RESEARCH ARTICLE

Hesperetin ameliorates lipopolysaccharide-induced acute lung injury in mice through regulating the TLR4–MyD88–NF- κ B signaling pathway

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Abstract Hesperetin, a major bioflavonoid in sweet oranges and lemons, exerts an anti-inflammatory effect in pulmonary diseases; however, its effect on lipopolysaccharide (LPS)-induced acute lung injury is unclear. This study investigated the effect of hesperetin on LPS-induced lung inflammatory response. Mice were intratracheally instilled with 5 mg/kg body weight LPS, and then were given hesperetin orally (10, 20, and 30 mg/kg body weight) 1 h later. Hesperetin dramatically suppressed the levels of interleukin-6 and tumor necrosis factor- α , as well as the number of inflammatory cells in bronchoalveolar lavage fluid. Besides, it reduced lung injury, wet weight/dry weight ratio, and myeloperoxidase and lactate dehydrogenase activities, and enhanced superoxide dismutase activity. In addition, hesperetin significantly downregulated the Toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 (MyD88) protein expression and suppressed nuclear factor-kappa B (NF- κ B) activation in lung tissue. Together, these results indicated that the anti-inflammatory effect of hesperetin is associated with the TLR4–MyD88–NF- κ B pathway, and that hesperetin shows therapeutic potential for LPS-induced acute lung injury.

Keywords Hesperetin · LPS · Inflammation · TLR4–MyD88–NF- κ B pathway

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are the key causes of acute respiratory failure which are associated with considerable morbidity and mortality (Sweeney and McAuley 2016). ALI is characterized by the disruption of endothelium and alveolar epithelial barrier, leading to increased microvascular permeability. At later stages, ALI can lead to numerous devastating complications (Reiss et al. 2012).

Inflammation is a highly regulated process characterized by the release of cytokines, chemokines and growth factors, as well as the transmigration of inflammatory cells, such as neutrophils, monocytes and lymphocytes, from blood to the affected tissues. The inflammation induced by these immune cells is modulated by the inflammatory mediators including interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , IL-12, IL-18, and nitric oxide (NO) (Robb et al. 2016). However, overexpression of these mediators can induce ALI and even cause death in severe cases. The mechanisms underlying ALI are still unclear.

Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, has been implicated in the pathogenesis of ALI (Rosadini and Kagan 2017). LPS is a specific ligand of Toll-like receptor 4 (TLR4), and ALI is associated with the activation of LPS-induced TLR4 signaling pathways such as myeloid differentiation primary response gene (Myd88)-dependent pathway (Wang et al. 2016). LPS promotes the excessive production of inflammatory mediators (cytokines, chemokines, and adhesion molecules) by activating the Myd88-dependent TLR4 signaling pathway, leading to pulmonary damage and the development of ALI (Zhang et al. 2017). Nuclear factor-kappa B (NF- κ B), a critical nuclear transcription factor, plays an important role in ALI by modulating various vital physiopathological processes,

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including inflammation (Cheng and Li 2016; Feng et al. 2015; Sun et al. 2016).

Hesperetin, a member of the flavanone subclass of flavonoids, is present in some citrus fruits. Several studies have identified the antioxidant, anticancer, and anti-inflammatory activities of hesperetin (Shih et al. 2012; Seyedrezazadeh et al. 2015; Wei et al. 2012). In addition, Ma et al. have suggested that hesperetin attenuates ventilator-induced ALI by suppressing NF- κ B-mediated inflammation (Ma et al. 2015). Furthermore, it has been reported that hesperetin suppresses the inflammatory response to LPS-induced injury in RAW 264.7 cells. Hesperetin ameliorates ALI by targeting MD2 via an unknown mechanism (Ye et al. 2019). Therefore, hesperetin may exert a protective effect against LPS-induced ALI. This study aimed to evaluate the effect of hesperetin on the LPS-induced inflammatory response and recovery of damaged lung tissue in mice with ALI, and the role of the TLR4–MyD88–NF- κ B pathway in this effect.

