Effect of epicatechin on inflammatory cytokines and MAPK/NF-κB signaling pathway in lipopolysaccharide-induced acute lung injury of BALB/c mice

Zelin Li1, Xiaoping Fu1, Yingrun Fan1, Chunyan Zhao1, Qiuting Wang1, Li Feng1, Xiaojing Shen1, Zhonghui Han2 and Jiangping Fan1

1 College of Food Science and Technology, Yunnan Agricultural University, Kunming, China
2 College of Food Science and Technology, Qilu University of Technology, Jinan, China

Abstract. This study evaluated the anti-inflammatory effect of epicatechin (EC) on acute lung injury (ALI) induced by lipopolysaccharide (LPS) of tracheal installation in BALB/c mice. It was observed that EC could alleviate not only the histopathological changes but also decrease the wet/dry weight (W/D) ratio of lung tissues. It also suppressed the release of IL-1β, IL-6, and TNF-α in serum, bronchoalveolar lavage fluid (BALF), and lung tissues, respectively. A quantitative real-time PCR-based study further indicated that EC also inhibited the levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA in lung tissues. In addition, the Western blot report suggested that EC was closely involved in the inhibition of phosphorylation of ERK, JNK, p38, p65, and IκB in mitogen-activated protein kinases (MAPK) and nuclear factor-κB (NF-κB) signaling pathway. These results provide an experimental and theoretical basis for treating pulmonary inflammation by EC.

Key words: (-)-Epicatechin — Acute lung injury — Inflammation — Phosphorylation

Introduction

Acute lung injury (ALI) is a form of severe respiratory disease. The characteristic symptoms of ALI are severe hypoxemia, dyspnea with infiltrated neutrophils, disruption of the endothelium, and alveolar injury in the pulmonary. This results in an uncontrolled inflammatory response, diffused interstitial pulmonary and noncardiogenic lung edema along with the increased release of inflammatory mediators, increased oxidative stress due to the release of reactive oxygen species, and neutrophil accumulation (Tan et al. 2018; Wang BQ et al. 2019; Ji et al. 2021). Further, the transmigration of neutrophils and endothelial cells promotes the production of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) (Huang et al. 2017). Also, the activated neutrophils are attracted by the proinflammatory cytokines. Moreover, the neutrophils bursting and continuous releasing of proinflammatory cytokines are aggravated the overall cellular inflammatory response of the pulmonary system (Tang et al. 2018; Du et al. 2021).

Lipopolysaccharide (LPS) is an effective proinflammatory agent tested in induced murine ALI, a suitable lung inflammatory model, as it shares many common features with human ALI, which is caused due to sepsis (Wang BQ et al. 2019). LPS not only promotes the release of inflammatory mediators but also reduces superoxide dismutase (SOD) activity in lung tissues (Zhang et al. 2019). In addition, the gene expressions of nitric oxide kinase (iNOS), cyclooxygenase-2 (COX-2), and TNF-α are increased, and the inflammatory signaling pathways,
Escherichia coli O55:B5) were obtained from Solarbio, Inc. (-)-Epicatechin (HPLC > 98%), lipopolysaccharide (from Reagents

Materials and Methods

Reagents

(-)-Epicatechin (HPLC > 98%), lipopolysaccharide (from Escherichia coli O55:B5) were obtained from Solarbio, Inc.
aggregation in alveolar cavity or blood vessel wall, alveolar wall thickening and/or hyaline membrane formation were four indicators used to assess the degree of lung tissue damage by referring to the Wolthuis's methods (Wolthuis et al. 2009).

**The W/D ratio of lungs**

The right upper lobe lung tissue of the slaughtered mice was taken, blotted using a filter paper to remove water and blood from the surface, mass determined (wet weight), and then dried at 60°C for 72 h using a drying oven and weighed (dry weight). The tissue edema of the lungs was assessed by calculating the wet/dry weight (W/D) ratio (Chu et al. 2018).

**Determination of TNF-α, IL-6, and IL-1β**

The contents of IL-1β, IL-6, and TNF-α in serum, BALF and lung tissue samples were determined by using a colorimetric sandwich ELISA kit according to the manufacturer's instructions, respectively.

