

Increased expression of bronchial epithelial transient receptor potential vanilloid 1 channels in patients with severe asthma

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Background: The airway epithelium is exposed to a range of physical and chemical irritants in the environment that are known to trigger asthma. Transient receptor potential (TRP) cation

channels play a central role in sensory responses to noxious physical and chemical stimuli. Recent genetic evidence suggests an involvement of transient receptor potential vanilloid 1 (TRPV1), one member of the vanilloid subfamily of TRP channels, in the pathophysiology of asthma. The functional expression of TRPV1 on airway epithelium has yet to be elucidated.

Objective: In this study we examined the molecular, functional, and immunohistochemical expression of TRPV1 in asthmatic and healthy airways.

Methods: Bronchial biopsy specimens and bronchial brushings were obtained from healthy volunteers (n = 18), patients with mild-to-moderate asthma (n = 24), and patients with refractory asthma (n = 22). Cultured primary bronchial epithelial cells from patients with mild asthma (n = 4), nonasthmatic coughers (n = 4), and healthy subjects (n = 4) were studied to investigate the functional role of TRPV1.

Results: Quantitative immunohistochemistry revealed significantly more TRPV1 expression in asthmatic patients compared with healthy subjects, with the greatest expression in patients with refractory asthma ($P = .001$). PCR and Western blotting analysis confirmed gene and protein expression of TRPV1 in cultured primary bronchial epithelial cells. Patch-clamp electrophysiology directly confirmed functional TRPV1 expression in all 3 groups. In functional assays the TRPV1 agonist capsaicin induced dose-dependent IL-8 release, which could be blocked by the antagonist capsazepine. Reduction of external pH from 7.4 to 6.4 activated a capsazepine-sensitive outwardly rectifying membrane current.

Conclusions: Functional TRPV1 channels are present in the human airway epithelium and overexpressed in the airways of patients with refractory asthma. These channels might represent a novel therapeutic target for the treatment of uncontrolled asthma. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: Ion channel, sensory, asthma, irritant, chemical, exacerbation, cough

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During an asthma exacerbation, the airways become hypersensitive, and patients are often troubled with bouts of cough and wheeze after exposure to relatively innocuous stimuli, such as aerosols, strong odors, or changes in air temperature. The precise mechanism for this airway hypersensitivity is not known, but upregulation of receptors responsible for sensing chemical and physical stimuli might be relevant. The recently described transient receptor potential (TRP) proteins, a family of Ca^{2+} -permeable

Abbreviations used

C _T :	Cycle threshold
ER:	Endoplasmic reticulum
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GINA:	Global Initiative for Asthma
IHC:	Immunohistochemistry
IQR:	Interquartile range
I-V:	Current-voltage
PBEC:	Primary bronchial epithelial cell
qRT-PCR:	Quantitative real-time PCR
TRP:	Transient receptor potential
TRPV1:	Transient receptor potential vanilloid 1

nonselective cation channels that sense a vast array of chemical and physical stimuli,^{1,2} are believed to have an important role in the regulation of airway function in both health and disease.^{3,4} Recent data have provided genetic evidence for the involvement of one subfamily member, transient receptor potential vanilloid 1 (TRPV1), in asthma pathophysiology.⁵

TRPV1 is activated by capsaicin (the pungent principle contained in hot chili peppers), and inhaled capsaicin causes cough⁶ and bronchospasm⁷ in asthmatic patients. The airway inflammatory events that accompany an asthma exacerbation include an increase in airway temperature,⁸ acidosis,⁹ and release of mediators derived from lipoxygenase metabolism of arachidonic acid.^{4,10} TRPV1 is primarily recognized as a heat thermal sensor, but it is also readily activated by low extracellular pH^{11,12} and lipoxygenase products, such as leukotriene B₄.¹³ TRPV1 receptors are expressed in several cell types in the human airway, including sensory neurones,¹⁴ smooth muscle cells,¹⁵ and epithelial cells from both the upper¹⁶ and lower¹⁷ airways. Because the bronchial epithelium has an important role in sensing and responding to noxious stimuli, we hypothesized that TRPV1 is overexpressed in the airway epithelium of asthmatic patients, particularly those with difficult-to-treat asthma. Furthermore, we considered that the inflammatory response after irritant activation of airway epithelial TRPV1 might contribute to the heightened neuronal reflexes responsible for cough and bronchospasm in asthmatic patients.