Materials and methods

Reagents

Hesperetin (Hesp, purity > 98%) and lipopolysaccharide (LPS coli 055:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Myeloperoxidase (MPO), lactate dehydrogenase (LDH), superoxide dismutase (SOD), NO determination kit and TNF- α , IL-6 enzyme linked immunosorbent assay (ELISA) kits were provided by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). Dexamethasone (DEX) was purchased from Chengdu Tiantaihan pharmaceutical Co., Ltd. (Chengdu, China). MyD88 (ab2094), iNOS (ab213987), TRAF6 (ab227560), NF- κ B p65 (ab207297), p-NF- κ B p65 (ab28856), I κ B- α (ab32518), p-I κ B- α (ab133462), TAK1 (ab109526), p-TAPK1 (ab109404) were all obtained from Abcam, Cambridge, UK; TLR4 (sc-293072) and β -actin (sc-81760) from Santa Cruz Biotechnology, Santa Cruz, California, USA. The bicinchoninic acid (BCA) Protein Assay Kit was obtained from Beyotime Institute of Biotechnology (Nanjing, China). All other reagents were of analytic cal grade.

Animal and treatment protocols

Male C57 mice (8 weeks old, 18–22 g) were purchased from Shandong University Laboratory Animal Center. All animal welfare and experimental procedures were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols used were approved by the Animal Ethics Committee of Shandong University. Mice were divided into six groups ($n = 8$ per group): control group, LPS group, LPS + hesperetin (Hesp,

10, 20, or 30 mg/kg) groups, and dexamethasone group (DEX, 2 mg/kg). Dex group was used as a positive control.

The mice received an equal volume of PBS in control group. Hesperetin (10, 20, or 30 mg/kg) and DEX groups was orally administered 1 h after LPS (5 mg/kg) intratracheal injection (Qu et al. 2003). All mice were sacrificed at 24 h post-induction, and bronchoalveolar lavage fluid (BALF) and lung tissues were collected for subsequent experiments.

Histological analysis of lung tissues

The lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μ m sections, then stained with hematoxylin and eosin (H&E). Lastly, the optical microscope (Olympus, Japan) was used to observe the pathological changes of lung tissues.

Lung wet/dry weight ratio

The right lung was collected to obtain the “wet” weigh. Then the lung was dried in an oven at 60 °C for 72 h and weighted to obtain the “dry” weight. Lung wet/dry (W/D) weight ratio was used to assess the lung edema.

MPO, LDH and SOD assay

Lung tissues were homogenized after collection. MPO activity in lung tissues was measured according to the manufacturer’s instructions. The enzymatic activity was detected at 460 nm using microplate spectrophotometer (BIO-RAD, USA). The kits used to determine SOD and LDH activities were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Proteins concentration and cell counts in bronchoalveolar lavage fluid (BALF)

In the BALF, leukocyte populations, macrophages were counted using Wright’s–Giemsa stain (Nanjing Jiancheng, China) following the manufacturer’s instructions. Briefly, the cell smear was stained with Wright–Giemsa solution for 1–2 min, immersed rapidly in phosphate buffer for 5 min, and then rinsed with running distilled water for 30 min to destain and air dried. Slides were examined and differential cells counts were performed on each slide.

BALF was performed 3 times with totally 1 ml PBS, and the recovery ratio of BALF was about 80%, followed by centrifugation at 3000 rpm for 10 min at 4 °C. Supernatants in BALF were used to determine total protein concentration by BCA Protein Assay Kit and to measure the cytokines in the BALF. The cells were then stained with Wright–Giemsa stain and 200-cell differential counts were determined.

ELISA assays for nitric oxide(NO, TNF- α , and IL-6)

The levels of TNF- α , NO and IL-6 in the BALF were measured using ELISA kits according to the manufacturer's instructions. All measurements were performed in duplicate and the optical density of each well was read at 450 nm using a microplate spectrophotometer.

