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Identification of Pendrin as a Common Mediator for Mucus Production in Bronchial Asthma and Chronic Obstructive Pulmonary Disease¹

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Excessive production of airway mucus is a cardinal feature of bronchial asthma and chronic obstructive pulmonary disease (COPD) and contributes to morbidity and mortality in these diseases. IL-13, a Th2-type cytokine, is a central mediator in the pathogenesis of bronchial asthma, including mucus overproduction. Using a genome-wide search for genes induced in airway epithelial cells in response to IL-13, we identified pendrin encoded by the *SLC26A4* (*PDS*) gene as a molecule responsible for airway mucus production. In both asthma and COPD mouse models, pendrin was up-regulated at the apical side of airway epithelial cells in association with mucus overproduction. Pendrin induced expression of MUC5AC, a major product of mucus in asthma and COPD, in airway epithelial cells. Finally, the enforced expression of pendrin in airway epithelial cells in vivo, using a Sendai virus vector, rapidly induced mucus overproduction in the lumens of the lungs together with neutrophilic infiltration in mice. These findings collectively suggest that pendrin can induce mucus production in airway epithelial cells and may be a therapeutic target candidate for bronchial asthma and COPD. *The Journal of Immunology*, 2008, 180: 6262–6269.

Bronchial asthma and chronic obstructive pulmonary disease (COPD)³ are chronic inflammatory airway diseases associated with infiltration of inflammatory cells and alterations of structural components (1). Mucus overproduction is often a common feature of bronchial asthma as well as COPD and is a major factor responsible for morbidity and mortality in these diseases (2–4). The precise mechanism of mucus overproduction in both diseases, however, is still poorly understood. Although a nonspecific antiinflammatory therapy such as inhalation of corticosteroids is effective for sup-

pressing mucus overproduction and local inflammation in bronchial asthma (5), it is critical to find a molecular target to specifically control mucus overproduction in these disorders (6).

IL-13, a Th2-type cytokine, is a central mediator in the pathogenesis of bronchial asthma (7–9). Notably, IL-13 signals are capable of inducing mucus production in bronchial epithelial cells (10). However, the molecular mechanism of IL-13-induced mucus production is still poorly understood. Because chloride transport regulates mucus production (11), anion transporters are suggested to be involved in mucus hypersecretion in bronchial asthma. Consistent with this idea, niflumic acid, a broad inhibitor of anion transport, suppresses the development of asthma in mice (12). Previous reports showed that Th2-type cytokines including IL-13 induce expression of gob-5 (mCLCA3), which had been thought to be a chloride transporter, suggesting a critical role for gob-5 in mucus overproduction in bronchial asthma (13, 14). However, the role of gob-5 in asthma remains somewhat elusive at the moment, because this transporter is indeed a secretory, but not transmembrane, protein (15).

The *SLC26A4* (*PDS*) gene encoding pendrin was originally identified as the defective gene in Pendred syndrome, characterized by deafness and goiter (16). Pendrin is a multispanning transmembrane protein and acts as an anion transporter. It is expressed at the apical membrane of the follicular epithelium in thyroid, acting as a transporter of iodide (17). Additionally, pendrin is localized in cochlea; disruption of the *Slc26a4* (*Pds*) gene causes auditory dysfunction due to dysplasia of the cochlea (18, 19). To date, however, nothing is known about the role of pendrin in airway mucus production.

In this study, we identified the *SLC26A4* gene as an IL-13-inducible gene, using a microarray approach, and found that pendrin is highly expressed at the apical membrane of bronchial epithelial cells (BECs) in animal models of bronchial asthma and COPD.

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³ Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; BEC, bronchial epithelial cell; BAL, bronchoalveolar lavage; EGFP, enhanced GFP; PAS, periodic acid-Schiff; Penh, enhanced pause.

Moreover, the enforced expression of pendrin in airway cells caused mucus overproduction *in vitro* and *in vivo*. These results suggest that pendrin is a critical mediator of mucus production in airway epithelial cells, and that it may serve as a potential therapeutic target candidate for bronchial asthma and COPD.

Materials and Methods

Culture of freshly prepared human tracheal epithelial cells

Trachea for cell culture were obtained 3–6 h after death from four patients (59–77 years of age, 3 men, 72 years of age, one woman) under a protocol passed by the Tohoku University Ethics Committee. None of the patients had a respiratory illness. Isolation and culture of human tracheal epithelial cells were performed as previously described (20). Briefly, the culture medium, composed of DMEM-Ham's F-12 medium (Invitrogen) containing 5% FCS, was added to both the mucosal surface side and the basolateral side (by immersion). This medium was replaced by DMEM-Ham's F-12 medium containing 2% ultrosor G (BioSeptra) on the first day with an air-liquid interface fashion, and 10 ng/ml IL-13 was added in the medium of the basolateral side. Human tracheal epithelial cells were cultured for 3 or 7 days after IL-13 addition for mRNA extraction and for 3 wk for histochemical staining.

Probe preparation and microarray analysis

The procedures of probe preparation and microarray analysis were performed as we previously reported (21), except that we performed the analysis with GeneChip Human Genome U95 Array (HG-U95, Affymetrix) representing ~10,000 full-length genes and expressed sequence tag clusters. The data were analyzed using GeneChip software (suite ver. 4.0, Affymetrix).

Culture of mouse trachea

Culture of mouse trachea was performed based on the previous report with minor modifications (22). Briefly, tracheas were dissected from anesthetized mice and longitudinally cut on the ventral side. The dissected tracheas were placed with the lumen side up on stainless steel wire mesh screens suspended in a dish filled with the culture medium. Then the screens were placed in the culture wells such that the top of the trachea was bathed in the medium.

Cells

TGMBE-02-3 cells and COS7 cells were cultured as previously described (23, 24). NCI-H292, a human lung carcinoma cell line (American Type Culture Collection, CRL-1848), was cultured with RPMI 1640 medium (Invitrogen) containing 10% FCS, 10 mM HEPES, 4.5 g/L glucose, 1.5 g/L NaHCO₃, 2 mM glutamine, 1.0 mM sodium pyruvate, 100 µg/ml streptomycin, and 100 U/ml penicillin G.

