

D-pinitol regulates Th1/Th2 balance via suppressing Th2 immune response in ovalbumin-induced asthma

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Received 18 September 2006; revised 10 October 2006; accepted 27 November 2006

Available online 11 December 2006

Edited by Beat Imhof

Abstract D-pinitol has been demonstrated to exert insulin-like and anti-inflammatory activities. However, its anti-allergic effect in the Th1/Th2 immune response is poorly understood. Recently, it was shown that T-bet and GATA-3 are master Th1 and Th2 regulatory transcription factors. In this study, we have attempted to determine whether D-pinitol regulates Th1/Th2 cytokine production, T-bet and GATA-3 gene expression in OVA-induced asthma model mice. We also examined to ascertain whether D-pinitol could influence eosinophil peroxidase (EPO) activity. After being sensitized and challenged with ovalbumin (OVA) showed typical asthmatic reactions. These reactions included an increase in the number of eosinophils in bronchoalveolar lavage (BAL) fluid, an increase in inflammatory cell infiltration into the lung tissue around blood vessels and airways, airway luminal narrowing, and the development of airway hyper-responsiveness (AHR). The administration of D-pinitol before the last airway OVA challenge resulted in a significant inhibition of all asthmatic reactions. Accordingly, this study may provide evidence that D-pinitol plays a critical role in the amelioration of the pathogenetic process of asthma in mice. These findings provide new insight into the immunopharmacological role of D-pinitol in terms of its effects in a murine model of asthma, and also broaden current perspectives in our understanding of the immunopharmacological functions of D-pinitol.

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Keywords: Asthma; Ovalbumin; D-pinitol; MMP-9; GATA-3; T-bet; IL-4; IL-5; AHR; Eotoxin

1. Introduction

Asthma is a chronic inflammatory lung disease that is characterized by airway hyper-responsiveness to allergens, airway edema, and increased mucus secretion. A propensity for aller-

gic responses, atopy, is associated with the development of asthma [1]. OVA-induced asthma is characterized by AHR and inflammation of the airways [2]. This inflammation is associated with the infiltration of eosinophils, neutrophils, and lymphocytes into the bronchial lumen and lung tissues [2,3]. These cellular infiltrates release various chemical mediators that can cause AHR [4–6]. Recruitment of these inflammatory cells from the blood to the site of inflammation is regarded as a critical event in the development and persistence of airway inflammation. Inflammatory cells have to cross the basement membrane and move through connective tissue until they finally reach inflammatory sites, and require the involvement of adhesion molecules, cytokines, chemokines, and enzymes such as matrix metalloproteinases (MMPs) in this journey. The MMPs are a family of zinc- and calcium-dependent endopeptidases capable of proteolytically degrading many of the components of the extracellular matrix [7]. MMPs are produced not only by structural cells [8,9], but also by inflammatory cells [10–13]. They are secreted in their latent forms, followed by proteolytic processing to the active forms [7]. Of the MMP family, MMP-2 (gelatinase A, 72-kDa gelatinase) and MMP-9 (gelatinase B, 92-kDa gelatinase) share similar domain structures and in vitro matrix substrate specificities [14], and appear to induce the migration of eosinophils, lymphocytes, neutrophils, and dendritic cells across basement membranes during tissue injury and repair [15,16]. Recruitment of leukocytes from the circulating blood into tissues requires a series of cell adhesion molecules, including ICAM-1 and VCAM-1, which are shown to play important roles in the induction of airway inflammation.

The flavonoids comprise a family of common phenolic plant pigments, which have been identified as dietary anticarcinogens and antioxidants [17]. We reported previously that a variety of phytochemicals exhibit profound immunoregulatory activity, particularly in DC [18–20]. D-pinitol (3-*O*-methyl-chiroinositol), an active component of the traditional antidiabetic plant, *Bougainvillea spectabilis*, reportedly exerts insulin-like effects. Pinitol is also known to exert insulin-like effects, via the driving of creatine and other nutrients into muscle cells [21]. In addition, D-pinitol has been suggested to possess multifunctional properties, including anti-inflammatory activity

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[22], and has also been implicated in the prevention of cardiovascular diseases [23].

In conclusion, it has been shown using animal models that allergic airway inflammation is impaired by Th2 cytokine production and decreased Th1 cytokine production. In a recent study, it was suggested that T-bet might protect against asthma through increased expression of GATA-3 mRNA in asthmatic airways [24–26]. This study investigated the role of D-pinitol on T-bet and GATA-3 in a murine model of asthma.

2. Materials and methods

2.1. Animals and experimental protocol

Female BALB/c mice, 6–8 weeks of age and free of murine-specific pathogens, were obtained from the Charles River Laboratories (Yokohama, Japan). All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the Pusan National University Medical School. Mice were immunized intraperitoneally (i.p.) with 20 µg of OVA (Sigma–Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) on days 1 and 15. Mice were challenged for 30 min via the airway with OVA (50 mg/ml of saline) each day from days 21 to 23 on consecutive days. BAL fluid was obtained at 24 h after the last challenge. At the time of lavage, the mice (six mice in each group) were killed with an overdose of ether. The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed saline solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4 °C. A part of each pool was then centrifuged, and the supernatants were kept at –70 °C until use (Fig. 1).

2.2. Administration of D-pinitol

Mice were injected i.p. with 10 or 20 mg/kg/day in 200 µl of D-pinitol (Sigma) each day from days 18 to 20 on consecutive days.

