



Isatin down-regulates expression of atrial natriuretic peptide receptor A and inhibits airway inflammation in a mouse model of allergic asthma

R. Kandasamy^{a,c}, S.J. Park^a, S. Boyapalle^{a,b}, S. Mohapatra^{a,b}, G.R. Hellermann^a, R.F. Lockey^{a,b}, S.S. Mohapatra^{a,b,*}

^a Division of Allergy and Immunology-Joy McCann Culverhouse Airway Disease Center, Dept. of Internal Medicine and Nanomedicine Research Center, College of Medicine, Tampa, FL, USA

^b James A. Haley, VA Hospital, Tampa, FL, USA

^c Department of Pharmaceutical Technology, Anna University, Tiruchinnappalli, Tamil Nadu, India

ARTICLE INFO

Article history:

Received 18 July 2009

Received in revised form 8 October 2009

Accepted 2 November 2009

Keywords:

Atrial
Natriuretic
Asthma
NPRA
Isatin
Nanoparticles

ABSTRACT

Isatin, an endogenous indole compound, prevents atrial natriuretic peptide (ANP) from signaling through its cell-surface receptor, NPRA. Allergic airway inflammation has been linked to natriuretic peptide signaling and blocking this signaling axis in the lung prevents allergen-induced pathology. In this study we encapsulated isatin in chitosan nanoparticles and tested them in a mouse model of allergic asthma by intranasal delivery to the lung. Isatin nanocapsules reduced lung pathology by blocking ANP signaling, but surprisingly also by reducing the expression of NPRA. Ovalbumin-allergic mice were treated intranasally with isatin-containing chitosan nanocapsules either before or after allergen challenge, and lung function, cytokine levels, histopathology and cellular infiltration were determined. ANP activity was quantitated by measuring changes in intracellular cyclic GMP and changes in NPRA levels were determined. For comparison with isatin's effects, the expression of the receptor was inhibited with small interfering RNA against NPRA mRNA. Isatin nanocapsules administered locally to the lung reduced cGMP production and NPRA expression and protected allergic mice from airway hyperreactivity and lung inflammation when given either before or after allergen challenge. Leukocyte infiltration was reduced and lung cytokine profiles showed a repolarization from a Th2-like to a Th1-like phenotype. Isatin nanocapsules administered locally to the lung inhibit NPRA signaling but also are capable of lowering the expression of NPRA, thus effectively reducing inflammation in a mouse model of allergic asthma. Pharmacological intervention to reduce NPRA activity through the inflammatory natriuretic peptide axis in the lung may be a useful adjunct therapy for treating lung disease.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Asthma is now one of the world's most common chronic illnesses, according to the *Global Burden of Asthma Report* [1]. The disease is estimated to affect as many as 300 million people worldwide—a number that could increase by a further 100 million by 2025. Asthma can be controlled reasonably well through inhaled corticosteroids and long-acting beta agonists, but these drugs can have serious side effects and do not correct the underlying causes of the disease [2,3]. Despite significant increases in understanding of the immune mechanisms involved in allergic disease over the past several decades, this knowledge has not been effectively translated into new therapies. Chronic airway inflammation is a major factor in asthma symptoms and decreased lung function, and increases susceptibility to exacerbation even in those patients who have mild disease.

The mechanisms that generate and maintain the inflammatory environment are complex and incompletely understood, but several signaling pathways such as those involving NF-kappa B, Erk1,2 (extracellular-regulated kinases 1 and 2) and the STATs (signal transducers and activators of transcription), have an influence on persistent inflammation and airway disease [4]. One of the current goals in asthma therapy is to promote endogenous anti-inflammatory systems to restore health.

One such system is the immunomodulatory pathway involving the atrial natriuretic peptide (ANP)-natriuretic peptide receptor A (NPRA) signaling axis that is highly expressed in airway epithelium [5]. In the cardiovascular system and the kidney, ANP regulates blood pressure and volume, sodium and water excretion through cGMP that is produced by the intrinsic guanylyl cyclase domain of the cell-surface receptor, NPRA (also known as GC-A). Ligand-mediated activation of GC-A produces the second messenger cGMP, which can turn on the cGMP-dependent protein kinase PKG. PKG in turn phosphorylates a number of species that results in the activation of ion transporters and transcription factors affecting cell growth and proliferation, apoptosis, and inflammation.

* Corresponding author. University of South Florida, College of Medicine, Department of Internal Medicine, Box MDC 019, 12908 Health Drive, Tampa FL 33612, USA.

E-mail address: smohapat@health.usf.edu (S.S. Mohapatra).

In the lung, NPRA signaling produces bronchorelaxation but also causes elevation of pro-inflammatory cytokines. Previous studies of allergic asthma in mouse and pig models have shown that although ANP promotes bronchodilation, its overproduction in the lung is associated with increased inflammation and polarization toward a T helper cell 2-like phenotype [6,7]. Thus, reducing ANP signaling through NPRA locally in the lung may be an effective strategy for eliminating the chronic inflammation of asthma and other respiratory diseases. A number of clinical trials testing the therapeutic efficacy of ANP signal modification in various disease conditions have been conducted with some success [8,9]. The ANP–NPRA signaling pathway is regulated at the transcriptional, translational and post-translational levels offering considerable latitude for intervention.

Isatin is a naturally occurring heterocyclic indole derivative, indole-2,3-dione, that is found in the brain, body fluids and other tissues. It is elevated in mammals including man under stressful conditions and interacts with a number of proteins/enzymes associated with energy metabolism, cytoskeleton, cell signaling, differentiation and survival [10]. Isatin has been shown to inhibit NPRA signaling [11] and studies with physiological doses revealed that its primary targets are the particulate guanylyl cyclase of NPRA [12–14] and the nitric oxide-activated soluble guanylyl cyclase [15]. When administered to rats by intraperitoneal injection, isatin blocked cGMP production under conditions of fluid overload that are known to induce endogenous ANP release and cGMP formation [16]. Isatin appears to have significant pharmacological potential [17,18] especially when used locally with targeted nanoparticle delivery systems.

Based on these studies, we hypothesized that lung-specific inhibition of ANP signaling by isatin might be a useful adjunct therapy for standard asthma treatment. We have demonstrated targeting of plasmids and small-molecule drugs to lung epithelial cells by encapsulation in nanoparticles produced from chitosan, a derivative of the natural biopolymer chitin [19–21]. In the present study, we evaluated the ability of intranasally administered isatin-chitosan nanocapsules to target the lung and inhibit inflammation in a mouse model of ovalbumin-induced allergic asthma. Treatments were done prophylactically to demonstrate the potential use of the adjunct treatment for prevention of airway inflammation and therapeutically to show its effectiveness in reducing exacerbation.