SLC26A4 cDNA inserted in pEGFP-C1 (pEGFP-C1-PDS) was prepared as described before (25). The coding region of enhanced GFP (EGFP) and pendrin in this plasmid was inserted into pIRESneo2 (pIRESneo2-EGFP-PDS, Clontech). pEGFP-C1-PDS and pIRESneo2-EGFP-PDS were used for transient and stable transfection into the cells, respectively. Expression of pendrin into COS7 or NCI-H292 cells was performed by TransFast transfection reagent (Promega). NCI-H292 cells stably transfected with pendrin were selected and maintained with the culture medium containing 300 µg/ml G-418 sulfate (Invitrogen).

For stimulation, cells were cultured with 20 ng/ml human IL-4 or IL-13 (Peprotech) or 10 ng/ml mouse IL-4 or 50 ng/ml mouse IL-13 (R&D Systems) for the indicated period. In some experiments, cells were incubated with 250 µM of niflumic acid (Sigma-Aldrich) for 24 h. The indicated concentration of scramble or EGFP siRNA (iGENE Therapeutics) was transfected into NCI-292 cells with lipofectamine 2000 (Invitrogen). The culture medium was exchanged 6 h after the transfection, followed by incubation for 48 h.

Model mice

Seven-week-old BALB/c mice (Japan SLC and Charles River Laboratories Japan) were used. Experiments were undertaken following the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animal Science (1987).

Asthma model mice were generated as previously reported (26). Mice were actively sensitized by i.p. injections of 50 µg OVA (Seikagaku Kogyo) with 1 mg alum on days 0 and 12. Starting on day 22, they were exposed to 1% OVA for 30 min, three times every fourth day. Prednisolone (Shionogi) was administered orally once a day for 10 consecutive days from the day before the first Ag challenge. Histological examination was performed 24 h after the final Ag challenge.

Intratracheal administration of IL-13 into mice was performed as previously described (14).

For expression of Sendai virus vector-coding proteins, 5×10^7 cell infectious units (CIU) of the fusion (F) gene-deleted Sendai virus (SeV/ΔF) vectors carrying the *Slc26a4* gene between the hemagglutinin-neuraminidase (HN) and large protein (L) genes, that is, SeV(HNL)-mPend/ΔF and SeV(HNL)-mPend/ΔF-GFP, or the control vector SeV/ΔF (SeV¹⁸⁺GFP/ΔF), in 50 µl 1% BSA/PBS was administered transnasally.

COPD model mice were generated as previously reported (27). Five units of porcine pancreas elastase (Elastin Products Company) in 50 µl PBS or 50 µl PBS alone was administered into mice via the trachea. Histological examination was performed after 1 or 2 wk.

RT-PCR

Total RNA was extracted by either ISOGEN (Nippongene) or RNeasy Mini Kit (Qiagen). The RT reaction primed with random hexamer was performed using GeneAmp RNA PCR Kit (Applied Biosystems). Quantitative RT-PCR analysis was performed using the ABI PRISM 7700 sequence detection system (PerkinElmer), as previously described (21). Primer sequences and PCR conditions are available upon request.

In situ hybridization

Paraffin-embedded mouse lung sections fixed with 4% paraformaldehyde (7 µm/section) were prepared for *in situ* hybridization. Hybridization was performed with 100 ng/ml digoxigenin-labeled anti-sense and sense probes corresponding to the 1605 to 2025 basic acids of the *Slc26a4* gene at 60°C for 16 h. The sections were washed with $5 \times$ SSC at 60°C for 20 min, then with $2 \times$ SSC in 50% formamide at 60°C for 20 min, followed by treatment of 50 µg/ml RNaseA for 30 min at 37°C. Then the sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin Ab (Roche Diagnostics), and the signals were detected with nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indoxyl phosphate (Roche Diagnostics) as substrates.

Immunohistochemical staining

The synthetic peptide corresponding to aa 630–643 of human pendrin (PTKEIEIQVDWNS) or 612–625 of rat pendrin (NNAFEPDEDVEEPE) was used to generate anti-pendrin serum. The peptides or recombinant GFP protein was injected together with complete Freund's adjuvant (Sigma-Aldrich) into New Zealand White rabbits.

Paraffin-embedded mouse lung tissues fixed with 3.7% formaldehyde were processed, and the Ag was detected using Histofine SAB-PO(R) Kit and anti-pendrin serum according to the manufacturer's procedure (Nichirei).

Recovery of Sendai virus vectors

We constructed SeV(HNL)-mPend/ΔF and SeV(HNL)-mPend/ΔF-GFP according to the methods described previously (28–30), with minor modifications. As a control vector, the GFP gene carrying SeV/ΔF (SeV¹⁸⁺GFP/ΔF, Ref. 30) was used. The virus vector titers were determined using infectivity and were expressed in CIU.

Western blotting

Pendrin-expressing NCI-H292 cells were lysed by PBS containing 1% SDS, and the cell lysates were boiled with SDS sample buffer containing 7 M urea. The samples were applied to SDS-PAGE and then electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Biosciences). The proteins were visualized by ECL (Amersham Biosciences).

