Nitric oxide production is stimulated by bitter taste receptors ubiquitously expressed in the sinonasal cavity

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ABSTRACT

Background: Bitter taste receptors (T2R) have recently been demonstrated to contribute to sinonasal innate immunity. One T2R, T2R38, regulates mucosal defense against gram-negative organisms through nitric oxide (NO) production, which enhances mucociliary clearance and directly kills bacteria. To determine whether additional T2Rs contribute to this innate defense, we evaluated two other sinonasal T2Rs (T2R4 and T2R16) for regulation of NO production and expression within the human sinonasal cavity.

Methods: Primary human sinonasal cultures were stimulated with ligands specific to T2R4 and T2R16, colchicine and D-salicin, respectively. Cellular NO production was measured by intracellular 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate fluorescence. For T2R expression mapping, sinonasal tissue was obtained from patients who underwent sinus surgery of the middle turbinate, maxillary sinus, ethmoid sinus, or sphenoid sinus. The expression of T2R4, T2R16, and T2R38 was evaluated by using immunofluorescence with validated antibodies.

Results: Similar to T2R38, T2R4 and T2R16 trigger NO production in a dose-dependent manner by using the canonical taste signaling pathway in response to stimulation with their respective ligands. All three receptors were expressed in the cilia of human epithelial cells of all regions in the sinonasal cavity.

Conclusion: These three T2Rs signaled through the same NO-mediated antimicrobial pathway and were ubiquitously expressed in the sinonasal epithelium. Additional T2Rs besides T2R38 may play a role in sinonasal immune defense. Mapping of T2R expression demonstrated the potential widespread role of T2Rs in sinonasal defense, whereas the genetics of these T2Rs may contribute to our understanding of specific endotypes of chronic rhinosinusitis and develop into novel therapeutic targets.

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Bitter taste receptors (T2R) have recently been identified as novel “players” in sinonasal innate immunity and in chronic rhinosinusitis (CRS). One human T2R isoform, T2R38, has been shown to regulate sinonasal defense against gram-negative organisms, *e.g.*, *Pseudomonas aeruginosa*, through detection of quorum-sensing molecules secreted by the pathogens.¹ This activation triggers the production of nitric oxide (NO), a potent antimicrobial defense molecule, in a calcium-dependent manner, which results in increased ciliary beat frequency and acceleration of sinonasal mucociliary clearance. NO also diffuses into the mucous and directly targets bacteria and viruses.^{2,3} The clinical importance of T2R38 as a biomarker for CRS has been further elucidated by studying polymorphisms in T2R38 that produce decreased receptor functionality and lead to an increased susceptibility to gram-negative infection and recalcitrant CRS.^{4,5} Furthermore, the genotype of T2R38 can predict surgical outcomes of patients with nonpolyposis CRS and even biofilm formation.^{6,7}

T2R38 is known to be expressed in the ciliated epithelial cells of the upper airway.⁸ However, the specific expression pattern of T2R38 in the sinonasal cavity has not been previously evaluated. Mapping the

expression of this receptor within the sinonasal cavity would further our understanding of the contribution that this receptor plays in sinonasal antimicrobial defense as well as determine its viability as a novel therapeutic target (*e.g.*, if the receptor is only discretely expressed in a small part of the upper airway, it may not be an ideal drug-development target). Additional T2R isoforms are expressed throughout the respiratory epithelium, and the evolving roles for other T2Rs found on sinonasal chemosensory cells are being investigated.^{9,10} However, the clinical relevance of other T2R isoforms in CRS and their roles in sinonasal immunity still need to be elucidated. To determine whether additional T2Rs contribute to this innate defense, we further evaluated additional sinonasal T2Rs for regulation of NO production and their expression within the human sinonasal cavity.

METHODS

Human Mucosal Tissue and Sinonasal Air-Liquid Interface Cultures

Sinonasal mucosal tissue specimens were acquired from patients who underwent sinonasal surgery at the Hospital of the University of Pennsylvania and the Philadelphia Veterans Affairs Medical Center, with full approval from the institutional review boards at both institutions. Exclusion criteria included a history of systemic diseases as previously described.¹ Air-liquid interface (ALI) cultures were established from human sinonasal epithelial cells from enzymically dissociated human tissue, as previously described.^{1,11,12}

Immunofluorescence and Confocal Microscopy

Human mucosal tissue specimens and ALI cultures were selected for immunostaining as previously described.¹ Two primary antibodies raised from different hosts were chosen for double immunofluorescent staining: mouse monoclonal anti- β -tubulin intravenous (IV) (1:1,000) (ab7792; Abcam, Cambridge, United Kingdom) and either rabbit polyclonal anti-T2R38 (1:500) (sc-67108; Santa Cruz Biotechnol-