2. Materials and methods

2.1. Reagents

Chitosan hydrochloride (>60 kDa) was obtained from TaeHoon Bio (Korea) and used as received. Isatin was purchased from Sigma-Aldrich (St Louis, MO, USA) and is the highest quality spectral grade, >99.9% pure. Chitosan and isatin were deemed to be endotoxin-free because they did not cause an inflammatory response compared to vehicle alone when administered to mice intranasally. All other reagents were obtained from Sigma.

2.2. Cell lines

The human embryonic kidney cell line, HEK293, was purchased from ATCC (Rockville, MD, USA) and grown in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/ml each of penicillin and streptomycin at 37 °C in a 5% CO₂ incubator.

2.3. Animals

Female BALB/c mice, 6–8 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in a pathogen-free environment, and all procedures were reviewed and approved by the University of South Florida Institutional Animal Care and Use Committee.

2.4. Preparation and characterization of chitosan nanoparticles containing isatin

Chitosan, a derivative of the natural polysaccharide chitin, was used as a nanoparticle carrier for isatin delivery because its mucoadhesiveness and positively-charged surface groups allow it to be readily taken up by the lung cells. Isatin was encapsulated in chitosan nanoparticles as previously described [19]. Briefly, chitosan hydrochloride (~60 kDa) was dissolved in 10 mL of Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, and isatin was added at a ratio of 1:2 (w/w). The mixture was vortexed vigorously for 12 h to form nanocomplexes. The mean size of the chitosan nanoparticles was determined by transmission electron microscopy to be around 100 nm (*data not shown*). Nanoparticles of this size are readily taken up by the epithelial cells in the lung.

2.5. Construction of small interfering RNA against NPRA (siNPRA or si9)

Potential DNA target sequences for siRNA against murine NPRA were determined by using the siRNA finder software from Ambion (Austin, TX) and the resulting sequences were cloned into the pSilencer-1.0-U6 vector (Ambion, Austin TX). The siRNAs were tested to determine which produced the greatest decrease in NPRA expression when transfected into HEK-GCA cells (HEK293 cells stably transfected with NPRA coding sequence to overexpress NPRA). The most effective siNPRA had the sequence, GGGCGCUGCUGCUGCUACCTdT (sense) and was designated si9. The vector carrying si9 was encapsulated in chitosan nanoparticles and administered to the mice (25 µg of plasmid per mouse) intranasally. A randomly generated sequence of the same base composition and length as si9 was used as an siRNA control.

2.6. Evaluation of isatin's effects on NPRA expression in vitro

Kidney cells normally express NPRA and are responsive to ANP. For that reason, we chose the human embryonic kidney cell line, HEK 293, to test the effects of isatin. Cells were seeded in 100 mm plates at a density of 1×10^6 cells/plate and incubated at 37 °C in a CO₂ incubator for 24 h. Cells were stimulated by incubation with 100 ng/ml bacterial lipopolysaccharide for 4 h. Cells were rinsed and a solution of isatin in DPBS was added to a final concentration of 25, 50 or 100 µM and incubation was continued for 12 h. After isatin treatment, whole-cell protein extracts were prepared by scraping cells from plates and resuspending in lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.5% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml leupeptin, 0.5 mM NaF, and 0.1 mM sodium vanadate. Protein aliquots (200 µg) were separated on 12% polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Blots were incubated with a goat polyclonal antibody against NPRA (Santa Cruz Biotech, Santa Cruz, CA, USA), washed and exposed to HRP-conjugated anti-goat IgG (Santa Cruz Biotech, Santa Cruz, CA, USA) secondary antibody. Beta-actin immunostaining was used as an internal protein-loading control. Immunoblot signals were developed using SuperSignal Ultra chemiluminescent reagents (Pierce, Rockford, IL, USA) and detected using X-Omat AR X-ray film.

2.7. Measurement of allergic airway inflammation in vivo: prophylactic protocol

BALB/c mice were divided into five groups of four mice each. This is the minimum number of mice per group that yields statistically meaningful results with each experiment being repeated twice ($n = 12$). One group of mice received no OVA sensitization or challenge as naïve control (NC) but was given intranasal chitosan nanoparticles alone. Previous experiments comparing mice given intranasal chitosan to those receiving vehicle have shown no effects of chitosan itself on lung inflammation. The second group also received chitosan nanoparticles

without isatin but was sensitized by i.p. injection of 50 µg OVA in 100 µl DPBS plus alum adjuvant (Imject, Pierce) on days 1 and 8 and challenged with 50 µg of OVA in 50 µl DPBS intranasally on days 20 and 21. Animals in the third to fifth groups were sensitized to OVA, then treated prophylactically with intranasal chitosan nanoparticles with encapsulated isatin at doses of 50 or 100 mg/kg (groups 3 and 4) or siNPRA plasmid (group 5) on days 16, 17, 18, and 19 prior to OVA challenge on days 20 and 21. Specific airway resistance, sRaw, in response to methacholine challenge was measured on day 22 by restrained, non-invasive airway mechanics in a dual-chamber plethysmograph (Buxco, Wilmington, NC). All mice were euthanatized and bronchoalveolar lavage (BAL) fluid was collected by rinsing with intratracheal injections of DPBS. The lungs were then perfused with 10% neutral buffered formalin, removed, paraffin-embedded, and 4 µm sections were stained with hematoxylin and eosin. The stained sections were examined for lung pathology and photographed under the microscope by personnel blinded to the treatment group. Representative digital photos are shown.

2.8. Differential cell enumeration in bronchoalveolar lavage (BAL) fluid

BAL fluid was collected and differential cell counts were performed as previously described [19]. Briefly, BAL fluid was centrifuged and the cell pellet was suspended in 200 µl of DPBS and counted using a hemocytometer. The cell suspensions were then centrifuged onto glass slides using a Shandon Cytospin centrifuge at 1000 rpm for 5 min at room temperature. Cytoentrifuged cells were air dried and stained with a modified Wright's stain (Leukostat, Fisher Scientific, Atlanta, GA, USA), which allows differential counting of monocytes and lymphocytes. At least 500 cells per sample were counted by direct microscopic observation by personnel blinded to the treatment group.

2.9. Quantification of cytokines in lung homogenates

OVA-allergic mice were treated prophylactically with chitosan nanoparticles loaded with 50 or 100 mg/kg isatin and 24 h later they were challenged with OVA. One day after the OVA challenge, lungs were removed, weighed, homogenized in buffer containing a protease inhibitor cocktail (see Section 2.6 above), and aliquots of the homogenates were assayed for IL-4, IL-5, and TNF- α by ELISA and cytokine bead array (BD Biosciences, San Jose, CA, USA). Values were expressed as pg cytokine per gram of lung.