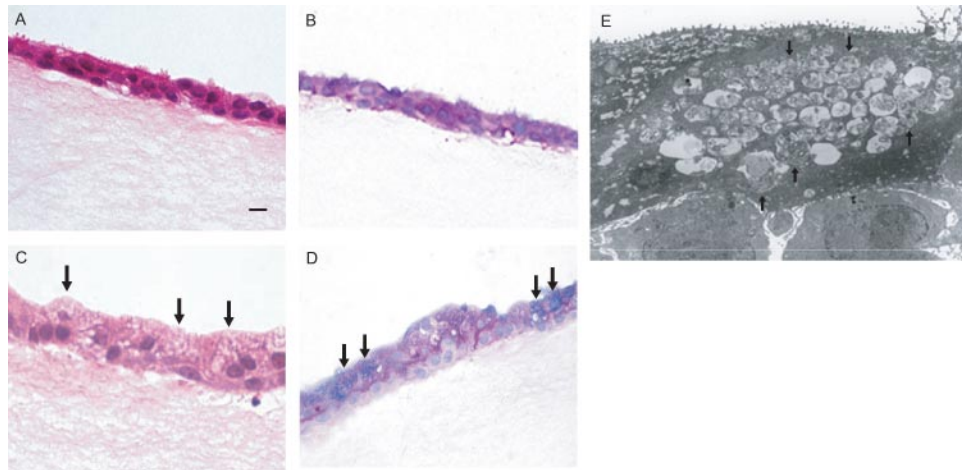
Transport assay of iodide

Iodide uptake and efflux were measured as previously described (31). COS7 cells (5×10^4 /well) transiently transfected with pEGFP-C1-PDS for 48 h or NCI-H292 cells (5×10^4 /well) incubated with 8×10^5 CIU/well of SeV(HNL)-mPend/ΔF or SeV/ΔF (SeV¹⁸⁺GFP/ΔF) for 48 h were used. Chloride-free buffer consisting of 140 mM sodium gluconate, 5.4 mM potassium gluconate, 1.8 mM CaOH₂, 0.5% BSA, and 10 mM HEPES-NaOH (pH 7.4) was used for incubation and washing. The incubation buffer contained 1 mM Na¹²⁵I (20 mCi/mmol).

ELISA

As for ELISA for Muc5ac/MUC5AC, we coated 96-well plates with 0.2 µg of anti-MUC5AC Ab (45M1, Lab Vision/Neomarkers) overnight at 4°C. After blocking with buffer (PBS containing 2% BSA and 0.05% Tween 20) for 1 h at room temperature, the indicated dilutions of bronchoalveolar lavage (BAL) fluid or supernatants were incubated for 1 h at room temperature. After extensive washing, 5 µg/ml peroxidase-conjugated soybean agglutinin (Sigma-Aldrich) was incubated for 1 h at room temperature, and then the signals were developed by the ABTS Peroxidase Substrate System

FIGURE 1. Freshly prepared human primary tracheal epithelial cells were differentiated into mucus-producing cells by IL-13. Freshly prepared human primary tracheal epithelial cells cultured in the absence (A and B) or presence (C and D) of 10 ng/ml IL-13 for 3 wk by air interface were stained with H&E (A and C) or PAS/Alcian blue (B and D) or subjected to the electron microscopic analysis (E). The arrows indicate the mucus produced in the cells (C and D) or the mucus granules produced in the cells (E); the bar in A indicates 10 μ m.



(KPL). Cxcl1 and Cxcl2 were quantitated using Quantikine Kits (R&D Systems), according to the manufacturer's instructions.

Measurement of airway reactivity

We measured airway reactivity in response to methacholine inhalation in mice by whole-body plethysmography using Buxco Electronics hardware and software 48 h after SeV(HNL)-mPend/ Δ F or SeV/ Δ F (SeV¹⁸⁺GFP/ Δ F) was exposed to mice. Mice were placed in chambers and exposed to aerosols of PBS or increasing concentrations of methacholine (0.25, 0.5, and 1 mg/ml) for 8 min. The average values of the enhanced pauses (Penh) between 3 and 6 min for each concentration were calculated.

Statistical analysis

Expression of pendrin, gob-5, MUC5AC, Cxcl1 and Cxcl2, the iodide transport, and Penh were compared by the two-sided, unpaired Student *t* test or the two-sided Welch test. A *p* value of <0.05 was considered to be statistically significant.

Results

Identification of pendrin as an IL-13-inducible molecule in human airway epithelial cells

To identify molecules involved in IL-13-induced mucus production, we used freshly prepared human primary tracheal epithelial cells (20), culturing them with IL-13 for 3 wk in an air-liquid interface fashion. As shown in Fig. 1, treatment of epithelial cells with IL-13 resulted in mucus production that was brightly colored by H&E staining and positive by PAS/Alcian blue staining. Additionally, electron microscopic analysis verified the existence of mucus granules at the apical side of the cytoplasm (Fig. 1E). These results suggest that IL-13 stimulation alone causes differentiation of airway epithelial cells into mucus-producing cells, as previously reported (32, 33). We then cultured freshly prepared primary tracheal epithelial cells derived from two different donors with IL-13 for 3 or 7 days and subjected them to microarray analysis. Among a number of IL-13-inducible genes, we were particularly intrigued with the *SLC26A4* gene encoding pendrin (expression compared with control: 18.8- and 118.3-fold at day 3 and 20.1- and 58.2-fold at day 7 for each donor). The *SLC26A4* gene was the most significantly induced gene encoding the ion channel family in our microarray analysis. We thus evaluated the potential role of pendrin in mucus overproduction in a bronchial asthma setting.

Expression of pendrin in the lungs of allergic airway disease

We first tested whether IL-4 or IL-13 can induce expression of pendrin in various airway epithelial cells. As shown in Fig. 2A–C, both IL-4 and IL-13 were capable of induction of pendrin in freshly prepared human primary tracheal epithelial cells as well as in mouse

cultured trachea, NCI-H292 cells (human lung carcinoma cell line), and TGMBE-02-3 cells (mouse tracheal epithelial cell line), in parallel with gob-5, a well-known IL-13-inducible product (14). Intratracheal administration of IL-13 into BALB/c mice up-regulated expression of pendrin, and the induction was abolished in STAT6^{−/−} mice, supporting the dependency of induction of pendrin on the IL-13/STAT6 pathway (Fig. 2D). To test whether expression of pendrin is induced in vivo in the pathophysiological setting of asthma, we used the OVA inhalation model (26). Mice inhaling OVA presented symptoms consistent with allergic airway disease, including mucus hyperproduction, infiltration of inflammatory cells including eosinophils into the bronchial epithelium (Fig. 3A), and enhanced airway hyperreactivity evaluated by acetylcholine-induced bronchial constriction (26). Treatment of such mice with prednisolone abrogated these symptoms (Fig. 3A). The epithelium collected from these OVA-induced asthmatic lungs produced a high amount of pendrin, whereas its level decreased in the presence of prednisolone as well as gob-5 (Fig. 3B). To perform immunohistochemical analysis, we generated anti-pendrin Ab, confirming the specificity of this Ab using pendrin-expressing NCI-H292 cells (Fig. 3C). Immunohistochemical staining and also in situ hybridization showed that the expression of pendrin was up-regulated, particularly at the apical side of BECs (Fig. 3D and data not shown). Immunohistochemical analyses using a limited number of human samples also showed that pendrin was expressed in BECs of asthma patients (data not shown). These results suggest a possible link between the induction of pendrin by IL-4/IL-13 in BECs and the overproduction of mucus in bronchial asthma.