**qPCR analysis**

Frozen lungs were homogenized to isolate the total RNA with TRIzol reagent (Xu et al. 2021). Step One Plus reverse transcription was employed to prepare cDNA and was amplified by a qPCR assay using the primer sets shown in Table 1. Reaction condition: 50°C 2 min, 95°C 2 min, 95°C 15 s, 60°C 32 s; read the plate, 40 cycles; analysis of melting curve at 60–95°C temperature. The levels of iNOS, COX-2 were detected.

**Western blot assay**

The lung tissues were homogenized and lysed using RIPA lysis buffer containing protease inhibitors. Homogenate was centrifuged at 15,000 × g for 15 min, and protein concentration was tested by Takara Biotechnology protein assay kit. The blotting assay was performed following the method described by Guo et al. (2019). Briefly, the protein sample was loaded onto a 10% SDS-PAGE gel and later transferred onto PVDF membranes. The membranes were then blocked for 2 h with 5% (w/v) non-fat milk, and incubated with primary antibody (containing antibodies for ERK 1:1000, P38 1:1000, JNK 1:2000, p-P38 1:1000, p-ERK 1:1000, p-JNK 1:2000, p65 1:1000, p-p65 1:2000, IκB 1:1000, p-IκB 1:1000 and β-actin 1:2000 prepared in non-fat milk) at 4°C for 48 h. Followed by this, they were incubated with secondary antibodies (HRP-conjugated, 1:5000) at 4°C for 2 h. Then, the expression of these proteins will be examined.

### Statistical analysis

All experiments were repeated thrice and the statistical analysis was done by using SPSS 18.0 (International Business Machines Corporation, NY, USA) and GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Data were expressed as mean ± standard deviation (SD). The bands were documented and band intensity quantified using ImageJ software (National Institute of Health, Bethesda, Maryland, USA). One-way analysis of variance (ANOVA) and repeated ANOVA with post hoc were employed to determine the differences among experimental groups. The p value < 0.05 and p value < 0.01 were regarded as significant (*p < 0.05 and **p < 0.01 vs. Control group; *p < 0.05 and **p < 0.01 vs. LPS group).

### Results

**Histopathological changes alleviated due to EC pretreatment**

Effects of EC on LPS-induced left lung lobe tissues in mice showed several histopathological changes in Figure 1. Mice

### Table 2. EC improved pulmonary edema in LPS-induced ALI mice

<table>
<thead>
<tr>
<th>Group</th>
<th>W/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.447 ± 1.09</td>
</tr>
<tr>
<td>LPS</td>
<td>6.970 ± 0.41</td>
</tr>
<tr>
<td>LPS+EC10</td>
<td>5.053 ± 0.75*</td>
</tr>
<tr>
<td>LPS+EC20</td>
<td>4.143 ± 0.25</td>
</tr>
<tr>
<td>LPS+EC40</td>
<td>4.046 ± 0.10</td>
</tr>
<tr>
<td>LPS+Dex</td>
<td>3.563 ± 0.82*</td>
</tr>
</tbody>
</table>

ALI, acute lung injury; EC, epicatechin; W/D, wet/dry ratio of lung tissue; Control, control group (treated by sterile saline); LPS, acute lung injury model group (treated by 10 mg/kg lipopolysaccharide); LPS+EC10, mice treated by 10 mg/kg LPS and 10 mg/kg epicatechin; LPS+EC20, mice treated by 10 mg/kg LPS and 20 mg/kg EC; LPS+EC40, mice treated by 10 mg/kg LPS and 40 mg/kg EC; LPS+Dex, mice treated by 10 mg/kg LPS and 5 mg/kg dexamethasone. *p < 0.05 vs. Control group; **p < 0.01 vs. LPS group.
treated with LPS alone showed neutrophil infiltration into the pulmonary vessel, edema of interstitial space, thickening of the alveolar wall, and alveolar epithelial cell damage in the lung tissue, along with inflammation indicative of ALI with a higher lung injury score (13.3 ± 0.58) by comparing the Control group ($p < 0.05$). These pathological processes were found to be alleviated in samples subjected to different concentrations (10, 20, and 40 mg/kg) of EC in mice. Compared with the LPS group, the lung injury score obviously decreased in a dose-dependent manner ($p < 0.05$).

