

Niflumic Acid Suppresses Interleukin-13–induced Asthma Phenotypes

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Rationale: Chloride channels have been implicated in the regulation of mucus production in epithelial cells. Expression of hCLCA1, a calcium-activated chloride channel, has been reported to be increased in the airway epithelium of patients with asthma. Interleukin (IL)-13 induces the cardinal features of bronchial asthma, and glucocorticoids are not sufficient to suppress IL-13–induced airway hyperresponsiveness or goblet cell hyperplasia.

Objectives: We studied the effects of chloride channel inhibitors in IL-13–induced asthma.

Methods: The effects of niflumic acid (NA), a relatively specific blocker of calcium-activated chloride channel (CLCA), on goblet cell hyperplasia, eosinophil accumulation, and airway hyperresponsiveness were evaluated after IL-13 instillation into the airways. Because IL-13–dependent features rely on JAK/STAT6 signaling, the effect of NA on phosphorylation of JAK2 and STAT6 after IL-13 stimulation was examined in airway epithelial cells *in vitro*. The expression of the mCLCA family in mouse lung after IL-13 local administration *in vivo* was analyzed using reverse transcription–polymerase chain reaction.

Measurements and Main Results: Treatment with NA inhibited not only IL-13–induced goblet cell hyperplasia but also airway hyperresponsiveness and eosinophilic infiltration. NA suppressed the eotaxin levels in bronchoalveolar lavage fluids and overexpression of the MUC5AC gene, a marker of goblet cell hyperplasia, in the lung after IL-13 instillation. NA suppressed JAK2 activation, STAT6 activation, and eotaxin expression in epithelial cells. The expression of mCLCA3 (mouse homolog hCLCA1), but not that of other CLCA family members, was up-regulated by IL-13.

Conclusions: These findings suggest that a chloride channel inhibitor can control IL-13–mediated airway features at least by suppressing JAK/STAT6 activation.

Keywords: airway hyperreactivity; calcium-activated chloride channel; goblet cell metaplasia; STAT6

Chloride channels play important roles in diverse processes, including volume regulation, cellular excitability, cell cycle, and muscle tone. In epithelial cells, chloride channels are fundamental for maintaining membrane potential and the movement of chloride ions for fluid and electrolyte transport and have been implicated in the regulation of mucus production (1, 2). A family

of calcium-activated chloride channels, termed CLCA, has recently been identified at the molecular level in many epithelial, endothelial, and smooth muscle cell types (3). Four (hCLCA1 to hCLCA4) and six (mCLCA1 to mCLCA6) family members of CLCA have been reported in human and mouse, respectively (3–6).

Bronchial asthma is characterized by chronic inflammation, eosinophilic infiltration, reversible airway narrowing, airway hyperresponsiveness (AHR) (1) to nonspecific stimuli, hyperplasia/metaplasia of goblet cells, and subepithelial fibrosis (7, 8). CD4⁺ Th2 lymphocytes and their cytokine products, including interleukin (IL)-4, IL-5, IL-9, and IL-13, are essential for generating these abnormalities. Among Th2 cytokines, IL-13 is considered particularly critical. The *in vivo* blockade of IL-13 markedly inhibits allergen-induced AHR, eosinophilia, and mucus overproduction (9, 10). Furthermore, local administration of recombinant IL-13 to nonimmunized mice induces the asthma phenotype (10, 11). Glucocorticoids are not sufficient to suppress IL-13–induced AHR or goblet cell hyperplasia (11). IL-13 binding to its receptor leads to phosphorylation of the signal transducer and activator of transcription-6 (STAT6), which translocates to the nucleus and affects the transcription of many genes. The lipoxygenase products arginase I, adenosine, and acidic mammalian chitinase are reported to contribute to IL-13–induced tissue responses (12–17). However, the precise mechanisms by which this cytokine mediates the pathophysiologic features of asthma are not clear.