2.10. Determination of specific airway resistance

The degree of bronchoconstriction, as determined by specific airway resistance, sRaw, was measured in conscious, restrained mice by non-invasive airway mechanics using a dual-chamber plethysmograph (Buxco, Wilmington, NC). Groups of mice were exposed for 5 min to nebulized DPBS to establish a zero-concentration baseline, then to 6.25 mg/ml (moderate dose) and 25 mg/ml (high dose) of nebulized methacholine (MCh; Sigma, St. Louis, MO) in DPBS. The number of MCh doses had to be limited to two to avoid harmful stress to the animals restrained tightly in the plethysmograph chambers. Challenges were done for 5 min followed by recordings of sRaw for 5 min. The sRaw values were averaged and mean $\pm$ SEM was reported for each MCh concentration.

2.11. Lung histopathology

A random number was assigned to each hematoxylin and eosin-stained lung section from the treatment groups. A pathologist blinded to the treatments examined the sections microscopically and scored them according to the extent of inflammation. The degree of peribronchial inflammation was evaluated on a subjective scale of 0, 1, 2, 3, and 4 corresponding to none, mild, moderate, marked, or severe inflammation, respectively, with an increment of 0.5 if the degree of inflammation

fell between two integers^{1,2}. Epithelial changes were scored as: 0 = no change, 1 = increased epithelial cell cytoplasm without desquamation, 2 = epithelial desquamation without bronchial exudates composed of inflammatory cells, and 3 = bronchial exudates composed of desquamated epithelial cells and inflammatory cells.

2.12. Effects of isatin treatment in vivo in the therapeutic model

Twenty BALB/c mice were divided into five groups of four mice each). One group served as naive control (NC) with no OVA sensitization or challenge, but mice were given intranasal chitosan nanoparticles alone or with empty vector, U6. The second group received only chitosan nanoparticles but was sensitized by i.p. injection of 50 µg OVA plus alum in 100 µl DPBS on days 1 and 8 and challenged with 25 µg of OVA in 50 µl DPBS intranasally on days 22, 23 and 24. Animals in the third and fourth groups were OVA-sensitized and challenged prior to intranasal treatment with 50 or 100 mg/kg isatin nanoparticles in 50 µl of DPBS respectively on days 31, 32, 33 and 34. The last group was OVA-sensitized and challenged, but treated intranasally with nanoparticles carrying 25 µg of plasmid encoding small interfering RNA against NPRA (si9) in 50 µl of DPBS on days 31, 32, 33 and 34. To prepare si9 chitosan nanoparticles, 250 µg of si9 was mixed with 750 µg of chitosan and vortexed vigorously for 30 s. As in the prophylactic procedure, mice were euthanatized and BAL fluid was collected from the lungs. Lungs were rinsed with intratracheal injections of DPBS, perfused with 10% neutral buffered formalin, removed, paraffin-embedded, and 4 µm sections were stained with H and E and examined under the microscope to determine lung pathology.

2.13. Immunofluorescent staining of lung sections for NPRA

Slides containing lung sections were deparaffinized in xylene, followed by decreasing concentrations of ethanol, rehydrated to DPBS and fixed with 4% paraformaldehyde for 25 min at room temperature. Sections were washed three times in DPBS, permeabilized for 15 min with 0.1% Triton X-100 and incubated with 5% rabbit serum at 37 °C for 1 h. Slides were washed with DPBS and incubated overnight with goat IgG polyclonal antibody against NPRA (Santa Cruz, CA, USA) at 4 °C. The stained slides were washed with DPBS and incubated with FITC-labeled anti-goat IgG for 1 h at RT. The lung sections were examined under a fluorescence microscope and photographed by personnel blinded to the treatment group. Representative digital photos are shown.

2.14. Measurement of cyclic guanosine monophosphate (cGMP) levels in lung homogenates

OVA-sensitized and -challenged mice were treated with isatin according to the therapeutic regimen and lung homogenates were prepared as described above. The cGMP levels were measured by ELISA according to manufacturer's instructions (BD Biosystems, San Jose, CA).

2.15. Statistics

A minimum of four mice was used in each test group. Experiments were repeated twice and measurements were expressed as means $\pm$ SEM. One-way ANOVA with Tukey's Multiple Comparison post-test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).

3. Results

3.1. Preparation and characterization of chitosan nanoparticles containing encapsulated isatin

Chitosan, a cationic polysaccharide derived from chitin [22,23] was used to generate nanoparticles for encapsulation and delivery of isatin.

Isatin has been shown by others to prevent natriuretic peptide signaling through surface NPRA receptors on cells from heart, kidney and brain [10]. NPRA activation by its ligands, ANP and BNP, generates cGMP through its intrinsic guanylate cyclase domain [24] and since isatin blocks signaling through NPRA, it is expected to reduce cGMP production. To characterize the activity of our isatin nanocapsule formulation in alleviating allergic lung inflammation, we administered it intranasally to OVA-sensitized and challenged mice and measured cGMP levels in the lung. As a control, one group of mice was given intranasal treatments with small interfering RNA (si9) that blocks expression of NPRA. The cGMP concentration was found to be significantly reduced ($p < 0.001$) in isatin-treated and si9-treated mice compared to non-treated controls (Fig. 1A).

Isatin is known to prevent ligand binding to NPRA, but to the best of our knowledge, there have been no reports describing its effects on NPRA gene expression. As a preliminary test we determined whether isatin could reduce NPRA levels *in vitro* using a model cell line, HEK293, human embryonic kidney cells. LPS-stimulated HEK 293 cells were treated with increasing concentrations of isatin for 12 h. We found that isatin at concentrations of 50 and 100 μ M reduced NPRA expression in a dose-dependent manner (Fig. 1B). This unexpected result demonstrates

a heretofore-unreported mode of action of isatin in suppressing NPRA signaling.

To determine whether isatin down-regulates NPRA *in vivo*, OVA-allergic mice were given intranasal treatments with isatin-chitosan nanocapsules either after (Fig. 1C) or before (Fig. 1D) OVA challenge. For comparison, one group of mice received nanoparticles containing siNPRA plasmid (si9) against NPRA instead of isatin prior to OVA challenge. Lungs were removed and sectioned, stained for NPRA and examined by immunofluorescence microscopy. The results show that intranasal treatment of mice with isatin reduced expression of NPRA when given either prophylactically or therapeutically. As expected, the small interfering RNA si9 down-regulated NPRA expression in lung cells.

