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Original Article

Anti-inflammation effect of epicatechin-gallate against lipopolysaccharide-induced acute lung injury via inhibition JAK/STAT1 pathway

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Abstract: Context: signal transducers and activators of transcriptions 1 (STAT1) plays a critical role in the inflammatory process in acute lung injury (ALI). Objective: This study sought to investigate the effects of epicatechin gallate (ECG), one component of green tea catechins, on mice with lipopolysaccharide (LPS)-induced ALI. Materials and methods: Mice were randomly allocated into three groups: saline (control group; n = 20), LPS-treated (LPS group; n = 20) and LPS plus ECG-treated (ECG group; n = 20). Lung wet/dry ratio, cellular and protein content in bronchoalveolar lavage fluid (BALF), myeloperoxidase (MPO) activity, and histo- pathologic damage in the lung were assessed. The levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α as well as total and phosphorylated protein levels of STAT1, JAK1, and JAK2 were also evaluated in vivo and vitro (RAW 264.7 cells). Results: The results showed that ECG alleviated pulmonary edema, attenuated neutrophil infiltration, reduced histopathologic damage in the lungs, and significantly inhibited release of pro-inflammatory cytokines IL-6 and TNF-α in the mice with LPS-induced ALI ($P < 0.01$). Moreover, administration of ECG significantly decreased total and phosphorylated levels of STAT1 in the lungs in vivo and in vitro ($P < 0.01$), and total ($P < 0.05$) and phosphorylated ($P < 0.01$) levels of JAK1 and JAK2 in vitro. Conclusion: These results indicated that ECG could alleviate pulmonary edema, ameliorate neutrophil infiltration, mitigate histopathologic changes, and attenuate inflammatory responses in the lungs of mice with LPS-induced ALI by inhibiting the JAK/STAT1 signal pathway.

Keywords: Epicatechin gallate (ECG), acute lung injury, lipopolysaccharide, pro-inflammatory cytokines, JAK/STAT signal pathway

Introduction

Acute lung injury (ALI) is characterized by acute respiratory distress, hypoxemia, and non-cardiogenic pulmonary edema. Although quantities of researches have been conducted on ALI and various standard therapies have been used, the mortality rate among ALI patients remains high. Clinically, gram-negative bacterial infection is the primary cause of ALI, and lipopolysaccharide (LPS), the major constituent of the outer membrane of gram-negative bacteria, is therefore considered an important pathological factor in ALI [1].

Understanding the mechanisms underlying LPS-induced inflammatory injury is essential for developing novel therapeutic strategies to treat ALI [2, 3]. Administration of LPS leads to acute and neutrophilic inflammation of the alveolar spaces of the lungs [4, 5] and induces production of numerous pro-inflam-matory cytokines [6, 7]. Inflammation plays a pivotal role in LPS-induced ALI, whereas JAK/STAT signal pathway promotes inflammation in ALI. Accumulating evidences suggests that JAK/STAT signal pathway is mediated by a variety of inflammatory cytokines and it plays an important role in lung injury and immune regulations [8-10]. STAT1 has been identified in various organs [11] as a transcription factor, which is the important part of the cell signal pathway JAK/STAT, and plays a critical role in inflammation reaction [12]. If STAT1 activation is time-spatially well regulated, the inflammatory response may proceed along its pathway with or without a minimum damage to the body. Moreover, considering STAT1 pathway a molecular target to develop new drugs may be helpful in

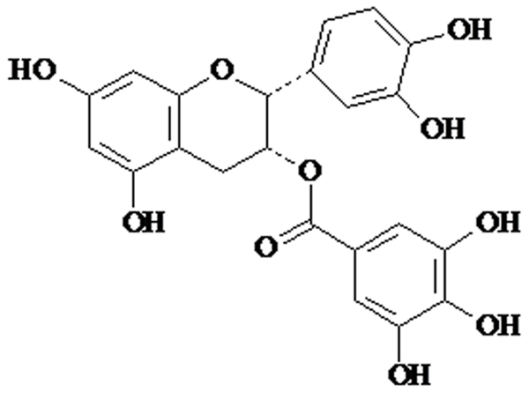

Figure 1. Structure formula of ECG.

inflammation correlated diseases with lower toxic side effects.

Catechins are polyphenols mainly present in green tea, comprising epigallocatechin gallate (EGCG), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), catechin gallate (CG), epicatechin gallate (ECG), gallocatechin gallate (GCG) and catechin (C) [13]. Catechins are considered to be strong antioxidants exerting protective effects against cancer and inflammatory diseases [14]. The best studied catechin is EGCG. Previous studies have demonstrated that EGCG had beneficial effects on inflammation in vitro and attenuated LPS-induced lung injury [15]. Besides, EGCG has been reported to reduce STAT1 phosphorylation in various human cells [16]. Different chemical structures of anti-STAT1 compounds have been used to confirm the presence of multiple points leading to inhibition of STAT1 activity, even if a similar chemical structure of epigallocatechin gallate (EGCE), myricetin and delphinidin, indicated these three substances recognized a possible common pathway [17]. Epicatechin gallate (ECG; **Figure 1**) has a similar chemical structure with EGCG, and thus may have similar effects on STAT1 activity. Accordingly, this study was designed to investigate effects of ECG on LPS-induced acute lung injury in mice and the underlying mechanisms of any effects.

