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Short-chain fatty acids induce tissue plasminogen activator in airway epithelial cells via GPR41&43

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Summary

Background: Chronic rhinosinusitis (CRS) is a heterogeneous chronic inflammatory disease generally divided based on the presence or absence of nasal polyps (NPs). One of the features of NPs is excessive fibrin deposition, which is associated with down-regulation of tissue plasminogen activator (t-PA) in NPs. As t-PA is expressed in epithelial cells, and epithelium is readily accessible to topical therapies, identifying compounds that can mediate the induction of t-PA would be a potential new strategy for the treatment of NPs.

Objective: The objective of this study was to determine whether short-chain fatty acids (SCFAs) can induce t-PA in airway epithelial cells via their known receptors GPR41 and GPR43.

Methods: We performed immunohistochemistry (IHC) to determine whether receptors for SCFAs, known as G protein-coupled receptor 41/free fatty acid receptor 3 (GPR41/FFAR3) and GPR43/FFAR2, are expressed in nasal tissue. Primary normal human bronchial epithelial (NHBE) cells were stimulated with different concentrations of SCFAs to test induction of t-PA, which was analysed by expression of mRNA and protein. Mediation of responses by SCFA receptors was evaluated by specific receptor gene silencing with siRNA.

Results: Immunohistochemistry study revealed that airway epithelial cells expressed GPR41 and GPR43. Acetic acid, propionic acid, butyric acid and valeric acid significantly induced t-PA expression from two- to tenfolds. The strongest inducer of t-PA from NHBE cells was propionic acid; cells stimulated with propionic acid released t-PA into the supernatant in its active form. Gene silencing of GPR41 and GPR43 revealed that induction of t-PA by SCFAs was dependent upon both GPR41 and GPR43.

Conclusions and Clinical Relevance: Short-chain fatty acids were shown to induce airway epithelial cell expression of t-PA via GPR41 and GPR43. Topical delivery of potent compounds that activate these receptors may have value by reducing fibrin deposition and shrinking nasal polyp growth.

KEYWORDS
chronic rhinosinusitis, fibrin, GPR41, GPR43, t-PA

1 | INTRODUCTION

Chronic rhinosinusitis (CRS) is a disease that is defined as inflammation of the nose and paranasal sinuses of at least 12 weeks of duration. CRS is one of the most common chronic diseases in the United States, which affects more than 10 million Americans and greatly impairs quality of life in affected patients.[1-4] CRS is a heterogeneous disease and is frequently divided into 2 groups based on the presence or absence of nasal polyps (NPs): CRS with NPs (CRSwNP) and CRS without NPs (CRSsNP). Inflammatory patterns in these 2 diseases are different and also show heterogeneity.[5,6] Pathologically, CRSwNP tissue often displays intense eosinophilic infiltration and a Th2-biased cytokine profile, especially in western countries.[7] It is well known that severe asthma shows high comorbidity with CRSwNP.[8] Furthermore, patients with CRSwNP often require repeated surgical intervention, despite the use of oral and/or topical corticosteroids. New medical approaches that can effectively shrink or eliminate NPs are clearly needed.

NPs usually present around the middle nasal meatus or paranasal sinuses as oedematous masses. Histologically, NPs consist of intense oedematous stroma and the formation of pseudocyst filled with plasma proteins accompanied by infiltration of inflammatory cells.[9] Although plasma exudation from capillaries occurs as a result of profound inflammation, the precise mechanisms of retention of extravascular plasma proteins in NP submucosa have not been fully elucidated. A previous report from our laboratory demonstrated excessive fibrin deposition, and low levels of d-dimer, a fibrin degradation product, in NPs, suggesting that fibrinolysis is impaired in NPs.[10] Profound fibrin deposition may mediate the retention of exuded plasma proteins and the formation of intense oedema in NP tissue. Activation of the coagulation cascade and deposition of fibrin are consequences of inflammation for host defence and may confine infections and support repair processes.[11] However, dysregulation of the coagulation cascade is involved in the aetiology of many diseases in which excessive fibrin deposition occurs, including rheumatoid arthritis, adult respiratory distress syndrome and severe asthma.[12-14] Ordinarily, fibrin degradation is mediated by plasmin, which is generated through cleavage of plasminogen by urokinase plasminogen activator (u-PA) and/or tissue plasminogen activator (t-PA). Our previous study demonstrated profound down-regulation of t-PA expression in NPs, but not u-PA.[10] The data showed that t-PA was most prominently observed in epithelial and glandular cells in normal uncinate tissue (UT), but expression was lost in NP epithelium. Tissue plasminogen activator converts plasminogen to plasmin, which is then responsible for the degradation of cross-linked of fibrin. Loss of t-PA in NP tissue thus may contribute to the accumulation of cross-linked fibrin in NP tissue. As therapies for the management of polyp growth in patients with CRSwNP are at present limited, a new strategy to reduce NP through the induction of t-PA is worthy of exploration. Published studies of t-PA expression by endothelial cells show that cytokines,[15,16] hormones [17] and drugs [18] can induce t-PA from endothelial cells or fibroblasts; regulation of t-PA expression in airway epithelial cells has not been studied extensively.

Short-chain fatty acids (SCFAs) are carboxylic acids with 1 to 6 carbon atoms. SCFAs are produced by microbiota in the distal small intestine and the colon by the anaerobic fermentation of non-digestive dietary residues.[19] Acetic acid, propionic acid and butyric acid are important species of SCFAs and are present at millimolar levels in the gut.[20] SCFAs produced in the gut lumen are readily absorbed by intestinal epithelial cells, circulate in the body and can be used as an energy source in peripheral tissues.[21] Many recent reports have discovered diverse functions of SCFAs in inflammation, carcinogenesis, metabolism and allergic diseases.[22–28] SCFAs are now recognized to be key molecules that regulate beneficial effects on human health.[29] They can perform their functions via intermediary metabolism or via specific SCFA receptors, namely G protein-coupled receptor 41/free fatty acid receptor 3 (GPR41/FFAR3) and GPR43/FFAR2, which are 7-transmembrane-spanning G protein-coupled receptors. These receptors are expressed on the surface of a variety of cells including immune cells, adipocytes and gut epithelial cells.[30,31] The activation of SCFA receptors can have various effects, depending on the disease state and the expressing cell type.[32] Although butyric acid has been shown to induce t-PA in endothelial cells,[33] there have not been any reports on SCFA regulation of t-PA in airway epithelial cells.

Based on the foregoing introduction, we believe that induction of t-PA expression in nasal or sinus epithelial cells would have a beneficial effect in blocking or reducing nasal polyp formation. The goal of these studies was therefore to test the hypothesis that SCFAs will induce t-PA expression by airway epithelial cells from the lungs or nose. The main finding that we report here is that SCFAs induce t-PA in human airway epithelial cells by a mechanism that is dependent on the known SCFA receptors GPR41 and GPR43.

2 | MATERIALS AND METHODS

2.1 | Patient recruitment and clinical sample collection

Patients with CRS were recruited from the Allergy-Immunology and Otolaryngology Clinics of the Northwestern Medical Group (NMG) and the Northwestern Sinus Center at NMG. All subjects gave informed consent, and the protocol and consent forms governing procedures for study have been approved by the Institutional

Review Board of Northwestern University Feinberg School of Medicine. All subjects met the criteria for CRS as defined by the American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force.[34,35] Sinonasal and NP tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. Details of the subjects' characteristics are included in Table 1. Further details are provided in the Supporting Information.

2.2 | ELISA

Concentrations of t-PA in tissue homogenate and the supernatants of cultured cells were determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (Assaypro, St. Charles, MO). The absorbance was measured using a Bio-Rad Spectrophotometer Model 680 Microplate Reader (Bio-Rad, Hercules, CA) with associated software applied to the sandwich enzyme immunoassay technique.

2.3 | t-PA activity assay

The activity of t-PA was determined with a commercially available Human tPA Chromogenic Activity Assay Kit according to the manufacturer's protocol (Assaypro). The absorbance was measured using the Bio-Rad Spectrophotometer Model 680 Microplate Reader (Bio-Rad).

2.4 | Cell viability assay

One hundred μL of NHBE cells (1×10^6 cells/well) was seeded in a collagen-coated 96-well tissue culture plates. Cells were cultured in BEGM without hydrocortisone for at least 24 hours before stimulation. Cells were exposed to concentrations of SCFA used in the activation studies, and viability was assayed as described below. A solution of 1% Triton X-100 (Sigma-Aldrich) was used as a positive control. Before stimulation, the medium was replaced, and NHBE cells were incubated with SCFAs. After 24 hours, 20 μL of MTS reagent (Promega, Madison, WI, USA) was added. The absorbance was measured after 2 hours at 490 nm using the Spectra max Gemini EM (Molecular Device, Sunnyvale, CA).

TABLE 1 Characteristics of the subjects

	Control (n = 46)	CRSwNP (n = 52)
Gender, male/female	18/28	30/22
Ethnicity, White/non-White	32/14	43/9
Age, y	44.4 + 13.0	46.4 + 13.5
Comorbidity, yes/no/unknown		
Asthma	2/44/0	31/21/0
Atopy, yes/no/unknown	8/35/3	30/14/8
Steroid use, yes/no/unknown	6/38/2	27/23/2

2.5 | pH measurement

The pH of medium containing SCFAs was measured using Accumet Basic (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. The mean pH value was assessed using triplicate measurements for each sample.

2.6 | Transepithelial electrical resistance measurement

NHBE cells (0.5×10^6 per plate) were plated in Corning 3460 Transwells (Corning, NY), and submerged cells were stimulated with 10 mmol/L of propionic acid for 48 hours. The transepithelial electrical resistance (TEER) was measured using an Epithelial Electronical Resistance Meter (Kanto Reagents, Tokyo, Japan) according to the manufacturer's protocol.