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ogies, Inc., Dallas, TX), which was previously validated,¹ rabbit polyclonal anti-T2R4 (1:1000) (NB110-74890; Novus Biologicals, Littleton, CO), or rabbit polyclonal anti-T2R16 (1:500) (ab75106; Abcam). Visualization was achieved by using Alex Fluor 488-conjugated (green) donkey antimouse immunoglobulin G for tubulin IV and Alex Fluor 594 (red) conjugated donkey antirabbit immunoglobulin G for T2R38, T2R4, and T2R16. Both secondary antibodies (Invitrogen, Carlsbad, CA) were diluted at 1:1000. The incubation time was overnight at 4°C for primary antibodies and 60 minutes at room temperature for secondary antibodies, respectively. Counterstaining was done using Hoechst (blue), a nuclear dye. Confocal images were acquired using an Olympus (Center Valley, PA) Fluoview System. A sequential scanning module was used to prevent bleed-through of fluorophores into other channels.

Live-Cell Imaging of Fluo-4 and 4-Amino-5-Methylamino-2',7'-Difluorofluorescein in Human ALI Cultures

Fluo-4 (Invitrogen) imaging was performed according to standard methods by using the 488-nm argon laser line of a Fluoview FV1000 laser scanning confocal system and IX-81 microscope (×10) (0.3 NA UPlanFLN objective; Olympus) as previously described.¹ In brief, cells were loaded with Fluo-4 by incubation at room temperature in the dark with an apical solution that contained 10 μM Fluo-4 AM (Invitrogen) for ~90 minutes. After loading with Fluo-4, the cultures were washed three times with Dulbecco's phosphate-buffered saline, followed by incubation for 15–20 minutes to allow for de-esterification of the loaded dye. The cultures were mounted in a custom-made chamber with a no. 1 coverslip bottom and basolateral solution volume of ~300 μL (changed after each experiment). Fluo-4 images were captured at ~5-second intervals. Fluo-4 fluorescence changes were normalized after subtraction of the background fluorescence, which was estimated for each experiment by measuring unloaded ALIs at identical settings. Baseline Fluo-4 fluorescence (F_0) was determined by averaging the first 10 frames of each experiment.

Cellular NO production was measured by using the fluorescent reactive nitrogen species indicator 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) (Invitrogen) and was imaged as previously described.⁸ DAF-FM fluorescence images were acquired at 5-second intervals. The phospholipase C β 2 (PLC β -2) inhibitor U73122 and its inactive analog U73343 (Cayman Chemical, Ann Arbor, MI) were used at 5 μM, and the TRPM5 blocker triphenylphosphine oxide (TPPO) (Sigma-Aldrich, St. Louis, MO) was used at 100 μM; working solutions were prepared fresh daily in phosphate-buffered saline from ×1000 dimethyl sulfoxide (DMSO) stock solutions. The nitric oxide synthase inhibitor, L-N^G-nitroarginine methyl ester (Cayman Chemical), was used at 100 μM and was prepared fresh daily from a ×1000 DMSO stock solution. The apical side of the cultures was incubated with the inactive analogs or inhibitors for 15 minutes before DAF-FM imaging.

Heterologous Expression

Constructs for three bitter taste receptor genes (*hTAS2R38*, *hTAS2R4* and *hTAS2R16*) were generated and inserted into a pCDNA3.1 vector according to Bufe *et al.*¹³ and were verified by sequencing. HEK293 cells were obtained from American Type Culture Collection, Manassas, VA (CRL-2828) and cultured at 37°C in opti-MEM (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen). For transient transfection, the cells were seeded onto 96-well plates with coverslips coated with polylysine at 40,000 cells per well. The cells were transfected with 0.2 μg *hTAS2R38*, *hTAS2R4*, or *hTAS2R16* per well. After another 24 hours, the cells were washed three times with Hanks' balanced salt solution, and immunofluorescent staining was performed as detailed above by comparing transfected and nontransfected HEK293 cells.

Statistics

Statistical analyses were performed by using GraphPad Prism (Graph Pad Software, La Jolla, CA) (mean, standard error of mean, Student's *t*-test, analysis of variance [ANOVA], and Bonferroni post-tests). ANOVA was used for comparing multiple data sets. When ANOVA denoted significant differences, Bonferroni posttests were used to determine individual significance. A $p < 0.05$ was considered statistically significant.

