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Casticin, an active compound isolated from Vitex Fructus, ameliorates the cigarette smoke-induced acute lung inflammatory response in a murine model

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ABSTRACT

The aim of this study was to determine of the effect of casticin, as an anti-inflammatory agent, on an acute lung inflammation in vivo model established through exposure to cigarette smoke (CS). Casticin is a phytochemical from *Vitex* species such as *Vitex rotundifolia* and *Vitex agnus-castus* that was recently shown to exert an anti-inflammatory effect in vivo. To demonstrate the effects of casticin, C57BL/6 mice were whole-body exposed to mainstream CS or fresh air for two weeks and treated with 1, 2, and 10 mg/kg casticin via an i.p. injection. Immune cell infiltrations and cytokine productions were assessed from bronchoalveolar lavage Fluid (BALF), and lung histological analysis was performed. Treatment with casticin was observed to significantly inhibit the numbers of total cells, neutrophils, macrophages, and lymphocytes and reduce the levels of proinflammatory cytokines and chemokines in the BALF. In addition, casticin significantly decreased the infiltration of peribronchial and perivascular inflammatory cells and the epithelium thickness. The results of this study indicate that casticin has significant effects on the lung inflammation induced by CS in a mouse model. According to these outcomes, casticin may have therapeutic potential in inflammatory lung diseases, such as chronic obstructive pulmonary disease (COPD).

1. Introduction

Chronic obstructive pulmonary disease (COPD) represents severe and important airway disease in global health that has constant abnormal inflammatory response and show accelerated decline in lung function leading to airflow limitation. [1].

Although the etiology and pathogenesis of COPD are poorly understood, it is generally agreed that cigarette smoke (CS) is the most important etiologic factor in the development of COPD [2]. CS exposure induces lung inflammation, and the established animal models exhibit several morphological and pathophysiological features similar to human COPD. These include the release of cytokines and chemokines from structural cells and alveolar macrophages, leading to an influx of inflammatory cells, such as neutrophils, lymphocytes and macrophages [3]. Additionally, CS contains excessive concentrations of reactive oxygen species, such as superoxide anion (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2), which can induce barrier dysfunction in lung epithelial cells and immune tolerance toward lung homeostasis likely due to the activation of transcription factors such as nuclear factor (NF)-κB. An exacerbated immune response with an imbalance of proinflammatory cytokines and reactive oxygen metabolites leads to tissue damage [4].

Roflumilast is an approved drug for the treatment of lung diseases, particularly COPD. However, side effects, such as vomiting, diarrhea, nausea, weight loss, emesis, headache, insomnia, dizziness, and decreased appetite, potentially limit the utility of roflumilast [5].

Recently, many researchers who are related in complementary and alternative medicine (CAM) are getting interested in investigating the components of medicinal herbs [6]. Casticin is one of the principal components of the herbs such as Vitex and Viticis Fructus and has been reported to exert anti-oxidant and anti-inflammatory effects and exhibit anticancer activities [7,8]. We hypothesized that casticin has potent anti-inflammatory effects on a CS-induced lung inflammation in vivo model. Herein, we show the first evidence the anti-inflammatory effects of casticin in terms of both immunological changes, such as decreased levels of proinflammatory cytokines (TNF-α and IL-6) and chemokines (KC and MCP-1), and histological changes in the lung. This study demonstrates that casticin is a potent therapeutic agent against lung inflammation in a CS-induced in vivo model.

2. Materials and methods

2.1. Animals

Female C57BL/6 (littermates, 7 to 8 weeks of age, weighing 20–25 g) wild-type mice were purchased from The Charles River Korea (Seung

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nam, South Korea). The mice were maintained under pathogen-free conditions with air conditioning and a 12-h light/12-h dark cycle. In addition, all of the mice had free access to food and water during the experiments. The study was conducted according to the Rules for Animal Care and the Guiding Principles for Animal Experiments Using Animals by the University of Kyung Hee Animal Care and Use Committee and in accordance with the recommendations of the "The use of non-human primates in research" report published by Weather all (KHUASP (SE)-12-015).

2.2. Reagent

The natural compound casticin, which is a flavonoid isolated from Vitex Fructus was purchased from Chromadex, Inc. (Irvine, CA, USA). It has a molecular weight of 374.34, appears as yellow crystals, and has a purity of 99.0%. Casticin was dissolved in 4% HCl and then diluted with phosphate-buffered saline (PBS). Roflumilast (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), which was used as a positive control, was also dissolved in 0.1% DMSO and then diluted with PBS.

2.3. Experimental protocol and design

The mice were placed into chamber (total volume = 170 L) (Live Cell Instrument, Seoul, South Korea) and exposed to 3R4F reference (without filter; University of Kentucky, Lexington, KY, USA) for 2 weeks with four times a day (5 cigarettes per time). Exposure to the smoke from each time lasted for 30 min and followed by a 30 min smoke-free interval [9]. The mice were exposed with fresh air or CS. The mice were randomly divided into six groups, namely, Air; fresh air exposed, CS; CS exposed, CS + Rofl; roflumilast (5 mg/kg, oral) treated with CS exposure, and CS + (casticin 1, 2 and 10 mg/kg); casticin treated (i.p.) with CS exposure. Roflumilast or casticin were treated on day 2 to 5 and 7 to 12. On day 13, the mice were sacrificed to acquire samples of the BALF and lung tissues for subsequent analyses.