Materials and methods

In vivo studies

Animals: Sixty male C57BL/6 mice (6-8 wk-of-age, 20-22 g) were obtained from Experimental Animal Center of Suzhou Aiermaite Technology Co. Ltd. (Suzhou, China). All mice were housed in a controlled pathogen-free environment maintained at 21 ± 2°C and with a relative humidity of 75-80% and a 12-hr light/dark cycle. All mice had ad libitum access to standard rodent chow and filtered tap water. All the experiments were performed in accordance with protocols and international guidelines for care and use of laboratory animals and were approved by the local experimental ethics committees.

After a 1-wk acclimatization period, mice were randomly allocated into three groups, i.e., a saline group (control; n = 20), an LPS-treated group (LPS; n = 20) and an LPS plus ECG-treated group (ECG; n = 20). In general, 10 mice of each group were used for Western blot analyses, and the remaining 10 for all other analyses. Mice in ECG group were to be injected with LPS (Escherichia coli, Type 055: B5; Sigma Chemical Company, St. Louis, USA) in the tail vein (at 25 mg/kg) 30 min before the intra-gastric administration of ECG (50 mg/kg, dissolved in normal saline; Sigma). Mice in LPS group were to receive only injected LPS (25 mg/kg); control mice received nothing but normal saline in place of LPS, in an equivalent injection volume. Both the LPS and control mice were also gavaged, but with vehicle only. Over the next 7 consecutive days, mice in ECG group were given ECG (50 mg/kg) once per day by gavage, while the other two groups received normal saline with equivalent volume instead. The gavage volume never exceeded 200 μL/mouse. At 24 hr following the final gavaging, mice were euthanized with a lethal injection of pentobarbital (150 mg/kg, intraperitoneal) and their lungs were immediately removed en bloc.

Lung wet/dry ratios: Wet/dry weight ratios of lungs were measured to evaluate the magnitude of pulmonary edema. Upper lobes of right lung of each mouse were weighed immediately after removal, then subjected to desiccation in an oven at 70°C until a stable dry weight was achieved (after 72 hr). Wet:dry weight ratios were then calculated.

BALF analysis: Total cellular counts of polymorphonuclear neutrophils (PMN) and proteins in the lungs were measured to evaluate the anti-inflammatory property of ECG. After removal from the chest, the left lung of each mouse was lavaged with ice-cold phosphate buffered saline (PBS, pH 7.4) five times (1 ml/wash). Fluid recovery was routinely ≈ 90%. The bronchoal-

veolar lavage fluids (BALF) were pooled and then centrifuged at 500 × g for 15 min at 4°C; resulting supernatant was collected and placed at -80°C for use in analyses of cytokines. Levels of TNF-α and IL-6 in BALF were measured with commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer instructions. The level of sensitivity of each kit was 3 pg TNF-α/ml and 3 pg IL-6/ml.

The remaining pelleted cells were re-suspended in 2 ml PBS. From this suspension, 1 m was used to perform cell counts (by hemocytometer) and the other 1 ml was used for measures of protein content using a Bradford assay.

Myeloperoxidase (MPO) analysis: After the BALF collection, the left upper lung lobe was homogenized in HEPES buffer (pH = 8.0) containing 0.5% cetyltrimethyl ammonium bromide (CTAB) using an IKA homogenizer (Staufen, Germany). The homogenate was then centrifuged (4°C, 13 000 ×g, 30 min) and the cell-free extracts in the supernatant were isolated and then stored at -20°C for later analysis. MPO activity was determined using a MPO detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer instruction. Protein content in the material was assessed using a Bradford assay. Samples were diluted in 50 mM phosphate citrate buffer (pH = 6.0) and the absorbance of the sample was measured at 460 nm using a SpectraMax Paradigm microplate reader (Molecular Devices, Sunnyvale, USA). All data were reported as U/mg lung tissue.

Histopathologic analysis: The right lower lung lobes of each mouse was fixed in 4% paraformaldehyde for 24 hr, then embedded in paraffin and cut to 5-μm sections. Hematoxylin-eosin (HE) staining of the sections was then performed using standard protocols. Thereafter, the sections were evaluated in a blinded manner using a LV150N microscope (Nikon, Tokyo, Japan).