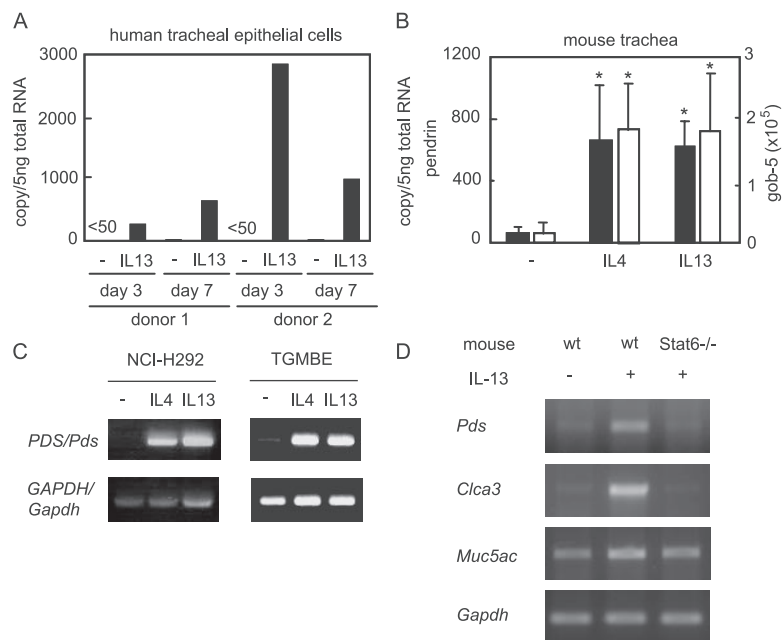
Expression of pendrin in the lungs of elastase-induced lung injury

Mucus hypersecretion is also a feature of COPD, a disease that can be the result of excess elastase activity. We thus tested the expression of pendrin in an experimental model (27) of mucus overproduction, inflammation, and alveolar wall destruction induced by administration of elastase. As shown in Fig. 4, intratracheal administration of pancreatic elastase induced inflammation, mucus overproduction, and increased expression of pendrin. Expression of the *Slc26a4* gene was detectable after a week and increased thereafter (Fig. 4B). Immunohistochemical analysis showed that pendrin was highly induced at the apical side of BECs (Fig. 4C). These results indicate an association between increased pendrin expression and mucus induction in the lungs by elastase, an inflammatory stimulus other than IL-13.

Pendrin induces mucus production in airway epithelial cells

To directly examine the function of pendrin in airway epithelial cells, we transfected NCI-H292 cells with a pendrin-expressing

FIGURE 2. Pendrin was induced in airway epithelial cells by IL-4 or IL-13. mRNA was extracted from freshly prepared human primary tracheal epithelial cells (A), mouse cultured trachea (B), or NCI-H292 cells or TGMBE-02-3 cells (C) cultured with IL-4 or IL-13 for 3 or 7 days (human tracheal epithelial cells), 24 h (mouse trachea), 48 h (NCI-H292 and TGMBE-02-3 cells), or lung tissues of intratracheally IL-13-administered to wild-type or Stat6^{-/-} mice (D) ($n = 4$). The quantitative RT-PCR analysis (A and B) and RT-PCR (C and D) for *SLC26A4/Slc26a4* (*PDS/Pds*), *Clca3*, *Muc5ac*, and *Gapdh* genes are depicted. In A and B, filled and open boxes represent *PDS/Pds* and *Clca3* genes, respectively. *, $p < 0.001$ (vs no stimulant).



vector. Pendrin can transport iodide, bicarbonate, and chloride (17, 34, 35). We first confirmed that pendrin expressed on plasma membranes acted as an anion transporter, using COS-7 cells and NCI-H292 cells (data not shown). Both cells transiently expressing

pendrin showed enhanced uptake and efflux activities for iodide. We then generated the NCI-H292 cells stably expressing EGFP-pendrin. Two clones of pendrin-expressing transfectants (no. 1 and no. 2–3) were generated; higher expression of pendrin (no. 2–3)