RESULTS

T2R38 Was Ubiquitously Expressed in the Sinonasal Cavity

In this study, we evaluated the expression pattern of T2R38 in human sinonasal tissue explants from different regions of the nasal cavity and sinuses, including the middle turbinate, inferior turbinate, maxillary sinus, ethmoid sinus, and sphenoid sinus. Tissues were obtained regardless of the presence of CRS, with the goal of the study to produce a qualitative analysis of T2R staining patterns. β -tubulin IV, a cilia marker (green), and T2R38 (red) fluorescence expression patterns were analyzed, and colocalization was noted in the ciliated cells of all sinonasal tissue regions (Fig. 1). T2R38 expression was found along the entire length of the cilia in our human sinonasal biopsy samples. In certain samples, there was a gradient of red fluorescence noted below the base of the cilia, previously shown to reflect T2R38 in the process of trafficking to the plasma membrane.¹ The specificity of our T2R38 expression was confirmed by staining similar samples for T2R47 (previously shown to not be present in ciliated cells¹⁴), which did not colocalize with β -tubulin (data not shown). Analysis of our mapping data indicated that T2R38 was expressed on ciliated epithelial cells throughout all regions of the sinonasal cavity.

T2R4 and T2R16 Were Ubiquitously Expressed in the Sinonasal Cavity

No previous studies evaluated the protein expression of other T2R isoforms in human sinonasal tissue. In this study, we evaluated, by immunofluorescence, the expression of T2R4 and T2R16, two receptors located closely to T2R38 on chromosome 7 and also found on ciliated cells of the lower airway.⁸ Commercially available T2R4 and T2R16 antibodies were validated in HEK293 cells through heterologous expression (Supplemental Fig. 1). T2R16 antibody specificity was demonstrated by the specific staining of only T2R16-transfected HEK293 cells with no cross-reactivity with other T2Rs (Supplemental Fig. 2). T2R4 was previously validated by Shah *et al.*⁸ Again, there was colocalization of both T2R4 and T2R16 with β -tubulin in all the regions of the sinonasal cavity, which indicated that these receptors were ubiquitously expressed in the ciliated cells of the nose (Figs. 2 and 3). Thus, sinonasal ciliated cells may coexpress multiple T2R receptors.

T2R4 and T2R16 Activation Trigger Ca²⁺-mediated NO Production

T2Rs are G-protein-coupled receptors that activate an intracellular Ca²⁺ signaling cascade that involves PLC β -2 and the transient receptor potential M5 ion channel.^{15–17} T2R38 interacts with gram-negative acyl-homoserine lactone molecules to increase intracellular Ca²⁺ levels and mediate NO production.¹ Because T2R4 and T2R16 are expressed in ciliated sinonasal epithelial cells, we were interested to see if they too promote Ca²⁺ signaling to trigger an NO response. As previously described, the fluorescent calcium indicator Fluo-4 was used to detect elevated Ca²⁺ levels in sinonasal cells in response to the T2R4 agonist, colchicine (depicted as the change in Fluo-4 fluorescence from baseline [Fluo-4 F/ F_0]).¹⁸ Apical (air side) stimulation with colchicine (100 μM and 1 mM) induced low-level, sustained intracel-

Figure 1. T2R38 is ubiquitously expressed in the cilia of human sinonasal passages. Representative images of β -tubulin (green [a ciliary marker]), T2R38 (red), and Hoechst (blue [a nuclear stain]) in a human sinonasal air-liquid interface (ALI) culture (A) and in primary human sinonasal tissue from different regions of the nasal cavity and sinuses (B-F), with colocalization of β -tubulin and T2R38 staining.

lular Ca^{2+} elevations in a dose-dependent manner (Fig. 4 A). The addition of TPPO, an inhibitor of the downstream signaling ion channel, transient receptor potential M5, subsequently reduced Ca^{2+} signaling.

We then evaluated the potential of T2R4 and T2R16 to individually activate the antimicrobial NO pathway in response to the low-level increases in intracellular Ca^{+2} . By using the fluorescent probe, DAF-FM,¹⁹ which reacts with NO-derived reactive nitrogen species to form

Figure 2. T2R4 is ubiquitously expressed in the cilia of human sinonasal passages. Representative images of β -tubulin (green [a ciliary marker]), T2R4 (red), and Hoechst (blue [a nuclear stain]) in a human sinonasal air-liquid interface (ALI) culture (A) and in primary human sinonasal tissue from different regions of the nasal cavity and sinuses (B–F) with colocalization of β -tubulin and T2R4 staining.

a fluorescent benzotriazole, we measured the cellular production of NO after treatment with known T2R4 and T2R16 agonists (Figs. 4 B and 5 A). Stimulation with T2R4 agonists colchicine (10 μ M, 100 μ M,