**Effect of EC on pulmonary W/D ratio**

The effects of EC on pulmonary W/D ratio demonstrated that pretreatment of EC resulted in decreased W/D in a dose-dependent manner. Mice pre-treated with EC at concentrations of 10, 20, and 40 mg/kg (i.e., subsequent addition of LPS) showed the pulmonary W/D ratio to be 5.05 ± 0.75, 4.14 ± 0.25, 4.05 ± 0.10 ($p < 0.05$), respectively, while the ratio value in the Dex-treated group to be 3.56 ± 0.82 (Table 2).

**Effect of EC on inflammatory cytokine levels in ALI mice**

Colorimetric sandwich ELISA kit detection of TNF-α, IL-1β, and IL-6 in serum, BALF, and lung tissues in LPS-stimulated mice showed that TNF-α was drastically up-regulated (56.7 ± 1.5 pg/ml) in serum, BALF (430 ± 68 pg/ml) and lung tissues (885 ± 1.5 pg/ml) ($p < 0.01$). Also, the IL-1β significantly increased in serum (67.9 ± 0.65 pg/ml), BALF (279 ± 6.3 pg/ml) and lung tissue (745 ± 5.0 pg/ml); IL-6 was significantly increased in serum (1116 ± 28.4 pg/ml), BALF (767 ± 2.2 pg/ml) and lung tissues

Figure 1. Histopathological assessment of lung tissue. A. Hematoxylin-eosin staining of lung tissue. Images are representative of 6 experiments, magnification ×100. The arrows were presented the characteristic of acute lung injury. B. The score of lung injury assessment. Control, control group (treated by sterile saline); LPS, acute lung injury model group (treated by 10 mg/kg lipopolysaccharide); LPS+EC10, the mice treated by 10 mg/kg LPS and 10 mg/kg epicatechin; LPS+EC20, the mice treated by 10 mg/kg LPS and 20 mg/kg EC; LPS+EC40, the mice treated by 10 mg/kg LPS and 40 mg/kg EC; LPS+Dex, the mice treated by 10 mg/kg LPS and 5 mg/kg dexamethasone. **$p < 0.01$ vs. Control group; * $p < 0.05$ vs. LPS group.
Epicatechin influence on inflammatory}

Contrary to the above findings that the TNF-α was induced by LPS could be inhibited when pre-treated with different concentrations of EC, maximum at 40 mg/kg, the levels could be prominently decreased in serum (31.6 ± 1.2 pg/ml), BALF (259 ± 1.2 pg/ml) and lung tissue (767 ± 12 pg/ml) (p < 0.01). Similarly, when the mice tissues were pre-treated with 40 mg/kg EC, the levels of IL-1β production were decreased in serum (31.5 ± 2.1 pg/ml), BALF (88.4 ± 1.4 pg/ml), and lung tissues (617 ± 7.7 pg/ml). The levels of IL-6 were significantly decreased in serum (399 ± 1.1 pg/ml), BALF (250 ± 0.70 pg/ml), and lung tissues (1217 ± 53 pg/ml), and these indicators were also drastically down-regulated in Dex-treated groups in Figure 2.

**Effect of EC on iNOS and COX-2 mRNA level**

The levels of iNOS and COX-2 mRNA were found to be up-regulated in the LPS-stimulated group by 77.6% and 60.2% more than Control groups. However, iNOS levels were significantly decreased to 31.3%, 45.1%, and 46.4% in 10, 20, and 40 mg/kg of pre-treated EC, respectively, compared to the LPS group (p < 0.05). Further, pretreatment with EC could also inhibit the level of COX-2 mRNA from...
Effect of EC and Dex on inflammation

Examination of EC-mediated inhibition of inflammation by modulation of MAPK and NF-κB signaling pathway in LPS-induced ALI of mice by testing for p38, ERK, JNK, p-p38, p-ERK, p-JNK, p65, p-p65, IκB, and p-IκB expressions. The results showed the LPS stimulation could significantly enhance phosphorylated expression of p38, ERK, and JNK in mice lung tissues, compared to the Control group. Furthermore, p-p38, p-ERK, and p-JNK could be inhibited by EC and Dex pretreatments in a dose-dependent manner (Fig. 4). In addition, the LPS stimulation was also shown to increase the phosphorylated expression of p65 and IκB in mice lung tissues compared with the Control group (Fig. 5). Unexpectedly, the expression of p-p65 and p-IκB were seen in the Control group, which might have been due to the defense mechanism of the immune system by itself. Interestingly, the LPS-induced expression of p-p65 and p-IκB could also be significantly inhibited by EC and Dex pretreatments in a dose-dependent manner.