2.7 | Immunohistochemistry, cell culture and treatments, real-time PCR, and Western blot analysis

Details can be found in the Supporting Information section in the online repository.

2.8 | Statistical analysis

All data are presented as the mean ± SEM, unless otherwise mentioned. The data from culture experiments were analysed using the paired Student's *t* test. Differences between the groups were analysed with the Kruskal-Wallis ANOVA with Dunnett *post hoc* testing and a Mann-Whitney *U* test. In all cases, $P < .05$ was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) software.

3 | RESULTS

3.1 | Epithelial cells expressed both GPR41 and GPR43 in sinonasal tissue

To assess the expression of GPR41 and GPR43 in sinonasal tissues and in NP tissues, we performed IHC analysis of surgical specimens from uncinate tissue (UT) of control and NPs from CRSwNP subjects (Figure 1). Expression of both GPR41 and GPR43 was observed in sinonasal epithelial cells as well as resident and infiltrating cells within the lamina propria.

3.2 | GPR41 and GPR43 mRNA expressions were up-regulated in nasal polyp tissue

Next, we wanted to know the expression levels of both GPR41 and GPR43 in sinonasal tissue. The mRNA from whole tissue extracts was analysed to compare the expression levels of both GPR41 and GPR43. As shown in Figure 2A,B, the expression of both GPR41 and

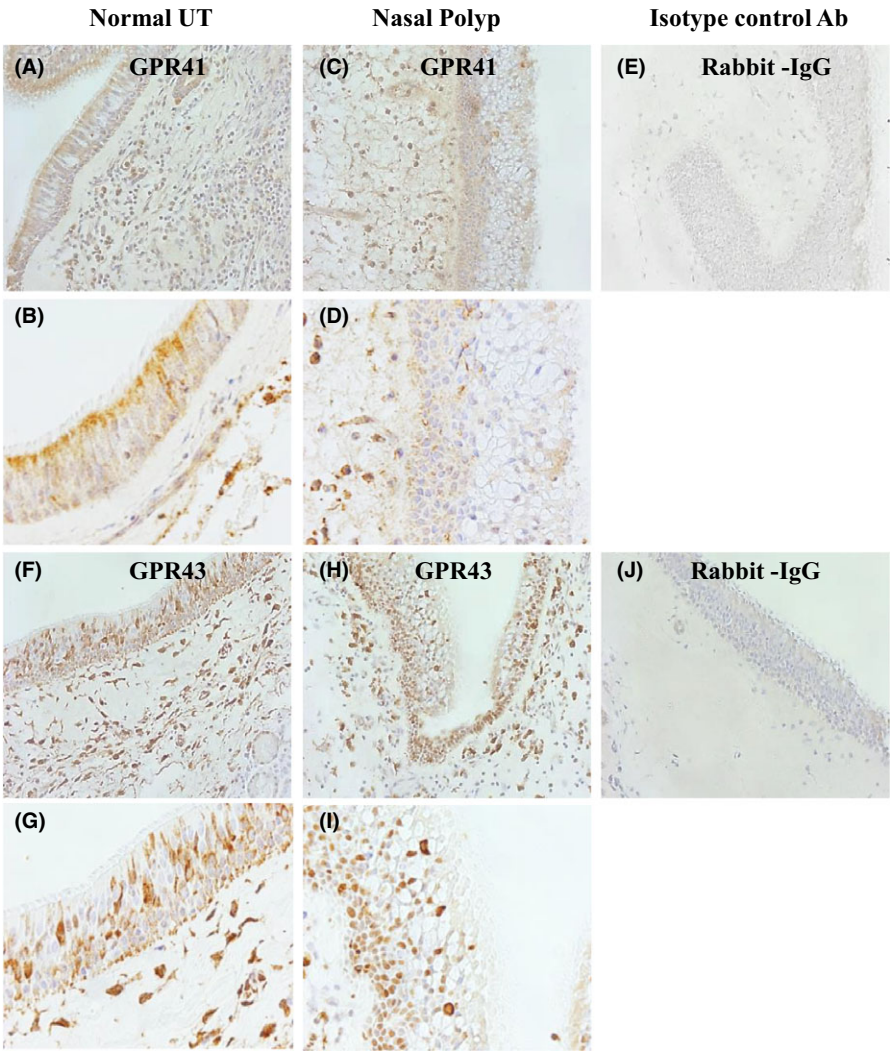

FIGURE 1 Immunohistochemical (IHC) detection of GPR41 (A-E) and GPR43 (F-J) in human sinonasal tissue. Representative immunostaining for GPR41 in UT from control subject (A, B) and in NP tissue (C, D), and staining for GPR43 in UT from a control subject (F, G) and in NP tissue (H, I). Representative isotype control antibody staining in NP tissue is shown (E, J). Magnification ×200 (A, C, E, F, H, J) and ×400 (B, D, G, I)

GPR43 mRNA was ten- to 100-fold higher in NPs compared to control UT samples (GPR41; $P < .001$, GPR 43: $P < .0001$). As IHC analysis showed that both GPR41 and GPR43 were expressed in epithelial cells, we evaluated expression of both genes in fresh nasal cells collected by scraping. As with whole tissue extracts, the expression of both GPR41 and GPR43 was significantly higher (two to ten-fold) in nasal scraping-derived epithelial cells from NPs compared to normal UT epithelial scrapings (GPR41; $P < .05$, GPR 43: $P < .001$, Figure 2C,D).

3.3 | SCFAs induced t-PA from NHBE cells

We stimulated cultured submerged NHBE cells with SCFAs in vitro. As shown in Figure 3A, propionic acid, butyric acid and valeric acid strongly induced t-PA expression in a concentration-dependent manner, whereas acetic acid only weakly induced t-PA and hexanoic acid did not induce t-PA. The maximal concentrations of SCFAs to significantly induce t-PA in NHBE cells were 10 mmol/L for acetic acid ($P < .05$), 1 mmol/L for propionic acid ($P < .05$), 0.5 mmol/L for butyric acid ($P < .05$) and 1 mmol/L for valeric acid ($P < .05$); none of these concentrations induced harmful effects on the viability of NHBE cells (Figure S1). We measured t-PA protein levels in the supernatants from SCFA stimulated NHBE cells by ELISA. A 48-hour stimulation with SCFAs induced t-PA protein release from NHBE cells into the supernatant (Figure 3B). The levels of t-PA released into the supernatant by stimulation with propionic acid ($P < .001$), butyric acid ($P < .05$) and valeric acid ($P < .05$) were significantly higher (three- to fivefold) than levels in supernatants of non-stimulated NHBE cells. In the presence of SCFA in the medium, there were no significant pH changes (Figure S2).

3.4 | Epithelial-derived t-PA was in the active form

To assess whether released t-PA was in the active form, we measured t-PA activity with a commercially available t-PA activity kit. As propionic acid was the strongest inducer of t-PA in NHBE cells, we used propionic acid in further experiments. We found that a significant amount of t-PA released from non-stimulated NHBE cells was in the active form. Supernatants from propionic acid stimulated

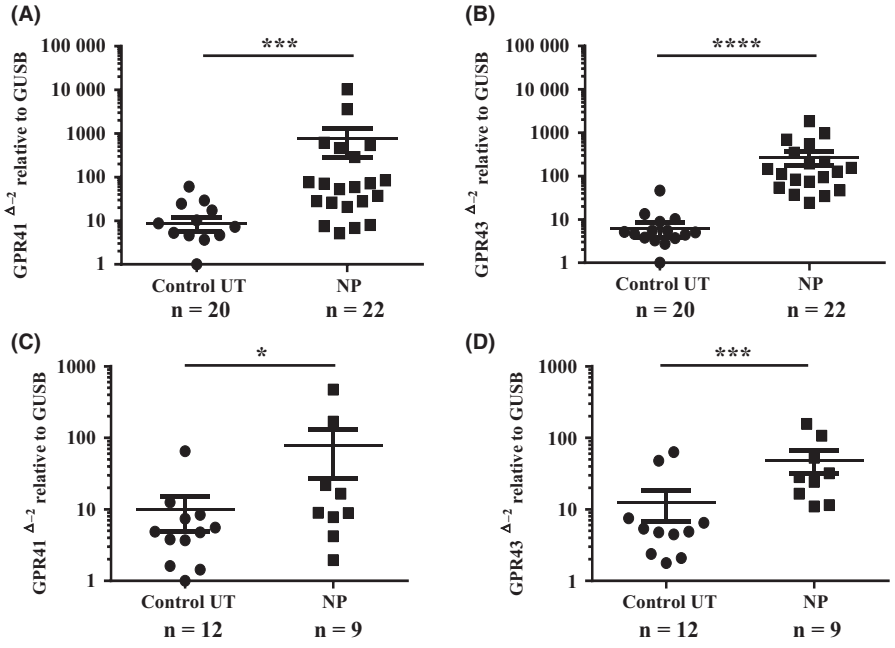

FIGURE 2 Total RNA was extracted from nasal UT and NP tissues (A, B) and nasal epithelial cell scrapings from UT and NP tissues (C, D). Expression of GPR41 and GPR43 mRNA was analysed by RT-PCR. The expression of mRNA was normalized to the housekeeping gene β-Glucuronidase (GUSB). *$P < .05$, ***$P < .001$, ****$P < .0001$. (●; control UT, ■; NP tissue)

NHBE cells showed significantly higher t-PA activity compared to non-stimulated NHBE cells and the increase in activity was proportionally similar to the increased level of t-PA mRNA (approx. three-fold) (Figure 3C). These results indicate that at least a proportion of the t-PA released into the supernatant by both unstimulated and stimulated epithelial cells is in the active form.