CLCA family members may be involved in asthma. mCLCA3/Gob-5 up-regulation in a murine model of asthma is strongly associated with mucus overproduction (18), and the expression of its human homolog hCLCA1 is up-regulated in the airway epithelium of patients with asthma (19, 20). mCLCA3 expression is reportedly induced by intratracheal administration of Th2 cytokines (21), and mCLCA3 is also increased in the lung of IL-13 transgenic animals, depending on STAT6 signals in airway epithelial cells (22). IL-13 inhibits ciliated cell differentiation of airway epithelial cells and increases secretory cells *in vitro* (23). IL-13 induces a hypersecretory ion transport phenotype in airway epithelial cells *in vitro* associated with enhanced calcium-activated chloride conductance (24). However, the role of CLCAs in IL-13–induced AHR and eosinophilia is not clear.

Although specific antagonists for CLCAs are lacking, several potent chloride channel inhibitors, including 4,4′-diisothiocyanostilbene-2, 2′-disulfonic acid (DIDS), diphenylamine-2-carboxylic acid, and niflumic acid (NA), are available. DIDS and diphenylamine-2-carboxylic acid have low selectivity for different chloride channel subtypes (25). NA, on the other hand, is considered to be a more specific blocker of CLCA (26–28). Here we show that NA inhibited all pulmonary effects of IL-13 through suppressing JAK/STAT6 activation and that mCLCA3 expression, but not that of other CLCAs, in the lung was up-regulated by IL-13 local administration. Some of the results of this study have been previously reported in the form of an abstract (29).

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METHODS

Protocol

Male 6-wk-old A/J mice received an intratracheal instillation of 0.5 μ g recombinant murine IL-13 solution (Sigma, St. Louis, MO) or phosphate-buffered saline on Days 1, 3, and 5 as described previously (11). NA (Sigma) was dissolved in 0.4 M NaHCO₃ in 5% glucose and administered intraperitoneally (30 mg/kg) 24 h before and 1 h before the first instillation of IL-13 on four consecutive days (Day 0 to Day 5), and 0.4 M NaHCO₃ in 5% glucose was used as the vehicle. The dose of NA used in this study was chosen on the basis of previous *in vivo* experiments (28).

Measurements of airway responsiveness, bronchoalveolar lavage, histologic assessment, and total RNA extraction from lung tissues were performed on Day 6, 24 h after the last instillation.

Measurement of Airway Responsiveness

Mice were anesthetized, and their tracheas were cannulated via tracheostomy. The animals were ventilated to measure AHR to acetylcholine aerosol as described previously (11). The data are expressed as the provocative concentration 200 (PC₂₀₀; i.e., the concentration at which airway pressure was 200% of its baseline value). Further details are provided in the online supplement.

BAL and Cell Counting

Mice were given a lethal dose of pentobarbital, and lungs were gently lavaged once with 1.0 ml saline via the tracheal cannula. Total cell counts and differential counts were performed (11).

ELISA

Mouse eotaxin in the supernatant of bronchoalveolar lavage fluid (BALF) and mouse and human eotaxin in the supernatants of cell culture were measured using ELISA kits (R&D Systems, Inc., Minneapolis, MN).

Histologic Assessment

Lungs were fixed with 10% formalin, and tissue sections were stained with Alcian blue/periodic acid-Schiff (AB/PAS) to determine the presence of mucin glucoconjugates. Numeric scores for the abundance of PAS-positive, mucus-containing cells in each airway were determined as described previously (30).

Determination of mRNA Expression of CLCAs and MUC5AC

Expression of mCLCA genes and MUC5AC was analyzed using reverse transcription–polymerase chain reaction (RT-PCR). Further details are provided in the online supplement.

Cell Culture and Reagents

TGMBE-02-3 cells, a mouse tracheal epithelial cell line, and BEAS-2B cells, a transformed human airway epithelial cell line, were cultured as described in the online supplement.