2.3. Total cell counting

The total cell numbers were counted with a hemacytometer. Smears of BAL cells prepared with Cytospin II (Shandon, Runcorn, UK) were stained with Diff-Quik solution (Dade Diagnostics of P.R. Inc., Aguada, PR) for differential cell counting. Two independent, blinded investigators counted the cells, using a microscope. Approximately, 200 cells were counted in each of four different random locations.

2.4. T cell counting by flow cytometric analysis

Equal amount of BAL fluids in each group, 5×10^5 cells were incubated in staining buffer (PBS with 2% FBS and 0.1% sodium azide) containing FITC-conjugated anti-CD3e Ab (BD Pharmingen) for 15 min on ice to block the non-specific binding. Cells stained with the appropriate isotype-matched Ig were used as negative controls. After staining, the cells were fixed with 4% w/v paraformaldehyde and analyzed by FACSCalibur, using CellQuest software (BD Biosciences).

T cell number = BAL Fluids Total cell $\times$ T cell (%)

T cell (%) = T cell number/FACS reading cells (10^4) $\times$ 100

2.5. Measurement of eosinophil peroxidase

The suspension of BAL cells and the pulmonary homogenates were frozen/thawed three times using liquid nitrogen and a water bath at 37 °C to obtain the EPO. The BAL fluid was centrifuged to 4 °C for 10 min and serially diluted in a 96-well plate (75 µl/well) followed by the addition of 150 µl of substrate (1.5 mM o-phenylenediamine and 6.6 mM H₂O₂ in 0.05 M Tris–HCl, pH 8.0). After 30 min at room temperature, the reaction was stopped by the addition of 75 µl of 30% H₂SO₄, and the absorbance of the samples was determined at 492 nm on an ELISA reader.

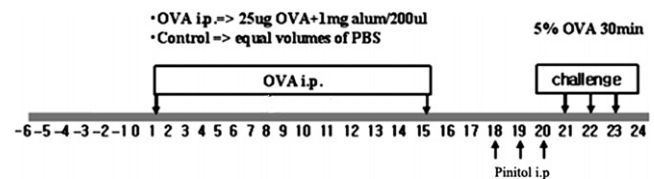


Fig. 1. Schematic diagram of the experimental protocol. Mice were immunized intraperitoneally (i.p.) with 20 µg of OVA emulsified in 1 mg of aluminum hydroxide on day 1 and 15. Mice were challenged for 30 min via the airway with OVA (50 mg/ml of saline) each day from days 21 to 23 on consecutive days. D-pinitol was administered i.p. with 10 mg/kg/day, 20 mg/kg/day on days 18–20.

2.6. Immunohistochemistry

At 48 h after the last challenge, lungs were removed from the mice after they had been sacrificed. Prior to the removal of the lungs, the lungs and trachea were filled intratracheally with a fixative (4% paraformaldehyde) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 µm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and sequentially stained with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). An inflammation score was graded by three independent investigators who were not associated with this study.

2.7. RNA preparation and quantitative RT-PCR

The total RNA from lung tissues was isolated with the use of a rapid extraction method (TRI-Reagent) (Invitrogen Life Technologies, CA, USA), as previously described [14]. Real-time PCR was performed on cDNA samples using the SYBR Green system (Bio Rad, Richmond, CA). Primers used were T-bet sense 5'-CAA CAA CCC CTT TGC CAA AG-3', T-bet antisense 5'-TCC CCC AAG CAG TTG ACA GT-3'; GATA-3 sense 5'-GAG GTG GAC GTA CTT TTT AAC ATC G-3', GATA-3 antisense 5'-GGC ATA CCT GGC TCC CGT-3'. Cycling conditions were 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles each corresponding to 15 s at 95 °C and 1 min at 60 °C. Analysis used the sequence detection software supplied with the instrument. The relative quantitation value is expressed as $2^{\pm \text{DCT}}$, where DCT is the difference between the mean CT value of duplicates of the sample and of the GAPDH control.

2.8. Measurement of cytokines

Levels of IFN-γ, IL-4, IL-5 and Eotaxin were quantified in the supernatants of BAL fluids by enzyme immunoassays performed according to the manufacturer's protocol of the manufacturer (IFN-γ, IL-4, IL-5, Eotaxin; R&D Systems, Inc., Minneapolis, MN).

2.9. Measurement of Th1/Th2 cytokine production in BLAB/c spleen cells

Flow cytometric analysis was performed, as described by Kim et al. [18]. OVA-induced spleen cells were pre-treated before 2 h with D-pinitol 10 µg/ml, 20 µg/ml and then stimulated for 24 h with 2 µg/ml OVA peptide (OVA 323–339). Briefly, 5×10^5 cells were incubated in staining buffer (PBS with 2% FBS and 0.1% sodium azide) containing anti-CD4 Ab (BD Pharmingen) for 15 min on ice to block the non-specific binding of Igs. Cells stained with the appropriate isotype-matched Ig were used as negative controls. After staining, the cells were fixed with 4% w/v paraformaldehyde and analyzed by FACSCalibur, using CellQuest software (BD Biosciences). For intracellular cytokine staining, the cells were treated with brefeldin A (10 µg/ml; 4 h; 37 °C), washed with 1% v/v FBS-PBS (staining buffer), and fixed in 4% w/v paraformaldehyde (20 min for 4 °C). Subsequently, the cells were washed twice in staining buffer, permeabilized in 100 µl of 0.1% saponin, 1% FBS-PBS (permeabilization buffer), and incubated with PE-conjugated anti-IL-4 mAb and stained with FITC-conjugated anti-IFN-γ mAb, (30 min; 4 °C). Appropriate isotype-matched, irrelevant mAbs served as negative controls.