3.5 | SCFA-induced t-PA is secreted by NHBE cells onto both apical and basolateral surfaces

Most of the fibrin deposition that has been described in NP is found in the lamina propria within NPs.[10] To test whether t-PA induced by SCFAs is released at the site of cross-linked fibrin, we tested whether the release was basolateral or apical. To clarify this point, NHBE cells were cultured in collagen-coated transwells. Cell lysates, basolateral media and apical supernatants were all collected after 48 hours of stimulation with 10 mmol/L propionic acid, and t-PA was measured by ELISA. Propionic acid significantly enhanced t-PA protein levels found in cell lysates as well as in both apical and basolateral media (Figure 3D). The results demonstrate that 78.5% of the total amount of t-PA produced by propionic acid stimulated NHBE was released and the remainder was retained in the cells. Although 66.8% of the released t-PA was found in the apical supernatants, a substantial amount (33.2%) was released to the basolateral side. To clarify whether the administration of propionate affects TEER, which might affect the passage of t-PA between the apical and basolateral layers, we also measured TEER. We found that the administration of propionate did not have any significant effect on TEER (Figure S3). These results indicate that induction of t-PA by SCFAs in vivo might be expected to increase the amounts of t-PA found within the tissue both above and below the basement membrane.

3.6 | Induction of t-PA expression by SCFAs in NHBE cells is dependent on GPR41 and GPR43

GPR41 has been shown to signal through the PTX-sensitive Gαi/o family, whereas GPR43 signals through both Gαi/o and Gαq protein families which are PTX-insensitive.[30,31] To clarify whether t-PA induction by SCFA was mediated by GPR41 or GPR43, NHBE cells were pretreated with PTX prior to stimulation by propionic acid. Figure 4A shows that PTX produced a dose-dependent inhibition of propionic acid-induced t-PA expression in NHBE cells, partially implicating GPR41. The minimum concentration of PTX to significantly attenuate propionic acid-induced t-PA expression was 1 ng/mL ($P < .001$); however, higher concentrations of PTX did not fully suppress t-PA expression, implying that some t-PA induction by propionic acid might be mediated by a PTX-sensitive (e.g Gαi/o family) signalling pathway. We next tested whether some of the propionic acid t-PA induction utilized a PTX-insensitive Gαq protein. YM-254890, an experimental compound that was isolated from the culture broth of *Chromobacterium* sp., is a selective Gαq/11 inhibitor that blocks the exchange of GDP for GTP during Gαq/11 activation.[36,37] NHBE cells were pretreated with YM-254890 prior to stimulation with propionic acid. Pretreatment with 10 nmol/L of YM-254890 attenuated t-PA expression induced by propionic acid in NHBE cells ($P < .05$, Figure 4B), without affecting t-PA mRNA expression or cell viability (Figures S4 and S5). When NHBE cells were pretreated with both PTX (1 ng/mL) and YM-254890 (1000 nmol/L), the combined effect revealed greater inhibition of t-PA induction by propionate than either compound alone (Figure 4C).

Although experiments with PTX and YM-254890 implicate GPR41 and GPR43, they provide only circumstantial support for the involvement of these 2 SCFA receptors. To confirm the involvement of GPR41 and GPR43 in propionic acid-mediated induction of t-PA,

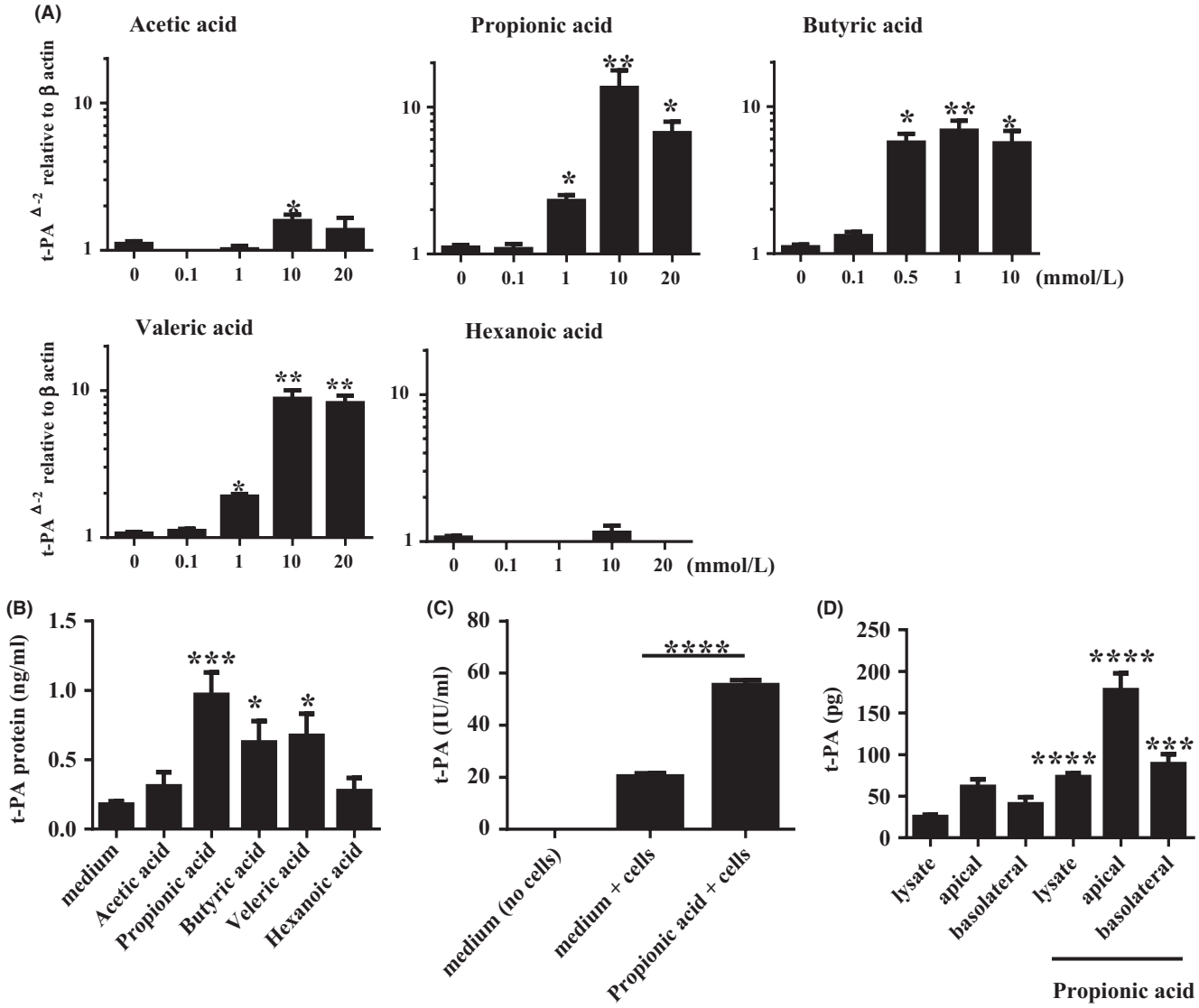

FIGURE 3 Submerged normal human bronchial epithelial (NHBE) cells were stimulated with indicated concentrations of SCFAs for 24 h. A, Cell lysates were harvested for RNA extraction to analyse t-PA mRNA expression by RT-PCR (n = 4-8); B, Submerged NHBE cells were stimulated with 10 mmol/L acetic acid, 10 mmol/L propionic acid, 1 mmol/L butyric acid, 10 mmol/L valeric acid and 10 mmol/L hexanoic acid. Supernatants were collected after 48 h of stimulation to analyse t-PA protein by ELISA (n = 6); C, Submerged NHBE cells were stimulated with 10 mmol/L propionic acid for 48 h. Then t-PA activity in the supernatants was measured by chromogenic activity kit (n = 5-6); D, NHBE cells were harvested from transwells after stimulation with 10 mmol/L of propionic acid for 48 h. Whole cell lysates and apical and basolateral medium were collected. Levels of t-PA in the cell lysates and medium were measured by ELISA (n = 8). Data shown are mean ± SEM of 4 independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ when compared to non-stimulated cells

a small interfering RNA (siRNA) knock-down approach was employed. Concentrations of 40 nmol/L of one siRNA targeting GPR41 and another targeting GPR43 significantly suppressed expression of GPR41 and GPR43 proteins in NHBE cells as detected by Western blot analysis after transfection ($P < .05$, Figure 5A,B). Although the genes were not completely suppressed, the reduction of either gene alone was sufficient to partially inhibit t-PA induction by propionic acid (Figure 5C,D). The combination of siRNA against both receptors leads to nearly complete suppression of t-PA expression, as shown in Figure 5E, supporting an involvement of both GPR41 and GPR43 in SCFA-mediated induction of t-PA in NHBE cells.