In vitro studies

Cell culture and treatment: The mouse macrophage RAW 264.7 cell line was obtained from Shanghai Institute of Cells (Chinese Academy of Sciences, Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMTU) supplemented with heat-inactivated 10% FBS, penicillin (100 U/ml), and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2 at 37°C. All tissue culture reagents were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). For these experiments, the cells were plated in six-well plates (at 2 × 10^6 cells/well). At ≈ 90% confluence, cells were washed with cold PBS and then received serum-free DMEM for 2 hr before being treated with ECG (30 μM in PBS) and then 2 hr later with LPS (100 ng/ml) or LPS alone. Cells were then cultured at 37°C for 24 hr before well supernatants were collected for ELISA analyses of TNF-α and IL-6 (done as with BALF), and then the cells were harvested for analyses. All treatments were performed in triplicate.

Western blot

For these studies, the lungs of 10 mice/group were perfused with PBS (pH 7.4) to remove erythrocytes/white blood cells trapped in the vasculature, and then the tissue samples were collected. Total proteins from the tissues were extracted using a Total Protein Extraction Kit (Thermo Fisher Scientific, Massachusetts, USA), according to manufacturer protocols. In similar assays, the harvested RAW cells were also processed as above for use in Western blot analyses.

Protein levels in the suspensions were subsequently determined using a BCA protein assay kit (Thermo Fisher Scientific, Massachusetts, USA). Samples were then loaded onto (30 μg/well) and then resolved over a denaturing 8% SDS-polyacrylamide gel. The protein contents were then electro-transfered to a nitrocellulose membrane that, after blocking with Tris-buffered saline containing 0.1% Tween20 (TBST buffer) and 2% bovine serum albumin (BSA), was incubated overnight at 4°C (with rocking) with mouse and rabbit monoclonal antibodies against STAT1, p-STAT1, JAK1, JAK2, p-JAK1, p-JAK2, or β-actin. Specifically, anti-STAT1 (1:500; Cell Signaling Technology, Boston, MA), anti-p-STAT1-Tyr-701 (1:500; Cell Signaling), anti-JAK1 (1:1000; Epitomics Biotechnology Co., Burlingame, VT), anti-JAK2 (1:1000; Epitomics), anti-p-JAK1 (PY1022/1033, 1:1000; Epitomics), anti-p-JAK2 (PY1007/1008, 1:1000;

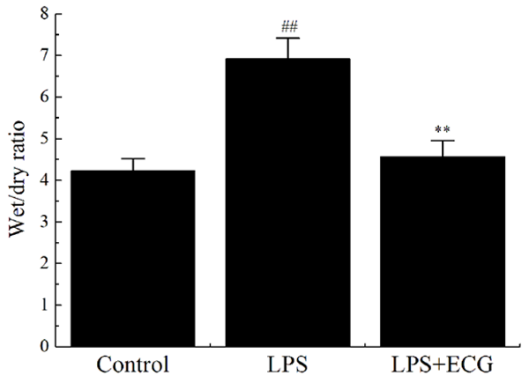

Figure 2. Effect of ECG on wet/dry ratio in mice with LPS-induced ALI. Data shown are means ± SD (n = 10/group). ##P < 0.01 vs. LPS only; **P < 0.01 vs. control.

Epitomics) or β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) were employed.

After washing with TBST buffer to remove unbound primary antibody, the membrane was then incubated for 90 min at room temperature with secondary antibody (anti-mouse or anti-rabbit, 1:5000 dilutions; Santa Cruz Biotechnology). The membrane was then washed three times for 5 min each. Quantification of the level of protein was done by using a Bio-Rad ChemiDoc XRS system (Bio-Rad, Hercules, USA) and Image J software (NIH, Bethesda, MD). Standards of each protein of interest were run in parallel (on the PAGE gel/on membrane) to allow for the simultaneous detection protocol to succeed.

Statistic analyses

All data were presented as mean ± SD. Statistic analyses between the groups were performed using a one-way analysis of variance (ANOVA) provided in the Statistical Analysis System software packet (v.9.0, SAS Institute Inc., NC, USA). If $P < 0.05$ in the ANOVA test, then post hoc test analysis was used to determine the difference between each two group. A *p*-value < 0.05 was considered significant.

Results

Effect of ECG on the pulmonary edema in mice with LPS-induced ALI

Lung wet/dry ratio was measured to evaluate the extent of pulmonary edema in the mice. As shown in **Figure 2**, LPS administration caused a significant increase in the ratio compared to that in control mice (from 4.23 [± 0.29] to now 6.92 [± 0.49]). Treatment with ECG significantly decreased the wet/dry ratio compared with the LPS group (to now 4.57 [± 0.37]; $P < 0.01$).

Effect of ECG on cellular counts and protein in BALF

Mice treated with LPS had a significant increase in total cells (**Figure 3A**) and total protein (**Figure 3B**) in their BALF compared with the control mice (from 7.23 [± 0.56] to 13.17 [± 0.91] × 10^4/mL and from 50.32 [± 5.52] to 2753.12 [± 167.66] μg/mL, respectively). Due to being treated with ECG, the total cells and protein values were respectively decreased to 10.09 [± 0.81] × 10^4/mL and 1895.3 [± 114.76] μg/mL ($P < 0.01$ compared with the LPS group).

