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The immunomodulation of endotoxin-induced acute lung injury by hesperidin *in vivo* and *in vitro*

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Abstract

To investigate the modulation of lung local immune responses of hesperidin (HES) on the acute lung inflammation induced by LPS *in vivo*. Mice were challenged with intratracheal lipopolysaccharide (100 μg) 30 min before with treatment hesperidin (200 mg/kg oral administration) or vehicle. After 4 and 24 h, bronchoalveolar lavage fluid was obtained to measure proinflammatory (TNF-α, IL-1β, IL-6), anti-inflammatory (IL-10, IL-4, IL-12) cytokines, chemokines (KC, MCP-1 and MIP-2), total cell counts, nitric oxide production, and proteins. Lung histology was performed in inflated-fixed lungs. Hesperidin downregulate the LPS-induced expression of TNF-α, IL-1β, IL-6, KC, MIP-2, MCP-1, and IL-12. It also enhanced the production of IL-4, IL-10. Total leukocyte counts; nitric oxide production, iNOS expression, and proteins were significantly decreased by hesperidin. *In vitro*, HES suppressed the expression of IL-8 on A549 cells and THP-1 cells, the expression of TNF-α, IL-1β, and IL-6 on THP-1 cells, the expression of ICAM-1 and VCAM-1 on A549 cells which effect cell adhesion function. The suppression of those molecules is controlled by NF-κB and AP-1, which are activated by IκB and MAPK pathways. HES inhibits those pathways, thereby suppressing the expression of IL-8, TNFα, IL-1β, IL-6, IL-12, ICAM-1 and VCAM-1. This study indicates that HES had a markedly immunomodulatory effect in a clinically relevant model of ARDS. Nevertheless, further investigations are required to determine the potential clinical usefulness of HES in the adjunctive therapy of ARDS.

Keywords: LPS; Proinflammatory cytokines; Anti-inflammatory cytokines; Chemokines; Immunomodulation; ICAM-1; VCAM-1

Introduction

Acute respiratory distress syndrome (ARDS) can lead to sepsis, trauma, and severe pneumonia, among which sepsis and in particular pneumonia are leading causes of acute lung injury. Bacterial superinfection of the lung is a common complication of acute lung injury (Dreyfuss and Ricard, 2005). ARDS is characterized by acute lung inflammation involving the local recruitment and activation of polymorphonuclear neutrophils (Chignard and Balloy, 2000) and the release of proinflammatory mediators (Shinbori et al., 2004; Wright et al., 2004), proteases, and reactive oxygen and nitrogen species (Haddad et al., 1994; Matthay et al., 1999). Eventually, these processes can cause alveolar-capillary damage with high permeability pulmonary edema and alteration of lung mechanics, resulting in severe gas exchange abnormalities (Dreyfuss and Ricard, 2005). Several animal models have been developed to investigate the pathophysiologic mechanisms of ARDS. In particular, the *in vivo* intratracheal administration of lipopolysaccharide (LPS), a component of the wall of gram-negative bacteria, has gained wide acceptance as a clinically relevant model of severe lung inflammation (Antonicelli et al., 2004). The inhalation of LPS results in acute, neutrophilic inflammation of the distal air

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spaces of the lungs. The molecular mechanisms underlying the inflammatory response to LPS involve the detection of LPS by pattern recognition receptors, followed by the coordinated expression of proinflammatory cytokines (TNF-α and IL-1β), chemokines (KC or IL-8), and adhesion molecules (ICAM-1 and VCAM-1), these then direct the emigration of neutrophils across the endothelial and epithelial barriers that separate the bloodstream from the pulmonary air spaces (Martin, 2000; Martin et al., 1992; Puneet et al., 2005). The presence of infiltrating leukocytes is the hallmark of pulmonary inflammation associated with acute lung injury (Heflin and Brigham, 1981). β-chemokines (CXC) such as IL-8 (in human), and keratinocyte-derived chemokine (KC) (in mice), mostly attract neutrophils, monocytes, T cells, basophils, and eosinophils (Gerard et al., 1997). In addition, the production of anti-inflammatory cytokine might limit the severity of the inflammatory response without interfering with the beneficial components of host defense and immunity (Varley et al., 1995). These cytokines and adhesion molecules can amplify this response by stimulating the NF-κB or AP-1-dependent induction of proinflammatory cytokines in cells (Li and Verma, 2002; Chen et al., 2004; Sadikot et al., 2003).