2.10. Determination of airway responsiveness to methacholine

Airway responsiveness was measured in mice 24 h after the last challenge in an unrestrained conscious state, as described previously [24]. Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (2.5–50 mg/ml) was nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization. Enhanced pause (Penh), calculated as (expiratory time/relaxation time⁻¹) × (peak expiratory flow/peak inspiratory flow), according to protocol of the manufacturers, is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure of airway responsiveness to methacholine. Results were expressed as the percent increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) was expressed as 100%. Penh values were averaged for 3 min after each nebulization and evaluated.

2.11. Zymography

Gelatin zymography was used to assess the activity of MMP-9. The supernatant was diluted three times with non-reducing loading buffer (400 mmol/L Tris–HCl, 5% sodium dodecyl sulfate, 20% glycerol, 0.006% bromophenol blue). Fifteen microlitres of equal amounts of the sample were mixed with the loading buffer, and proteins were separated by polyacrylamide gel electrophoresis (0.75 mm; constant current 20 mA) consisting of an 8% sodium dodecyl sulfate solution with 1% gelatin (Bio-Rad). The gels were incubated in a renaturing buffer (2.5% Triton X-100 buffer) for 30 min to remove the sodium dodecyl sulfate. After rinsing, the gels were incubated (37 °C for 20 h) in an enzyme activation buffer (50 mmol/L Tris–HCl [pH 7.3], 200 mmol/L NaCl, and 0.02% Tween 20). The gels were then stained with Coomassie brilliant blue R250 stain and destained (5% methanol, 7% acetic acid in PBS solution), and the gelatinolytic activity was detected as clear bands. The molecular weight of the gelatinolytic bands was estimated relative to the prestained molecular-weight markers (see BluePlus2 Prestained Standard; Invitrogen, Carlsbad, CA).

2.12. Densitometric analysis and statistics

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the means ± S.E.M. ANOVA was used to compare experimental groups to control values while comparisons between multiple groups were performed using Tukey's Multiple Comparison test. Statistical significance was indicated by a *P* value less than 0.05. *P**** < 0.001, *P*** < 0.05, *P** < 0.01.

3. Results

3.1. D-pinitol decreases the increased numbers of inflammatory cells in BAL fluids of OVA-sensitized and OVA-challenged mice

The total cell numbers in BAL fluids were significantly increased, about 10-fold, compared to those in the control group 2 days after the last OVA challenge. The number of neutrophils, eosinophils, lymphocytes, and Macrophages in BAL fluids was increased 16-, 94-, 3-, and about 8-fold, respectively, compared to those in the control group at 2 days after the OVA challenge. Interestingly, the number of neutrophils, eosinophils, lymphocytes, and monocytes observed in BAL fluids in the D-pinitol treated group of mice decreased to the level of 16-, 9-, 2-, 2-fold of OVA-challenge group. The administration of the D-pinitol significantly reduced the increase in neutrophils, eosinophils, lymphocytes, macrophages and total cells elicited in the airway lumen 2 days after OVA inhalation (Fig. 2).

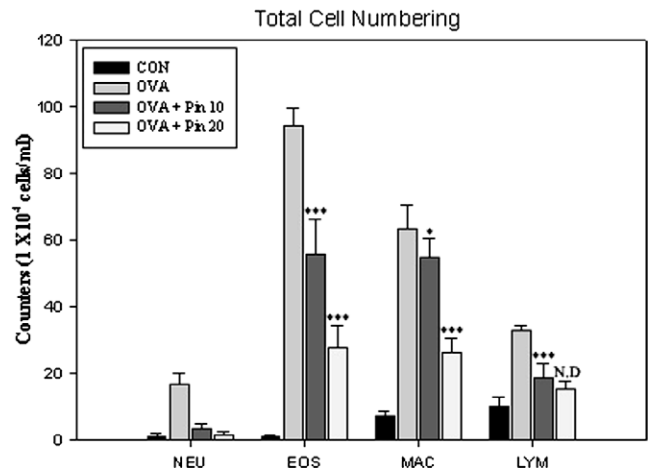


Fig. 2. Effect of Curcumin on total and differential cellular components of BAL fluids of OVA-sensitized and OVA-challenged mice. Mice were treated with the Con (PBS), OVA plus D-pinitol 10 mg/kg/day, 20 mg/kg/day (OVA + Pin 10, OVA + Pin 20) and OVA (OVA), respectively, as described in Section 2. The BAL cells were collected 1 day after the OVA challenge. The different cell types were enumerated. The arrow is pointed at eosinophils. The results were from one representative experiment out of 5 performed. This experiment used five mice (*n* = 5). ****P* < 0.001 vs. OVA, **P* < 0.01 vs. OVA. NEU, neutrophil; EOS, eosinophil; LYM, lymphocyte; MAC, macrophages; TOT, total cell.

3.2. D-pinitol inhibits CD3e⁺ T cell number in BAL Fluids

Numbers of CD3e⁺ T cell in BAL fluids were increased significantly at 24 h after OVA inhalation (24.27%) compared with the numbers after saline inhalation (6.04%) (Fig. 3, Table 1). The increased numbers of CD3e⁺ T cell were significantly reduced by the administration of D-pinitol (19.32%).