Western blotting analysis

Protein was extracted in cold RIPA buffer from lung tissues. Equal amounts of protein were separated by 12% SDS-PAGE, and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Protein blots were blocked with 5% BSA for 2 h and incubated with primary antibodies against TLR4, MyD88, TRAF6, iNOS, I κ B- α , phosphorylated I κ B- α , NF- κ B p65, phosphorylated NF- κ B p65, TAK1 and phosphorylated TAK1 at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibodies (1:10,000). Finally, the bands were detected by enhanced chemiluminescence (ECL) kit and analyzed using the Image Lab for quantification.

Statistical analysis

All statistical analysis were performed using GraphPad Prism (version 6.0) and expressed as the mean $\pm$ SD. Statistical significance was analyzed using one-way analysis of

variance (ANOVA), followed by Dunnett's test. $p < 0.05$ was considered as statistically significant for all tests.

Results

Hesperetin ameliorates LPS-induced histopathologic changes

As shown in Fig. 1a, the lung tissues in the control group represented normal structure without histopathologic changes. However, widespread alveolar wall thickness caused by edema, severe hemorrhage in the alveolus, alveolus collapse, and obvious inflammatory cell infiltration were observed in the LPS group, which were ameliorated after the administration of hesperetin (20, 30 mg/kg) and Dex (2 mg/kg) (Fig. 1b).

Hesperetin suppresses LPS-induced wet/dry weight ratio

In this study, the lung edema was detected by lung wet/dry weight ratio. The results showed that lung wet/dry weight ratio was significantly higher in the LPS group than that in the control group. However, treatment of hesperetin (30 mg/kg) and Dex notably inhibited LPS-induced lung wet/dry weight ratio ($p < 0.05$, $p < 0.01$, respectively) (Fig. 2).

Fig. 1 Hesperetin ameliorates LPS-induced pathological changes in lung tissue of ALI mice. Representative histological changes of lung obtained from mice of different groups. **a** Control group, LPS group, LPS + hesperetin (10 mg/kg) group, LPS + hesperetin (20 mg/kg) group, LPS + hesperetin (30 mg/kg) group, Dex group (hematoxylin and eosin staining, magnification $\times 200$). **b** Lung inflammatory scores were calculated according to the sum of the levels of cell infiltration and damage levels as assessed from the lung sections. ## $p < 0.01$ versus control group, ** $p < 0.01$ versus LPS group, $n = 8$. Data are presented as mean $\pm$ SD.

Fig. 2 Hesperetin (30 mg/kg) and Dex inhibits LPS-induced lung wet/dry weight ratio. Effects of hesperetin (10 mg/kg, 20 mg/kg, 30 mg/kg) and positive control Dex (2 mg/kg) on the lung W/D ratio of LPS-induced ALI mice. ## $p < 0.01$ versus control group, * $p < 0.05$ and ** $p < 0.01$ versus LPS group, $n = 8$. Data are presented as mean $\pm$ SD.

Hesperetin lowers LPS-induced MPO, LDH activity and enhances SOD activity

The effects of hesperetin on LPS-induced MPO and LDH activities were detected in this study. As shown in Fig. 3, MPO and LDH activities were significantly enhanced and SOD activity was decreased in LPS group compared with those of the control group. Nevertheless, treatment of

hesperetin (30 mg/kg) and Dex significantly reversed LPS effect.

Hesperetin inhibits LPS-induced inflammatory cytokines levels and NO production

The levels of inflammatory cytokines (TNF- α and IL-6) and NO production in the BALF at 24 h post-LPS-treatment were shown in Fig. 4. The results showed that LPS stimulation induced significant increases in NO, TNF- α , IL-6 production, while hesperetin (10, 20, or 30 mg/kg) and Dex reduced TNF- α production; hesperetin (20, 30 mg/kg) and Dex diminished IL-6 production; hesperetin (30 mg/kg) and Dex attenuated NO level in BLAF.

Hesperetin diminishes the inflammatory cells counts in BALF

To examine the effects of hesperetin on LPS-induced pulmonary inflammation, the numbers of inflammatory cells were analyzed at 24 h after LPS injection. From Fig. 5, the numbers of total cells, neutrophils and macrophage significantly augmented in the LPS group compared with control group. However, hesperetin (20, 30 mg/kg) and Dex reduced total cells and neutrophils, and hesperetin (30 mg/kg) and Dex reduced macrophage.