Hesperidin (HES, 5,7,3′-trihydroxy-4′-methoxy-flavanone 7-rhamnoglucoside, CAS number: 520-26-3) inhibits inflammation, hypotension and analgesia, and nitric oxide synthase (Boisseau, 2002; Olszanecki et al., 2002). A study recently reports that HES could reduce TNF-α production and inhibits infection-induced lethal shock (Kawaguchi et al., 2004). The mechanisms for those effects are not clear, but the results of the aforementioned studies support the concept that HES could be an immunomodulator agent in severe systemic inflammation. Although the anti-inflammatory effects of HES *in vitro* have been characterized on rodent cell lines and human cell lines (Sakata et al., 2003; Choi et al., 2003). There is no information regarding the influence of HES treatment on lung inflammation. We therefore evaluated the preventive effects of HES on acute lung inflammation induced by the intratracheal instillation of LPS *in vivo*.

Materials and methods

Materials

Unless otherwise specified, all reagents and media were purchased from Sigma Chemical (Deisenhofen, Germany). Recombinant human TNF-α was purchased from BD Pharmingen (San Diego, CA, USA) and IL-1β was from R&D Systems (Minneapolis, MN, USA). Hesperidin (HES) was dissolved in dimethyl sulfoxide (DMSO: Wako Pure Chemical Industries) (stock concentration is 80 mM) for *in vitro* and suspended in saline (1 mM) for *in vivo* experiments.

Mice

Male BALB/c mice, 6 weeks old and weighing 30 g, were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (30 mg/kg) given intraperitoneally. Intratracheal administration of LPS (*Escherichia coli*, 0055:B5) was performed with a bent 27G tuberculin syringe (in a volume of 50 μL). The cervical incision was closed with 5-0 silk suture and the mice were returned to their cage. The animals recovered rapidly after surgery. Animals were challenged with intratracheal LPS (100 μg/kg), or treated with 100 μL HES (200 mg/kg), administered orally 30 min before LPS-treated. In addition, a group of naïve group was not challenged.

Lung histology

Histopathologic changes induced by LPS were evaluated in five mice treated with HES and five mice treated with vehicle. 24 h after surgery, the animals were anesthetized and killed by exsanguinations, and the lungs were inflated-fixed with 4% paraformaldehyde. Paraffin embedded lungs were sectioned at 3 μm and stained with hematoxylin and eosin for morphologic analysis.

Bronchoalveolar lavage

Twenty-four hours after the surgery, the mice were reanesthetized. The animals were bled by transaction of the inferior vena cava to reduce hemorrhage into the lungs. Bronchoalveolar lavage was performed by the intratracheal instillation of 1.5 mL PBS into the exposed lungs. The lavage fluid was infused a total of two times into the lungs before final collection. The bronchoalveolar lavage fluid (BALF) was then centrifuged and the cell-free supernatant was frozen at −80 °C until further analysis. The cells were resuspended in a volume of 0.5 mL (0.08% Trypan blue) and total cell counts were performed with a hemocytometer.

Protein assay

The amount of proteins in the BALF was assayed using the Bradford assay. Proteins are expressed in mg protein/mL BALF.

Nitrate/nitrite concentration

The pulmonary production of nitric oxide was determined by the measurement of nitrate and nitrite, the stable end products of nitric oxide metabolism, in the BALF. After 1 mmol/L NaNO2 was diluted by means of doubling series, it was mixed with equal Griess reagent (Sigma Chemicals, St. Louis, MO), and then shaken for 10 min lightly at room temperature. At last, absorbance value (*A*) of triplicate samples was read on ELISA reader using a test wavelength of 550 nm.

Cell culture

Culture of A549 cells (cell line with alveolar type II epithelial cell characteristics, CCL 185, American Type Culture Collection, Manassas, VA) was seeded on the upper or lower side of polycarbonate filter inserts (5 mm pore size, diameter, 6.5 mm, Costar, Cambridge, MA) and cultured in DMEM medium (Life Technologies, Eggenstein, Germany) containing