3.7 | SCFAs induced t-PA from PNECs

Induction of t-PA by SCFA or synthetic agonists for their receptors, given therapeutically topically in the nose, could theoretically shrink nasal polyp tissues in which fibrin deposition plays a role in polyp growth. After demonstrating that SCFAs induced t-PA from NHBE cells, we next tested whether SCFAs can induce t-PA in primary nasal epithelial cells (PNECs) from nasal polyp. PNECs from nasal polyp were stimulated with varied concentrations of SCFAs. As shown in Figure 6, propionic acid stimulation of PNECs from nasal polyp enhanced the expression of t-PA. The approximate efficacy of SCFA as inducers of t-PA in PNECs was propionic acid acid > butyric

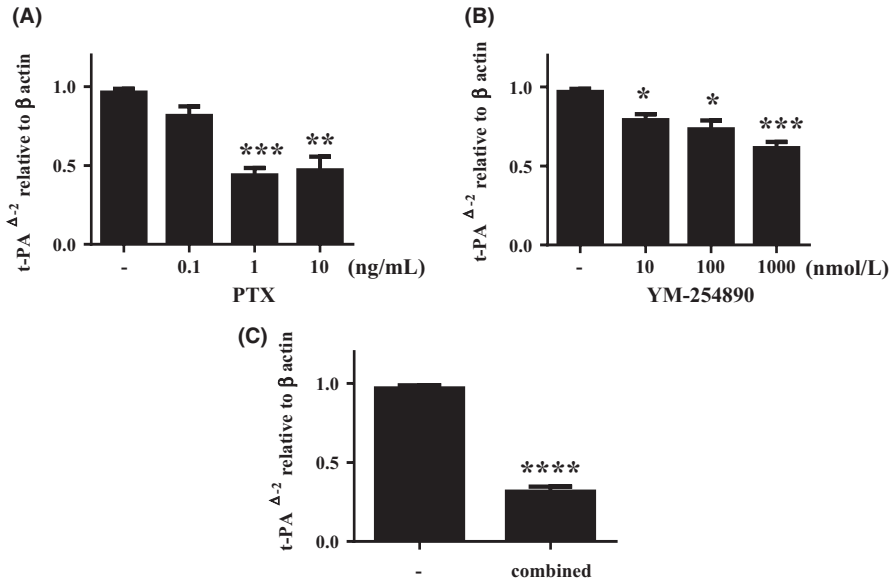

FIGURE 4 Pertussis toxin and YM-254890attenuate propionic acid-mediated t-PA induction in NHBE cells. Submerged NHBE cells were pretreated with PTX or PBS (A), YM-254890 or DMSO (B), PTX (1 ng/mL) and YM-254890 (1000 nmol/L) (C) (n = 5) prior to stimulation with 10 mmol/L of propionic acid for 24 h. Cell lysates were harvested for RNA extraction to analyse t-PA mRNA expression by RT-PCR. Data shown are mean ± SEM of independent experiments. *$P < .05$, **$P < .01$, ***$P < .001$

acid = valeric acid. The minimum concentrations that significantly enhanced t-PA expression were 1 mmol/L of propionic acid ($P < .05$), 0.5 mmol/L of butyric acid ($P < .05$) and 10 mmol/L of valeric acid ($P < .05$), respectively.

4 | DISCUSSION

Mucosal epithelium presents a first line of defence against many pathogens, allergens and environmental exposures. Epithelial cells have many functions that maintain homeostasis such as mucociliary action, maintenance of airway surface liquid, production of mucus and antimicrobial peptides, ion transport and maintenance of a structural barrier.[38] Fibrin is produced during the process of epithelial repair,[39] and epithelial-derived t-PA can take part in the repair process via LRP-1(Low density lipoprotein receptor-related protein 1).[40] Dysregulation of epithelium and morphological changes in NPs have been shown.[3,41] Prolonged inflammatory states also disrupt the balance of the coagulation and fibrinolysis cascades, leading to excessive fibrin deposition in the tissue. Takabayashi et al showed that excessive fibrin deposition is seen in NPs and that t-PA is downregulated in NPs, further demonstrating that IL-4 and IL-13 inhibit t-PA induction in airway epithelial cells.[10] They also reported that FXIIIA, a transglutaminase that cross-links fibrin in the final stage of the coagulation cascade, is elevated in NPs.[42] Activated FXIII cross-links fibrin between γ-glutamyl and ε-lysyl residues on adjacent fibrin chains in polymerized fibrin to yield the mature clot, and also cross-links α2-plasmin inhibitor with fibrin. The cross-linking of fibrin reinforces its stiffness and rigidity that allows retention of plasma proteins. Notably, Takabayashi et al showed that M2 macrophages were major FXIII-A-expressing cells in NPs. Their findings align well with the view that the Th2-polarized inflammatory state facilitates fibrin deposition by decreasing fibrinolysis and enhancing the coagulation cascade in NPs. Together, these findings suggest that it is feasible to diminish NPs formation by inducing t-PA, causing removal of fibrin and decreased disease development and symptoms.

Induction of the t-PA gene and t-PA activity is regulated by several mechanisms.[43] The regulation of t-PA gene transcription has thus far been investigated in a variety of cell types. Retinoic acid, butyric acid and statins are known to increase t-PA gene expression in endothelial cells, but the precise mechanism is not well understood.[18,43,44] In the present study, we demonstrate that SCFAs can induce t-PA in NHBE cells and PNECs without any harmful effects on cell viability. Among several SCFAs, propionic acid tended to be the most effective agonist inducing t-PA in both NHBE cells and PNECs. Although the SCFAs induced t-PA at concentrations in the mmol/L level, these levels are similar to levels found in vivo and are in the same range as reports in other cell types.[33] This level of potency may restrict the activation mechanism of both GPR41 and GPR43 by SCFAs to specific locations in the body. We showed that a significant amount of t-PA released from propionic acid stimulated NHBE cells was in the active form, and t-PA from transwell cultured NHBE cells was released both apically and basolaterally, indicating that some t-PA released from airway epithelial cells should reach the submucosal layer and effect fibrin degradation in the tissue.

SCFAs are generated in part by bacterial fermentation of dietary fibres by gut microbiota. SCFAs are produced at concentrations of ~100 mmol/L in the gut lumen, and the major components are acetic acid (C2), propionic acid (C3) and butyric acid (C4).[45] Previous studies have highlighted the variety of functions of SCFAs in inflammation, carcinogenesis, metabolism and allergy.[22–28] There is interest in SCFA receptors as attractive pharmaceutical targets having a broad range of potential therapeutic applications.[46] Thus, it is important to clarify the signalling partners associated with SCFA beneficial effects. We showed that the receptors, GPR41 and GPR43, are present in epithelial cells of both normal uncinate tissue and in NPs. This is the first report that these GPRs exist in the upper airway epithelium.

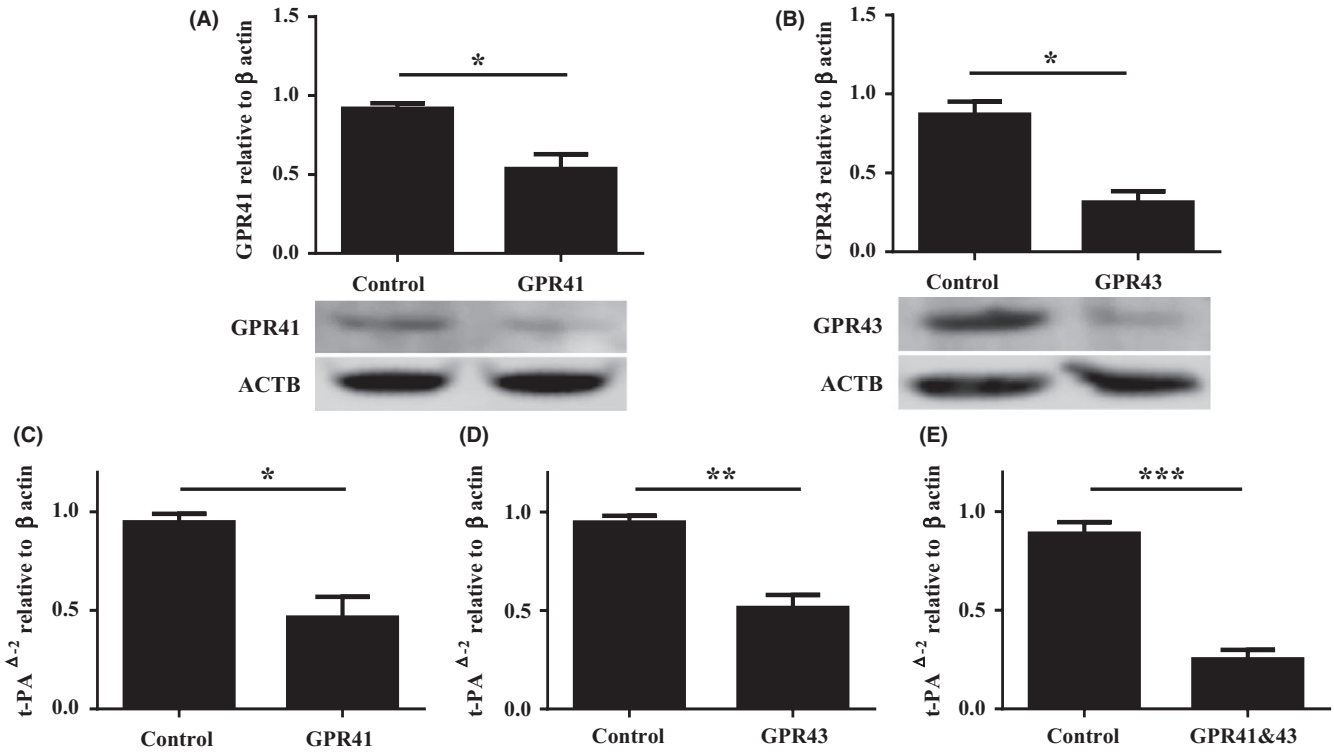

FIGURE 5 Small interfering RNA (siRNA) attenuate propionic acid-mediated t-PA induction in NHBE cells. (A, B) Submerged NHBE cells were transfected with 40 nmol/L of control siRNA and siRNA targeting human GPR41 (A) or GPR43 (B). Whole cell lysates were electrophoresed and transferred onto a PVDF membrane. Representative Western blot with anti-human GPR41 or anti-human GPR43 antibody is shown (bottom). Semiquantitative densitometry of 4 independent experiments. (C, D, E) Submerged NHBE cells were transfected with 40 nmol/L of control siRNA or siRNA targeting human GPR41 (C), GPR43 (D), or GPR41 and GPR43 (E) as indicated. Forty-eight hours after transfection, cells were stimulated with 10mM of propionic acid for 24 h (n = 6). Data shown are mean ± SEM of independent experiments. *$P < .05$, ** $P < .01$, ***$P < .001$