NA or DIDS was prepared as a stock solution of 100 mM in dimethyl sulfoxide (DMSO) and diluted in a culture medium. Cells were pretreated with 100 μ M NA, 100 μ M DIDS, or vehicle DMSO for 24 h, and after that mouse or human IL-13 (Peprotech, Rocky Hill, NJ) was added for the indicated period. The dose of NA or DIDS was chosen on the basis of previous *in vitro* experiments (31, 32).

Western Blotting

Immunoblotting was performed using antiphospho JAK2 (Calbiochem, San Diego, CA), antiphospho STAT6 (New England Biolabs, Beverly, MA), anti-JAK2, or anti-STAT6 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies as described (33).

Data Analysis

Groups were compared by analysis of variance or the Mann-Whitney U test. Values of $p < 0.05$ or less were considered significant. Details are described in the online supplement.

RESULTS

Effects of NA on IL-13–induced Goblet Cell Hyperplasia

Chloride channels have been implicated in the regulation of mucus production in epithelial cells (1, 2). We first studied the inhibitory effects of NA, a relatively selective antagonist of CLCA on goblet cell hyperplasia induced by IL-13. AB/PAS staining cells were rare in the airways of naive mice and in animals that had received vehicle instillations. In mice that had received IL-13, there were prominent AB/PAS staining cells in the trachea, the main bronchi, and in the intrapulmonary bronchi. Treatment with NA caused marked decreases in the levels of AB/PAS staining cells after IL-13 (Figures 1A and 1B).

We also analyzed the expression of the MUC5AC gene, a marker of goblet cell hyperplasia. There was no difference in MUC5AC expression after vehicle instillation compared with that in naive animals (data not shown). IL-13 instillation markedly enhanced the expression of MUC5AC mRNA in the lung, and NA treatment suppressed IL-13–induced overexpression of MUC5AC (Figure 1C). Effects of NA on MUC5AC expression were also analyzed after single IL-13 instillation, and NA suppressed overexpression of MUC5AC 24 h after IL-13 (Figure E1 of the online supplement).

Effects of NA on AHR and on Eosinophils in BALF after IL-13

Intratracheal administration of IL-13 caused decreases in PC₂₀₀ values (Figure 2A). IL-13 also increased significantly the concentration of eotaxin in BALF and the number of eosinophils compared with the control (Figure 2B). Treatment with NA significantly suppressed the decrease in PC₂₀₀ values after IL-13 instillation (Figure 2A). NA also caused significant decreases in IL-13–induced eotaxin levels in BALF and eosinophil accumulation (Figure 2B). Treatment with NA did not change the number of macrophages, neutrophils, and lymphocytes in BALF after IL-13 instillation. There were no significant differences in baseline values of airway pressure among the control, IL-13 instillation alone, and IL-13 plus NA groups. We conclude that NA inhibited not only goblet cell hyperplasia but also AHR and eosinophilic inflammation induced by IL-13.

It has been reported that NA has an inhibitory effect on cyclooxygenase (34). We studied the effect of indomethacin on IL-13–induced airway responses. Indomethacin (1.0 mg/kg) was administered intraperitoneally 24 h before and 1 h before the first instillation of IL-13 and on four consecutive days. Treatment with indomethacin did not affect IL-13–induced eosinophil accumulation or AHR (Figure 2) and caused no increase in AB/PAS staining cells (data not shown).

To assess whether NA may modulate expression of IL-13 receptor (IL-13R), we examined mRNA expression of IL-13R in the lung *in vivo*. NA did not affect the expression of soluble IL-4R, membrane IL-4R, IL-13R α 1, or IL-13R α 2 (data not shown).

