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Asiatic acid inhibits pulmonary inflammation induced by cigarette smoke

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ABSTRACT

Asiatic acid (AA) is one of the major components of Titrated extract of *Centella asiatica* (TECA), which has been reported to possess antioxidant and anti-inflammatory activities. The purpose of this study was to investigate the protective effect of AA on pulmonary inflammation induced by cigarette smoke (CS). AA significantly attenuated the infiltration of inflammatory cells in bronchoalveolar lavage fluid (BALF) of CS exposure mice. AA also decreased ROS production and NE activity, and inhibited the release of proinflammatory cytokines in BALF. AA reduced the recruitment of inflammatory cells and MCP-1 expression in lung tissue of CS exposure mice. AA also attenuated mucus overproduction, and decreased the activation of MAPKs and NF-kB in lung tissue. Furthermore, AA increased HO-1 expression and inhibited the reduced expression of SOD3 in lung tissue. These findings indicate that AA effectively inhibits pulmonary inflammatory response, which is an important process in the development of chronic obstructive pulmonary disease (COPD) via suppression of inflammatory mediators and induction of HO-1. Therefore, we suggest that AA has the potential to treat inflammatory disease such as COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a serious global health problem, which has a high risk of morbidity and mortality [1]. COPD is characterized by chronic airway inflammation, mucus overproduction and progressive airflow limitation, and is a common preventable and treatable disease [2]. Cigarette smoke (CS) is the major environmental risk factor of COPD [3]. CS is a rich source of oxidants and free radicals [4], and induces oxidative stress and airway inflammation resulting in neutrophilic inflammation and mucus overproduction [5,6].

Increased neutrophils have been found in the bronchial wall and lumen of COPD patients [6,7]. Neutrophils secrete various stimulatory mediators including cytotoxic proteins, reactive oxygen species (ROS), proinflammatory cytokines and proteolytic enzymes such as elastase, resulting in aggravation of airway inflammation, emphysema and mucus overproduction [8]. ROS are key signaling molecules that play an important role in the progression of oxidative stress and inflammation [9,10]. Increased generation of ROS accelerated the development of COPD through NF-kB signaling pathway [11]. Increased neutrophil elastase (NE) activity enhanced emphysema-like phenotype resulting in destruction of alveolar structure [12]. Tumor necrosis factor α (TNF-α) is a major inflammatory cytokine in many immune-mediated diseases [13], and constitutive overexpression of TNF-α in lung of animal model show pathological features of COPD such as infiltration of the inflammatory cells and emphysema [14]. Interleukin 6 (IL-6) is a proinflammatory cytokine that plays an important role in the systemic inflammation of COPD [15]. Monocyte chemoattractant protein-1 (MCP-1) is one of the key chemokine which triggers the infiltration of inflammatory cells such as neutrophils and macrophages into sites of immune response [16–18]. Airway mucus overproduction is common feature of chronic airway inflammatory diseases [19]. In COPD patients, mucus is viscous, and mucociliary transport impairment leads to local stagnation of mucus causing bacterial infections and other recurrent airway infections [20]. Mucus overproduction is inhibited by the pharmacological agents that block prominent signaling molecules such as mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-kB) [3,21–23]. MAPKs signaling pathway plays a key regulatory

Abbreviations: COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid; TECA, Titrated extract of Centella asiatica; AA, Asiatic acid; IL-6, interleukin 6; TNF-α, tumor necrosis factor α;; ROS, reactive oxygen species; NE, neutrophil elastase; MCP-1, monocyte chemoattractant protein-1; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-kappa B;; IκB, inhibitor of NF-κB; SOD, superoxide dismutase; HO-1, heme oxygenase-1.

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role in inflammation and is molecular target for inflammation-associated diseases [24,25]. NF-kB also plays a major role in mediating mucus overproduction and proinflammatory cytokines [26]. Heme oxygenase 1 (HO-1) is the inducible stress protein that implicates a cytoprotective role against the toxic agents [27]. HO-1 has been reported as a protective mediator in CS-induced COPD [27]. Superoxide dismutase 3 (SOD3) reduce oxidative stress and tissue damage, and has been shown to protect pulmonary emphysema [28]. Decreased SOD3 activity has been reported in a rat model of smoke-induced emphysema [29].

Titrated extract of *Centella asiatica* (TECA) is used as a therapeutic agent in wound healing, and has been reported to possess anti-cancer [30], anti-aging [31,32], antioxidant and anti-inflammatory effects [33]. Asiatic acid (AA) is one of major components of TECA, which is a well-known herbal medicine for its excellent pharmacological activities such as anti-tumor and antibacterial activities in China and in the West [34–36]. Ramachandran et al. have reported that AA possesses antihyperglycemic effect on streptozotocin-induced diabetic rat by modulating the key regulatory enzymes [37]. AA also has been shown to possess antioxidant and anti-inflammatory activities in human bronchial epithelial cells [38]. Recently, it has been reported that AA has neuroprotective effect on oxidative stress-mediated apoptosis in SH-SYS5Y cells [39]. However, protective effect of AA is not investigated in pulmonary inflammation induced by CS.