1 mM) and dapsone (100 μ M, 1 mM) resulted in NO-derived reactive species production (DAF-FM fluorescence) (Fig. 4 B). Treatment with T2R16-specific agonists D-salicin (4 mM) and phenyl β -D-glucopyra-

Figure 3. T2R16 is ubiquitously expressed in the cilia of human sinonasal passages. Representative images of β -tubulin (green [a ciliary marker]), T2R16 (red), and Hoechst (blue [a nuclear stain]) in a human sinonasal air-liquid interface (ALI) culture (A) and in primary human sinonasal tissue from different regions of the nasal cavity and sinuses (B-F), with colocalization of β -tubulin and T2R16 staining.

noside (4 mM) also showed an increase in NO production (Fig. 5 A). Next, we assessed the T2R dependence of the NO response by blocking components of the T2R canonical signal transduction pathway.

We found that NO stimulation by colchicine was attenuated with the addition of TPPO. Similarly, the PLC β -2 inhibitor, U73122, blocks T2R4-mediated NO production, an effect not seen with its inactive

Figure 4. T2R4 induces Ca^{2+} responses and T2R38-independent NO production in sinonasal ALI cultures. (A) Ca^{2+} responses after stimulation with 100 μM or 1 mM colchicine and 100 μM ATP stimulation (means $\pm$ SE). Ca^{2+} response to 1 mM colchicine is attenuated with the addition of TrpM5 inhibitor, TPPO (100 μM). (B) DAF-FM fluorescence increases after stimulation with two T2R4 agonists, colchicine and dapsone. The addition of taste signaling inhibitors (U73122, TPPO, and L-NAME) attenuates the DAF-FM response to colchicine and dapsone, not observed with U73343 (inactive analog of U73122). (C) Increase in DAF-FM fluorescence after stimulation by colchicine is independent of T2R38 genotypes. There was no difference in the increase of DAF-FM levels between PAV/PAV (165.6 ± 33.8 and 98.2 ± 13.4 DAF-FM units) and AVI/AVI (176.8 ± 15.3 and 89.8 ± 10.2 DAF-FM units) cultures after 1 mM and 100 μM colchicine treatment (mean $\pm$ SE). Significance was determined by 1-way ANOVA with the Bonferroni posttest. NO = Nitric oxide; ALI = air-liquid interface; ATP = adenosine triphosphate; TrpM5 = transient receptor potential melastatin isoform 5; TPPO = triphenylphosphine oxide; DAF-FM = 4-amino-5-methylamino-2, 7-difluorofluorescein; L-NAME = L-N^G-nitroarginine methyl ester; ANOVA = analysis of variance; n.s. = no statistical significance.

analog U73343 (Fig. 4 B). The dapsone-T2R4-activated NO response was similarly diminished by NOS inhibitor L-N^G-nitroarginine methylester and U73122 but not by U73343. We also demonstrated that NO production by T2R16 agonists D-salicin and β -D-glucopyranoside was attenuated after blocking the T2R signaling pathway with U73122 (Fig. 5, A and B).

T2R4-mediated NO Production Was Independent of T2R38

T2R38 has two well-studied polymorphisms that encode either a functional (PAV) or a nonfunctional (AVI) receptor, which lead to differing antibacterial NO responses. The NO response stimulated by a T2R4 agonist (colchicine) was evaluated in primary ALI cultures from patients with PAV/PAV and patients with AVI/AVI. The T2R4-mediated NO production (DAF-FM fluorescence) was not a function of the T2R38 genotype because cultures from patients with PAV/PAV and those with AVI/AVI exhibited the same levels of response (Fig. 4 C), which indicated that T2R4 independently mediated NO produc-

tion in a dose-dependent manner. A similar T2R38-independent mechanism is hypothesized for T2R16.

DISCUSSION

As the contribution of T2Rs to sinonasal innate immunity becomes more evident, mapping of their expression patterns adds important pieces of information. T2R38 expression has been observed in the apical membrane and cilia of sinonasal respiratory epithelial cells.¹ Our findings revealed that T2R38 was ubiquitously expressed among the ciliated cells of the human sinonasal cavity, along with two other T2Rs, T2R4 and T2R16. Furthermore, T2R4 and, most likely, T2R16 signal through the same NO-mediated antimicrobial pathway independently of T2R38. Our study was a qualitative study and not designed to quantify levels of T2R expression. We also could not make conclusions in this study about differences in receptor expression among patients with and those without CRS. These results demonstrated widespread expression of T2R38, T2R4, and T2R16 throughout the sinonasal cavity.