2.4. Analysis of inflammatory cell profiles

The lungs were lavaged and pooled three times with 1 ml of ice-cold PBS (mean volume 2.0 ± 0.2 ml). Bronchoalveolar Lavage Fluid (BALF) was centrifuged at 300 g for 10 min at 4 °C. The supernatants were stored in - 80 °C in an ultraslow freezer. The pellet the BALF cells were resuspended in 1 ml of PBS. The total viable cells in the resulting pellet were counted. To count the differential cells (neutrophils, macrophages and lymphocytes), BALF cells adhered to glass slides using cytospin (Sandon, Waltham, MA, USA) with Diff-Quick staining (Life Technologies, Auckland, New Zealand). When performing the BALF cell counts under a light microscope, one researcher randomly selected a BALF cell slide and a blinded researcher performed the BALF cell count. The results are expressed as the cell number $\times 10^4$.

2.5. Assessment of proinflammatory cytokines and chemokines

The concentrations of the proinflammatory cytokines (TNF-α, IL-6 and IL-1β) and chemokines (KC and MCP-1), were measured using a quantitative sandwich enzyme-linked immunoassay kit (BD Pharmingen, San Diego, CA, U.S.A. for TNF-α, IL-6, IL-1β and MCP-1; R&D, Minneapolis, MN, USA for Mouse CXCL1/KC) according to the manufacturer's protocols. The optical density (OD) was measured at 450 nm using a microplate reader (SOFT max PRO software, Sunnyvale, CA, USA). For standardization purposes, the optical densities obtained for TNF-α, IL-6, IL-1β, KC and MCP-1 were each divided by the total protein concentrations of the respective BALF samples. The total protein concentrations were determined using a BCA kit (Pierce Biotechnology Inc., IL, USA) according to the manufacturer's protocol.

2.6. Histologic analysis

Hematoxylin and eosin (H&E) staining was performed for inflammation index, and periodic acid Schiff (PAS) staining was performed for quantification of the epithelium thickness. After the lung tissues were removed from the mice, the right lower lobes of the lung were removed for histological analysis, and 4% paraformaldehyde fixing solution was infused into the lungs. The specimens were dehydrated and embedded in paraffin. For histological examination, 4-μm-thick sections of the embedded tissue were cut on a rotary microtome, placed on glass slides, deparaffinized, and stained sequentially with H&E and PAS. Images of the lung tissue sections were acquired with an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus, Tokyo, Japan) under 200× and 400× magnification. Lung inflammation of H&E stained lung sections was evaluated on a subjective score 0 to 5 on randomized, blinded sections by five independent readers. All sections were scored from 0 to 5 by readers according to the following criteria: 0 = normal; 1 = very mild; 2 = mild; 3 = moderate; 4 = marked; 5 = severe inflammation [10]. For quantification of the epithelium thickness in the lung, all sections were obtained under 400× magnification, and epithelium thickness was determined by taking sections measurements from randomly selected four representative areas per bronchus to determine that bronchus average using the Image Pro-Plus 5.1 software (Media Cybernetics, Inc., Silver Spring, MD, USA) [11]. The slides were mounted with Canadian balsam (Showa Chemical Co. Ltd., Tokyo, Japan).

2.7. Analysis of systemic toxicity by casticin

Blood samples from mice were used for toxicological diagnostic reasons. Fresh air exposed mice were received casticin (1, 2, and 10 mg/kg body wt, i.p.) on days 2 to 5 and 7 to 12. On day 13, the mice were sacrificed and blood samples were collected. Blood samples were left at room temperature for 1 h and then centrifuged for 10 min 2500 *g* to separate the serum. The serums were directly analyzed and then stored at −80 °C. The levels of serum blood urea nitrogen (BUN), Creatinin, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvate transaminase (GPT) were determined by FUJI DRI-CHEM 3500i. instrument (Fuji Photo Film Ltd., Tokyo, Japan) according to instructions given by the manufacturer.

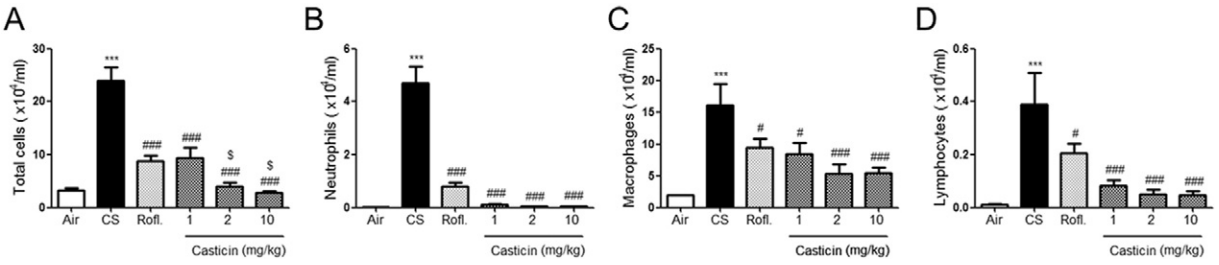

Fig. 1. Total and cellular profiles in the BALF. The (A) total cells, (B) neutrophils, (C) macrophages and (D) lymphocytes in the BALF from the lungs of the mice were counted. The statistical analyses were conducted by one-way ANOVA followed by Newman–Keuls multiple comparison test ($^{***}p < 0.001$ vs. air, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ vs. CS, $^{\$}p < 0.05$ vs. Rofl.; n = 5–8).