3.2. Prophylactic isatin treatment decreases airway hyperreactivity

Airway hyperreactivity is characteristic of asthma, and asthmatic mice deficient in NPRA are resistant to methacholine-induced bronchoconstriction [24]. Prophylactic intranasal treatment with isatin nanoparticles was tested to determine if its down-regulation of NPRA and

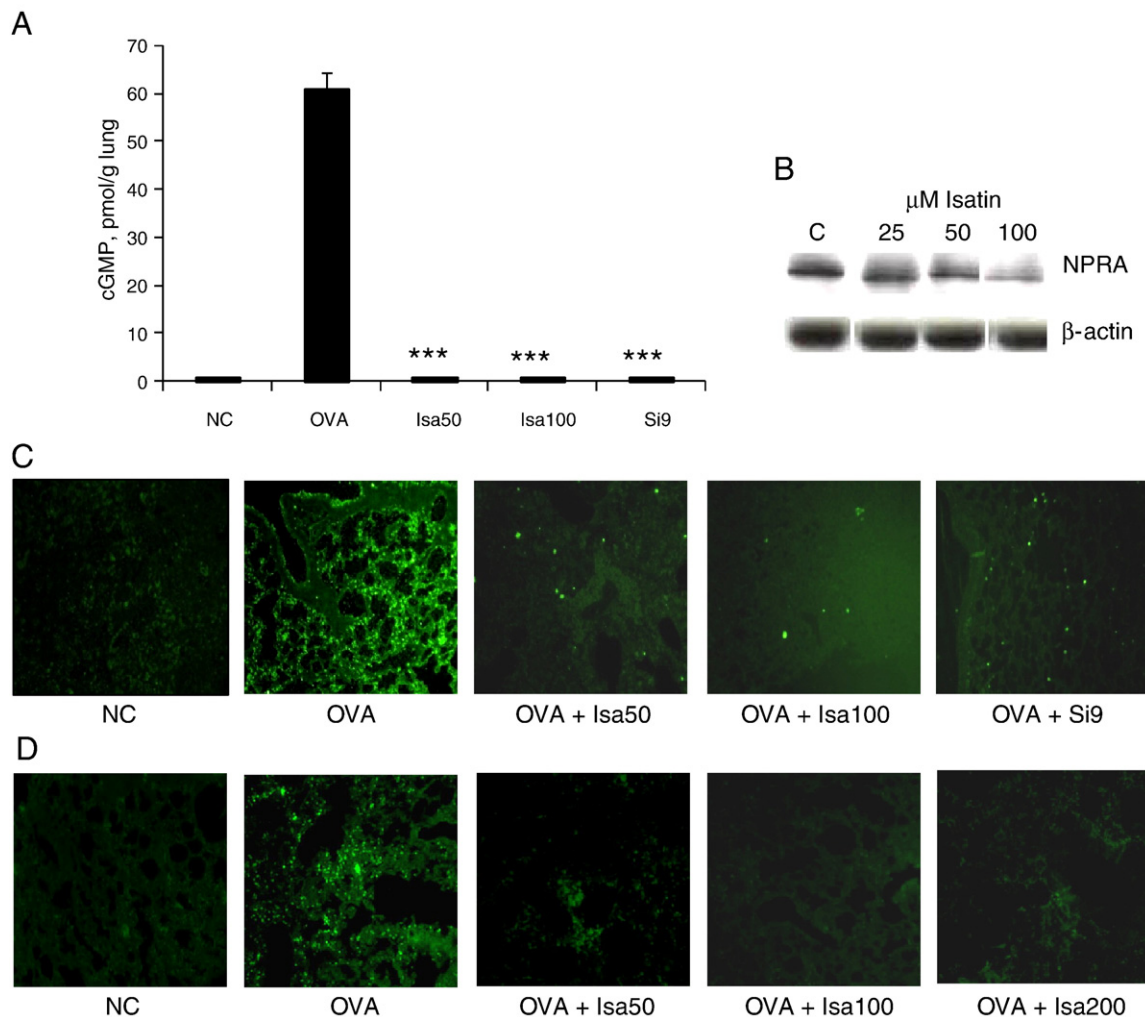


Fig. 1. Isatin nanocapsules block cGMP production and down-regulate NPRA expression. (A). Therapeutic isatin reduces cGMP production. BALB/c mice were sensitized and challenged with ovalbumin (OVA), and treated intranasally with chitosan nanoparticles encapsulating isatin (Isa) at 50 or 100 mg/kg or with si9 vector that expresses siNPRA. Naive control (NC) mice were treated with chitosan or chitosan-vector alone. Lungs were collected, homogenized and cGMP was measured by ELISA. *** $p < 0.001$, OVA vs Isa50, Isa100 and Si9. (B) Isatin inhibits expression of NPRA in kidney cells. HEK293 cells were incubated with or without isatin for 12 h, then harvested and lysed. Equal aliquots of total protein were separated by SDS-PAGE, western blotted and probed with antibodies against NPRA and against beta actin as loading control. (C and D) Isatin inhibits NPRA expression *in vivo*. Mice were treated with intranasal isatin either after OVA challenge (therapeutic model; C) or before OVA challenge (prophylactic model; D) and lungs were fixed, sectioned, stained for NPRA and examined by immunofluorescence microscopy. Representative fields from three separate experiments are shown (200 $\times$).

inhibition of NPRA signaling could attenuate the airway restriction seen after methacholine challenge in OVA-allergic mice. Four groups of mice were tested. The first group served as naive control while the second group received OVA sensitization and challenge but no isatin as a control. OVA-sensitized mice in the third and fourth groups received two different concentrations of isatin prior to OVA challenge. Airway constriction to aerosol methacholine challenge (6.25 and 25 mg/ml) was measured as specific airway resistance (sRaw) by restrained non-invasive airway mechanics, 24 h after OVA challenge. The mice receiving isatin prophylactically had lower sRaw readings than the control group (Fig. 2A). The apparent increase in airway resistance at 100 mg/kg isatin was not statistically significant.

3.3. Prophylactic isatin treatment reduces IL-4, IL-5 and TNF- α

The asthmatic lung has a cellular milieu characterized by a Th2-like phenotype and chronic airway inflammation leading to reversible episodes of airway obstruction [25,26]. Data from animal models have implicated the T helper cell 2 (Th2) cytokines IL-4, IL-5, and IL-13 in the pathogenesis of asthma and allergic airway disease [26]. To examine whether isatin can alter this Th2 bias, OVA-allergic mice were treated prophylactically with chitosan-isatin nanoparticles, lungs were removed and lung homogenates assayed for the indicated cytokines. Significant reduction of IL-4 and IL-5 was observed in the isatin-treated

groups (Fig. 2B) indicating a possible repolarization from a Th2-like phenotype to Th1. TNF- α was inhibited, but no change in IL-2 or INF- γ could be detected in any of the groups.