Effect of Chloride Channel Inhibitors on Eotaxin Expression and JAK/STAT6 Activation in Airway Epithelial Cells after IL-13 Treatment

Treatment with NA unexpectedly suppressed eosinophil infiltration and eotaxin production *in vivo*. Airway epithelial cells are known to be one of the important sources of eotaxin *in vivo*, and eotaxin expression has been demonstrated *in vitro* using cultured epithelial cells (35, 36). We evaluated the effect of NA on eotaxin expression in murine airway epithelial cells *in vitro*. In TGMBE-02-3 cells, IL-13 increased eotaxin production. Treatment with NA or with another chloride channel inhibitor (DIDS) significantly suppressed eotaxin expression induced by

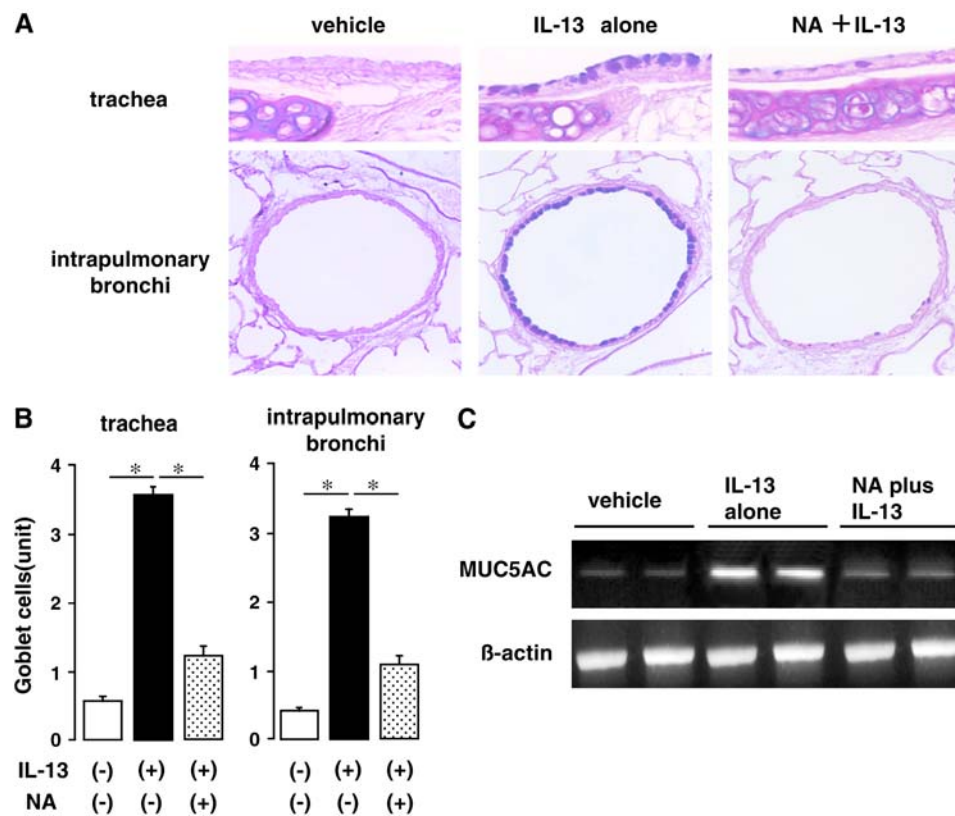


Figure 1. (A) Representative light photomicrographs of mouse lung tissue stained with Alcian blue–periodic acid–Schiff (AB/PAS) to identify goblet cells (original magnification: $\times 200$). There are marked increases in goblet cells in the epithelium of the trachea and of the intrapulmonary bronchus 24 h after interleukin (IL)-13 instillation compared with those in vehicle-treated animals. Treatment with niflumic acid (NA) caused marked decreases in the levels of AB/PAS staining cells after IL-13 instillation. (B) Semiquantitative analysis of the abundance of PAS-positive, mucus-containing cells. Lung tissue sections were obtained from formalin-fixed, paraffin-embedded lung tissue prepared and stained with AB/PAS. The numeric scores for the abundance of PAS-positive, mucus-containing cells in each airway were determined as follows: 0, $< 5\%$ PAS-positive cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, $> 75\%$. * $p < 0.05$ by Kruskal-Wallis test with Mann-Whitney U test. (C) MUC5AC expression in vehicle-treated, IL-13-treated, and NA plus IL-13-treated mice. Whole-lung total RNA was isolated, and MUC5AC levels were amplified by reverse transcription–polymerase chain reaction. Reverse transcription–polymerase chain reaction products for β -actin are shown for comparison. Amplified DNA was separated by electrophoresis on an agarose gel containing ethidium bromide, illuminated with ultraviolet light, and photographed.