Effect of ECG on MPO activity in lung

The activity of MPO, an index for neutrophil infiltration that plays a pivotal role in the progress of ALI, was significantly increased in lungs of LPS-treated mice compared with values in control hosts (**Figure 4**). ECG treatment efficiently suppressed the increase in MPO activity from 2.17 [± 0.11] U/mg lung tissues in the LPS group to 1.55 [± 0.08] ($P < 0.01$).

Effect of ECG on histopathologic changes in lung

Figure 5 depicts the histopathologic assessment of lung sections from the mice. In the control group, both clear pulmonary alveoli and intact structure were observed (**Figure 5A**). In LPS only group, histopathologic staining revealed pulmonary edema, infiltration of inflammatory cells in the tissues and alveoli, hemorrhage, bronchiole epithelia desquamation, and alveolar collapse (**Figure 5B**). Due to administration of ECG, the histopathologic changes in the lung tissues expected from the LPS were mitigated (**Figure 5C**).

Effect of ECG on TNF-α and IL-6 production in lungs and RAW 264.7 cells

Pro-inflammatory TNF-α and IL-6 in BALF and RAW 264.7 cultures were measured using ELISA. In the absence of ECG, LPS challenge resulted in a burst of TNF-α and IL-6 release in BALF and cell cultures (**Figure 6**). However, the administration of ECG significantly attenuated these increases. With TNF-α levels in BALF

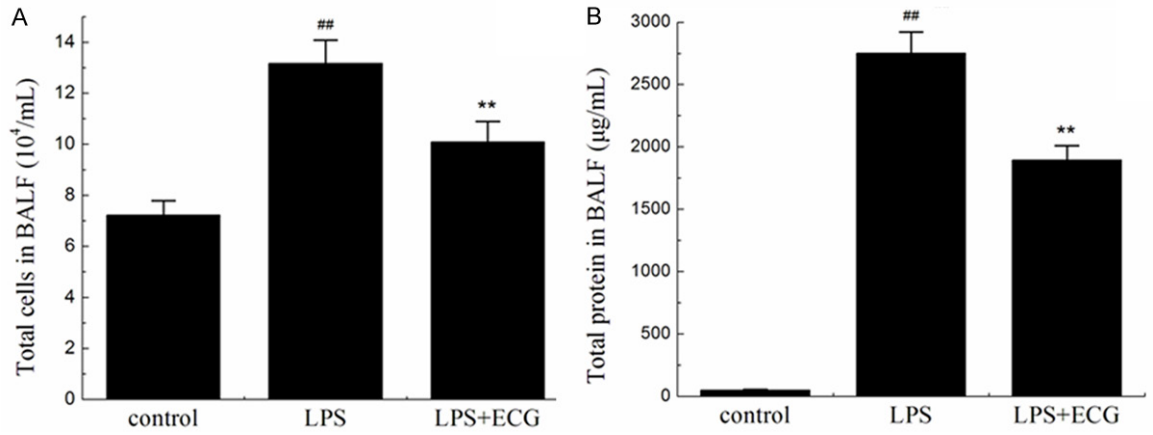

Figure 3. Effect of ECG on BALF parameters. (A) Total cellular counts and (B) total protein. Data shown are means ± SD (n = 10/group). ##P < 0.01 vs. LPS only; **P < 0.01 vs. control.

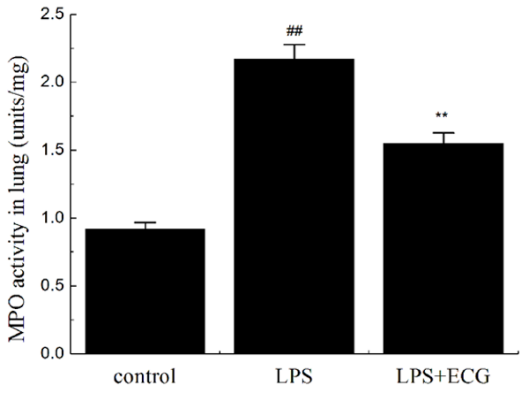

Figure 4. Effect of ECG on MPO activity. Data shown are means ± SD (n = 10/group). ##P < 0.01 vs. LPS only; **P < 0.01 vs. control.

were reduced to 161.82 [± 12.09] pg/ml from 220.94 [± 18.61] pg/ml for the LPS-only mice; with the RAW 264.7 cells, these levels were, respectively, 38.32 [± 2.64] and 65.07 [± 5.21] pg/ml (P < 0.01). With IL-6, levels in BALF were reduced to 189.05 [± 15.23] ng/ml from 257.13 [± 20.79] pg/ml in BALF; with the RAW 264.7 cells, the levels were, respectively, 69.81 [± 4.88] and 110.46 [± 7.84] pg/ml (P < 0.01). Control mouse BALF and untreated RAW 264.7 cell levels of the cytokines were below level of kit detection (1000 pg/ml).