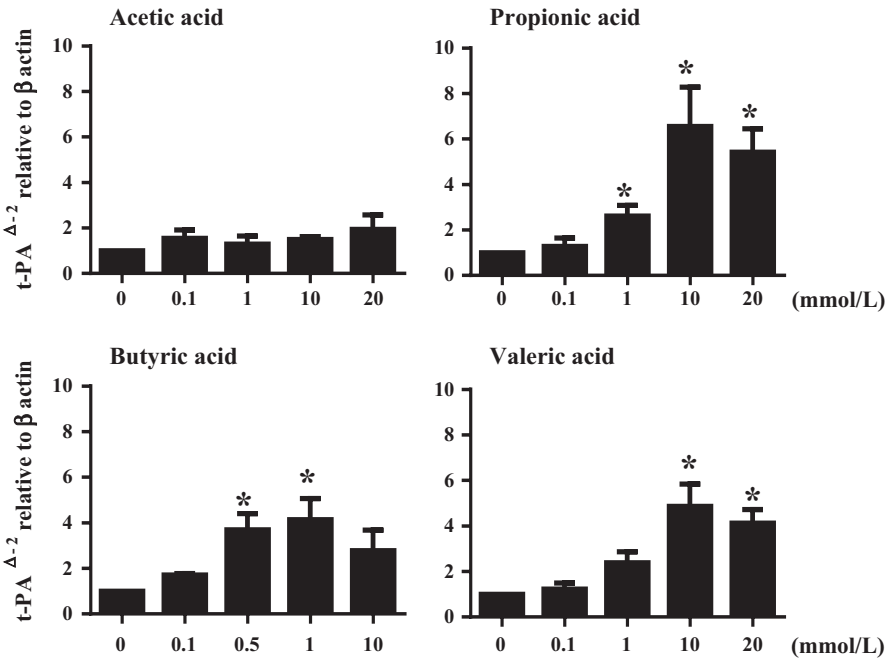

FIGURE 6 SCFAs induced t-PA expression from human primary nasal epithelial cells (PNECs). Submerged PNECs were stimulated with different concentrations of SCFAs for 24 h. Cell lysates were harvested for RNA extraction to analyse t-PA mRNA expression by RT-PCR (n = 4). Data shown are mean ± SEM of 4 independent experiments. *$P < .05$ when compared to non-stimulated cells

Furthermore, the expression of both GPR41 and GPR43 was significantly higher in nasal scraping-derived epithelial cells from NPs compared to normal UT epithelial scrapings (Figure 2C,D). These results support the rationale of using topical administration of SCFAs to patients with NPs to induce t-PA and potentially decrease the size of the NPs.

GPCRs constitute the largest class of cell surface receptors, consisting of Gα, Gβ and Gγ subunits. GPCR is classified into 4 families according to their α subunit: Gi, Gs, G12/13 and Gq.[47] The Gq family activates phospholipase and consists of 4 members: Gq, G11, G14 and G15/16 and their respective α subunits are thus Gαq, Gα11 and Gα14.[48] On the other hand, the Gα15/16, and Gs and Gi families regulate adenylyl cyclase activity.[43] PTX inhibits the α subunit of Gi proteins by locking the α subunits in a GDP-bound inactive state. Although GPR41 and GPR43 display 43% amino acid identity, they have different affinities for SCFAs.[49] The rank order of potency for GPR41 is propionic acid ~ butyric acid ~ valeric acid > acetic acid, whereas the corresponding rank for GPR43 is acetic acid ~ propionic acid > butyric acid > valeric acid.[30,31] These potency rank orders for both GPRs are consistent with our results that propionic acid was, for the most part, the strongest inducer of t-PA in NHBE, and the response was inhibited by both Gαi/o and Gαq/11 inhibitors. We pretreated NHBE cells with both PTX and YM-254890 to test the combined inhibitory effect on t-PA induction. The result showed greater inhibition than either drug alone but revealed some residual t-PA that was not suppressed (Figure 4C), indicating either that the drugs did not completely block their targets or that other mechanisms might be present. Other potential mechanisms by which SCFAs could affect the cells might be via effects mediated by a transporter or pursuant to direct passage of the SCFAs across the cell membrane. Once inside the cell, SCFAs might affect signal transduction or transcriptional events, as reported elsewhere.[26,45] It has been reported that SCFAs can act as histone deacetylase inhibitors (HDACi),[26] and HDACi can also enhance t-PA induction in endothelial cells. We therefore tested whether trichostatin A, a broad HDACi, induces t-PA in NHBE cells. The results showed that trichostatin A increased t-PA expression (Figure S6). We also observed that t-PA induction by SCFAs was at least partially receptor dependent based on reduced response following siRNA gene knock-down of each receptor and an even greater reduction when both receptors were knocked down. We used primary nasal epithelial cells as well as NHBE cells, and found similar responses, suggesting that SCFAs can theoretically activate t-PA expression and release in the nose and sinuses of patients with CRS. Furthermore, we attempted to measure levels of SCFAs in nasal lavage fluids by gas chromatography; however, we could not detect SCFAs (data not shown). This could possibly reflect the large dilution of nasal lining fluid during lavage with a large volume of saline, could reflect partitioning of SCFAs in tissue vs the airway lumen or could reflect lower levels of SCFAs in nasal tissues than in the circulation. Nonetheless, based on the present findings, we suggest that it may be worthwhile to test whether intranasal administration of SCFAs, or other GPR41 and GPR43 agonists, can induce t-PA release and decrease nasal polyp size in vivo. Synthetic allosteric agonists for GPR41 and GPR43 have been reported, and agonists with much higher potency or pharmacodynamic attributes amenable to topical nasal use would be particularly promising in this regard.[32]

In NPs, infiltration of eosinophils accompanies other immune cells such as mast cells, basophils, dendritic cells and neutrophils.[7,50–52] Mast cells in NPs have distinct phenotypes by tissue location, and are thought to contribute to the pathogenesis of NPs.[50,53] These cells release cytokines and chemokines that orchestrate and accelerate inflammatory response in NPs. Importantly, these inflammatory cells also express GPR41 and/or GPR43.[30,31] We performed immunofluorescence double staining of ECP (eosinophil cationic protein) for eosinophils and either GPR41 or GPR43. We found that eosinophils in NPs express GPR43 and weakly express GPR41 (Figure S7). SCFAs are known to modulate and control inflammatory responses via these receptors. Importantly, Trompette et al showed that high fibre diet or propionic acid administration in mice impaired Th2 polarization via GPR41 by suppressing expression of IL-4, IL-5, IL-13 and IgE, as well as reducing eosinophil infiltration in the lung.[24] Another group also reported that high fibre diet or SCFA administration modified the development of allergic airway disease. This report provided evidence for a role of diet and acetic acid in the development of airway disease in humans.[28] As Th2 cytokines are elevated in NPs and IL-13 can down-regulate t-PA, SCFAs might also indirectly be able to decrease the size of NPs by dampening type 2 inflammation in addition to inducing release of t-PA. We found that expression of both GPR41 and GPR43 mRNA was elevated in NPs in surgical samples (Figure 2A,B), which might reflect the infiltration of GPR41 or GPR43 expressing inflammatory cells in NPs, as well as increased expression by epithelial cells. Although further studies are required, we speculate that the administration of SCFAs might produce beneficial effects for treatment of NPs by more than one mechanism.