3.4. Prophylactic isatin treatment reduces lung pathology and infiltration of immune cells into the lung

The most direct indicator of allergic airway inflammation is lung histopathology. In order to ascertain treatment-associated changes, lungs were removed 24 h after OVA challenge from OVA-allergic mice treated prophylactically with isatin nanoparticles. Lung sections were stained with hematoxylin and eosin and several fields from each slide were examined by blinded observers and photographed. Representative fields are shown (Fig. 2C). Isatin reduced goblet cell hyperplasia and epithelial denudation.

In order to determine the effects of isatin nanoparticles on cell infiltration into the lungs, bronchoalveolar lavage (BAL) fluid was collected and BAL cells were stained using a differential cell staining kit. Total cells were counted in at least four fields and averaged. The effect of prophylactic isatin on the numbers of lymphocytes, eosinophils, and neutrophils is shown in Fig. 2D. Treatment with isatin nanoparticles significantly ($p < 0.001$) reduced eosinophil and neutrophil recruitment into the lungs relative to the untreated control. Treatment with si9 had effects similar to those of isatin at 100 mg/kg.

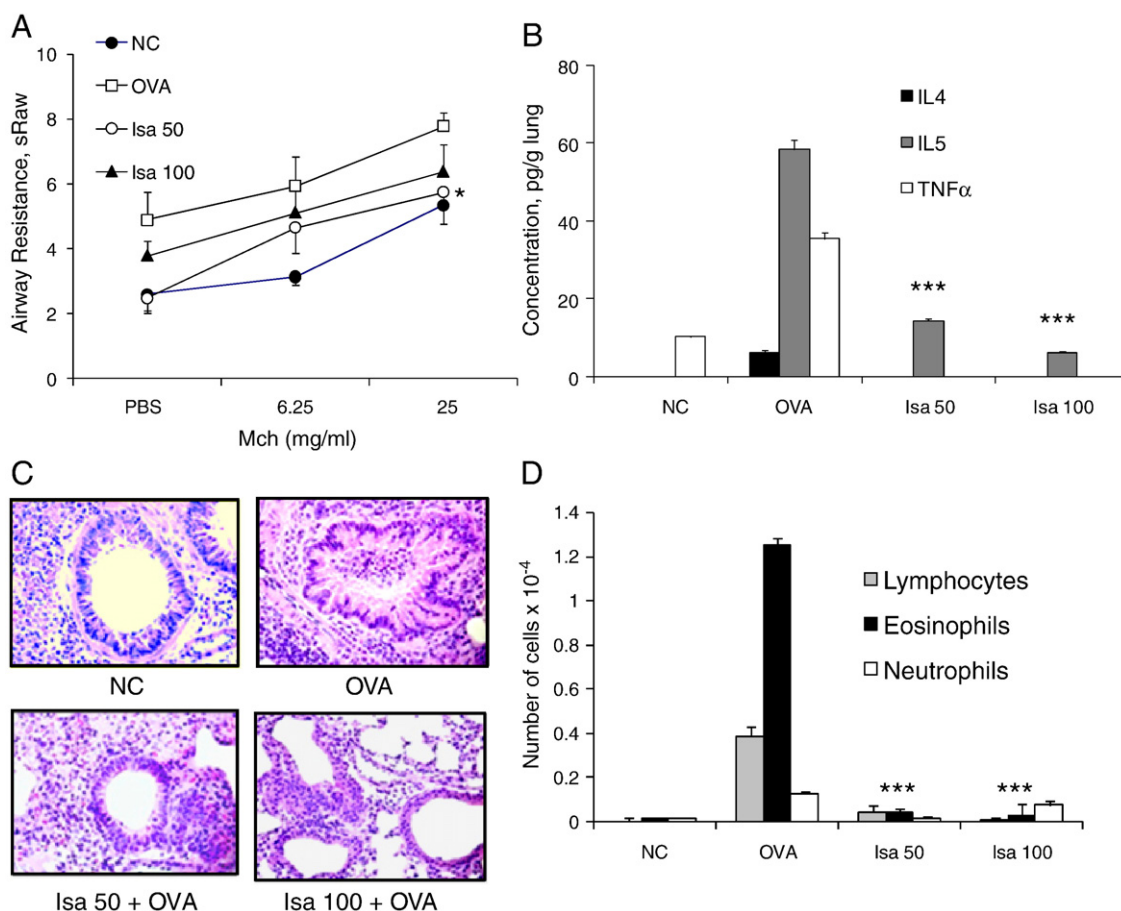


Fig. 2. Prophylactic isatin treatment reduces allergen-induced lung hyperreactivity and inflammation. Mice were sensitized to ovalbumin (OVA) then treated intranasally with isatin encapsulated in chitosan nanoparticles (Isa) at 50 or 100 mg/kg. After prophylactic isatin treatment, airway inflammation was induced by intranasal challenge with 50 μ g OVA. Naïve control (NC) mice were not OVA-sensitized but were treated with chitosan alone. (A) Isatin prevents airway hyperreactivity. Airway hyperreactivity to methacholine (Mch) was measured as specific airway resistance (sRaw) using non-invasive dual-chamber plethysmography. * $p < 0.05$, OVA vs Isa100. (B) Isatin inhibits production of pro-inflammatory cytokines. Lungs were collected, homogenized and cytokines were measured by ELISA. *** $p < 0.001$, OVA vs Isa50, Isa100. (C) Isatin prevents lung histopathology. Lungs were fixed, sectioned and stained with hematoxylin and eosin. Representative photos from three separate experiments are shown (200 $\times$). (D) Isatin reduces eosinophilia. BALF was collected, cells were stained and differential cell counts were performed. *** $p < 0.001$, OVA vs Isa50 and Isa100.

3.5. Therapeutic isatin treatment decreases airway hyperreactivity

Chitosan nanoparticles complexed with isatin at 50 and 100 mg/kg were administered therapeutically after OVA challenge to test their effectiveness in reducing bronchoconstriction and alleviating an asthmatic exacerbation. Five groups of mice were tested. The first group served as naive control while the second group received OVA sensitization and OVA challenge but no isatin as the positive control. Animals in the third and fourth groups were isatin-treated while the fifth group received si9. Airway constriction in response to aerosol methacholine challenge (6.25 and 25 mg/ml) was measured as sRaw (specific airway resistance) 24 h after isatin therapy. The isatin-treated mice had significantly lower sRaw readings than controls (Fig. 3A).

3.6. Therapeutic isatin treatment reduces cellular infiltration into the lung and protects against lung pathology

After methacholine challenge and measurement of sRaw, the mice were euthanized and lungs were lavaged to recover infiltrated cells. Cytoцентрифугed BAL cells were fixed and stained for differential cell counting. Fig. 3B shows the total numbers of lymphocytes, eosinophils and monocytes averaged for each group. Therapeutic treatment with isatin nanoparticles significantly reduced the recruitment of eosinophils into lungs of OVA-challenged mice. H and E-stained lung sections from

mice treated with isatin showed a substantial decrease in lung inflammation, goblet cell hyperplasia and infiltration of inflammatory cells compared to the non-treated OVA group (Fig. 3C).