Fig. 3 Hesperetin (30 mg/kg) and Dex reversed changes of MPO, LDH and SOD activity induced by LPS treatment. Effects of hesperetin (10 mg/kg, 20 mg/kg, 30 mg/kg) and positive control Dex (2 mg/kg) on MPO (a), LDH (b) and SOD (c) activity in lung tissues of LPS-induced ALI mice. ## $p < 0.01$ versus control group, * $p < 0.05$ and ** $p < 0.01$ versus LPS group, $n = 8$. Data are presented as mean $\pm$ SD.

Fig. 4 Hesperetin and Dex reversed LPS-induced inflammatory cytokines production and NO level in the BAL Fluid. Treatment of Hesperetin markedly decreased NO content (a), decreased TNF- α (b) and IL-6 content (c) in BALF. $^{##}p < 0.01$ versus control group $^{*}p < 0.05$ and $^{**}p < 0.01$ versus LPS group $n = 8$. Data are presented as mean $\pm$ SD.

Fig. 5 Hesperetin and Dex reduced inflammatory cells infiltration in the BAL Fluid. Number of total cells (a), neutrophil (b) and macrophages (c) were counted in 1 ml BALF. $^{##}p < 0.01$ versus control group $^{*}p < 0.05$ and $^{**}p < 0.01$ versus LPS group $n = 8$. Data are presented as mean $\pm$ SD.

Effect of hesperetin on LPS-induced signaling pathways

In this study, the expressions of TLR4–MyD88–NF- κ B pathways in lung tissues were detected by western blotting.

As shown in Fig. 6, the levels of TLR4, MyD88, TRAF6 proteins showed obvious increase in LPS group at 24 h post-LPS-administration, which was attenuated after treatment of hesperetin (30 mg/kg) and Dex. In addition, LPS stimulation

Fig. 6 Effect of hesperetin-mediated TLR4/NF- κ B pathway related proteins in lung tissues of LPS-induced ALI mice. Mice in different groups were treated with LPS, LPS + hesperetin (30 mg/kg), and LPS + Dex (2 mg/kg), respectively. **a** The protein expression of TLR4, MyD88, TRAF6 and iNOS. **b** The protein expression of phospho-I κ B- α , phospho-NF- κ B p65 and phospho-TAK1. **c** The quantitative analysis of protein blots. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus control group, $^*p < 0.05$ and $^{**}p < 0.01$ versus LPS group, $n = 8$. Data are presented as mean $\pm$ SEM.

apparently induced phosphorylation of NF- κ B p65, I κ B- α and TAK1, whereas hesperetin and Dex administration reversed this result. The activation of NF- κ B could induce the expression of many pro-inflammatory genes, including iNOS, and the increased expression of iNOS further promoted NO expression.

Discussion

ALI/ARDS is characterized by damage to the alveolar epithelium, lung edema, and infiltration of neutrophils, and there has been no effective treatment to date (Diaz et al. 2010). Although some promising pharmacological therapies have been studied in patients with ALI and ARDS, none of them have effectively reduced mortality (do Nascimento Xavier et al. 2019). ALI represents a state of hyperinflammation involving a variety of mediators and cell types. IL-6 is a cytokine with multiple immunomodulatory functions, which can stimulate the proliferation of B cells, T cells and stem cell, promote the production of immunoglobulin via B cell, and facilitate the differentiation of cytotoxic lymphocyte and stem cell (Zhou et al. 2017). TNF- α generated by macrophages is implicated in the cytotoxic function in certain tumors, and in the development and procession of immunoregulatory effects of ALI (El-Awady et al. 2018). The inflammatory response in ALI is triggered by IL-6 and TNF- α , which are released during in the early phase of the disease and induce the production of other proinflammatory cytokines (Butt et al. 2016). LPS plays an important role in the pathogenesis of ALI, and a single intratracheal instillation of LPS has been used to establish animal models of ALI (Du et al. 2018; Wan et al. 2018; Zhang et al. 2018). In this study, the levels of IL-6 and TNF- α in BALF were increased after LPS treatment, which were reversed by hesperetin and Dex administration, suggesting that hesperetin alleviated ALI.