In summary, we showed that SCFAs, especially propionic acid, butyric acid and valeric acid, induced the expression and release of t-PA by airway epithelial cells, and that released t-PA was in the active form. We also found that the SCFA receptors GPR41 and GPR43 are expressed by nasal epithelial cells and can mediate the induction of t-PA following stimulation with SCFAs. Our results indicate that allosteric agonists for GPR41 and GPR43, including SCFAs, might be worthy of consideration as a new strategy for treatment of NPs.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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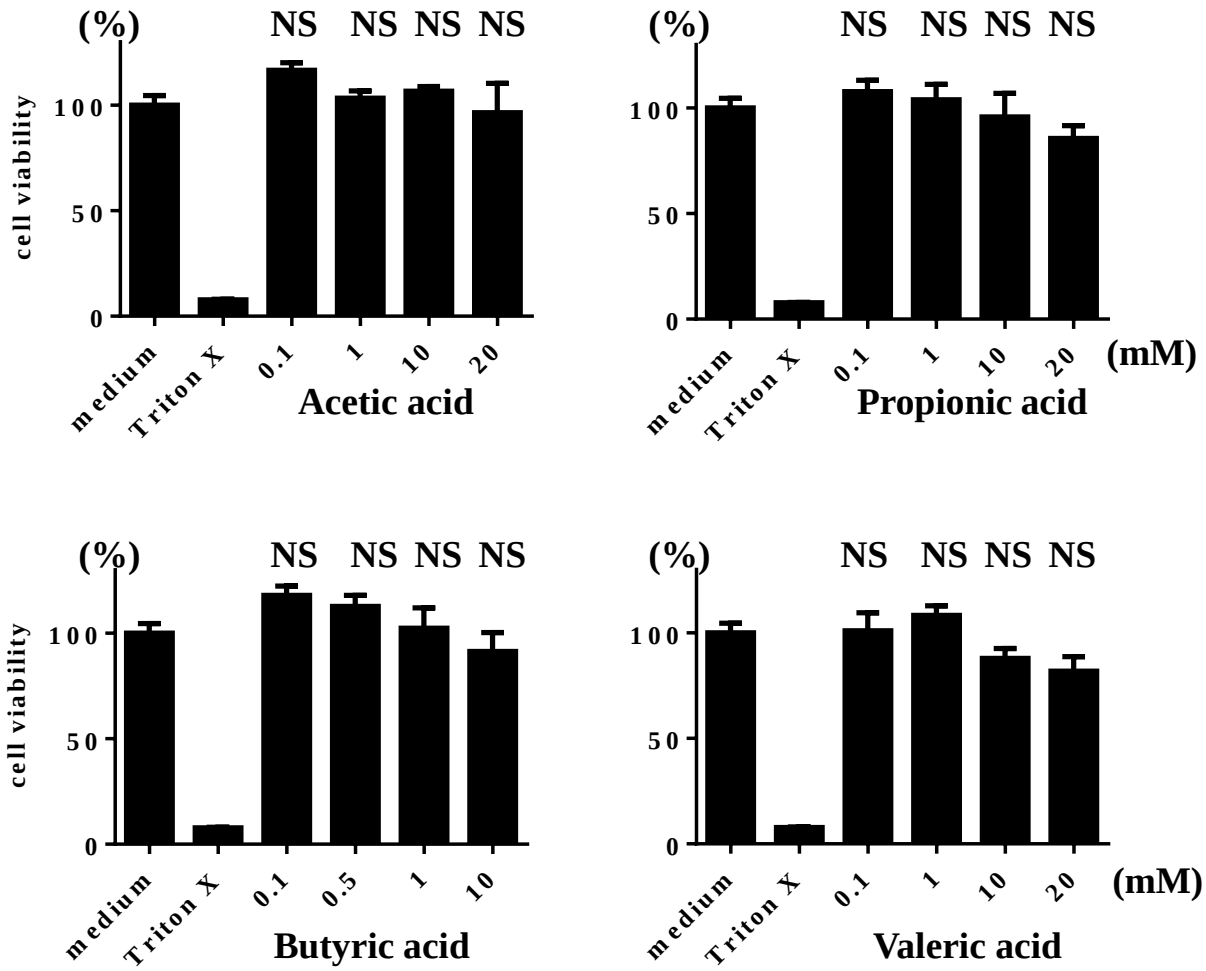
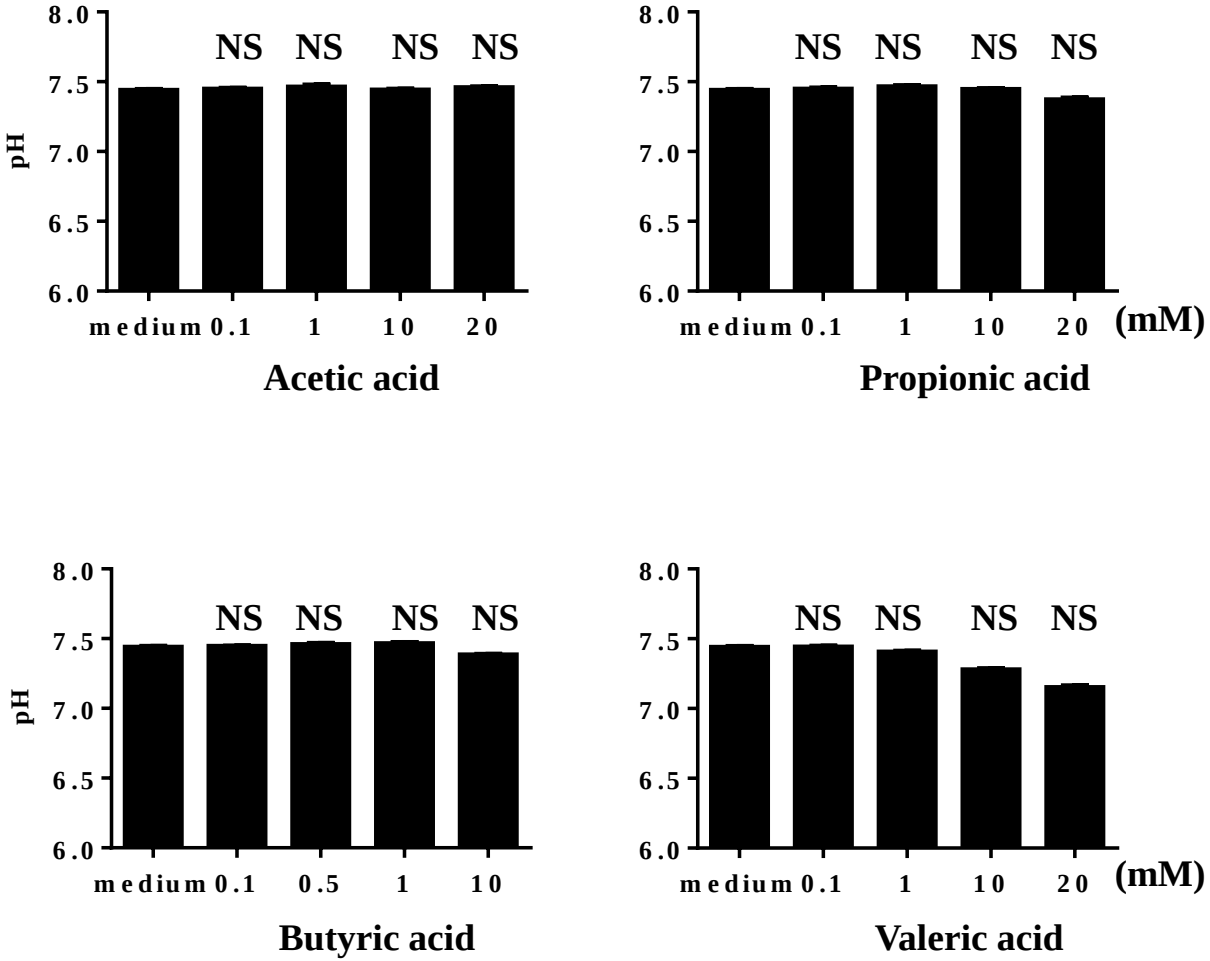
Supplementary Figure 1



Supplementary Figure 2



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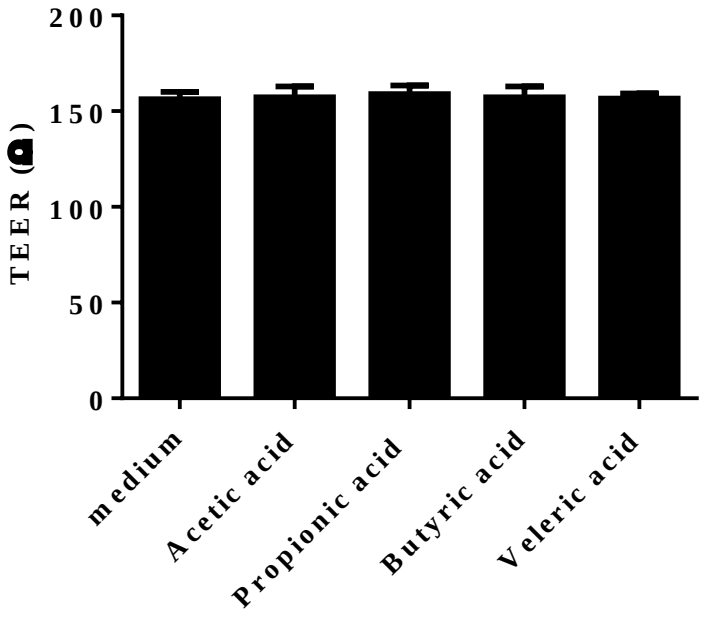

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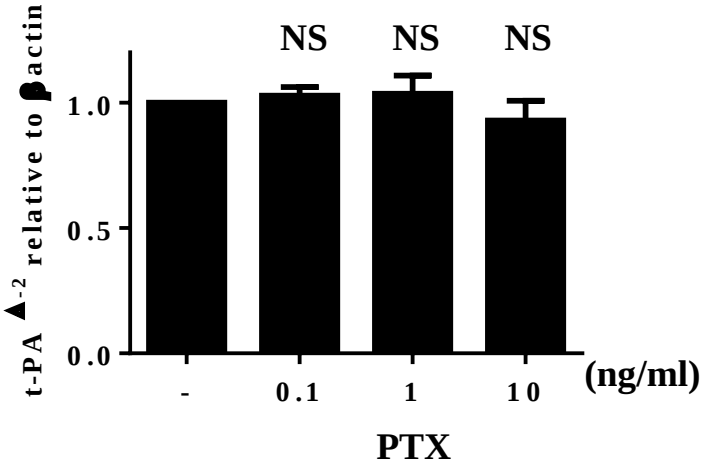
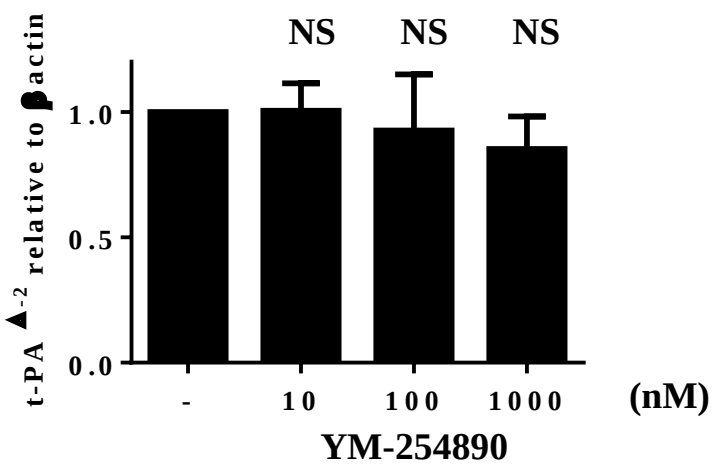
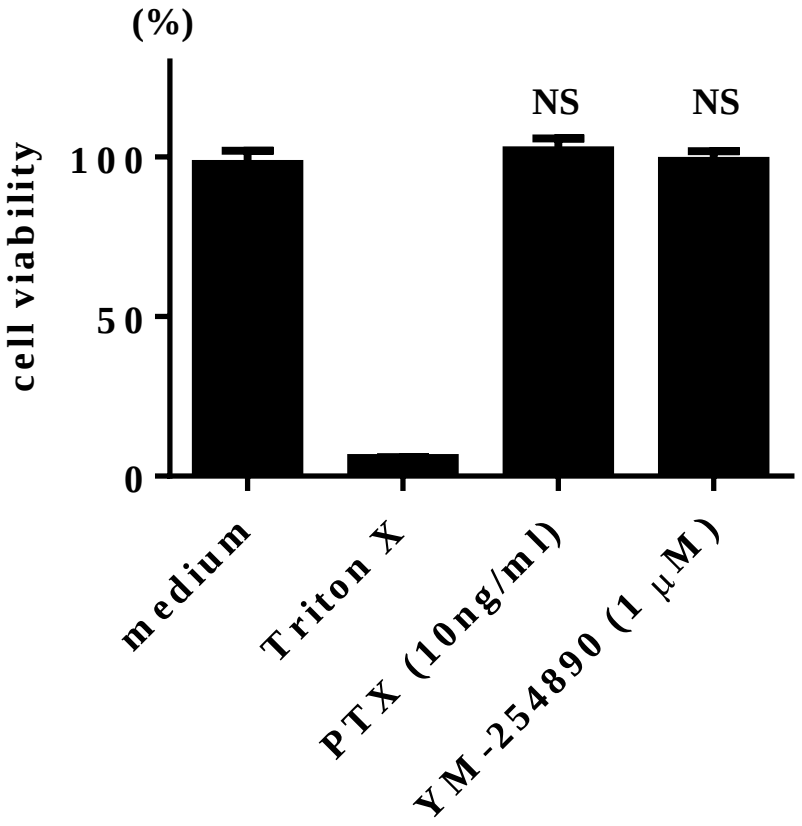