3.3. Effect of D-pinitol on changes in eosinophils in BAL fluids

The levels of eosinophils in BAL fluids were significantly increased at 24 h after OVA inhalation compared with the levels after saline inhalation (Fig. 4). The increased levels of these cells were significantly reduced by about 53% (Pin 10 mg) and 71% (Pin 20 mg) upon administration of D-pinitol.

3.4. D-pinitol turns on pathological changes of OVA-induced asthma

Histological analyses revealed typical pathologic features of asthma in the OVA-exposed mice, as compared with the control, with the OVA-exposed mice showing numerous inflammatory cells, including eosinophils infiltrated around the bronchioles (Fig. 5). Mice treated with D-pinitol showed marked reductions in the infiltration of inflammatory cells in the peribronchiolar and perivascular regions (Fig. 5). The increased total lung inflammation and cell infiltration were significantly reduced by the administration of D-pinitol. These results suggest that D-pinitol inhibits antigen-induced inflammation in the lungs, including the influx of eosinophils.

3.5. Effect of D-pinitol on expression of MMP-9 mRNA, T-bet and GATA3 mRNA levels in lung tissues of OVA-sensitized and -challenged mice

Quantitative RT-PCR analysis revealed that expression of MMP-9 and GATA3 mRNA in lung tissues was significantly increased at 24 h after OVA inhalation compared with the

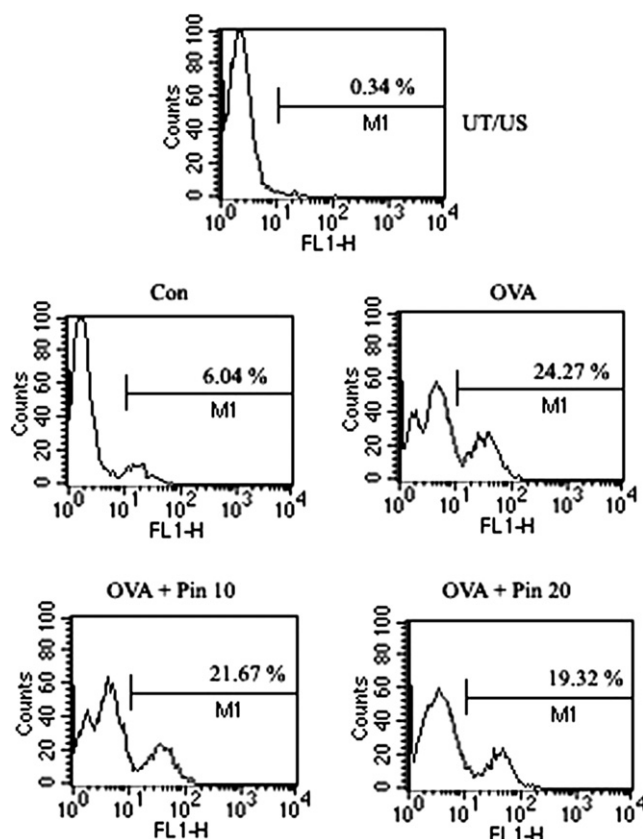


Fig. 3. D-pinitol decreased CD3e⁺ T cell levels in BAL fluids of OVA-sensitized and OVA-challenged mice. The cells were harvested and analyzed via two-color flow cytometry. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (Con), OVA-sensitized mice administered saline (OVA), and OVA-sensitized mice administered Pin 10 mg/kg/day (OVA + Pin 10) and Pin 20 mg/kg/day (OVA + Pin 20). The BAL fluids were stained PE-conjugated anti-CD3 mAb. The Dot plots shown are gated on BAL cells. Data represent means $\pm$ S.E.M. from three independent experiments.

levels after saline inhalation (Fig. 6B, C). The increased mRNA expression of MMP-9, GATA-3 was decreased by the administration of D-pinitol. Conversely, D-pinitol treated group increase T-bet mRNA in Th1 transcription factor T-bet (Fig. 6A).

3.6. D-pinitol regulates on levels of IL-4, IL-5, Eotaxin and IFN- γ in lung tissues of OVA-sensitized and -challenged mice

BAL fluids were obtained 4 h after the last airway challenge. The levels of IL-4, IL-5 and Eotaxin in the BAL fluids were

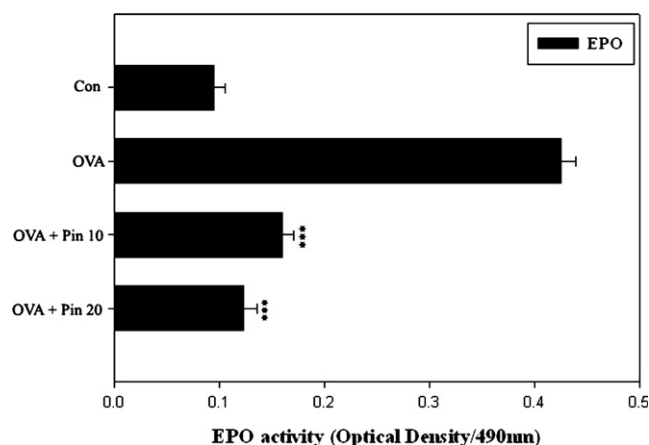


Fig. 4. Eosinophil peroxidase (EPO) activity in BAL fluids of OVA-sensitized and -challenged mice. EPO is an indicator of the numbers of eosinophil levels. The increased levels of EPO were significantly reduced about 53% (OVA + Pin 10 mg/kg/day), 71% (OVA + Pin 20 mg/kg/day) compared with OVA-treated mice. The results were from one representative experiment out of 5 performed. This experiment used five mice ($n = 5$). *** $P < 0.001$.

significantly increased by airway challenge with OVA when compared with that of the control. The administration of D-pinitol reduced the concentration of IL-4, IL-5, and Eotaxin secretion (Fig. 7A, C, D). As shown in Fig. 5, the levels of Th2 cytokines, IL-4 and IL-5, were found to be increased in OVA-sensitized and -challenged mice, but that of the Th1 cytokine, IFN- γ (Fig. 7B) was significantly decreased as compared to saline-sensitized and -challenged mice. These results indicate that D-pinitol treatment not only selectively inhibits Th2 cytokine levels but also increases Th1 cytokine in the BAL fluids.

