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Genipin inhibits allergic responses in ovalbumin-induced asthmatic mice

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ABSTRACT

Genipin is a natural compound isolated from the fruit of *Gardenia jasminoides* with various pharmacological effects. In this study, we investigated whether genipin effectively alleviates allergic responses in a murine model of ovalbumin (OVA)-induced asthma. The mice were administered an intraperitoneal injection of OVA on day 0 and 14 to boost the immune response; genipin was then administered from day 18 to 23 by oral gavage. On days 21 to 23, mice were OVA-challenged using am ultrasonic nebulizer, and airway hyperresponsiveness (AHR) was determined on day 24 by plethysmography. Genipin significantly reduced the inflammatory cell count in bronchoalveolar lavage fluids (BALF) and AHR, which were accompanied by lower interleukin-5 (IL-5), IL-13 and OVA-specific immunoglobulin (Ig) E levels in the BALF or serum from OVA-induced asthmatic mice. In histology, genipin significantly decreased airway inflammation and mucus hypersecretion in OVA-induced asthmatic mice. Additionally, genipin inhibited OVA-induced increases in the expression of inducible nitric oxide synthase and cyclooxygenase-2 proteins. Further, genipin reduced the activity and protein levels of matrix metalloproteinase-9 in lung tissue from OVA induced asthmatic mice. Overall, genipin effectively alleviated the asthmatic inflammatory response in an OVA-induced asthmatic model. Therefore, our results suggest that genipin has therapeutic potential for treating asthma.

1. Introduction

Asthma is an important pulmonary disease that has markedly increased in recent decades [1] and is commonly induced by allergen exposure, including house dust, pollen, animal dander, and smoking [2,3]. Further, asthma is characterized by eosinophil accumulation, mucus hypersecretion, and airway hyperresponsiveness (AHR) [4]. These pathophysiological changes are closely associated with elevated allergen-specific type 2 T helper (Th2) cytokines, including interleukin (IL)-4, -5, and -13. Th2 cytokines are involved in eosinophil maturation, infiltration, and activation, as well as IgE and mucus production, which eventually induce the asthmatic responses [5]. Other signaling molecules related to the inflammatory response, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and matrix metalloproteinase (MMP)-9, also play a critical role in the progression of airway inflammation in allergic asthma [6,7]. In addition to inducing airway inflammation, COX-2 and iNOS aggravate the inflammatory response in damaged lesions, while increased MMP-9 expression plays a role in the development of asthma by inducing inflammatory cell infiltration into lung tissue, and finally, airway remodeling [7]. Therefore, suppression of the inflammatory response is considered an important step for controlling asthma.

Genipin is an aglycon of geniposide and a natural iridoid compound extracted from the fruit of *Gardenia jasminoides* that has various pharmacological properties, including antioxidative, anti-inflammatory, anticancer, and antifungal activities [8,9]. According to previous studies, the anti-inflammatory effects of genipin are exerted via iNOS and COX-2 suppression [10]. It has also been reported that MMP-9, a protein related to inflammation and tissue remodeling, is regulated by genipin [9] and the protective effects of genipin against acute lung injury have been previously investigated [11,12]. Despite the known anti-inflammatory and pharmacological properties of genipin against pulmonary disease, the antiasthmatic of genipin has not been previously explored.

Therefore, we investigated the protective effects of genipin in an ovalbumin (OVA)-challenged asthmatic mouse model by measuring inflammatory cells, cytokine levels and mucus secretion. Additionally, we elucidated the underlying mechanisms associated with the effects of genipin by focusing on the anti-inflammatory response, specifically the expression of iNOS, COX-2 and MMP-9.

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2. Materials and methods

2.1. Animals

BALB/c female mice (6-week-old, Koatech Co., Pyeongtaek, Republic of Korea) were used after 1 week of quarantine and acclimatization. The mice were housed under standard condition and provided sterilized tap water and standard rodent chow (Samyang Feed, Wonju, Republic of Korea) ad libitum. The Institutional Animal Care and Use Committee of Chonnam National University approved the protocols for the animal study (CNU IACUC-YB-R-2016-19), and the animals were cared for in accordance with the National Institutes of Health Guidelines for the care and use of laboratory animals and Korean national laws for animal welfare.

2.2. Ovalbumin-induced asthma and experimental groups

The animals were treated with 20 μg OVA (Sigma-Aldrich Co., St. Louis, MO, USA) with 2 mg aluminum hydroxide (Sigma-Aldrich Co.) by intraperitoneal injection on day 0 and 14 and then airway challenged using an OVA solution (1% w/v) with a nebulizer (NE-U12, Omron Co., Tokyo, Japan) for 1 h from day 21 to 23. Dexamethasone and genipin were dissolved in phosphate buffered saline (PBS) and administered to the mice by oral gavage from day 18 to 23. The animals were grouped into five groups (n = 7) as follows: NC (normal control, PBS sensitization/challenge), OVA (OVA sensitization/challenge); DEX (OVA sensitization/challenge + dexamethasone 3 mg/kg); and GEN-10 or 20 (OVA sensitization/challenge + genipin 10 or 20 mg/kg, respectively).