Effect of ECG on JAK/STAT1 pathway

Levels of STAT1 and p-STAT1 in lung BALF (**Figure 7**) and RAW 264.7 cells (**Figure 8**), and the levels of JAK1, JAK2, p-JAK1 and p-JAK2 in the RAW 264.7 cells only (**Figure 9**) were evaluated. In the *in vivo* and *vitro* studies, the protein levels of STAT1 and p-STAT1 significantly increased in the LPS-only groups; such effects were efficiently inhibited by ECG administration (P < 0.01). In the in vitro study, LPS induced an increase in JAK1, JAK2, p-JAK1, and p-JAK2 levels in the RAW 264.7 cells; these changes were also significantly attenuated by use of ECG (JAK1 and JAK2, P < 0.05; p-JAK1 and p-JAK2, P < 0.01).

Discussion

Acute lung injury, characterized by pulmonary edema, disruption of endothelial and epithelial integrity, release of pro-inflammatory mediators and extensive neutrophil infiltration [17], is a common clinical problem associated with significant morbidity and mortality in shock, sepsis, ischemia reperfusion, etc. [18, 19]. This study demonstrated that ECG could alleviate pulmonary edema, attenuate neutrophil infiltration, and mitigate the lung histopathological changes, and also could regulate the inflammatory cytokines release in the lung of mice with LPS-induced ALI. Moreover, the administration of ECG decreased the total and the phosphorylated protein levels of STAT1 in lungs *in vivo* and *in vitro* and the phosphorylated protein level of JAK1 and JAK2 in vitro. These results could imply that ECG might be able to ameliorate LPS-induced acute lung injury in mice via inhibiting JAK/STAT1 pathway.

Pulmonary edema, a typical symptom of inflammation both in systemic inflammation and local inflammation, can cause reduction of lung compliance and deterioration of pulmonary gas exchange [20] LPS injury associated with micro-

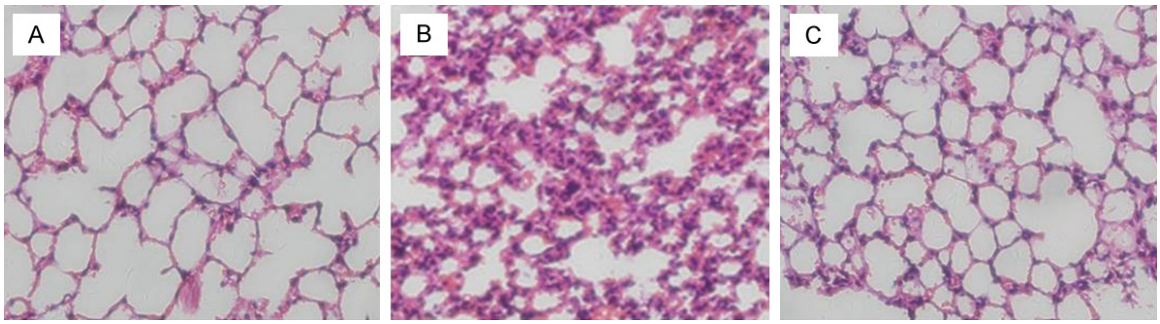

Figure 5. Effect of ECG on lung histopathology. Representative sections are shown. A. Control group lung structure. B. LPS group lung structure. C. ECG group lung structure. Magnification 200 ×; H&E stain.

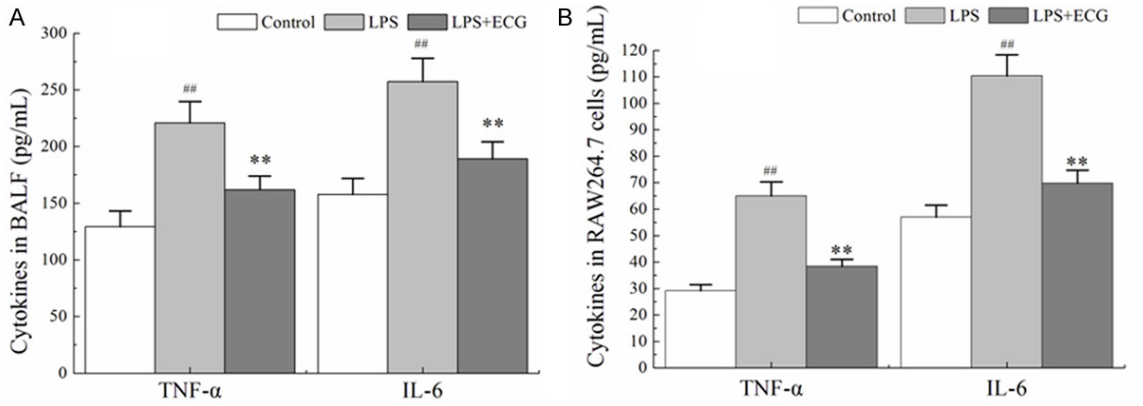

Figure 6. Effect of ECG on pro-inflammatory cytokines. Levels of TNF-α and IL-6: (A) in BALF and (B) produced by RAW 264.7 cells. Data shown are means ± SD (n = 10 mice/group for BALF). $^{\#\#}P < 0.01$ vs. LPS only; $^{**}P < 0.01$ vs. control.

vascular leakage and reduction of pulmonary surfactant were considered the main contributors of pulmonary edema [21]. The current study found that ECG attenuated LPS-induced pulmonary edema *in vivo*; this suggested to us that ECG could protect mice with LPS-induced ALI by alleviating edema. Further, ECG also mitigated LPS-induced histopathological changes in the lungs. Classical ALI histopathological changes were seen in mice with LPS-induced ALI, but these were diminished by ECG administration.