Hesperetin is a flavanone glycoside compound naturally occurring in the fruit peel of *Citrus aurantium* L. (Rutaceae), which exhibits anti-inflammatory, antitumor, and antioxidant activities (Parhiz et al. 2015). Chen et al. have found that hesperetin derivative-14 significantly attenuates inflammation in LPS-treated RAW 264.7 cells (Chen et al. 2017). In addition, hesperetin can suppress the airway hypersensitivity response and reduce the number of inflammatory cells in patients with allergic asthma (Shih et al. 2012).

In patients with ALI, the lung wet/dry ratio, an indicator of systemic and local inflammation, is used as an index of pulmonary edema. In the present study, hesperetin decreased the LPS-induced lung wet/dry ratio. Macrophages exuded from inflamed lung are key players in host defense against pathogen invasion. Neutrophils are the first immune cells to be recruited into the lungs, where they modulate the

production of proinflammatory cytokines and induce pulmonary injury. In this study, the number of neutrophils and macrophages in bronchoalveolar lavage fluid (BALF) was increased by LPS, while decreased by hesperetin and Dex.

Neutrophils are also vital components of the inflammatory response that characterizes ALI, and are considered to be the final effector cells that responsible for lung injury due to their ability to express multiple cytotoxic products (Li et al. 2019; Pinheiro et al. 2019). Myeloperoxidase (MPO), which is localized in the primary granules of neutrophils (Lee et al. 2019), reflects the adhesion and migration of neutrophils in lung tissues. In this study, LPS promoted the level of MPO, but hesperetin and Dex reversed the result. Overproduction of NO may exert a cytotoxic effect via the formation of peroxynitrite. Accordingly, inhibiting NO production could ameliorate LPS-induced ALI. In this study, decreased NO level in BALF was noticed after hesperetin and Dex treatment. The above findings were confirmed by the results of a histological analysis showing that LPS increased pulmonary edema and neutrophil infiltration.

LPS receptor TLR4 triggers activation of NF- κ B via the MyD88-dependent pathway, resulting in upregulation of proinflammatory mediators. MyD88 activates transcription factors in the NF- κ B family that regulate immunity, oxidative stress, and inflammation (Hoesel and Schmid 2013). Activation of NF- κ B induces the expression of proinflammatory factors encoded by genes, including TNF- α and iNOS; and the upregulation of iNOS expression results in an increase of NO level (Cheng et al. 2014). NF- κ B also regulates the expression of proinflammatory cytokines. Inactive NF- κ B exists either in a complex with I κ B or as two polymers with the precursor protein. Following LPS stimulation, NF- κ B p65 is translocated from the cytoplasm into the nucleus to regulate the expression of proinflammatory cytokines, including TNF- α and IL-6, both of which activate NF- κ B. To find out whether the anti-inflammatory activity of hesperetin is mediated by the TLR4–MyD88–NF- κ B pathway, we evaluated the expression of TLR4/NF- κ B pathway related proteins. In this study, hesperetin and Dex suppressed the expression of TLR4, MyD88, TRAF6, pTAK1, p-NF- κ Bp65, and p-I κ B α which were upregulated by LPS. Furthermore, western blotting showed that hesperetin decreased the levels of TLR4 MyD88, TNF- α , and IL-6. These findings indicated that hesperetin inhibited the activation of TLR4–MyD88–NF- κ B pathway in LPS-treated lung tissue, thereby suppressing NF- κ B activation.

In summary, hesperetin ameliorated LPS-induced ALI, as indicated by improvement of the pathologic changes in lung tissue and decreased infiltration of inflammatory cells and the release of proinflammatory cytokines. The efficacy of hesperetin may be mediated by inhibition of the TLR4–MyD88–NF- κ B signaling pathway. Therefore, hesperetin may exhibit therapeutic potential for ALI therapy, but

the involvement of other signaling pathways in these effects needs further study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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