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Hesperetin attenuates ventilator-induced acute lung injury through inhibition of NF-κB-mediated inflammation

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ABSTRACT

Hesperetin, a major bioflavonoid in sweet oranges and lemons, has been reported to have anti-inflammatory properties. However, the effect of hesperetin on ventilator-induced acute lung injury has not been studied. In present study, we investigated the protective effect of hesperetin on ventilator-induced acute lung injury in rats. Rats were orally administered hesperetin (10, 20, or 40 mg/kg) two hour before acute lung injury was induced by mechanical ventilation. Rats were then randomly divided into six groups: the lung protective ventilation group (n=20, LV group), injurious ventilation group (n=20, HV group), vehicle-treated injurious ventilation group (n=20, LV+vehicle group), hesperetin (10 mg/kg)-treated acute lung injury group (n=20, HV+Hsp (10 mg)), hesperetin (20 mg/kg)-treated acute lung injury group (n=20, HV+Hsp (20 mg)), and hesperetin (40 mg/kg)-treated acute lung injury group (n=20, HV+Hsp (40 mg)). The lung tissues and bronchoalveolar lavage fluid were isolated for subsequent measurements. Treatment with hesperetin dramatically improved the histology of lung tissue, and reduced the wet/dry ratio, myeloperoxidase activity, protein concentration, and production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and MIP-2 in the bronchoalveolar lavage fluid of rats with ventilator-induced acute lung injury. Additionally, our study indicated that this protective effect of hesperetin results from its ability to increase the expression of peroxisome proliferator-activated receptor (PPAR)-γ and inhibit the activation of the nuclear factor (NF)-κB pathway. These results suggest that hesperetin may be a potential novel therapeutic candidate for protection against ventilator-induced acute lung injury.

1. Introduction

Acute lung injury (ALI), a form of hypoxemic respiratory failure, is characterized by pulmonary inflammation, accumulation of neutrophils, disruption of epithelial integrity, and severe impairment of gas exchange (Reddy et al., 2009). Despite significant progress being made in recent decades, ALI and acute respiratory distress syndrome (ARDS) caused by shock, sepsis, ischemia reperfusion, or viral pneumonia, still has a high mortality rate (Raghavendran and Napolitano, 2011). Therefore, it is extremely urgent to discover effective drugs and clinical therapies for ALI and ARDS.

Majority of the patients with acute lung injury (ALI) require mechanical ventilation (MV) for life support. However, during the repetitive opening and closing of the lung units, MV may generate excessive force that can lead to alveolar overdistension and ventilator-induced lung injury (Rubenfeld et al., 2005). Ventilator-induced lung injury is frequently associated with multiple distal organ dysfunctions because of biotrauma (Tremblay et al., 1997).

Previous studies regarding ventilator-induced lung injury have reported excess activation of nuclear factor (NF)-κB and production of inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and MIP-2 (Ding et al., 2013; Held et al., 2001). Additionally, phosphorylation and degradation of IκBα result in the activation of NF-κB and subsequent inflammatory responses (Schabbauer et al., 2004).

Previous study has been reported that the transcription factor, peroxisome proliferator-activated receptor (PPAR)-γ could be activated by its ligand (Evans, 1988). Meanwhile, PPAR-γ is widely distributed in adipose tissue, it plays a significant role in many tissues and cells, including airway epithelial cells, macrophages, and smooth muscle tissue (Pawliczak et al., 2002; Wang et al., 2001). PPAR-γ activation alters the activation and proliferation of cells, as well as inflammatory reactions (Vamecq and Latruffe, 1999). PPAR-γ activation can also regulate NF-κB activation and

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inhibit the production of the inflammatory cytokines that are mediated by the NF-κB pathway (Bailey and Ghosh, 2005).

As a member of the flavanone subclass of flavonoids, hesperetin (3′,5,7-trihydroxy-4-methoxyflavanone, Hsp) is present in some citrus fruits (Garg et al., 2001). Hsp has been demonstrated to have anti-cancer and hypolipedemic properties (Aranganathan and Nalini, 2009; Morin et al., 2008). It also has anti-oxidant properties since it promotes anti-oxidant enzyme activity and has radical scavenging effects (Choi, 2008; Choi and Ahn, 2008; Hwang and Yen, 2008; Kim et al., 2004; Pollard et al., 2006). Furthermore, Hsp has been found to be neuroprotective (Choi and Ahn, 2008) and has anti-inflammatory potential (Hirata et al., 2005). Therefore, based on these previous stidies, we hypothesized whether Hsp has a potentially protective effect on ventilator-induced lung injury by its anti-inflammatory acitivity.

In the present study, we investigated whether Hsp has a protective effect on ventilator-induced ALI in a rat model. We treated rats with ventilator-induced ALI with Hsp and assessed the effect. Hsp treatment improved the histology of the lung tissues, and reduced the wet/dry ratio, inflammatory cell infiltration, and cytokine production in the bronchoalveolar lavage fluid (BALF). It attenuates ventilator-induced ALI by activating PPAR-γ expression, and reduces the production of inflammatory cytokines by blocking the NF-κB pathway *in vivo*.