Neutrophils (PMN) and macrophages are key cells in inflammatory response in ALI. Neutrophilic granulocytes are important inflammatory cells [22] and alveolar macrophages are one of the main sources of inflammatory cytokines [23]. In the current study, ECG significantly decreased the total cells, as well as specifically levels of PMN and macrophages, in the mouse BALF. Furthermore, MPO, the most abundant granule enzyme in PMN, has been linked to tissue damage in many diseases, especially those characterized by acute inflammation [24]. MPO activity is an index for infiltration of PMN that plays a key role in the progression of ALI; down-regulation of MPO activation is known to protect hosts from ALI [21]. Accordingly in the present study, the finding that MPO activity was significantly suppressed by ECG indicated that this compound could attenuate PMN infiltration and so restrain ALI progression.

With respect to potential mechanisms of effect for the ECG, the JAK-STAT (Janus kinase-signal transducers and activators of transcription) cascade, an essential inflammatory signaling pathway that mediated immune responses [25], was evaluated. STATs were reported to be involved in the inflammatory signaling cascades triggered by LPS [26]. STAT1 exists in an inactive form in the cytoplasm and can be

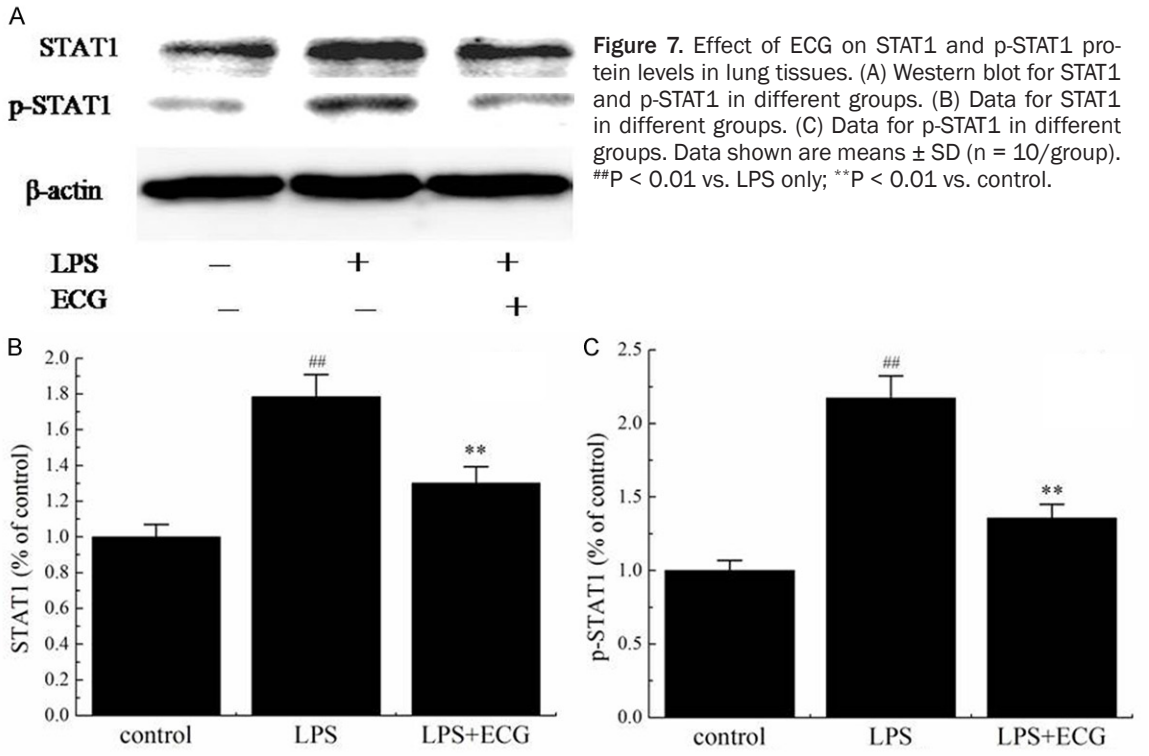

Figure 7. Effect of ECG on STAT1 and p-STAT1 protein levels in lung tissues. (A) Western blot for STAT1 and p-STAT1 in different groups. (B) Data for STAT1 in different groups. (C) Data for p-STAT1 in different groups. Data shown are means ± SD (n = 10/group). ##P < 0.01 vs. LPS only; **P < 0.01 vs. control.