2.3. Airway hyperresponsiveness

AHR was evaluated by whole-body plethysmography (OCP3000, Allmedicus, Seoul, Republic of Korea) 24 h after the final OVA challenge. Each animal was placed on a plastic chamber and exposed to methylcholine (Sigma-Aldrich) aerosols via a nebulizer at increasing concentrations (0, 10, 20, and 30 mg/mL) for 3 min. Finally, enhanced pause (Penh) value was measured for 3 min after the methylcholine exposure.

2.4. Analysis of bronchoalveolar lavage fluid

The animals were anesthetized with Zoletil 50® (10 mg/kg, i.p.; Virbac Laboratories, Carros, France) 24 h after measuring AHR, and blood was collected via the caudal vena cava. To separate serum, blood was incubated for 1 h at room temperature and the supernatant collected after centrifugation at 200*g* for 10 min. BALF was collected as previously described [13]. Specifically, a tracheotomy was performed, and an endotracheal tube was inserted into the trachea. Ice-cold PBS (0.7 mL) was infused into the lung and withdrawn two times (total volume 1.4 mL). To count inflammatory cells, 100 μL BALF was centrifuged (200*g*, 4 °C, 10 min) onto slides using a cytospin (Hanil Science Industrial, Seoul, Korea). The slides were dried, fixed, and stained with Diff-Quik® reagent (Sysmex Co., Kobe, Japan). The remaining BALF was centrifuged (200*g*, 4 °C, 10 min), and its supernatant was stored at − 80 °C for Th2 cytokine analysis. IL-5 and IL-13 levels were determined in BALF via a commercial enzyme-linked immunosorbent assay (ELISA, R & D System Inc., Minneapolis, MN, USA).

2.5. Analysis of OVA-specific immunoglobulin (Ig) E in serum

The production of OVA-specific IgE in serum was evaluated using an ELISA kit (BioLegend, San Diego, CA, USA). Specifically, 96-well microtiter plates were coated overnight with OVA (10 μg/mL in PBS-Tween-20). After incubation, the 100 times diluted samples were added to each well and the plates were washed, blocked and incubated for an additional 2 h. The plates were then washed, horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgE antibodies were added, and the plates were washed again. *o*-Phenylenediamine dihydrochloride (200 μL, Sigma-Aldrich) was then added, and the plates were incubated for 10 min in the dark. Absorbance was measured at 450 nm using an ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The sigmaplot (Systat software, Inc., San Jose, CA) was used to plot the logistic, 4-parameter graph which was used to calculate the measured absorbaces.

2.6. Histopathological analysis of the lung tissue

After BALF collection, the lung tissue was fixed in neutral buffered formalin, embedded in paraffin, and sectioned 4-μm thick. The tissues were stained with hematoxylin and eosin (H & E) solution (Sigma-Aldrich) to evaluate airway inflammation and periodic acid Schiff (PAS) (IMEB Inc., San Marcos, CA, USA) to measure mucus production. Each slide was examined manually in a totally blinded manner using a light microscope (Leica, Wetzlar, Germany) with 10 and 20 × objective lenses and a 100 × oil immersion lens. Ten randomly selected non-overlapping areas per slide were captured with digital camera (IMTcamCCD5; IMT Inc., Daejeon, Republic of Korea), and quantitative analyses of airway inflammation and mucus production were performed using an image analyzer (IMT i-Solution software, Vancouver, BC, Canada).

2.7. Analysis of MMP-9 in lung tissue

To estimate MMP-9 activity, gelatin zymography was carried out as described previously [14]. Briefly, equal amounts of total cellular protein (30 μg) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 1% gelatin (Sigma-Aldrich) as an MMP substrate. Quantitative analysis of MMP-9 activity was performed via a ChemiDoc (Bio-Rad Laboratories).

To determine MMP-9 expression, we performed immunohistochemistry (IHC) per a previous report [13]. Paraffin-embedded sections were deparaffinized, dehydrated, washed with PBS containing 0.3% Triton X-100, and incubated for 10 min at room temperature with 10% goat serum to block non-specific staining. The slides were incubated overnight with primary mouse MMP-9 antibodies (1:200 dilution, Santa Cruz Biotechnology, Dallas, TX, USA). After incubating with the primary antibodies, the slides were washed and incubated with biotinylated secondary antibodies at 37 °C for 1 h and then incubated with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The excess complex was removed and slides were washed with PBS prior to incubating in 0.05% diaminobenzidine (1:200, Millipore Co., Bedford, MA) for 10 min. The slides were counterstained, rinsed with PBS to terminate the reaction, and protected with coverslips prior to microscopic examination. Each slide was examined manually in a totally blinded manner using a light microscope (Leica) with 10 and 20 × objective lenses and a 100 × oil immersion lens. Ten randomly selected non-overlapping areas per slide were captured with digital camera (IMTcamCCD5; IMT Inc.), and quantitative image analysis of MMP-9 expression was conducted via IMT i-Solution software.

2.8. Western blot analysis

Lung tissue was homogenized with tissue lysis reagent (Sigma-Aldrich) containing a protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations in the samples were determined using Bradford reagent (Bio-Rad Laboratories). Western blotting was performed as described previously [13], and following the antibodies were used: iNOS (Cell Signaling, Danvers, MA, USA), COX-2 (Cell Signaling), β-actin (Cell Signaling) and MMP-9 (Santa Cruz Biotechnology). To determine protein expression, band densities were quantified using a ChemiDoc (Bio-Rad Laboratories).

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