IL-13 (Figure 3A). Indomethacin did not affect IL-13-induced eotaxin expression.

IL-13 exerts its actions by binding to the IL-13R on the cell surface, the heterodimer composed of the IL-13R α 1 chain and the IL-4R α chain. IL-13 binds to IL13R α 1 and then recruits IL-4R α to the complex. Heterodimerization of IL-13R causes activation of Janus kinases JAK1, JAK2, and TYK2, followed by activation of STAT6 (37). IL-13-dependent features depend on STAT6 signaling (38). We next tested the effect of channel inhibitors on JAK2 and STAT6 phosphorylation by IL-13 in a human airway epithelial cell line. STAT6 was activated and eotaxin levels in supernatant were increased after IL-13 stimulation, and NA suppressed STAT6 activation and eotaxin expression (Figures 3B and 3C). NA also suppressed JAK2 activation induced by IL-13 (Figure 3D). DIDS also suppressed eotaxin expression induced by IL-13. The solvent DMSO at equivalent concentrations to those used to dissolve NA or DIDS did not affect the phosphorylation of JAK2 or STAT6 or eotaxin expression (data not shown). These findings indicate that the inhibitory action on IL-13-induced chemokine expression of NA is due, at least in part, to a JAK/STAT6-dependent pathway.

mCLCA Expression after IL-13

We analyzed the mRNA expression of the CLCA family in the lung after IL-13 instillation into the airways. IL-13 instillation markedly enhanced mCLCA3 expression in the lung (Figure 4). In contrast, the expressions of other mCLCA family members were not affected by IL-13. Because there were no detectable signals of mCLCA2 and mCLCA6 in the lung, the expression

of these genes was analyzed in the intestine as a positive control. There was no difference in any mCLCA gene expression after vehicle instillation compared with that in naive animals (data not shown). Expression of mCLCA3 on earlier time points was also analyzed by real-time RT-PCR after single IL-13 instillation, and there was an increase in mCLCA3 expression 12 h and 24 h after IL-13 (Figure E2).

In BEAS-2B cells, hCLCA1 expression was rapidly increased after IL-13 stimulation (Figure E3).

DISCUSSION

In this study, we demonstrated that treatment with the chloride channel blocker NA inhibits all pulmonary effects of IL-13, including goblet cell hyperplasia, AHR, eosinophilic infiltration, and overexpression of eotaxin and a mucin gene MUC5AC. NA also suppressed JAK/STAT6 activation and eotaxin expression in cultured airway epithelial cells. These findings indicate that NA can control IL-13-induced asthma at least through suppressing STAT6 activation.

We used two different chloride channel blockers in this study. DIDS has low selectivity for different chloride channel subtypes (25). NA, on the other hand, is considered to be a more specific blocker of CLCA channels (26–28). Both chloride channel blockers suppressed IL-13-induced eotaxin expression. NA attenuated JAK/STAT6 activation and eotaxin expression in epithelial cells *in vitro*. Therefore, chloride channel blockers may act upstream of JAK2/STAT6 activation. NA did not affect IL-13 receptors expression, and we could not identify the site(s) of