3.7. Effect of D-pinitol on Th1/Th2 cytokine production in CD4⁺ T cells

The levels of IL-4-positive CD4⁺ T cell in the spleen cells were significantly increased by stimulating with OVA (37.7%) when compared with that of the control (5.76%) (Fig. 8). Following D-pinitol 10, 20 μ g/ml treatment, there was increased in the number of IFN- γ positive CD4⁺ T cell (43.1%) when compared with that of the OVA stimulating group (17.69%).

3.8. Effect of D-pinitol on airway hyper-responsiveness

Airway responsiveness was assessed as the percent increase of Penh in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose–response curve of percent Penh was shifted to the left compared with that of

Table 1
D-pinitol inhibits CD3e⁺ T cell numbers in BAL Fluids

Group	CON	OVA	OVA + Pin 10 mg/kg/day	OVA + Pin 20 mg/kg/day
% CD3e ⁺ Positive cell				
Percentage of total	6.04 %	24.27 %	21.67 %	19.32 %
T cell number	3×10^5 cells	1.25×10^6 cells	1.1×10^6 cells	9.5×10^5 cells

D-pinitol decreased CD3e⁺ T cell levels in BAL fluids of OVA-sensitized and OVA-challenged mice. The cells were harvested and analyzed via two-color flow cytometry. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (Con), OVA-sensitized mice administered saline (OVA), and OVA-sensitized mice administered Pin 10 mg/kg/day (OVA + Pin 10) and Pin 20 mg/kg/day (OVA + Pin 20). The BAL fluids were stained PE-conjugated anti-CD3e mAb. The Dot plots shown are gated on BAL cells. Data represent means $\pm$ S.E.M. from three independent experiments. Total cells were 5×10^6 cells in each group.

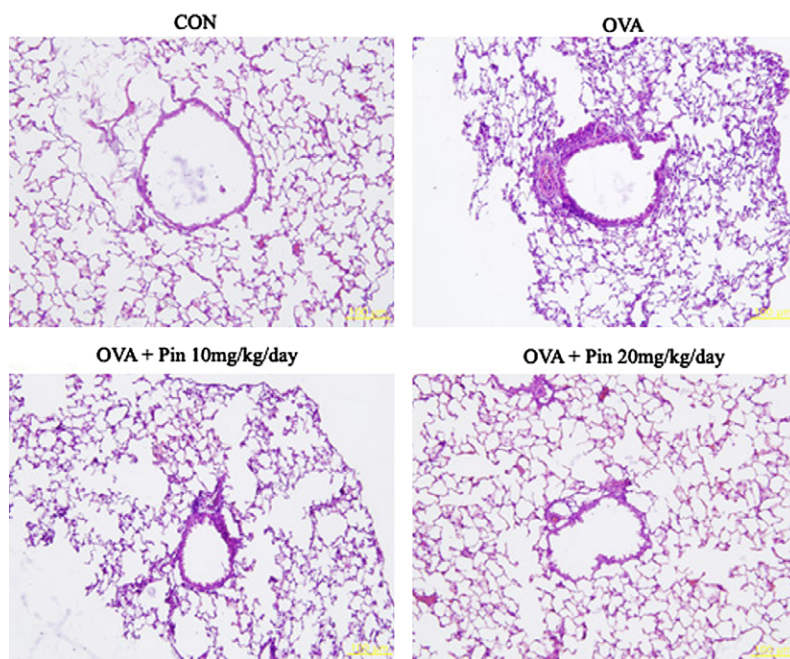


Fig. 5. D-pinitol inhibits lung inflammation and inflammatory cells infiltration. Mice were sensitized and challenged as described in Section 2. Sections were obtained from the lungs of mice receiving the control (CON), OVA plus D-pinitol (OVA + Pin 10 mg/kg/day, 20 mg/kg/day) and OVA (OVA). Lungs were removed 2 days after the last airway challenge. Sections were stained by haematoxylin and eosin staining (200 $\times$).