Figure 5. T2R16 also stimulates NO production in sinonasal ALI cultures. (A) DAF-FM fluorescence increases after stimulation with two T2R16 agonists, salicin (4 mM) and β -D-glucopyranoside (4 mM). The addition of U73122, an inhibitor of PLC β -2 in the T2R pathway, results in lower DAF-FM production. Mean traces $\pm$ SE (B) RNS measured 5 minutes after ALI stimulation with salicin or glucopyranoside alone or with the addition of U73122. DAF-FM fluorescence with salicin alone (164.8 ± 20.9 DAF-FM units) versus salicin + U73122 (39.4 ± 10.5) and glucopyranoside (15.6 ± 17.2) versus glucopyranoside + U73122 (18.2 ± 9.2). Significance determined by 1-way ANOVA with Bonferroni posttest. NO = Nitric oxide; ALI = air-liquid interface; DAF-FM = 4-amino-5-methylamino-2,7-difluorofluorescein; PLC β -2 = phospholipase C β -2; RNS = reactive nitrogen species; ANOVA = analysis of variance. $^{**}p < 0.05$.

The ability of T2R38 to mediate the antimicrobial NO pathway makes it an attractive therapeutic target to promote endogenous immune responses in patients with upper respiratory infections. However, patients with polymorphisms that render a nonfunctional T2R38 (*i.e.*, individuals with AVI/AVI) would be less responsive to the treatments with T2R38 agonists. It, thus, still is important to identify other T2Rs that activate similar responses. Although their clinical roles in sinonasal innate defense have yet to be elucidated, other T2R isoforms have been hypothesized to play important roles in upper respiratory innate immunity.^{20,21} Our findings revealed that T2R4 and T2R16 signal through the same NO pathway, independent of T2R38 and thus may be additional targets for novel therapeutic interventions.

These results supported further investigation into other T2R-mediated responses in addition to the T2R38-mediated defense against gram-negative bacteria. Gram-positive pathogens such as *Streptococcus* and *Staphylococcus* species are commonly implicated in CRS and can signal through quorum-sensing molecules that may also be endogenous ligands for certain T2Rs.^{22,23} We hypothesized that the upper-airway epithelium is able to detect gram-positive bacteria and mount an NO response. Further T2R studies, including that of T2R4 and T2R16, are warranted to explore any potential candidates that may pair with gram-positive ligand or other microbial class to stimulate an NO-dependent immune defense in sinonasal cells.

Although analysis of the current data indicated that T2R38, T2R4, and T2R16 were ubiquitously expressed in ciliated cells of the sinonasal cavity, analysis of our data was not able to resolve whether multiple T2Rs are expressed within the same ciliated cells or whether it is one cell-one receptor. In the human oral cavity, a single taste receptor cell can express multiple taste receptors.^{8,24,25} Ongoing work

by using direct conjugation of primary antibodies to different dyes as well as single-cell transcriptome analysis, it is hoped, will directly address this question in sinonasal cells. T2R38 has recently been indicated as a genetic marker for CRS with loss of function polymorphisms of T2R38, which results in decreased defensive response to bitter compounds and serve as a predictor of increased susceptibility to gram-negative infections.⁵ The same loss of function polymorphism (AVI) of T2R38 has been linked to increased nicotine and alcohol dependence in African Americans.²⁶ There have also been polymorphisms found in T2R4 and T2R16 that alter receptor sensitivity to bitter compounds. In particular, the K172N variant of T2R16 affects a patient's response to β -D-glucopyranoside and is associated with increased alcohol dependence in the African American population.²⁷ These known functional polymorphisms of T2R4 and T2R16 may be potential targets in assessing genetic variability and one's predisposition to specific upper respiratory infections if these receptors do indeed contribute to our innate sinonasal defense.

CONCLUSION

This study sheds light on the ubiquitous expression of T2R38 and two other T2Rs, T2R4 and T2R16, in the ciliated cells of the human sinonasal epithelium. This information supports an innate defensive role for respiratory T2Rs throughout the sinonasal cavity as well as putative targets for novel topical therapeutic interventions in the treatment of CRS. Moreover, we showed that T2R4 and T2R16 also signaled through an NO-mediated pathway, independent of T2R38. NO production and increased ciliary beat frequency are not unique defenses against gram-negative bacteria, and there is much to be elucidated in the role of T2R signaling against other pathogens. These

results pave the way for further investigation into other T2Rs, such as T2R4 and T2R16, and the clinical implications of their similar NO-mediated pathways in sinonasal defense.

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