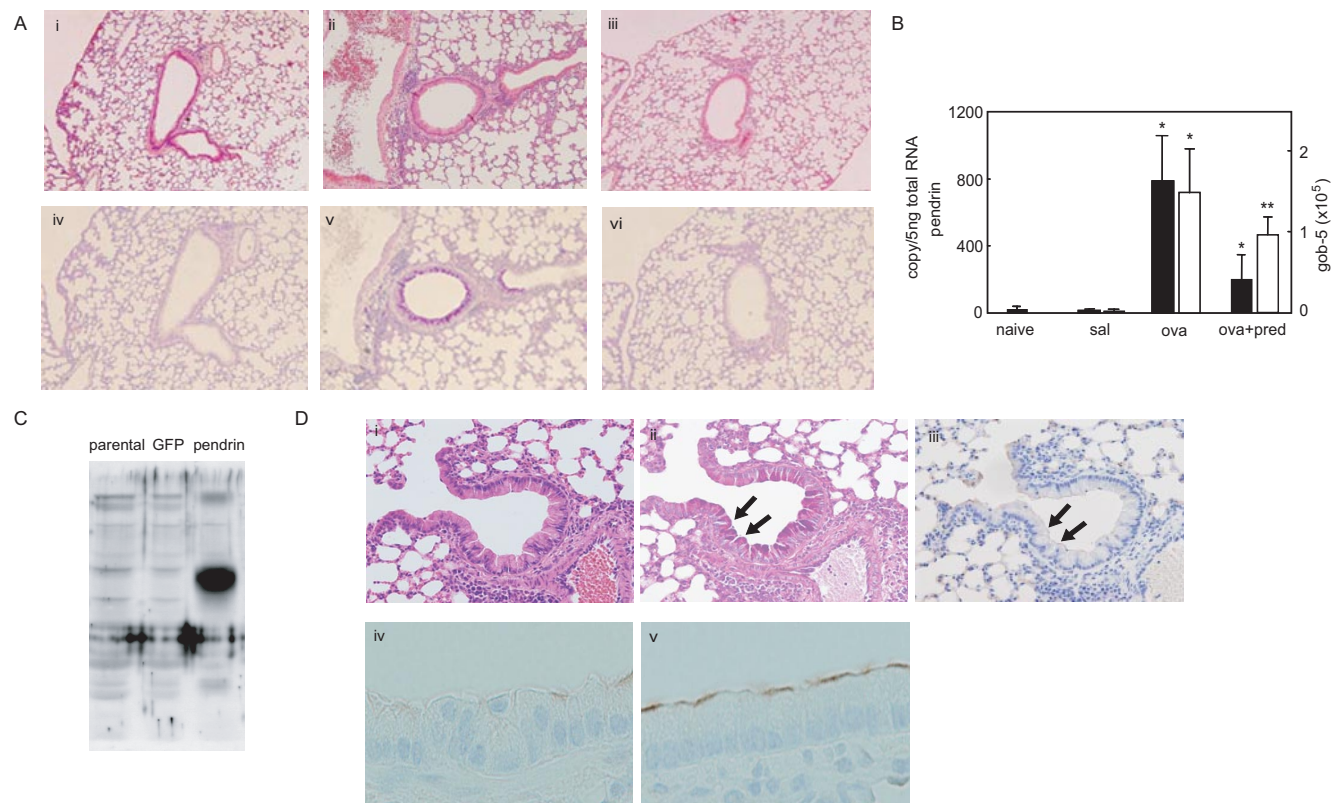
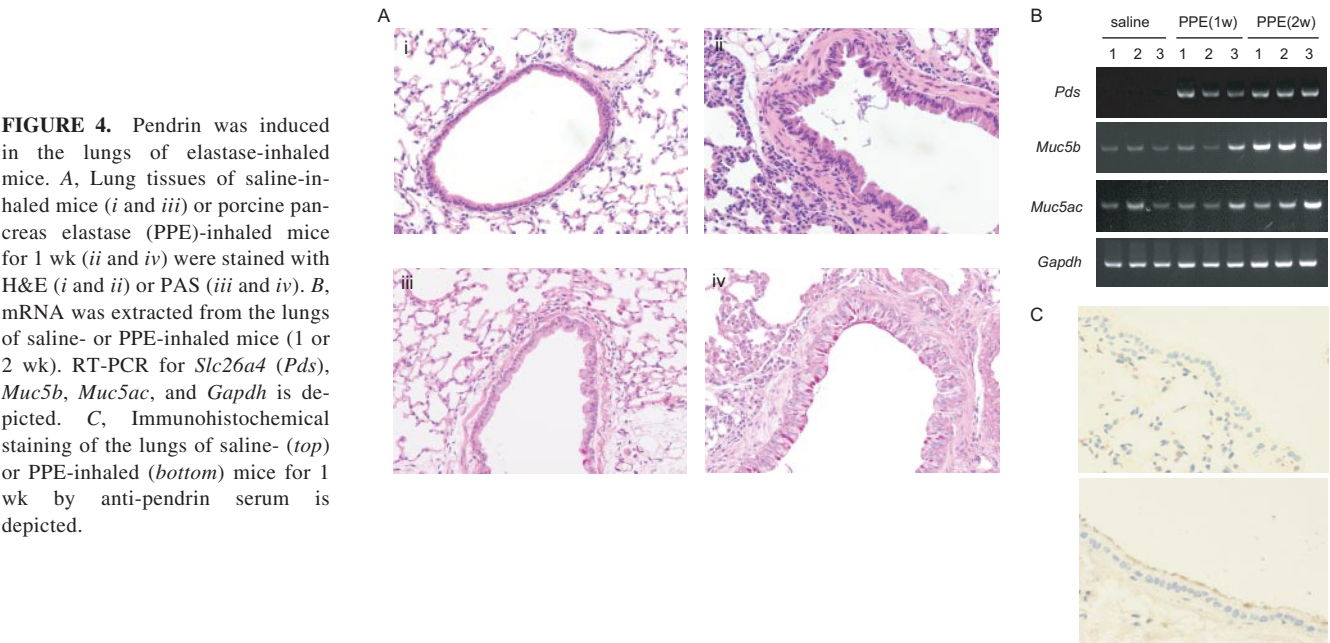


FIGURE 3. Pendrin was induced in asthmatic lung. A, The lung tissues of saline-inhaled mice (i and iv) or OVA-inhaled mice without (ii and v) or with treatment of prednisolone (iii and vi) were stained with H&E (i–iii) or PAS (iv–vi). B, mRNA was extracted from the lungs of naive or saline- or OVA-inhaled mice without or with treatment of prednisolone. Quantitative RT-PCR analysis for *Slc26a4* (■) or *Clca3* (□) gene is depicted. *, $p < 0.001$; **, $p < 0.05$ (vs saline- or OVA-inhaled mice). C, Western blotting using anti-pendrin Ab in parental NCI-H292 cells or NCI-H292 cells incubated with SeV/ΔF (SeV¹⁸⁺GFP/ΔF) (GFP) or SeV(HNL)-mPend/ΔF (pendrin) is depicted. D, The lung tissues of OVA-inhaled (i–iii and v) or saline-inhaled (iv) mice stained with H&E (i) or PAS (ii) or immunostaining with anti-pendrin serum (iii–v) are depicted. The arrows indicate PAS-positive or pendrin-expressing cells; iv and v show high-magnification views.



correlated with more pronounced iodide uptake (Figs. 5A–C). These transfectants up-regulated the expression of MUC5AC, a major mucus protein generated in asthma and COPD patients (36),

at both the mRNA and protein levels, in parallel with the expression level of pendrin (Fig. 5D). Because pendrin expressed in these transfectants was a fusion protein with EGFP, transfection with