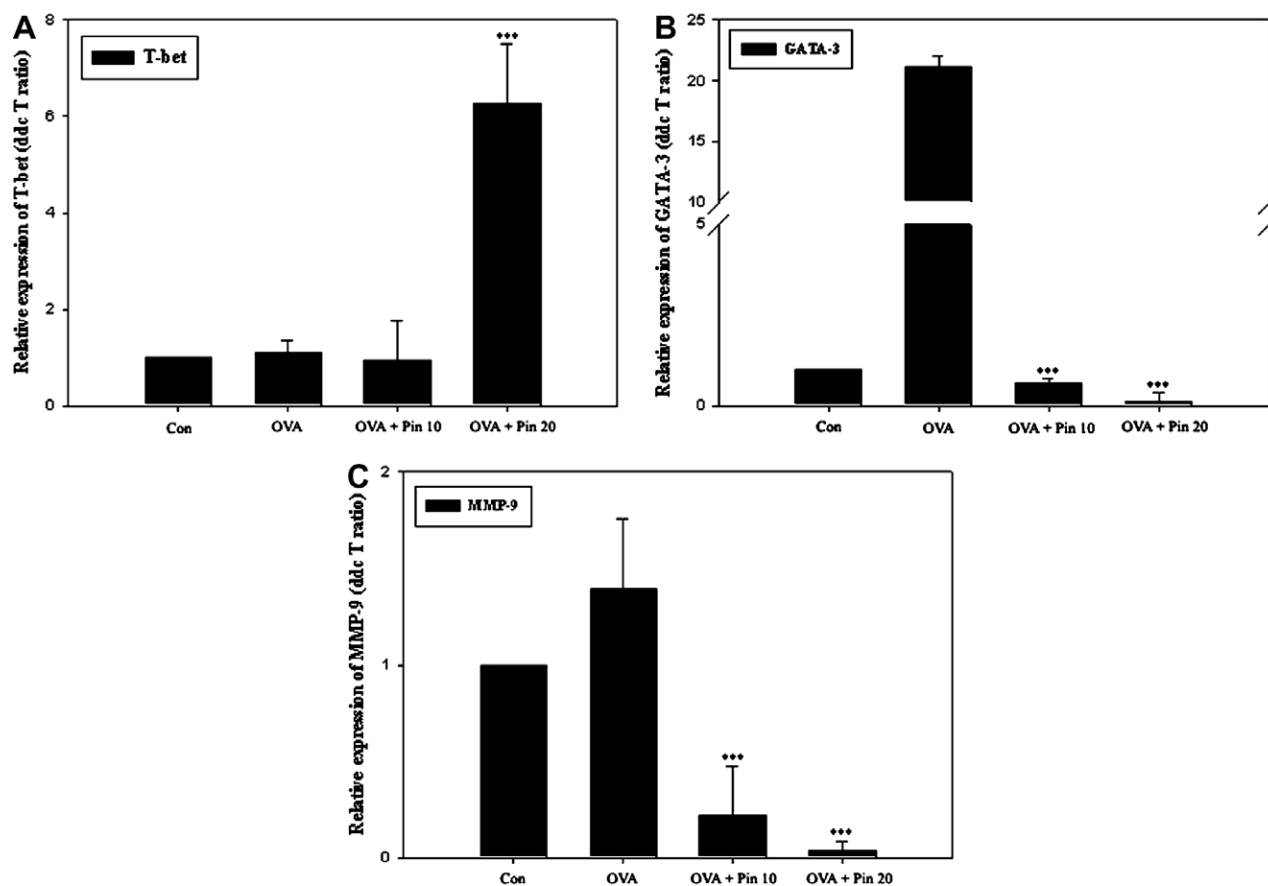


Fig. 6. Effect of D-pinitol on MMP-9, T-bet, GATA3 and GAPDH mRNA expression in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed at 24 h after the last challenge in saline-inhaled mice administered saline (CON), OVA-inhaled mice administered saline (OVA), OVA-inhaled mice administered D-pinitol 10 mg/kg/day (OVA + Pin 10) and OVA inhaled mice administered D-pinitol 20 mg/kg/day (OVA + Pin 20).