Lung pathology was quantitated by blinded observers who scored peribronchial inflammation according to the criteria outlined in Methods. Naïve animals had no detectable lung inflammation, whereas the OVA group without isatin treatment had significant peribronchial and perivascular inflammation (Table 1). Compared with the untreated group, lung inflammation was significantly ($p < 0.001$) inhibited in the isatin-treated groups.

3.7. Therapeutic isatin treatment reduces inflammatory cytokines

Lung pathology and eosinophilia are accompanied by increased levels of pro-inflammatory cytokines in the lung. Measurements of IL-4, IL-5, and TNF- α in lung homogenates revealed that therapeutic isatin treatment of OVA-challenged mice reduced the levels of these pro-inflammatory cytokines (Fig. 3D).

4. Discussion

Asthma symptoms can be controlled by the currently used medications, but chronic inflammation can cause asthma to become progressively worse and new therapeutic strategies that circumvent this are

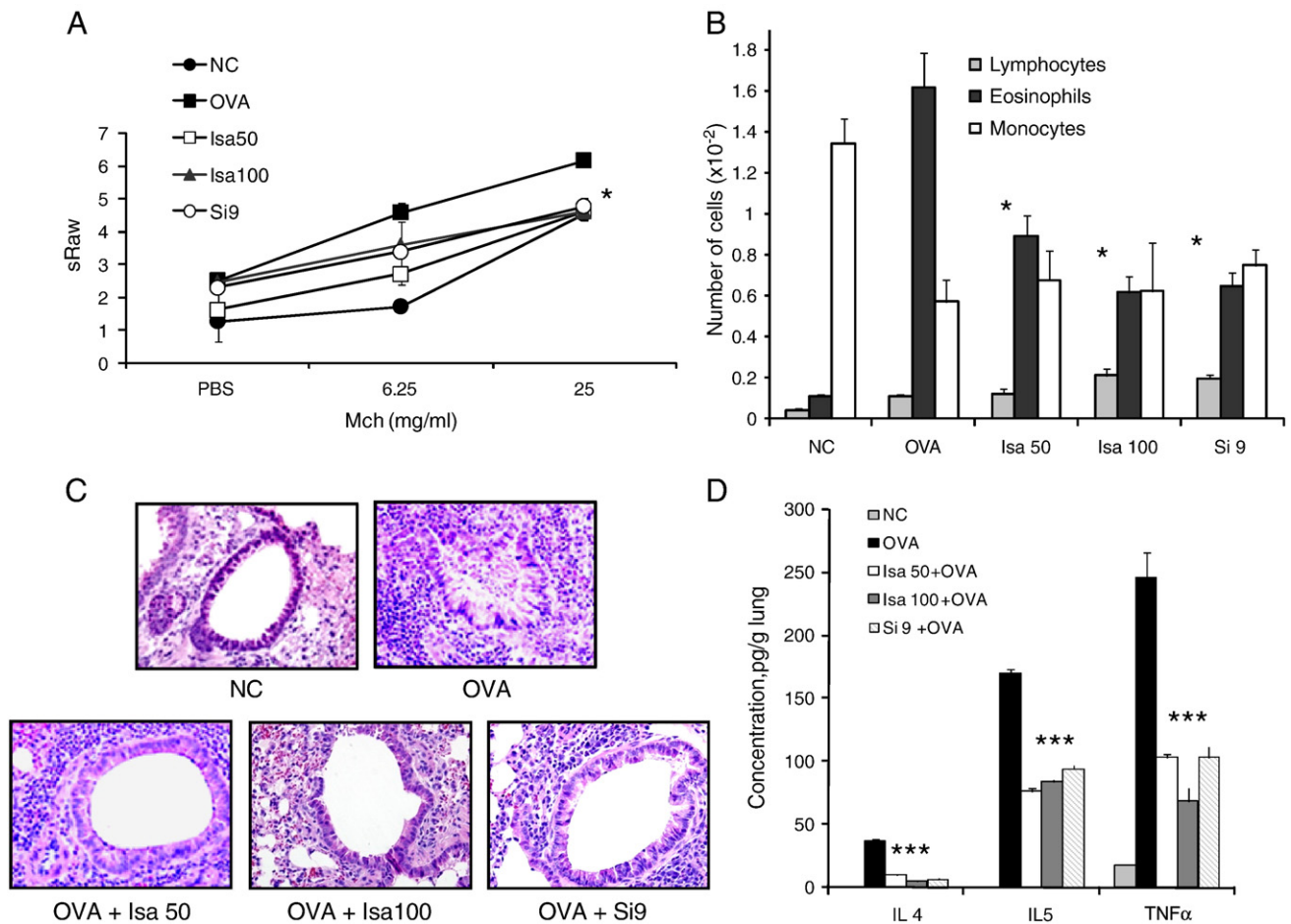


Fig. 3. Therapeutic isatin treatment reduces allergen-induced lung pathology. Mice were sensitized to ovalbumin (OVA) and challenged with 50 μ g OVA to trigger airway inflammation. They were then treated intranasally with chitosan-encapsulated isatin nanoparticles carrying isatin at 50 or 100 mg/kg or a plasmid encoding small interfering RNA against NPRA (Si9). Naïve control (NC) mice were not OVA-sensitized but were treated with chitosan or chitosan-vector alone. (A) Therapeutic isatin reduces airway hyperreactivity. Airway hyperreactivity to methacholine (Mch) was measured as specific airway resistance (sRaw) using non-invasive dual-chamber plethysmography. (B) Isatin reduces lung eosinophilia. BALF was collected, cells were stained and differential cell counts were performed. * $p < 0.05$. (C) Isatin prevents OVA-induced lung histopathology. Lungs were fixed, sectioned and stained with hematoxylin and eosin. Representative photos from three separate experiments are shown. (D) Isatin therapy reduces production of pro-inflammatory cytokines. Lungs were collected, homogenized and cytokines were measured by ELISA. *** $p < 0.001$, OVA vs Isa50 and Isa100.

Table 1
Semiquantitative analysis of lung damage by histopathological scoring.