2. Materials and methods

2.1. Materials

Asiatic acid (AA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). AA was solubilized with 1% dimethyl sulfoxide (DMSO) with 1% tween 20 in PBS.

2.2. Mouse models of pulmonary inflammation induced by cigarette smoke (CS) exposure

Specific pathogen-free male C57BL/6 (6 weeks) mice were purchased from Koatech Co (Pyeongtaek, Korea) and used after 1 week of quarantine and acclimatization. Mice were randomly divided into six groups ($n = 7$ each group): NC; normal control, Vehicle; 1% DMSO with 1% Tween-20 in PBS, CS; cigarette smoke, ROF; CS + roflumilast (10 mg/kg, p.o), and AA15 or 30; CS + asiatic acid (15 or 30 mg/kg, p.o). CS exposure was performed as previously described [3]. In brief, CS exposure was generated by exposing to 8 sticks of 3R4F research cigarettes, containing 11.0 mg of total particulate matter, 9.4 mg of tar, and 0.76 mg of nicotine per cigarette (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) for 1 h a day for 21 consecutive days. LPS was instilled intranasally on 19 (3 μg dissolved in 30 μl distilled water). AA and ROF were given by an oral injection for 11 days (11 − 21) 1 h before CS exposure [3]. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology and performed in compliance with the National Institutes of Health Guidelines for the care and use of laboratory animals and Korean national laws for animal welfare.

2.3. Measurement of inflammatory cells in BALF

BALF collection was performed as previously described [40]. In brief, mice were given an intraperitoneal injection of a pentobarbital (100 mg/kg; Hanlim Pharm, Co., Seoul, Korea) 24 h after the last challenge, and a tracheostomy was performed. To obtain BALF, ice-cold PBS (0.7 ml) was infused into lung and withdrawn via tracheal cannulation two times (total volume, 1.4 ml). The numbers of inflammatory cells were counted using a hemocytometer. To determine differential cell counts, 100 μl of BALF was centrifuged onto slides using a Cytospin (Hanil Science Industrial, Seoul, Korea) for 5 min at 1500 rpm. The slides were dried, and the inflammatory cells were fixed and stained using Diff-Quik® staining reagent (B4132-1A; IMEB Inc., Deerfield, IL) according to the manufacturer's instruction.

2.4. Measurement of the levels of ROS production and NE activity in BALF

The effect of AA on ROS generation was determined 2′,7′-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich). Briefly, the cells in BALF washed with PBS and incubated with 20 μM DCF-DA for 10 min at 37 °C. Then the activity of intracellular ROS was observed under the fluorescence microscope at 488 nm excitation and 525 nm emission (Perkin-Elmer, Waltham, MA, USA) [6]. The activity of neutrophil elastase (NE) was measured using *N*-succinyl-(Ala)3-*p*-nitroanilide (Sigma-Aldrich) in 37 °C for 90 min, according to the protocol described by Sakuma et al. [41].

2.5. Measurement of the releases of proinflammatory cytokines in BALF

The levels of proinflammatory cytokines (TNF-α and IL-6) in BALF were measured according to the manufacturer's protocols (R&D System, china). The absorbance was measured at 450 nm using an ELISA reader (Molecular Device) [6].

2.6. Western blotting

Lung tissues were obtained 6 or 24 h after the last challenge with AA. The levels of MAPKs and NF-kB activation were assessed using lung tissues that were obtained 6 h after the last challenge with AA. The expression of MCP-1, HO-1 and SOD3 were determined with lung tissues that were obtained 24 h after the last challenge with AA. Protein samples were denatured and resolved on 12% SDS polyacrylamide gels and transferred to Hyond PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated with blocking solution (5% skim milk) in TBST for 1 h at room temperature. Specific antibodies against phosphorylated (*p*)-ERK, ERK, *p*-p38, *p*-38, *p*-JNK, JNK (rabbit polyclonal antibody, Cell signaling Technology; 1:1000), HO-1, MCP-1 (rabbit polyclonal antibody, Abcam, 1;1000), p65, *p*-p65, *p*-IkB, SOD3 (rabbit polyclonal antibody, Santa Cruz, 1;1000), and β-actin (rabbit polyclonal antibody, Cell signaling Technology; 1:2500) were incubated overnight at 4 °C with 5% skim milk. The membranes were washed three times with TBST, and then developed with enhanced Chemiluminescence (ECL) Kit (Thermo).

2.7. Histology

After BALF samples were collected, lung tissues were detached from the mice and fixed in 10% (v/v) neutral buffered formalin solution. For histological examination, lung tissues were embedded in paraffin, sectioned at 4 μm thickness using a rotary microtome, and stained with a hematoxylin and eosin (H&E) solution (Sigma-Aldrich) and periodic acid-Schiff (PAS) to estimate inflammatory response and mucus production, respectively.

2.8. Statistical analysis

All values shown in the figures are expressed as the mean ± SD obtained from at least three independent experiments. Statistical significance was carried out by two-tailed Student's *t*-test. Data with values of $p < 0.05$ were considered as statistically significant. Single (*) and double (**) marks represent statistical significance in $p < 0.05$ and $p < 0.01$, respectively.