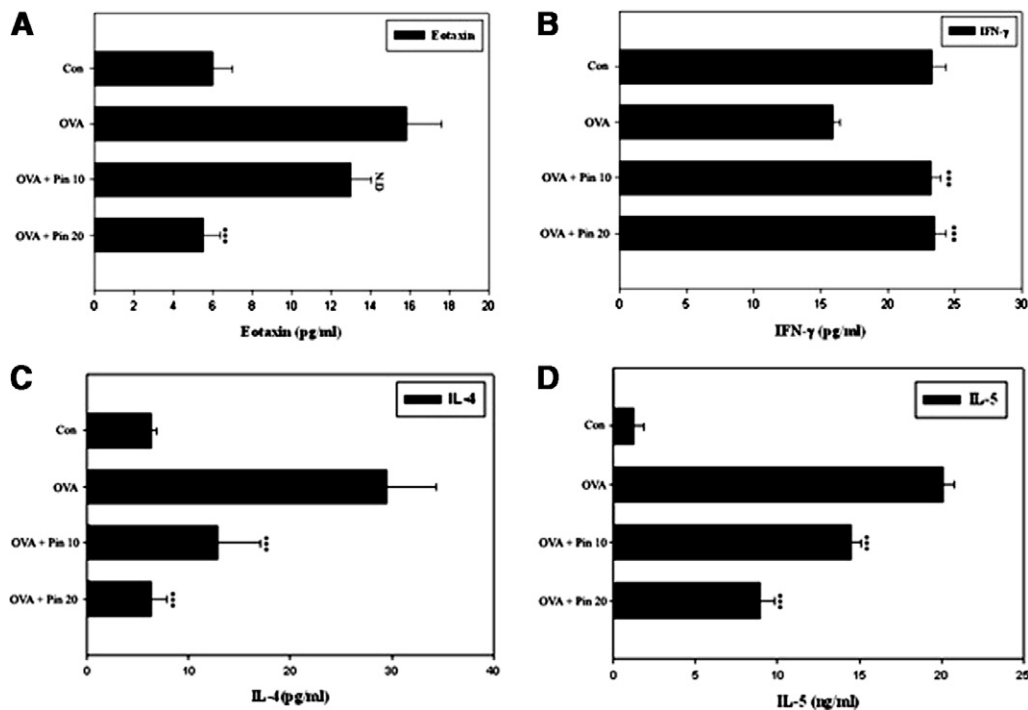


Fig. 7. Effect of D-pinitol treatment on Th1, Th2 cytokine. OVA-sensitized mice were treated as described in Fig. 1. (A) BAL fluid was performed 4 h after the last airway challenge as described by the manufacturer. Eotaxin levels in the BAL fluids were measured by ELISA Kit. Saline-inhaled mice administered saline (CON), OVA-inhaled mice administered saline (OVA), OVA-inhaled mice administered D-pinitol 10 mg/kg/day (OVA + Pin 10) and OVA inhaled mice administered D-pinitol 20 mg/kg/day (OVA + Pin 20). (B) The mice were bled 4 h after the last airway OVA challenge. The detection limit of the IFN- γ assay was 5 pg/ml, and the assay is assured by the manufacturer to be specific for the IFN- γ ELISA kit. (C) BAL fluid was performed 4 h after the last airway challenge as described by the manufacturer. IL-4 cytokine levels in the BAL fluids were measured by ELISA Kit. (D) BAL fluid was performed 4 h after the last airway challenge as described by the manufacturer. IL-5 cytokine levels in the BAL fluids were measured by ELISA Kit. Data represent means $\pm$ S.E.M. from five independent experiments of the response in 4–8 animals. **** $P < 0.001$.

control mice (Fig. 9). In addition, the percent Penh produced by methacholine administration (at doses from 2.5 mg/ml to 50 mg/ml) increased significantly in the OVA-sensitized and -challenged mice compared with the controls. OVA-sensitized and -challenged mice treated with D-pinitol showed a dose-response curve of percent Penh that shifted to the right compared with that of untreated mice. The shift was dose-dependent. These results indicate that D-pinitol treatment reduces OVA-induced airway hyper-responsiveness.

3.9. D-pinitol inhibited on MMP-9 gelatinolytic activity

OVA-challenge induced a marked induction of matrix metalloproteinase-9 activity in BAL fluids in comparison to control mice (Fig. 10). When the administration D-pinitol 10 mg/kg/day, this increased MMP-9 gelatinolytic activity was significantly inhibited (Fig. 10). These results indicate that D-pinitol regulates MMP-9 gelatinolytic activity in lung tissues.

4. Discussion

This study is the first to provide experimental evidence demonstrating that D-pinitol inhibits OVA-induced airway inflammation in a murine model of asthma. D-pinitol profoundly inhibited asthmatic reactions such as leukocytic recruitment into the airway and lung inflammation. We also demonstrated that D-pinitol regulates the Th1/Th2 balance, which can be mediated by the level of T-bet and GATA3 levels.

OVA-induced asthma has been recognized as a disease that results from chronic airway inflammation characteristically associated with the infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen [3,4]. In our experiment, we demonstrate that OVA-induced asthma increased levels of eosinophil infiltration, eosinophils peroxidase activity, eotaxin, and the thicknesses of bronchial wall and area of smooth muscle. But they were significantly decreased by administration of D-pinitol.

It was recently demonstrated that the administration of an MMP inhibitor reduces the migration of inflammatory cells through the endothelial and epithelial basement membranes [27]. Additionally, an MMP inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma [28]. In our murine model of asthma, intraperitoneally injected mice with 10 mg/kg/day, 20 mg/kg/day D-pinitol to evaluate the effect of D-pinitol on the expression of MMP-9, T-bet and GATA3 mRNA. In this study, D-pinitol reduced levels of MMP-9, GATA3 in lung tissues of D-pinitol treated mice. Conversely, D-pinitol treatment resulted in increase in Th1 transcription factor T-bet.

T-bet, a member of the T-box family of transcription factors, is a master determinant of Th1 lineage [28,29]. Indeed, T-bet deficient mice exhibit a profound lack of Th1 immune responses [30] and ectopic expression of T-bet in murine Th2 cells directs activation of IFN- γ , as well as the upregulation of IL-12R β [31,32]. Th1 cytokines are known to inhibit allergic responses [33,34]. GATA-3 belongs to the GATA family of