Discussion

(-)-Epicatechin is one flavonoid that recently received attention as an anti-inflammation compound (Qu et al. 2020b). There are very few reports describing the mechanism of the anti-ALI by EC in vivo. In the present study, the effects of EC on LPS-induced ALI in mice and its underlying anti-inflammatory mechanism were evaluated.

ALI is characterized by intensive diffuse inflammation, tissue damage, and increased microvascular permeability, which leads to multi-organ dysfunction and a high mortality...
Epicatechin influence on inflammatory

rate (Hu et al. 2019). Although studies show a suitable mechanical ventilation technology, it might cause secondary damage and, therefore, could be ineffective (Popat et al. 2018). There is few effective ways to manage the ALI issue. In this study, thus, an intratracheal instillation method was employed to establish the ALI model, and the effects of different concentrations of EC and Dex pretreatments were evaluated on it. The aim is to provide an idea for the regulation of ALI. As ALI developed, the tissue integrity and functional characteristics were imbalanced along with the endothelial and epithelial cell injury, the release of proinflammatory cytokines, as well as infiltration of a large number of neutrophil cells. Finally, pulmonary edema and increased W/D ratio were observed (Li et al. 2021; Zhang et al. 2021). The current study was detected that the LPS caused inflammatory responses in mice lung tissue, including neutrophil infiltration, diffusion of interstitial tissues, and pulmonary edema, with a higher score (Fig. 1). However, the mice pre-treated with EC have presented a drop in scores and W/D values of pulmonary in a dose-dependent manner (Fig. 1B and Table 2).

Cytokines belonging to a low molecular weight protein superfamily are involved in regulating inflammatory responses (Zhang et al. 2017). Proinflammatory cytokines play a crucial role in the development of early inflammatory diseases and are released by macrophages (Zhao et al. 2019). ALI causes release of TNF-α, IL-1β, IL-4, IL-6, IL-10, and other cytokine mediators. Elevated proinflammatory cytokines can be observed in almost all patients with inflammation (Lee et al. 2020; Park et al. 2020). Among them, TNF-α is a key pleiotropic cytokine that can induce inflammatory reactions through high particle concentration (Szondy and Pallai 2017). Mononuclear cells and macrophages release many cytokines by the inflammatory response, which accumulated results in excessive systemic inflammation and releases IL-6 (Norelli et al. 2018). Moreover, it was seen that increasing IL-6 levels in the serum of ALI patients were predicted with increased mortality in a clinical trial (Chen et al. 2019). IL-1β is a cytokine produced by endothelial cells and fibroblasts that bind to the immunoglobulin superfamily and are closely related to inflammation regulation (Shukla et al. 2018). TNF-α, IL-1β, and IL-6 induced agglutination of vascular endothelial cells eventually result in the enrichment of leukocytes into the inflammatory site and amplifies the inflammation process due to excessive NO and superoxide forms peroxynitrite which contains EC 11.6 mg/g could decrease the expression of iNOS and COX-2 mRNA were significantly increased after LPS-induced, and their levels are showed concentration-dependent decrease (Fig. 3). The levels of iNOS and COX-2 in our study were in line with the previous findings on the effect of flavonoid extracts of Quercus gilva Blume in RAW 264.7 murine macrophage cells, also related to the suppression of iNOS and COX-2 levels (Youn et al. 2017). High levels of these genes will activate inflammation pathways (MAPK and NF-κB pathway) and damage tissues.