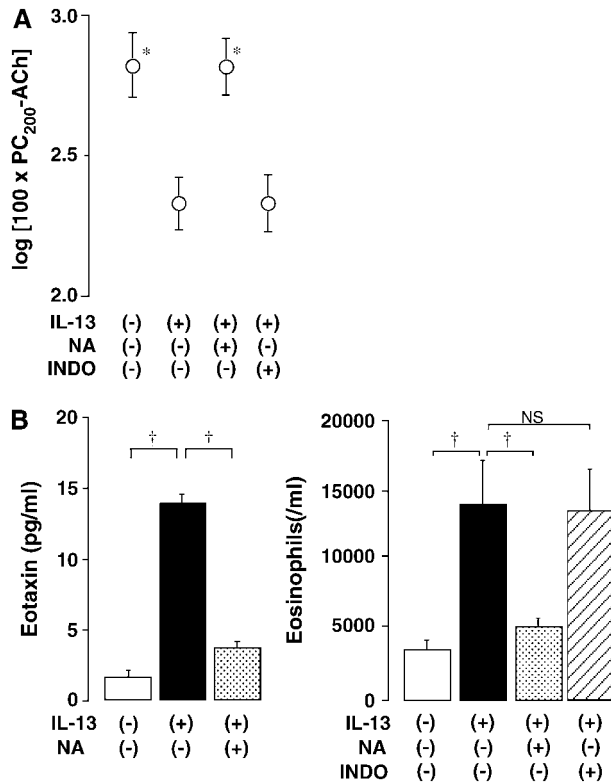


Figure 2. (A) Effect of treatment with NA or with indomethacin (INDO) on airway responsiveness to inhaled acetylcholine (ACh) after intratracheal instillation of IL-13. Airway responsiveness to inhaled ACh was measured 24 h after the last instillation. IL-13 was dissolved in vehicle (phosphate-buffered saline), and this vehicle was used for control. Under anesthesia with a mixture of ketamine and xylazine intraperitoneally, animals were given vehicle or cytokine solution intratracheally. Treatment with NA significantly suppressed the decrease in PC₂₀₀ values after IL-13. $n = 9-13$. * $p < 0.05$ compared with IL-13 instillation alone. (B) Effect of treatment with NA or with INDO on eosinophil counts and on eotaxin levels in bronchoalveolar lavage fluid after IL-13 instillation. IL-13 induces marked eosinophilia and up-regulates eotaxin expression. NA, but not INDO, significantly inhibits IL-13-induced eotaxin production and abolished eosinophilia. $n = 9-13$. † $p < 0.01$. NS = not significant.

action of chloride channel inhibitor in IL-13 signaling. It has been demonstrated that impaired cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel activity contributes to nuclear factor- κ B activation and IL-8 expression in cell with CFTR mutation (39). To our knowledge, there is no report regarding the effect of CLCA on cytokine signaling. IL-13 activates the JAK/STAT signaling cascades and the insulin receptor substrate family/phosphatidylinositol 3-kinase pathway (40). In addition, p42/p44 ERK and p38 MAPK pathways are involved in IL-13-dependent eotaxin release (41). The ligation between mCLCA1 and β_4 integrin is reported to trigger focal adhesion kinase-mediated downstream activation of ERK (42). Chloride channels may interact with IL-13 signaling cascades directly or indirectly.

IL-13 has a variety of proinflammatory effects that are relevant to asthma and other Th2-dominated inflammatory disorders, including the ability to induce IgE production and endothelial cell VCAM-1 expression. Previous studies have demonstrated that IL-13 is a potent inducer of mCLCA3/Gob-5 gene expression (21, 22). The administration of antisense gob-5 sequence markedly suppressed