	Naive control	OVA	OVA + Isa 50	OVA + Isa 100	Si9
Epithelial changes	0.5 ± 0.166	2.3 ± 0.21	1.2 ± 0.24***	0.9 ± 0.1***	1.2 ± 0.13***
Peribroncho-vascular infiltrate	0.6 ± 0.1	2.2 ± 0.2	1.2 ± 0.2**	1.28 ± 0.2*	1.2 ± 0.2*

Epithelial changes were scored as: 0 = no change, 1 = increased epithelial cell cytoplasm without desquamation, 2 = epithelial desquamation without bronchial exudates composed of inflammatory cells, and 3 = bronchial exudates composed of desquamated epithelial cells and inflammatory cells. Peribronchial inflammation was evaluated on a subjective scale of 0, 1, 2, 3, and 4 corresponding to none, mild, moderate, marked, or severe inflammation, respectively, with an increment of 0.5 if the degree of inflammation fell between two integers^{1,2}. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the untreated group.

needed. The ANP–NPRA signaling axis in the lung has been implicated in causing persistent pro-inflammatory effects and promoting a Th2-like phenotype. Methods for blocking ANP's pro-inflammatory signaling have centered on the ANP receptor, NPRA, which is a membrane-bound protein that contains a guanylyl cyclase domain. Activation of NPRA on epithelial cells through binding of ANP or the similar natriuretic hormone, BNP (brain natriuretic peptide), results in an increase in cytoplasmic cGMP that activates protein kinase G and cGMP-gated ion channels for calcium influx.

Here we show in a mouse model that the acute inflammation of an allergic asthma exacerbation can be reversed through local administration of isatin-chitosan nanoparticles. The unique feature of isatin's effect, which to our knowledge has not been reported before, is the down-regulation of the ANP/BNP receptor NPRA. The mechanism of this action is unknown. Isatin is an endogenous indole widely distributed in mammalian tissues and fluids. Its concentration increases under stress and it exhibits a variety of biological and pharmacologic activities. It is a monoamine oxidase inhibitor, a cholinesterase inhibitor, an anticonvulsant and antihypertensive [18]. It has been demonstrated *in vitro* and in animal studies that isatin inhibits natriuretic peptide signaling through NPRA which in turn reduces the intracellular level of cGMP; thus isatin can act as a natural brake on the activity of ANP.

A peptide, NP73-102, derived from the N-terminal fragment of the cleaved atrial natriuretic peptide prohormone prevented lung inflammation and airway hyperreactivity when administered intranasally in a mouse model of asthma [27]. Subsequently it was found that NP73-102 interferes with ANP signaling in part by down-regulating NPRA expression, establishing this as a potentially viable approach for inhibiting ANP-mediated persistent lung inflammation. In support of this hypothesis, we showed that NPRA-deficient mice sensitized and challenged with ovalbumin had significantly decreased lung eosinophilia, lymphocyte infiltration, goblet cell metaplasia and mucus production compared to wild type mice [24]. In addition, knock down of NPRA expression through the use of small interfering RNA against NPRA (siNPRA) in OVA-sensitized and -challenged BALB/c mice reduced pro-inflammatory Th2 cytokine production [28] further suggesting a potential therapeutic use of inhibiting NPRA signaling for treating inflammatory disease.

The physiological regulation of NPRA expression is not well understood. NPRA is up-regulated by glucocorticoids [29] and by changes in osmolarity in the kidney [30]. ANP down-regulates NPRA and it has been shown that cGMP is required for this process [31]. Mice that have been genetically altered to be deficient in ANP show much higher levels of NPRA mRNA in the lungs than their wild type counterparts [32]. The NPRA gene promoter has at least 3 regions containing transactivating response elements located within 1500 base pairs upstream of the start site and one is associated with both elevated ANP and cGMP [12]. Isatin can bind to a number of proteins including some involved in energy generation and carbohydrate metabolism, cytoskeleton formation, regulation of gene expression and cell differentiation, but no isatin-responsive genes have been found [33].

Increased airway inflammation is associated with activation of the transcription factors nuclear factor-kappa B (NFκB), activator protein-1 (AP1), and the extracellular-regulated kinases 1 and 2 (Erk1,2). NP73-102, the peptide inhibitor of NPRA, decreased ANP-mediated activation of NFκB, AP-1 and Erk 1, 2 in human lung epithelial cells [25]. Cane et al. [34] studied the effects of isatin *in vitro* and found that it dose-dependently reduced Erk-1,2 phosphorylation. Thus, isatin's anti-inflammatory properties, like those of NP73-102, may in part be attributable to its inhibition of Erk-1,2 signaling.

In our study, isatin was complexed with chitosan nanoparticles and administered intranasally. Chitosan is a cationic polysaccharide derived from the natural biopolymer, chitin. Chitosan alone was previously tested to determine if it had any effect on inflammation in our model of allergic asthma [21]. Mice were given intranasal chitosan nanoparticles and compared with a group that received only vehicle. Chitosan had no effect on lung function, inflammatory markers, cellular infiltration or lung histopathology [21]. Chitosan nanoparticles are ideal for drug delivery to the lung because they can be administered in the form of nose drops or a nasal spray and their mucoadhesiveness and size of around 100 nm ensures that they are readily endocytosed by epithelial cells and can deliver a therapeutic dose of drug [21,23].

In acute asthma exacerbations, ANP levels are elevated and there is an increase in lung eosinophil numbers. The evidence that NPRA^{−/−} mice have less eosinophilia and lower levels of Th2-like cytokines compared to wild type indicate that the ANP pathway is pro-Th2, and this is consistent with a previous study that showed that exposing human dendritic cells to ANP promoted Th2-like cytokine expression [35]. Intranasal isatin nanoparticles decrease eosinophilia in OVA-allergic BALB/c mice, reduce airway hyperresponsiveness, and support a phenotypic change from a Th2-like cytokine profile to one characterized by Th1-like cytokines. Our current finding that isatin reduces NPRA protein levels, which decreases lung inflammation, further supports the hypothesis that ANP–NPRA signaling is involved in the inflammatory process.

In summary, we demonstrate that intranasal administration of isatin encapsulated in chitosan nanoparticles can effectively inhibit the ANP–NPRA pathway in the lung and reduce inflammation in a mouse model of asthma.

Acknowledgments

This research was supported by the Joy McCann Culverhouse Foundation, a VA Merit Award to SSM and an NIH grant (1R21HL088299-01A2). RK was supported by a BOYSCAST (Better Opportunities for Young Scientists in Chosen Areas of Science and Technology) fellowship from the Ministry of Science & Technology, Government of India.