2. Materials and methods

2.1. Animals

Male, specific pathogen-free Sprague-Dawley rats ($n=130$, 240–290 g) were acquired from the SLRC laboratory (Shanghai, China). Rats were housed in an animal facility under standard laboratory conditions (with water and standard chow *ad libitum*) for three days before the initiation of experiments. All experimental procedures were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals, and the animal handling followed the dictates of the National Animal Welfare Law of China.

2.2. Experimental design

Rats were randomly divided into six groups: the lung-protective ventilation group (the healthy control) ($n=20$, LV group); injurious ventilation group or ventilator-induced ALI group ($n=20$, HV group); vehicle-treated ALI group ($n=20$, HV+vehicle group); Hsp (10 mg/kg)-treated ALI group ($n=20$, HV+Hsp (10 mg) group); Hsp (20 mg/kg)-treated ALI group ($n=20$, HV+Hsp (20 mg) group); and Hsp (40 mg/kg)-treated ALI group ($n=20$, HV+Hsp (40 mg) group). Rats were killed at the indicated time points after receiving their treatment for the induced ALI.

2.3. Mechanical ventilation

Two h before ALI induction, rats orally received 10 mg/kg, 20 mg/kg, or 40 mg/kg Hsp, dissolved in phosphate buffered saline (PBS). Rats were then anesthetized by an intraperitoneal injection of thiopental (37 mg/kg), before receiving a dorsal penile vein injection of 5 mg/kg succinylcholine and a tracheostomy. A rodent volume ventilator (Harvard Apparatus, Ealing, Les Ulis, France) was employed to ventilate the animals for two h. Rats were either protectively ventilated by a low ventilation (40 breaths/min, 3 cm H_2O positive end-expiratory pressure [PEEP] and 7 ml/kg VT) (LV group) or injuriously by a high ventilation (40 breaths/min, zero end-expiratory pressure [PEEP] and 42 ml/kg VT) (HV group). Rats also had their blood pressure and pulse monitored during the process of mechanical ventilation using a rats blood pressure/pulse monitor (Kent Scientific, USA).

2.4. Samples collection

After receiving MV, rats were killed by an intravenous injection of thiopental. Their blood was collected by cardiac puncture, and the BALF was obtained from three bronchoalveolar lavages in the upper part of the trachea by using 5 ml of PBS. The BALF was centrifuged (2000 g for 10 min at 4 °C) and the supernatants were stored for further examination.

2.5. Histopathological examination

The right lung tissues were excised and fixed in 4% paraformaldehyde for three days at 4 °C, before being dehydrated in grade alcohol and embedded in paraffin wax. Hematoxylin and eosin (H&E) staining was performed and pathological changes were examined using an Olympus BX51 microscope system (Olympus, Japan). All microscopic sections were analyzed by a blinded pathologist. For each group of rats, 10 tissue sections were selected and 10 fields were examined for each section. According to the degree of interstitial and alveolar hemorrhage, edema, and infiltration of inflammatory cells, the histopathological lung changes were scored on a scale of 0–5 (Nishina et al., 1997). 0 score represents no injury, 1 score represent modest injury, 2 score represent intermediate injury, 3 score represent widespread injury, and 4 score represent severe injury (such as most prominent, marked congestion, and interstitial edema with neutrophilic infiltrate nearly filling the alveolar spaces). The average values of the lung injury were considered a measure of lung injury (Osman et al., 1998; Zheng et al., 2001).

2.6. Lung wet/dry ratio

After the rats were euthanized by an intravenous injection of thiopental, the middle lobe of the left lungs were removed and weighed to measure the wet weight. Lung tissues were then dried in an oven at 80 °C for two days to obtain the stable dry weight. The lung wet/dry ratios were obtained as follows: wet/dry ratio= (wet weight-dry weight)/dry weight (Numata et al., 1998). The wet/dry ratio was considered an index of the severity of pulmonary edema.

2.7. Myeloperoxidase (MPO) activity in lung tissues

MPO activity is a significant marker for neutrophil accumulation in inflammatory tissues. After induction of ALI, MPO activity was measured as previously described (Mo et al., 2014). Briefly, the lung tissues were weighed and homogenized. The homogenate was then centrifuged at 15,000*g* at 4 °C for 20 min, and the supernatant assayed with an MPO assay kit (Bluegene, China) according to the manufacturer's standard protocols. The absorbance of the samples was measured using a spectrophotometer at 450 nm. Results were expressed as units per gram of lung tissue. All the samples were assayed in triplicate.

2.8. BALF, protein concentration, and total cell count

After being anesthetized, the rats were exsanguinated, and the BALF was collected from lungs through a tracheal cannula with 5 mL PBS. The recovery rate was $>90\%$ and lavage samples were kept on ice. Next, the BALF samples were centrifuged at 2000*g* for 10 min at 4 °C and the supernatants were removed and stored for further use. The cells in the BALF were resuspended with 0.5 ml PBS and the number of infiltrated cells was counted. Protein