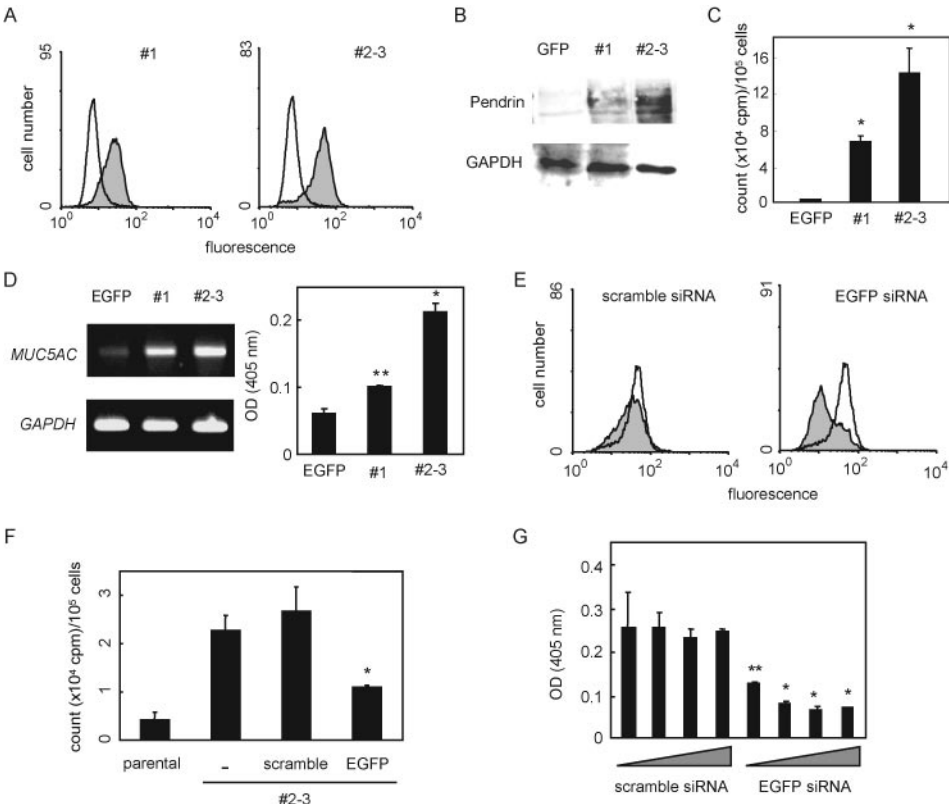


FIGURE 5. Mucus production was induced in pendrin-expressing transfectants of NCI-H292 cells. **A**, Expression of EGFP by flow cytometric analysis was shown in two clones (no. 1 and no. 2–3) of NCI-H292 cells stably expressing EGFP-PDS. Gray and white areas represent expression of the transfectants and parental cells, respectively. **B–D**, Western blotting of pendrin and GAPDH (**B**) or iodide uptake at 1 min (**C**) or RT-PCR for *MUC5AC* and *GAPDH* (**D**, left) or the amounts of MUC5AC in the cell lysates (**D**, right) in NCI-H292 cells stably expressing EGFP alone and EGFP-PDS (no. 1 and no. 2–3) are depicted. **E** and **F**, Expression of EGFP by flow cytometric analysis (**E**) or iodide uptake at 1 min (**F**) in NCI-H292 cells stably expressing EGFP-PDS (no. 2–3) transfected with either scramble or EGFP siRNA (10 pmol) is depicted. Gray and white areas represent expression of the transfectants with or without siRNA, respectively. **G**, The relative amounts of MUC5AC in the cell lysates of NCI-H292 cells stably expressing EGFP-PDS (no. 2–3) transfected with either scramble or EGFP siRNA (1–50 pmol) are depicted. *, $p < 0.01$; **, $p < 0.05$ (vs EGFP or scramble).

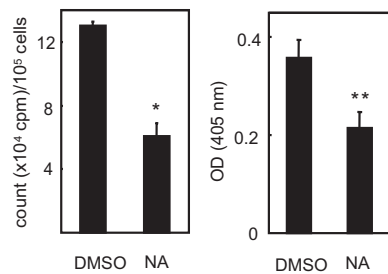


FIGURE 6. Niflumic acid inhibited mucus production by pendrin. Iodide uptake at 1 min (*left*) and the amounts of MUC5AC (*right*) in the cell lysates of NCI-H292 cells stably expressing EGFP-PDS (no. 2–3) incubated with either DMSO alone or niflumic acid (NA, 250 μ M) are depicted. *, $p < 0.01$; **, $p < 0.05$ (vs DMSO).

EGFP siRNA significantly knocked down expression of pendrin on the plasma membrane, followed by down-regulation of its anion transport activity (Fig. 5, *E* and *F*). Down-regulation of pendrin expression attenuated MUC5AC production in a dose-dependent manner (Fig. 5*G*). Thus, expression of pendrin directly induced enhanced mucus production in airway epithelial cells.

Effects of niflumic acid on mucus production by pendrin

Niflumic acid, a well-known blocker of anion transporters, has recently been shown to act as an inhibitor of pendrin (37). We thus

investigated whether niflumic acid inhibits anion transport by pendrin, followed by attenuated mucus production (Fig. 6). Addition of niflumic acid partially inhibited iodide transport at ~ 250 μ M; a higher concentration of niflumic acid showed cytotoxicity (data not shown). Treatment of pendrin-expressing transfectants with niflumic acid suppressed MUC5AC production by almost 50%, in parallel with the levels of anion transport. These results suggest that mucus production by anion transporters including pendrin is dependent on its anion transport activity and that niflumic acid acts as a weak inhibitor against such anion transporters. Alternatively, this result may suggest that an anion transporter insensitive to niflumic acid is involved in mucus production.