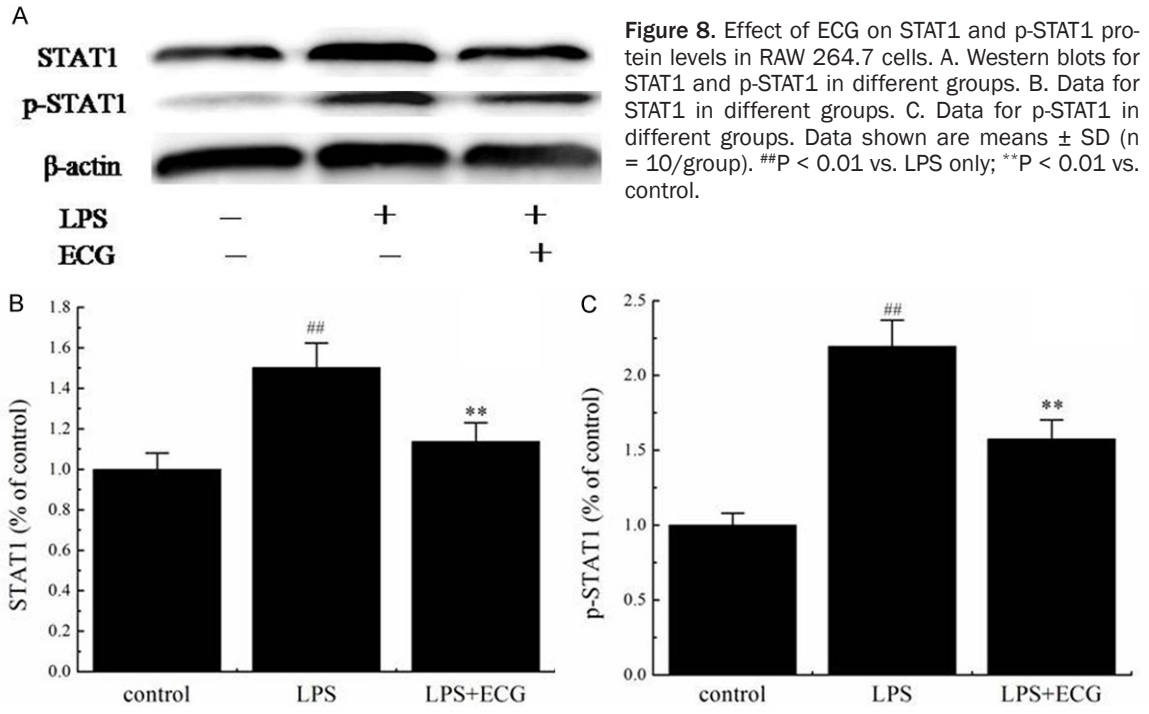

Figure 8. Effect of ECG on STAT1 and p-STAT1 protein levels in RAW 264.7 cells. A. Western blots for STAT1 and p-STAT1 in different groups. B. Data for STAT1 in different groups. C. Data for p-STAT1 in different groups. Data shown are means ± SD (n = 10/group). ##P < 0.01 vs. LPS only; **P < 0.01 vs. control.

activated by upstream molecule phosphorylated JAK1 and JAK2. Binding of ligands of its receptors induces the phosphorylation of receptor-associated Janus kinase (JAK) that, in turn, leads to STAT phosphorylation [27]. Phosphorylation of STAT1 is critical step initiat-