MAPK and NF-κB signaling pathways, including ERK, p38, JNK, p65, and IkB, have played a vital regulatory role in attenuating inflammatory responses in the in vitro experiment (Cao et al. 2019; Du et al. 2021). LPS-induced inflammation can activate its phosphorylated proteins with increased ERK, p38, JNK, p65, and IkB (Hu et al. 2019; He et al. 2021). In the MAPK family, the release of proinflammatory cytokines could be promoted by protein kinases such as ERK, p38, and JNK. The accumulation of small G proteins in the Ras family stimulates upstream proteins to activate the ERK pathway (Wang et al. 2020). In addition, p38 is also seen to be activated by LPS and G^+ bacterial cell wall components (Guo et al. 2018). Research indicates that the MAPK signaling pathway regulates the processes of ALI and participates in the production of proinflammatory cytokines (Li et al. 2020). The same trends were also found in the NF-κB signaling pathway. In our study, the activation of MAPK and NF-κB signal pathways in lung tissues was monitored by Western blot, and the results showed that EC could significantly inhibit the phosphorylation expression of ERK, p38, JNK, p65, and IkB (Figs. 4 and 5). The findings showed EC could attenuate LPS-induced activation of MAPK and NF-κB signaling pathway in LPS-induced ALI of BALB/c mice.

Conclusion

In conclusion, this study demonstrated that EC plays a regulatory role in LPS-induced mice. Compared to the LPS group,
the inflammatory neutrophil infiltration was significantly alleviated the TNF-α, IL-6, and IL-1β in serum, BALF, and lung tissue homogenate of mice after EC pretreatment. Further, the results of qPCR and Western blot revealed that EC could inhibit iNOS, COX-2 mRNA levels, and phosphorylation expression of ERK, p38, JNK, p65, and IκB proteins.

**Conflicts of interest.** The authors declare no conflict of interest.

**Acknowledgments.** This study was financially supported by national natural science foundation of China (31560457).

**Author contributions.** All the authors listed below were involved in this work and they have approved the manuscript submission. ZL, participated in the main experiment and edited the manuscript. XF participated in experiment design and statistical analysis. JF sponsored the research and was responsible for review and collection. XS provided literature. ZH participated in experiment. YF, CZ, QW and LF participated in the animal experiment, data collection. XS provided literature. ZH participated in experiment design. JF sponsored the research and was responsible for review and revision of manuscripts.

**References**


and quercetin exhibit in vitro antioxidant effect, improve biochemical parameters related to metabolic syndrome, and decrease cellular genotoxicity in humans. Food Res. Int. 142, 110101
https://doi.org/10.1016/j.foodres.2020.110101

https://doi.org/10.1016/j.intimp.2017.11.034

https://doi.org/10.1016/j.foodchem.2018.06.009

https://doi.org/10.3892/ijmm.2021.4919

Marimoutou M, Le Sage F, Smadja J, Lefehvre D Hellencourt C, Gonthier M, Robert-Da Silva C (2015): Antioxidant polyphenol-rich extracts from the medicinal plants Antirhea borbonica, Doratoxylon apetalum and Gouania mauritiana protect 3T3-L1 preadipocytes against H2O2, TNF-α and LPS inflammatory mediators by regulating the expression of superoxide dismutase and NF-kB genes. J. Inflamm. 12, 10
https://doi.org/10.1186/s12950-015-0055-6

https://doi.org/10.1038/s41591-018-0036-4

https://doi.org/10.1007/s10438-019-09883-x

https://doi.org/10.1016/j.amjms.2018.03.013

https://doi.org/10.1016/j.jff.2018.10.021

https://doi.org/10.1080/10408398.2020.1723057

https://doi.org/10.1007/s10753-017-0675-5

https://doi.org/10.1016/j.amjms.2018.03.013

https://doi.org/10.1007/s12272-017-0991-1

https://doi.org/10.4103/0366-6999.227618

https://doi.org/10.1039/C8FO01337C

https://doi.org/10.3390/molecules21081087

https://doi.org/10.1097/SHK.00000000000011120

https://doi.org/10.3390/3801010101

https://doi.org/10.1016/j.taap.2020.115150

Epicatechin influence on inflammatory...


Received: December 16, 2021
Final version accepted: May 3, 2022