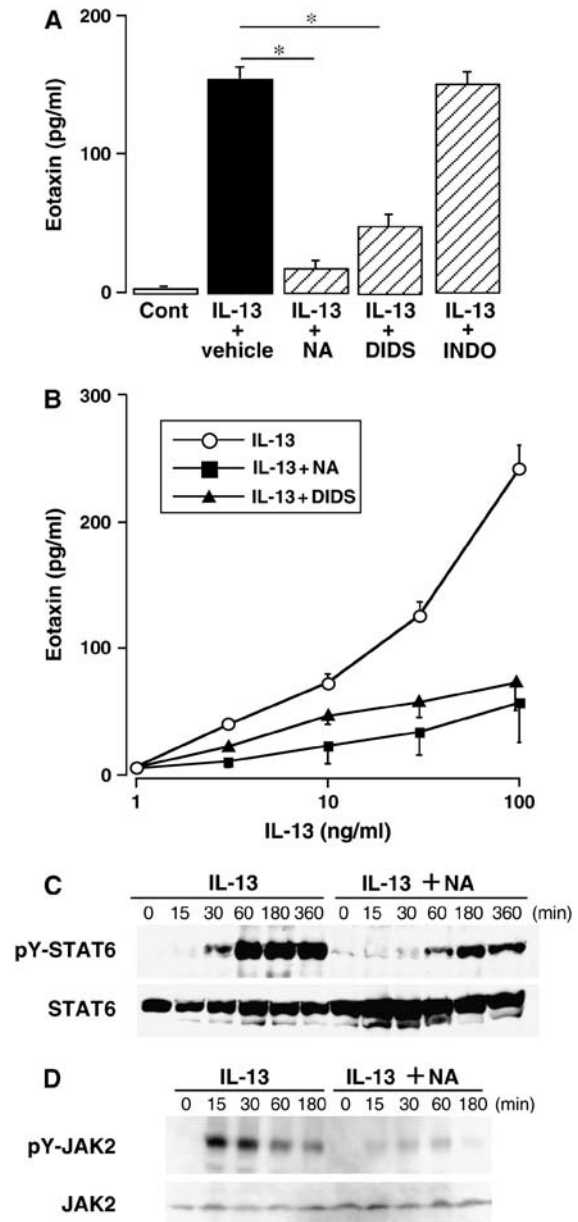


Figure 3. Effect of chloride channel inhibitors on eotaxin expression and STAT6 activation after IL-13 treatment. (A) IL-13 (50 ng/ml) increased eotaxin production from murine TGMBE-02-3 cells. Treatment with NA or with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) suppressed eotaxin expression induced by IL-13. $n = 5$. * $p < 0.05$. (B) In human BEAS-2B cells, eotaxin levels in the supernatant were increased after IL-13 stimulation dose dependently. NA and DIDS suppressed eotaxin expression. (C and D) STAT6 and JAK2 were activated by IL-13, and NA suppressed STAT6 activation.

eosinophilia, AHR, and goblet cell mucus production (18). The present work revealed that IL-13 selectively up-regulates mCLCA3 expression within the mCLCA family and that NA inhibits all pulmonary effects of IL-13 *in vivo* and eotaxin expression in cultured airway epithelial cells. Thus, it is speculated that mCLCA3 mediates an IL-13-induced asthmalike phenotype. However, a recent report suggested that mCLCA3/Gob-5 expression is not essential for mucin overproduction in murine models of allergic asthma using Gob-5 knockout mice (43). The CLCA family continues to grow (5, 6), and another novel CLCA