References

- [1] Farra Judith Rosen. The global burden of asthma and current approaches to its management. Business briefing: European pharmacotherapy director. Academic Services Connection, Inc.; 2005. p. 1–5.
- [2] Roland NJ, Bhalla RK, Earis J. The local side effects of inhaled corticosteroids: current understanding and review of the literature. *Chest* 2004;126:213–9.
- [3] O'Connor GT. Safety of long-acting beta-agonists. *N Engl J Med* 2006;354:1206–8.
- [4] Chatila TA. Interleukin-4 receptor signaling pathways in asthma pathogenesis. *Trends Mol Med* 2004;10:493–9.
- [5] Vollmar AM. The role of atrial natriuretic peptide in the immune system. *Peptides* 2005;26:1086–94.
- [6] Mohapatra S. Role of natriuretic peptide signaling in modulating asthma and inflammation. *Can J Physiol Pharmacol* 2007;85:754–9.
- [7] Ohbayashi H, Suito H, Takagi K. Compared effects of natriuretic peptides on ovalbumin-induced asthmatic model. *Eur J Pharmacol* 1998;346:55–64.
- [8] Pedram A, Razandi M, Levin ER. Natriuretic peptides suppress vascular endothelial cell growth factor signaling to angiogenesis. *Endocrinology* 2001;142:1578–86.
- [9] Thrane EV, Schwarze PE, Thoresen GH, Lag M, Refsnes M. Persistent versus transient MAP kinase (ERK) activation in the proliferation of lung epithelial type 2 cells. *Exp Lung Res* 2001;27:387–400.
- [10] Medvedev A, Buneva O, Glover V. Biological targets for isatin and its analogs: implications for therapy. *Biol Targets Ther* 2007;1:151.

- [11] Medvedev A, Crumeyrolle-Arias M, Cardona C, Sandler M, Glover V. Natriuretic peptide interaction with [³H]-isatin binding sites in rat brain. *Brain Res* 2005;1042:119–24.
- [12] Glover V, Medvedev A, Sandler M. Isatin is a potent endogenous antagonist of guanylate cyclase-coupled atrial natriuretic peptide receptors. *Life Sci* 1995;57:2073–9.
- [13] Medvedev AE, Sandler M, Glover V. Interaction of isatin with type A natriuretic peptide receptor: possible mechanism. *Life Sci* 1998;62:2391–8.
- [14] Sandler M, Medvedev A, Panova NG, Matta S, Glover V. Milestones in monoamine oxidase research: discovery of (–) deprenyl. Budapest: Meditcina Publishing House Co.; 2000.
- [15] Medvedev A, Bussygyna O, Pyatakova N, Glover V, Severina I. Effect of isatin on nitric oxide-stimulated soluble guanylate cyclase from human platelets. *Biochem Pharmacol* 2002;63:763–6.
- [16] Medvedev AE, Adamik A, Glover V, Telegdy G. Cyclic GMP excretion blocked by isatin administration under conditions of fluid overload. *Biochem Pharmacol* 2001;62:225–7.
- [17] Shvekhgeimer MG. Synthesis of heterocyclic compounds by the cyclization of isatin and its derivatives. *Chem Heterocycl Compd* 1996;32:249–76.
- [18] da Silva JF, Garden SJ, Pinto AC. The chemistry of isatins: a review from 1975 to 1999. *J Braz Chem Soc* 2001;12:273–324.
- [19] Lee DW, Shirley SA, Lockey RF, Mohapatra SS. Thiolated chitosan nanoparticles enhance anti-inflammatory effects of intranasally delivered theophylline. *Respir Res* 2006;7:112.
- [20] Zhang W, Yang H, Kong X, Mohapatra S, San Juan-Vergara H, Hellermann G, et al. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nat Med* 2005;11:56–62.
- [21] Kumar M, Behera AK, Lockey RF, Vesely DL, Mohapatra SS. Atrial natriuretic peptide gene transfer by means of intranasal administration attenuates airway reactivity in a mouse model of allergic sensitization. *J Allergy Clin Immunol* 2002;110:879–82.
- [22] Chen J, Yang WL, Li G, Qian J, Xue JL, Fu SK, et al. Transfection of mEpo gene to intestinal epithelium in vivo mediated by oral delivery of chitosan-DNA nanoparticles. *World J Gastroenterol* 2004;10:112–6.
- [23] Otterlei M, Varum KM, Ryan L, Espevik T. Characterization of binding and TNF- α -inducing ability of chitosans on monocytes: the involvement of CD14. *Vaccine* 1994;12: 825–32.
- [24] Mohapatra SS, Lockey RF, Vesely DL, Gower Jr WR. Mechanisms of asthma and allergic inflammation. Natriuretic peptides and genesis of asthma: an emerging paradigm? *J Allergy Clin Immunol* 2004;114:520–6.
- [25] Bousquet J, Jeffery PK, Busse W, Johnson M, Vignola A. Asthma. From bronchoconstriction to airway inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161: 1720–45.
- [26] Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. *J Clin Invest* 2003;111:291–7.
- [27] Hellermann GR, Kong X, Gunnarsdottir J, San Juan H, Singam R, Behera S, et al. Mechanism of bronchoprotective effects of a novel natriuretic hormone peptide. *J Allergy Clin Immunol* 2004;113:79–85.
- [28] Wang X, Xu W, Mohapatra S, Kong X, Li X, Lockey RF, et al. Prevention of airway inflammation with topical cream containing imiquimod and small interfering RNA for natriuretic peptide receptor. *Gen Vacc Ther* 2008;6:7.
- [29] Nuglozeh E, Mbikay M, Stewart DJ, Legault L. Rat natriuretic peptide receptor genes are regulated by glucocorticoids in vitro. *Life Sci* 1997;61:2143–55.
- [30] Santos-Araújo C, Roncon-Albuquerque Jr R, Moreira-Rodrigues M, Henriques-Coelho T, Quelhas-Santos J, Faria B, et al. Local modulation of the natriuretic peptide system in the rat remnant kidney. *Nephrol Dial Transplant* 2009;24:1774–82.
- [31] Cao L, Wu J, Gardner DG. Atrial natriuretic peptide suppresses the transcription of its guanylyl cyclase-linked receptor. *J Biol Chem* 1995;270:24891–7.
- [32] Vera N, Tse MY, Watson JD, Sarda S, Steinhilber ME, John SW, et al. Altered expression of natriuretic peptide receptors in proANP gene disrupted mice. *Cardiovasc Res* 2000;46: 595–603.
- [33] Fedchenko V, Globa A, Kaloshin A, Kapitsa I, Nerobkova L, Val'dman E, et al. The effect of short-term administration of (–)-deprenyl and isatin on the expressions of some genes in the mouse brain cortex. *Med Sci Monit* 2008;14:BR269–73.
- [34] Cane A, Tournaire MC, Barritault D, Crumeyrolle-Arias M. The endogenous oxindoles 5-hydroxyoxindole and isatin are antiproliferative and proapoptotic. *Biochem Biophys Res Commun* 2000;276:379–84.
- [35] Morita R, Ukyo N, Furuya M, Uchiyama T, Hori T. Atrial natriuretic peptide polarizes human dendritic cells toward a Th2-promoting phenotype through its receptor guanylyl cyclase-coupled receptor A. *J Immunol* 2003;170:5869–75.