Induction of mucus production by pendrin in mouse lungs

To examine the pathological role of pendrin in airway epithelial cells *in vivo*, we directly transfected the target gene specifically into the bronchial epithelial cells. To do so, we established the *Slc26a4* gene-expressing Sendai virus vector, because this vector possesses highly efficient gene transfer capability into airway epithelial cells *in vivo* (38). Inhalation of the vector induced expression of the coding product, specifically in lung epithelial cells (data not shown). Mice inhaling the pendrin vector developed periodic acid-Schiff (PAS)-positive mucus exudates containing cell components in the lumens of the lungs, whereas mice transfected with the control GFP vector did not display such abnormalities (Fig. 7*A*). Additionally, the expression of pendrin also caused

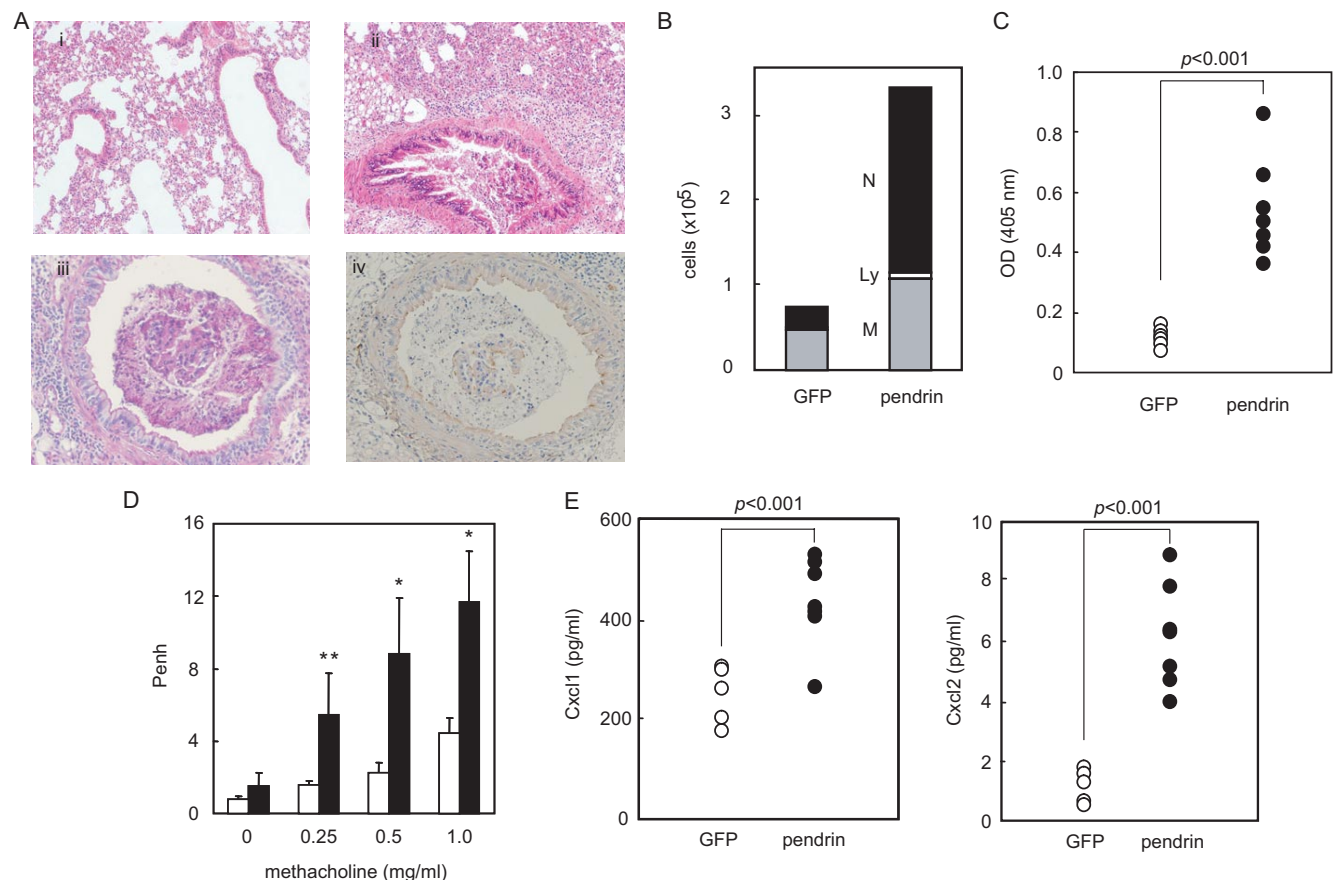


FIGURE 7. Enforced expression of pendrin caused mucus production in mouse lungs. Forty-eight hours after 5×10^7 CIU of SeV/ Δ F (SeV¹⁸⁺GFP/ Δ F) or SeV(HNL)-mPend/ Δ F was administered into mouse lungs, histochemical analysis (*A*), cell counts (*B*), ELISA for Muc5ac in the BAL fluid (*C*), analysis of airway reactivity (*D*), and ELISA for Cxcl1 and Cxcl2 in the BAL fluid (*E*) were performed. H&E staining (*i* and *ii*) or PAS staining (*iii*) or immunostaining with anti-pendrin serum (*iv*) of GFP-expressing (*i*) or pendrin-expressing (*ii–iv*) mouse lungs are depicted in *A*. N, Ly, and M represent neutrophil, lymphocyte, and macrophage, respectively, in *B*. EGFP-expressing mice ($n = 5$ or 6) and pendrin-expressing mice (filled boxes, $n = 6$ or 7) were used. Four identical experiments were performed, and the representative data are shown. *, $p < 0.01$; **, $p < 0.05$ (vs GFP-expressing mice).

neutrophilic infiltration in the lungs (Fig. 7, A and B). Furthermore, the protein level of Muc5ac in the BAL fluid, as evaluated by ELISA, was significantly elevated by the enforced expression of pendrin (Fig. 7C), which is associated with enhanced airway hyperreactivity (Fig. 7D), and with hyperproduction of Cxcl1 (KC) and Cxcl2 (MIP2), mouse chemoattractants for neutrophils, in BAL fluid (Fig. 7E). These results provide formal proof that pendrin overexpression can induce neutrophilic inflammation and mucus production in BECs in vivo.