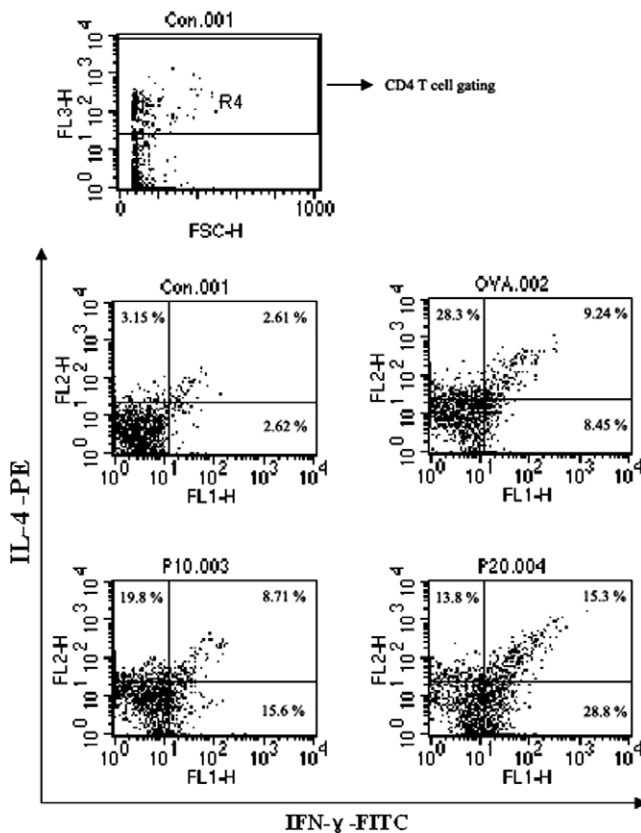


Fig. 8. D-pinitol increase the secretion of IFN- γ and decrease levels of IL-4 in OVA-induced BALB/c spleen cell. Spleen cells were stimulated by 2 μ g/ml OVA-peptide (OVA 323–339) for 24 h. P10, 20 indicates D-pinitol 10 μ g/ml, 20 μ g/ml pre-treated 2 h before OVA-peptide.

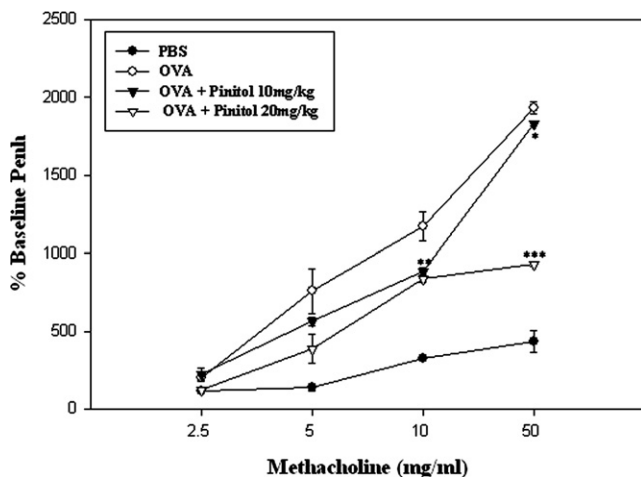


Fig. 9. Effect of D-pinitol on airway responsiveness in OVA-sensitized and OVA-challenged mice. Airway responsiveness was measured at 24 h after the last challenge in saline-inhaled mice administered PBS (PBS), OVA-sensitized mice administered saline (OVA) and OVA-sensitized mice administered D-pinitol (OVA + Pin 10 mg/kg/day, 20 mg/kg/day). Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed into the main chamber and were nebulized first with PBS, then with increasing doses (2.5–50 mg/ml) of methacholine for 3 min for each nebulization. Readings of breathing parameters were taken for 3 min after each nebulization during which Penh values were determined. Data represent means $\pm$ S.E.M. from five independent experiments. *** P < 0.001, ** P < 0.01, * P < 0.05.

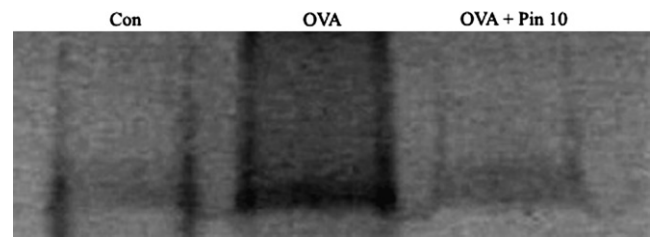


Fig. 10. The effect of D-pinitol on MMP-9 production in lung tissues of OVA-sensitized and OVA-challenged. Sampling was performed at 48 h after challenge with the PBS (Con), OVA (OVA), or OVA plus D-pinitol 10 mg/kg/day (OVA + Pin 10) and analyzed by zymography. All of the groups of the experiment showed MMP-9 production, but the OVA plus D-pinitol group only showed the active form of MMP-9.

transcription factors, Six members (GATA-1 to GATA-6) of this family have been identified in avians, with homologues in mammals and avians. Based on their expression profile and structure, the GATA proteins may be classified as haematopoietic (GATA-1 to GATA-3) [35] or non-haematopoietic (GATA-4 to GATA-6). Naive CD4⁺ T cells express low levels of GATA-3 mRNA [18]. The expression of GATA-3 is, however, markedly upregulated in cells differentiating along the Th2 lineage, and is downregulated in cells differentiating along the Th1 pathway [36].

Our data demonstrate that D-pinitol reduces the increased levels of GATA3 mRNA in OVA-sensitized and -challenged mice (Fig. 4). Also, it suggest that D-pinitol treatment is a novel, selective way to simultaneously suppress GATA-3 and increase T-bet expression in asthmatic reactions *in vivo* [24]. Also, we examined Th1/Th2 cytokine production in CD4⁺ T cell. D-pinitol reduces the increased levels of IL-4, Th2 cytokine production in OVA-sensitized and -challenged mice. The other side, it increases IFN- γ , Th1 cytokine production in D-pinitol administrated mice. Taken together these suggest that T-bet, GATA-3 might be a candidate gene for asthma, and a regulator of Th1/Th2 balance.

In conclusion, our results strongly indicate that D-pinitol reduces allergic airway inflammation and hyperresponsiveness due to the alteration of Th1/Th2 polarization via the suppression of GATA-3 and increase of T-bet expression. Therefore, our data suggest that D-pinitol might offer a new therapeutic approach to allergic airway diseases.

Acknowledgements: This work was supported by the Korea Research Foundation Grant funded by Korean Government (R05-2004-000-10600-0) and the Korea Science and Engineering Foundation through National Research Laboratory Program Grant M10500000008-06J000000810.

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