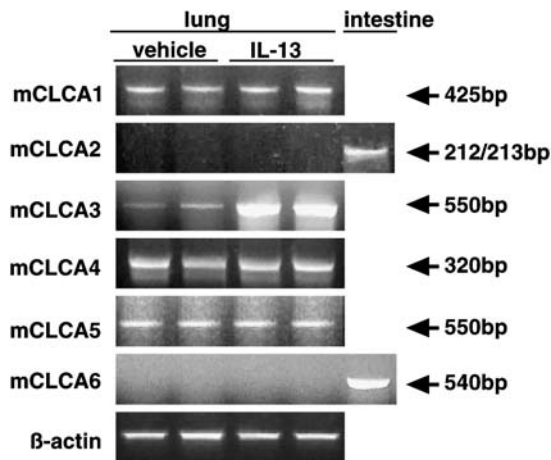


Figure 4. Reverse transcription-polymerase chain reaction analysis of mCLCA1, mCLCA2, mCLCA3, mCLCA4, mCLCA5, and mCLCA6 expression in the lung after airway instillation of vehicle or IL-13. Amplified DNA was separated by electrophoresis on an agarose gel containing ethidium bromide, illuminated with ultraviolet light, and photographed. For mCLCA1 and mCLCA2, cDNA was generated from lung and subjected to polymerase chain reaction using primers that recognize mCLCA1 and mCLCA2, spanning base pairs 650–1,075; the product was cleaved with EcoRI, taking advantage of an EcoRI site found at the center of this interval in mCLCA2 but not mCLCA1. mCLCA2 and mCLCA6 were not detected in the lung, and their expression in intestine was shown as positive control. β -actin was amplified as an internal control.

family member may be a site of action of NA. NA and DIDS are not completely specific blockers of chloride channel. It is not clear that the action of NA is on a chloride channel. A recent study concluded that hCLCA1 and mCLCA3 are not ion channels but rather secreted proteins (44). It is possible that the inhibitory effects of NA might not be attributable to its inhibition of mCLCA3 or any other chloride channel.

Several signaling cascades relevant to the IL-13-induced asthmatic phenotype have been reported, such as adenosine (12, 16), arginase I (13, 14), lipoxygenase products (15), and acidic mammalian chitinase (17). IL-13 and adenosine stimulate one another in an amplification pathway (16), and adenosine up-regulates another mucin gene (MUC2) through CLCA and epidermal growth factor receptor (45). It is possible that these pathways transactivate each other.

The exact mechanism of the development of IL-13-induced AHR and the precise contribution of the chloride channel are uncertain. The importance of the direct effect of IL-13 on epithelial cells in causing AHR has been demonstrated (22). Epithelial cells might produce secreted molecules that alter contraction of airway smooth muscle, and the chloride channel possibly mediates the production of secreted molecules. In addition, it is possible that the chloride channel on smooth muscle may contribute to muscle contraction. A report using murine tracheal rings showed that IL-13 enhanced agonist-induced contractility and calcium signals (46). The repolarization phase of slow waves in canine airway smooth muscle is mediated by calcium-dependent chloride currents (47), and slow waves are observed after stimulation with physiologic agonists. In the present study, the effects of NA on eotaxin production from epithelial cells were studied *in vitro*. It has been reported that chronic expression of IL-13 in the lung causes airway hyperresponsiveness, mucus production, and eosinophilic inflammation. All of these effects depend on

STAT6; reconstitution of human STAT6 only in epithelial cells in mouse is sufficient for IL-13-induced airway hyperresponsiveness and mucus production but not for eosinophilic inflammation (22). The direct IL-13 signals through STAT6 in other cell types may be required for eosinophilia, and it would be important to clarify the suppressive effects of NA in the cells contributing IL-13-induced eosinophilia directly.

An inhibitory effect on cyclooxygenase has been attributed to NA (34). We have studied the effect of another inhibitor of prostaglandin synthesis, indomethacin, on IL-13-induced asthma reaction, and indomethacin failed to attenuate the airway response to IL-13. Therefore, it is unlikely that the blockade of prostaglandins influences the inhibitory action of NA.

In conclusion, we provide evidence that NA-sensitive pathway is involved in the pathogenesis of eosinophilic airway inflammation, AHR, and mucus overproduction by contributing to the effector responses of IL-13, including the chemokine response. They also indicate that this is due, at least in part, to STAT6 activation. Asthma is a complex disorder, and the relevance of the present data to human asthma remains an important issue that cannot be fully addressed. It has been reported that IL-13 expression is increased in the airways of patients with asthma (48, 49) and that natural variation in the coding region of IL-13 is an important genetic determinant of susceptibility to allergy (50). Our findings add to our understanding of the pathogenesis of Th2 inflammation and asthma and suggest that NA or related agents might be of value in the development of new therapies for asthma and other IL-13-mediated diseases.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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