Discussion

Mucus overproduction is a hallmark of bronchial asthma and is closely related to morbidity and mortality of this disease (2). However, the underlying mechanism of this process is still poorly understood. Previous studies have shown that IL-13 is critical in mucus overproduction in bronchial asthma (7–9). In the present study, based on the findings that anion transporters play an important role in mucus production (11), we found pendrin to be a novel IL-13/IL-4-inducible molecule that is a multispanning transmembrane protein and acts as an anion transporter (Figs. 2, 3, and 5 and data not shown). OVA inhalation in mice caused asthmatic phenotypes, including mucus overproduction that was accompanied by increased pendrin expression at the apical surface of BECs (Fig. 3). Kuperman et al. previously showed that the *Pds* gene was included in all gene profiles of three different asthmatic models: wild-type mice exposed to OVA, mice expressing IL-13 in the epithelium, and mice expressing IL-13 in epithelium with restricted STAT6 expression in nonciliated airway epithelial cells (39). Likewise, Pedemonte et al. recently demonstrated that IL-4 induced pendrin expression in human BECs and that pendrin regulated thiocyanate transport that is essential for innate defense in bronchial mucosa (40). These findings are therefore consistent with our current finding that pendrin is a novel IL-13/IL-4-inducible gene in airway epithelial cells. However, no apparent abnormal lung phenotype has been reported in *Pds* knockout mice (19), and the functional role of pendrin in mucus production in bronchial asthma has not been addressed.

To formally test whether pendrin could directly regulate mucus production in BECs, we generated pendrin-expressing NCI-H292 cells in vitro. We found that pendrin functioned as an efficient anion transporter and remarkably induced the gene and protein expression of MUC5AC, a major mucus protein in asthma and COPD patients (Fig. 5). However, the precise molecular mechanism of mucus gene induction by pendrin is still unclear. *MUC5AC* has several putative *cis*-elements of transcription factors such as NF- κ B, Sp1, and AP1 within its promoter sequence (4). Thus, one possibility is that pendrin somehow directly activates these transcription factors to induce mucus gene expression. In contrast, another possibility is that pendrin induces several inflammatory mediators such as TGF- α , EGF, and TNF- α , which regulate the transcription of the *MUC5AC* gene via activation of the transcription factors, as mentioned above (4). We showed that the anion transport activity of pendrin was strictly correlated with its mucus production (Fig. 6). Thus, aberrant anion transport activities by pendrin in BECs may activate either pathway, inducing the expression of mucin genes. Further work is underway to test which possibility is the case in our system.

To further elucidate the role of pendrin in vivo, we forced the expression of pendrin in mouse lungs and found mucus overproduction in airway tracts accompanied by neutrophil-dominant inflammation (Fig. 7). In this system, mucus production may be induced not only by a direct effect of pendrin on airway epithelial cells, but also by an indirect effect of pendrin by recruiting inflammatory cells, particularly neutrophils, as occurred in COPD. The

precise mechanism whereby neutrophils are predominantly recruited into the lungs remains to be addressed. Based on our finding that Cxcl1 (KC) and Cxcl2 (MIP2) production were detected in BAL fluid, pendrin may directly or indirectly trigger the production of these chemoattractants toward neutrophils. Neutrophil elastase released from infiltrated neutrophils in the lungs enhances expression of mucin genes by both transcriptional and posttranscriptional mechanisms (4): neutrophil elastase activates the EGFR signals via several inflammatory mediators, including TGF- α , followed by activation of the MAP kinase pathway and the aforementioned transcription factors, thus leading to up-regulation of *MUC5AC* or *MUC2* genes; and neutrophil elastase prolongs the half-life of the *MUC5AC* mRNA. However, to firmly establish the contribution of pendrin in the pathogenesis of bronchial asthma and COPD, additional studies are needed that target patients and that use pendrin in knock-out mice.

Generally, infiltration of eosinophils, rather than neutrophils, is a typical feature of bronchial asthma. In severely exacerbated asthma, however, infiltration of neutrophils appears to be noted (1). It is often thought that neutrophil infiltration in asthmatic lungs is due to extrinsic factors other than allergens, such as viruses, LPS, and ozone (41); however, an allergen itself, such as OVA, could induce infiltration of neutrophils (42). Indeed, the profile of BAL cells and lung tissues in OVA-induced asthmatic mice exhibited a transition from neutrophil- to eosinophil-dominant inflammation in a time-dependent manner (H. Matsushita et al., unpublished data). These results clearly suggest that neutrophil infiltration is a cardinal feature of bronchial asthma itself in its early stage, after allergen exposure. Taken together, pendrin induced by IL-13/IL-4 signals could cause mucus overproduction dependent upon neutrophil inflammation.

Mucus overproduction is also a prominent feature of COPD along with metaplasia of submucosal glands and goblet cells, and *MUC5AC* protein expression is significantly higher in the bronchiolar epithelium of patients with COPD (3). In COPD, there is excessive activity of proteases as well as an imbalance between proteases and endogenous antiproteases. In particular, the aforementioned neutrophil elastase is a major constituent of lung elastolytic activity and also potentially stimulates mucus secretion (4). However, the molecular mechanisms connecting neutrophil elastase and mucus production have been poorly elucidated. In this study, we found that administration of elastase into mouse lungs caused COPD-like phenotypes along with pendrin expression (Fig. 4), just like IL-13. Interestingly, a recent study showed that in addition to IL-4, the inflammatory cytokine IL-1 β can induce expression of pendrin in BECs (40). Because elastase induces a strong inflammatory response in the lungs, elastase may induce such inflammatory mediators that in turn cause pendrin expression.

Given that proper control of mucus production is critical to alleviate the symptoms and decrease mortality from bronchial asthma (2, 4), pendrin may be a promising therapeutic target for this disease. Consistent with a recent study (40), we found that niflumic acid, which can inhibit pendrin activity (37), only partially inhibited anion transport and mucus production induced by anion transporters including pendrin (Fig. 6). Pedemonte et al. (40) mentioned that there is no available compound to inhibit the function of pendrin, having screened the Spectrum Library, which contains 2000 compounds. Further understanding of the molecular mechanisms of pendrin's function would pave the way for successful treatment of chronic inflammatory airway diseases such as bronchial asthma and COPD in the future.

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Disclosures

The authors have no financial conflicts of interest.

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