Supplementary Figure 5



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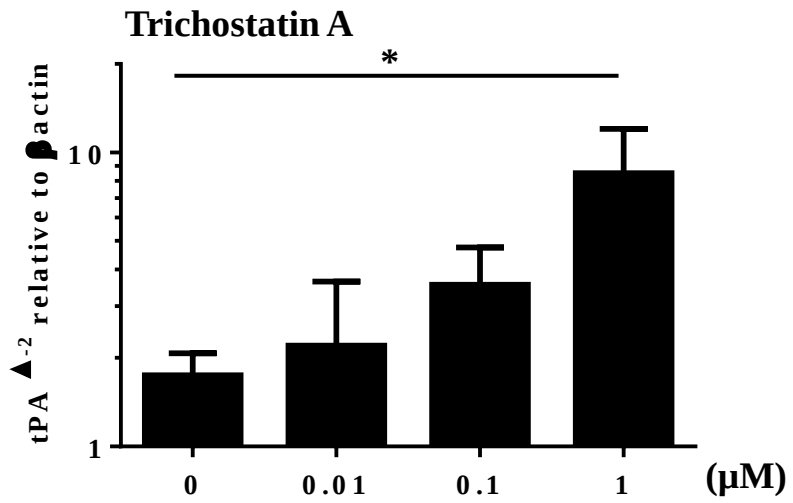
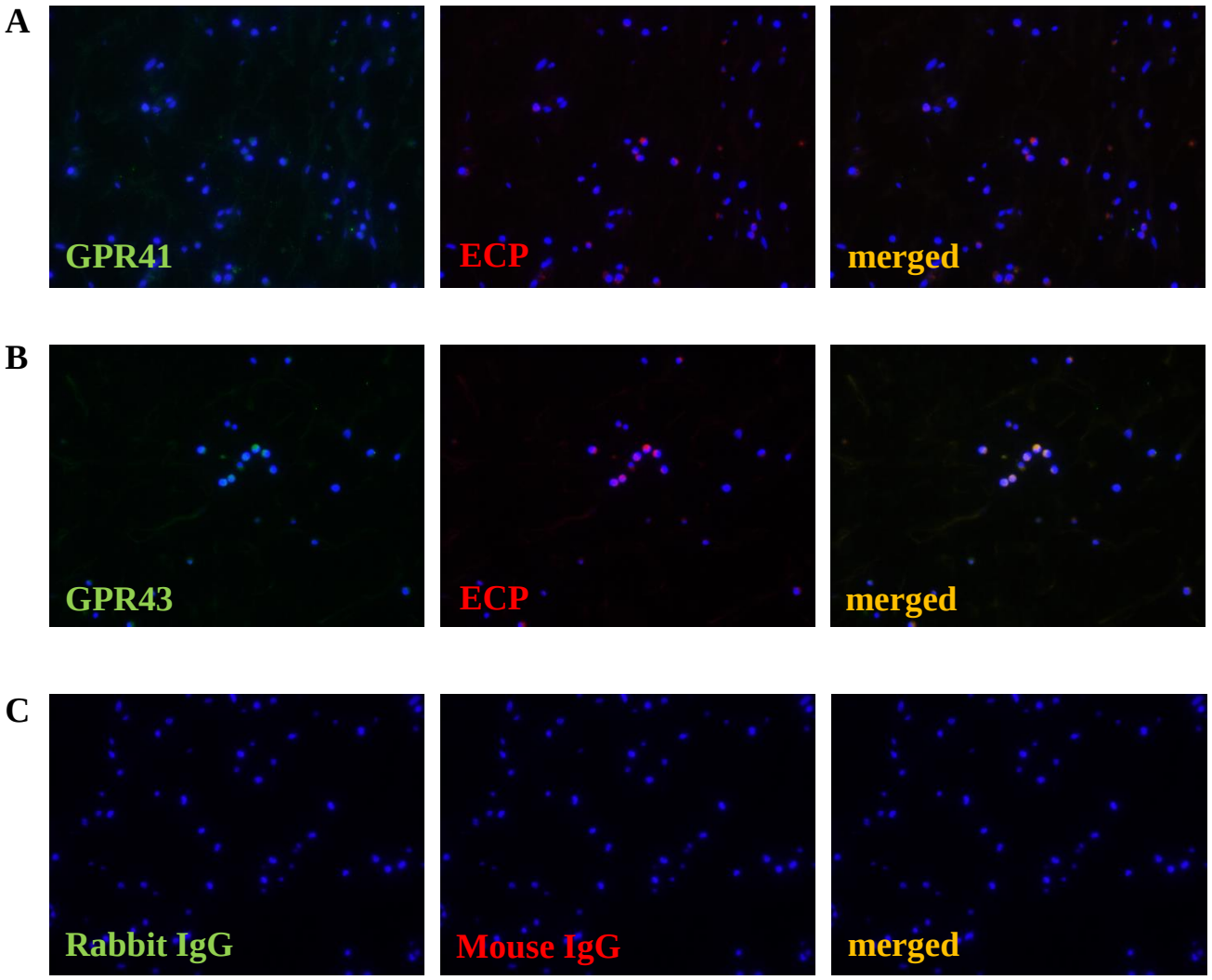


Supplementary Figure 7



Online Data Supplement

Title:

Short chain fatty acids induce tissue plasminogen activator in airway epithelial cells.

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Materials and Methods

Patients and biopsy specimens.

Patients with chronic rhinosinusitis (CRS) were recruited from the Allergy-Immunology and Otolaryngology Clinics at Northwestern University and the Northwestern Sinus Center. All subjects signed informed consent, and the protocol governing procedures for study have been approved by Institutional Review Board of Northwestern University Feinberg School of Medicine. All subjects met the criteria for CRS as defined by American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force (Meltzer OtolaryngolHeadNeckSurg 2004, Pearlman CurrPoinOtolaryngolHeadNeckSurg 2008). The presence of sinusitis or bilateral nasal polyps was confirmed by office endoscopy and computed tomography imaging. Patients with an established immunodeficiency, pregnancy, coagulation disorder, diagnosis of classic allergic fungal sinusitis, Churg-Strauss syndrome, or cystic fibrosis were excluded from the study. All patients scheduled for surgery had previously failed to respond to adequate trials of conservative medical therapy (prolonged antibiotic regimens, nasal steroid sprays, oral steroids, saline irrigations, and decongestants) for control of symptoms. Sinonasal and nasal polyp tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. Specimens from patients without CRS were obtained during the performance of skull base tumor excision, facial fracture repair, lacrimal duct surgery, and orbital decompression surgery. Two control subjects took oral steroid because of skull base surgery, two control subjects took oral or inhaled steroid because of asthma, the other two control subjects used nasal steroid spray because of allergic rhinitis. A portion of each sample for isolation of RNA was transferred in RNAlater (Ambion, Austin, TX) and stored at −20°C. Details of subjects' characteristics are described in Table 1.

Immunohistochemistry

Nasal tissue was dehydrated, infiltrated, and embedded in paraffin, and tissue was sectioned at 3 μm using a Leica RM2245 Cryostat (Leica Microsystems Inc, Bannockburn, IL) . Sections were rehydrated and endogenous peroxidase activity blocked with 3% H2O2/methanol, and then they were rinsed by 0.3% Tween-20/PBS for three times. Nonspecific binding was blocked with 3% goat serum/0.3% Tween-20/PBS. Tissue sections were incubated with rabbit anti-human GPR41/FFAR3 pAb (LS-A1737; LifeSpan Bioscience) at a dilution of 0.25μg/ml and with rabbit anti human GPR43/FFAR2 pAb (LS-B5986; LifeSpan Bioscience) at a dilution of 0.5 μg/ml overnight at 4°C. We used the same concentrations of rabbit control IgG (R&D Systems, Minneapolis. MN) as control experiments. Sections were rinsed by 0.3% Tween-20/PBS for three times, and then incubated in biotinylated secondary goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:500 dilution for 1 hour at room temperature. After another rinse as previous method, sections were incubated in ABC reagent (avidin–biotin–horseradish peroxidase complex; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Sections were rinsed again and incubated in diaminobenzidine reagent (Invitrogen, Carlsbad, CA) for 10 minutes at room temperature. Sections were rinsed in deionized H2O, and they were counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped using Cytoseal 60 (Richard- Allan Scientific, Kalamazoo, MI) in preparation for microscopic analysis. Microscopic analysis was performed with an Olympus DP-26inverted research microscope (Olympus) and MicroFire AR digital microscope camera (Optronics, Goleta, CA). For the immunofluorescence assay, rehydrated sections were blocked with 3% goat serum/0.3% Tween-20/PBS and then were incubated with rabbit anti-human GPR41/FFAR3

pAb (LS-A1737; LifeSpan Bioscience) at a dilution of 0.25μg/ml with rabbit anti human GPR43/FFAR2 pAb (LS-B5986; LifeSpan Bioscience) at a dilution of 0.5 μg/ml, and mouse anti human ECP mAB (mAb 593; Uppsala, Sweden) in blocking buffer overnight at 4°C. The same concentrations of isotype control IgG were used in control experiments. After washing, sections were incubated with 4 μg/mL of Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen), and 4 μg/mL of Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) for 1 hour at room temperature in the dark. Images from immunofluorescence slides were obtained with an Olympus BX53 microscope using ×40 objective lens.