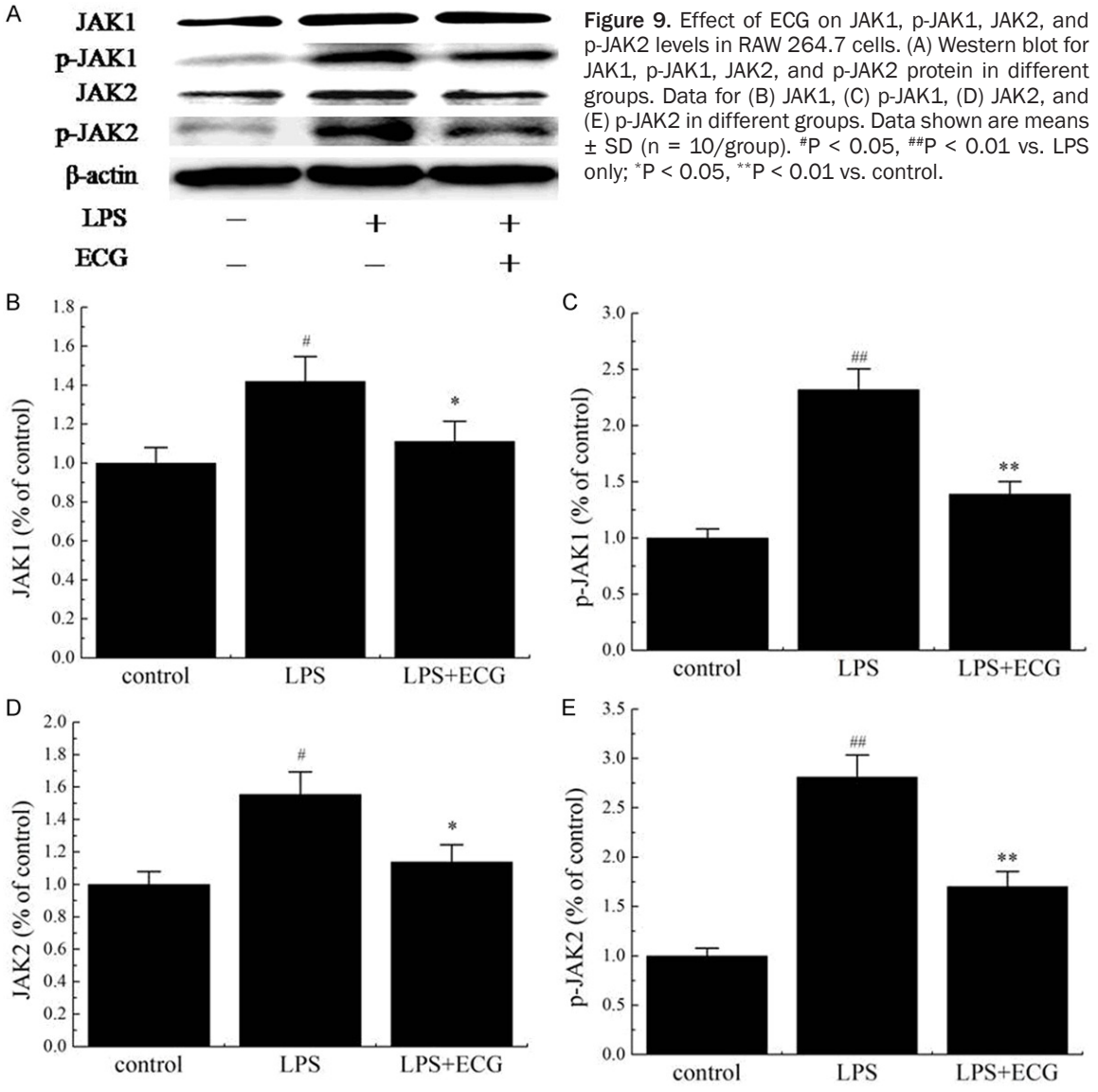

Figure 9. Effect of ECG on JAK1, p-JAK1, JAK2, and p-JAK2 levels in RAW 264.7 cells. (A) Western blot for JAK1, p-JAK1, JAK2, and p-JAK2 protein in different groups. Data for (B) JAK1, (C) p-JAK1, (D) JAK2, and (E) p-JAK2 in different groups. Data shown are means ± SD (n = 10/group). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. LPS only; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

ing pulmonary inflammation in LPS-induced ALI [28]. Therefore, the activated p-JAK1 and p-JAK2 induced the increase of p-STAT1 may be one of the most important mechanisms that cause inflammation and lung tissue collapse in LPS-induced ALI [26].

In keeping with those earlier studies, the *in vivo* portion of the present study, LPS increased the level of p-STAT1 in the lungs; and *in vitro* study, LPS-induced increases in p-STAT1 was accompanied by similar ones in both p-JAK1 and p-JAK2. The results here also showed that ECG could significantly reduce the levels of p-JAK1, p-JAK2 and p-STAT1 *in vitro*, and inhibit the LPS-induced increases in STAT1 and p-STAT1 *in vitro* and *vivo*. Taken together, these outcomes indicated to us that the protective effects of ECG might be by inducing an inhibition of JAK/STAT1 pathway.

Activated STAT1 is transported into nucleus and so promotes an up-regulation of expression of pro-inflammatory factors, including TNF-α and IL-6 [29, 30]. Several clinical studies have indicated that ALI is modulated by many of pro-inflammatory cytokines [31], notably TNF-α and IL-6 that participate in the early development of inflammation and that each can stimulate production of a host of other cytokines [32, 33]. TNF-α is an early primary mediator of the process of an inflammatory reaction that can elicit an inflammatory cascade that causes damage to the vascular endo-

thelial cells. TNF-α can also induce alveolar epithelial cells to produce other cell chemotactic factors, like IL-6 [34]. Therefore, if one is able to inhibit STAT1, the early formation/release of pro-inflammatory cytokines would be also put under control. The results in the current study showed that the ECG could significantly decrease the productions of LPS-induced TNF-α and IL-6 both *in vitro* and *vivo*, with the inhibitory effects occurring in the JAK/STAT1 pathway.

Conclusions

The present study reported an anti-inflammatory effect of ECG in mice with LPS-induced ALI and some of the underlying mechanisms for the effect. The results also demonstrated that ECG could alleviate pulmonary edema, attenuate PMN infiltration, improve lung histopathological changes, and suppress pro-inflammatory cytokines induced by LPS, and so ameliorate inflammation, by inhibiting the PTK/STAT1 signal pathway.

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Disclosure of conflict of interest

None.

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