40\textsuperscript{phox}, p67\textsuperscript{phox}). When appropriately stimulated, cytosolic subunits of NADPH oxidase translocate to cellular membranes, interact with activated small GTPase Rac2, and assemble into an active complex. NADPH oxidase is responsible for the abrupt consumption of oxygen, termed the respiratory burst in neutrophils, yielding superoxide anion, which is subsequently transformed into other ROS\textsuperscript{[27, 38]}. Interestingly, luteolin attenuated the fMLP-induced respiratory burst in neutrophils but had a negligible effect on PMA-mediated stimulation. Unlike fMLP, which triggers G protein-mediated signaling, PMA is a membrane-permeable protein kinase C activator\textsuperscript{[39]}. The interference of initial stimulation or intracellular signal transduction steps downstream of receptor activation is possible. Currently, the discrepancy between fMLP- and PMA-mediated responses has not been resolved.

Signals emanating from different cues converge on signaling molecules such as MAPK and Akt through the modulation of protein phosphorylation events, which in turn lead to the phosphorylation and activation of various downstream effectors and transcription factors. In general, fMLP stimulates a complex series of signal transduction events that culminate in the activation of neutrophils, in which MAPK cascades and PI3K/Akt pathways are essential for the respiratory burst and chemotaxis\textsuperscript{[32, 33, 38]}. Three major mammalian MAPK cascades have been identified, including ERK, p38 MAPK and JNK\textsuperscript{[38]}. Each MAPK cascade is activated by a distinct kinase cascade in which a MAPK-kinase kinase, also called MAPKKK or MEKKK, phosphorylates and activates a down-stream dual-specificity MAPK-kinase, also called MAPKK or MEK, which in turn stimulates MAPK activity through the dual phosphorylation of the Thr-Glu-Tyr motif within its activation loop. MEK is an upstream kinase of ERK\textsuperscript{[34]}. Several studies have shown that both the ERK pathway and p38 MAPK, but not JNK, are required for the fMLP-stimulated respiratory burst and chemotaxis\textsuperscript{[32, 33, 38]}. On the other hand, the PI3K/Akt pathway has been implicated in LPS-induced ALI\textsuperscript{[9]} and plays an important role in neutrophil chemotaxis and the respiratory burst\textsuperscript{[35]}. PI3Ks, which are lipid kinases, phosphorylate the 3-hydroxyl position of the inositol ring of phosphoinositides to generate phosphatidylinositol-3,4,5-trisphosphate\textsuperscript{[43]}. Akt, which functions downstream of PI3K, is activated by phosphatidylinositol-3,4,5-trisphosphate through serine and threonine phosphorylation\textsuperscript{[44, 45]}. The finding that a higher concentration of luteolin was required to inhibit the phosphorylation of p38 MAPK primarily precluded the involvement of p38 MAPK signaling. MEK, ERK, and Akt phosphorylation were attenuated by luteolin in fMLP-stimulated neutrophils in a parallel concentration-dependent manner, with IC\textsubscript{50} values similar to those observed in the suppression of the respiratory burst, suggesting that MEK/ERK- and Akt-related signaling play a pivotal role. In comparison with fMLP, LPS is not an effective chemoattractant.
for neutrophils but can trigger an inflammatory cascade\cite{46}. In the present study, we also found that luteolin attenuated LPS-induced MEK, ERK, and Akt phosphorylation in neutrophils. Recently, Schuh and Pahl\cite{47} used a specific inhibitor of MEK, U0126, to show that the ERK pathway plays an important role in LPS mediated pulmonary inflammation. These observations suggest that MEK/ERK and Akt could be critical targets for luteolin-mediated inhibitory effects in neutrophils.

In conclusion, luteolin has beneficial effects against LPS-induced ALI, as evidenced by an inhibition of leukocyte influx into the lung and lung permeability. Luteolin attenuates fMLP-induced neutrophil chemotaxis and the respiratory burst \textit{in vitro}. The inhibitory mechanism of luteolin seems to be multifactorial. It is reasonable to propose that the inhibition of MEK/ERK- and PI3K/Akt-related pathways by luteolin represents a critical mechanism to attenuate neutrophil activation. However, the initial interacting targets of luteolin and additional anti-inflammatory mechanisms require further investigation.

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Author contribution

Jen-pei LEE performed the research. Yi-ching LI performed the research and wrote part of the paper. Hung-yi CHEN performed part of the research. Ruey-hseng LIN analyzed the data and preparations. Shiang-suo HUANG performed part of the research. Hui-ling CHEN did part of the preparations. Pai-chuan KUAN wrote part of the introduction. Mao-fang LIAO wrote part of the discussion. Chun-jung CHEN designed the research and wrote the paper. Yu-hsiang KUAN designed the research, performed part of the research, and wrote the paper.

Abbreviations

ALL, acute lung injury; BAL, bronchoalveolar lavage; ERK, extracellular signal regulated kinase 1/2; fMLP, formyl-Met-Leu-Phe; HBSS, Hanks’ balanced salt solution; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase 1/2; NADPH, nicotinamide adenine dinucleotide phosphate; PI3K, phosphoinositide 3-kinase; PMA, phorbol myristate acetate; RFUs, relative fluorescence units; ROS, reactive oxygen species.

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Acta Pharmacologica Sinica

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