Cell culture and treatments

Primary normal human bronchial epithelial cells (NHBE) were purchased from Lonza (Walkersville, MD). We used NHBE cells from at least three different donors. Human primary nasal epithelial cells (PNEC) were collected from nasal polyp tissue by curettage with a Rhinoprobe (Arlington Scientific, Inc., Springville, UT) under a Northwestern University Feinberg School of Medicine Institution Review Board approved human subject research protocol. In past studies, the purity of epithelial cells collected with this method has been determined to be generally more than 95% based on cytokeratin staining. PNEC for *in vitro* cell culture experiments were collected from patients with CRS and expanded in 6-well culture plates. NHBE and PNEC were seeded in collagen coated 24 well plates, and were maintained in serum-free bronchial epithelial cell growth medium (BGEM, Lonza). Before stimulation, NHBE cells and PNECs were cultured in BEGM without hydrocortisone for either 24 or 48 hours. At confluency, submerged NHBE cells and PNECs were stimulated with varied concentrations of

acetic acid (Sigma-Aldrich, St. Louis, MO), propionic acid (Sigma-Aldrich), butyric acid (Sigma-Aldrich), valeric acid (Sigma-Aldrich) or hexanoic acid (Sigma-Aldrich). NHBE cells were also stimulated with trichostatin A (Sigma-Aldrich). Acetic acid, propionic acid, and butyric acid were dissolved in distilled water and stored at -20°C. Supernatants were collected, and centrifuged for 10 min at 14,000*g* at 4°C. The supernatants were collected for analysis and total RNA was isolated from the cells. For inhibition studies, NHBE cells were treated with Pertussis toxin (PTX; Sigma-Aldrich) or YM-254890 (Wako, Osaka, Japan) prior to stimulation. Dimethyl sulfoxide (DMSO) was used as a diluent for YM-254890 and a control where appropriate.

Transwell cell culture and whole cell lysate.

NHBE cells (0.5×10^6 per plate) were plated in Corning 3460 Transwells (Corning, NY). NHBE cells were cultured in BEGM without hydrocortisone for at least 24 hours before stimulation. At confluency, submerged NHBE cell were treated with 10mM acetic acid, 10mM propionic acid, 5mM butyric acid, 10mM valeric acid or 10mM hexanoic acid in apical layer for 48h. Basal medium and supernatant were collected for analysis, and centrifuged for 10 min at 14,000*g* at 4°C. NHBE cells were then washed twice with 1 ml of ice-cold phosphate-buffered saline (PBS) and lysed with 0.15ml Mammalian Protein Extraction Reagent (M-PER; Thermo Fisher Scientific, Waltham, MA) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Lysates were incubated on ice for 15 min and then clarified by centrifugation for 10 min at 14,000*g* at 4°C. Supernatants were collected for enzyme-linked immunosorbent assay (ELISA) and t-PA activity assays.

Real-time PCR

Total RNA from sinus tissue was extracted with QIAzol (Qiagen, Valencia, CA) and was cleaned and treated with DNase I (Invitrogen) using NucleoSpin RNA II (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's instructions. Under endoscopic guidance, epithelial cells were collected from the uncinate process by scraping with Rhinoprobe (Arlington Scientific, Arlington, TX). The cells were immediately placed in a stabilization reagent (RNA *later*; QIAGEN, Valencia, CA). Total RNA from nasal scrapings was extracted using NucleoSpin RNA XS (Macherey-Nagel) according to the manufacturer's instructions. Total RNA from cultured NHBE was extracted using NucleoSpin RNA II and was treated with DNase I according to the manufacturer's instructions. Quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using a RNA 6000 Nano LabChip (Agilent Technologies). Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers. Semiquantitative real-time RT-PCR was performed with a TaqMan method using an Applied Biosystems StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, CA) in 15 μl reactions (7.5 μl of 2× TaqMan Master mix [Applied Biosystems], 0.75 μl of 20× primer and probe mixture). Primer and probe sets for t-PA (Hs00263492_m1), β-actin (ACTB; sense, 5'-CTGGCCGGGACCTGACT-3'; antisense, 5'-GCAGCCGTGGCCATCTC-3'), and GUSB (human β- glucuronidase endogenous control, PN; 4326320E) were purchased from Applied Biosystems. A primer and probe set for β-Glucuronidase (GUSB) was chosen as the reference housekeeping gene in sinus tissue because previous studies have demonstrated no difference in the expression of this gene between patients and controls. To determine the exact copy number of the target genes, quantified aliquots of purified PCR fragments of the target genes were

serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 10 ng of total RNA were used for real-time PCR. The mRNA expression levels were normalized to the median expression of the housekeeping gene GUSB or ACTB.

GPR41 and GPR43 siRNA-mediated silencing.

NHBE cells were plated in the wells of a 12-well plate (5×10^5 cells/well). At 50 % confluency, cells were then transfected with siRNA targeting human GPR41 (SR301921OriGene; Rockville, MD) or GPR43 (SR301923; OriGene using siTran1.0 Transfection Reagent (OriGene) and Opti-MEM (Thermo Fisher Scientific)) according to the instructions of the manufacturer. Cells were harvested at 24h for qRT-PCR and 48 h for Western blot analysis.

Western blot analysis

Total proteins from NHBE for Western blotting were dissolved in LDL sample buffer (Invitrogen). To detect both GPR41 and GPR43, reduced samples were resolved on 4-12% NuPAGE Bis-Tris Gel (Invtrogen) in MES buffer system and then proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA).The membranes were blocked in blocking buffer for fluorescent western blotting (Rockland, Gilbertsville, PA) for overnight at 4°C, then the membrane were incubated with 1 μg/ml rabbit anti-human GPR41 polyclonal antibody (MBL), or 1 μg/ml rabbit anti-human GPR43 polyclonal antibody (Life Span Bioscience) overnight at 4°C. After primary antibody incubation, the membranes were washed repeatedly in 0.1% Tween

20/TBS and TBS, and then labeled by 60 minutes incubation at room temperature with IRDye 680 conjugated goat anti-rabbit IgG (1:1000, Li-Cor Bioscience, Lincoln, NE). After repeated washing of membranes, proteins were detected on an Odyssey CLx System (Li-Cor Bioscience). Membranes were then incubated with Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific) for 30 minutes. Membranes were washed repeatedly with PBS and were blocked in blocking buffer for fluorescent western blotting (Rockland) overnight at 4°C. Membranes were incubated with mouse anti-human ACTB monoclonal antibody (1 : 20000; MP Biomedicals, Solon, OH) overnight at 4°C. The membranes were washed repeatedly in 0.1% Tween 20/TBS and TBS, and then labeled by 60 minutes incubation at room temperature with IRDye 800 conjugated goat anti-mouse IgG (1:1000, Li-Cor Bioscience, Lincoln, NE). After repeated washing of membranes, ACTB was detected using the Odyssey CLx System.

Supplementary Figure Legend

Supplementary Figure 1. One hundred μl of NHBE cells (1×10^6 cells/well) were seeded in a collagen coated 96-well tissue culture plate, allowed to achieve confluence and then stimulated with respective SCFA at different concentration for 24h. 1% Triton X-100 (Sigma- Aldrich) was used as a positive control (n = 3). Data were shown as mean ± SEM of three independent experiments. NS, not significant when compared to non-stimulated cells.

Supplementary Figure 2. The pH of medium with or without SCFAs was measured. The mean pH value was assessed from three separate measurements (n = 3). Data are shown as mean ± SEM of three independent experiments. NS, not significant when compared to medium.

Supplementary Figure 3. Submerged NHBE cells were harvested from transwells after stimulation with 10 mM of propionic acid for 48 hours. The transepithelial electrical resistance (TEER) was measured. The mean TEER value was assessed from three separate measurements (n = 5). Data are shown as mean ± SEM of three independent experiments. NS, not significant when compared to medium.

Supplementary Figure 4. Submerged NHBE cells were exposed to PTX (100pg/ml, 1ng/ml. and 10ng/ml) and YM-254890 (10nM, 100nM, and 1 μM) respectively (n = 3). Data are shown as mean ± SEM of four independent experiments. NS, not significant when compared to non-stimulated cells.

Supplementary Figure 5. One hundred μl of NHBE cells (1×10^6 cells/well) were seeded in a collagen coated 96-well tissue culture plate and stimulated with Pertussis toxin (PTX; Sigma-Aldrich) and YM-254890 (Wako, Osaka, Japan) for 24h. 1% Triton X-100 (Sigma- Aldrich) was used as a positive control (n = 3). Data are shown as mean ± SEM of three independent experiments. NS, not significant when compared to non-stimulated cells.

Supplementary Figure 6. Submerged NHBE cells were stimulated with indicated concentrations of trichostatin A for 24 hours. Cell lysates were harvested for RNA extraction to analyze t-PA mRNA expression by RT-PCR (n = 4). Data shown are mean ± SEM. * $p < 0.05$ when compared to non-stimulated cells.

Supplementary Figure 7. Immunofluorescence of GPR41 and GPR43 in NP tissue. Immunofluorescence assay was performed with anti GPR41 (green fluorescence) and anti ECP mAb (red fluorescence) for eosinophils (A), anti GPR43 (green fluorescence) and anti ECP mAb (red fluorescence) for eosinophils (B), and control IgG (C). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue fluorescence). The results are representative of 6 separate subjects.