In this study we examined both TRPV1 expression and function in human bronchial epithelium. In addition, we provide evidence for the overexpression of TRPV1 in the airways of patients with severe asthma compared with those with mild asthma and healthy subjects. Some of the results of these studies have been reported in abstract form.¹⁸

METHODS

Full methodological details are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Study subjects

Participants with refractory asthma were recruited from Belfast City Hospital. All had persistent symptoms despite treatment at step 4 or 5 of the Global Initiative for Asthma (GINA) guidelines. Patients with mild-to-moderate asthma had a clinical diagnosis of asthma with a current history of recurrent wheezing and documented response to asthma medication. Bronchial biopsy samples from this Belfast cohort were analyzed by using immunohistochemistry (IHC). For examination of *in vivo* gene expression of TRPV1 in airway epithelium, an additional cohort of subjects from Leicester were recruited. Demographic details of both cohorts are shown in [Table I](#).

Primary bronchial epithelial cell (PBEC) cultures were grown from bronchial brushings obtained from patients with mild asthma and nonasthmatic patients recruited from a specialist cough clinic¹⁹ and healthy participants recruited by advertisement to investigate the functional role of TRPV1. Demographic details are shown in [Table II](#).

The study was approved by the appropriate Institutional Research Ethics Committees (details are provided in the [Methods](#) section in this article's Online Repository). Written informed consent was obtained from all participants before their involvement.

Bronchoscopy and sample processing

All asthmatic participants were clinically stable at the time of bronchoscopy and had an FEV₁ of greater than 60% of predicted value. Bronchial biopsy specimens and brushings were obtained from segmental airways by using standard techniques. Three biopsy specimens from each participant (Belfast cohort) were submitted for IHC studies. Gene expression microarray analysis (and quantitative real-time PCR [qRT-PCR]) was performed on fresh bronchial brushings (Leicester cohort). PBEC cultures from bronchial brushings were cultured, as described previously,²⁰ to investigate the functional role of TRPV1. These were obtained from patients with mild asthma (n = 4), nonasthmatic patients with chronic cough (n = 4), and healthy volunteers (n = 4).

IHC

Twenty-eight biopsy specimens (10 from healthy volunteers, 8 from patients with mild asthma, and 10 from patients with severe asthma) were processed for IHC by using local protocols, as detailed in the [Methods](#) section in this article's Online Repository. For TRPV1 and ER colocalization experiments, local protocols were used, as detailed in the [Methods](#) section in this article's Online Repository.

Gene expression analysis

Bronchial brushings and PBEC cultures. qRT-PCR analyses were performed with RNA from bronchial brushings by using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Calif), as previously described.²¹ RNA was amplified (Ambion, Austin, Tex) for Agilent (Santa Clara, Calif) 2-color Whole Human Genome 4 × 44k gene expression microarray analysis.

PBECs were trypsinized, total RNA was extracted by using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and qRT-PCR was performed by using a 7300 Real-Time PCR system (Applied Biosystems). More information, including details on glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *TRPV1* forward and reverse primers, is provided in [Table E1](#) and the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Western blotting

Protein concentration was determined by using the BCA protein assay kit (Thermo Scientific, Wilmington, Del), and protein expression was determined by using a rabbit polyclonal TRPV1 antibody (ab63083; Abcam, Cambridge, United Kingdom) at a dilution of 1:3000, followed by incubation for 1 hour with the secondary antibody horseradish peroxidase-labeled anti-rabbit (1:3000; Bio-Rad Laboratories, Hercules, Calif). More details are provided in the [Methods](#) section in this article's Online Repository.

TRPV1 agonist/antagonist experiments in PBECs

Capsaicin is a selective TRPV1 agonist, and in this study cultured PBECs were treated with either 10, 25, or 50 μmol/L capsaicin or control medium for 24 hours, and the concentration of IL-8 was measured by using ELISA (R&D Systems Europe, Abingdon, United Kingdom). For the antagonist experiments, PBECs were pretreated for 20 minutes with 10 μmol/L of the selective TRPV1 antagonist capsazepine.

Patch-clamp experiments. Whole-cell currents were recorded by using borosilicate heat-polished patch pipettes (2–3 MΩ resistance when filled with the pipette solution) and an Axopatch 200B amplifier (Molecular

TABLE I. Demographic characteristics of the asthmatic participants and healthy volunteers (Belfast and Leicester cohorts combined)

	Healthy volunteers (n = 22)	Patients with mild-to-moderate asthma (n = 30)	Patients with severe asthma (n = 31)	P value*
Age (y), median (IQR)	30 (22-45)	29 (24-39)	48 (38-54)†	<.0001
Sex (male/female)	10/12	14/16	22/9	.09
Duration of asthma (y), mean ± SD	NA	12 ± 10	22 ± 15††	.007
Atopy (n)	11	21	23	.16
FEV ₁ (L/min), mean ± SD	3.67 ± 0.78	3.32 ± 0.77	2.48 ± 0.67‡	<.0001
FEV ₁ (% predicted), mean ± SD	102.8 ± 11.9	93.5 ± 16.0	75.9 ± 16.9§	<.0001
FEV ₁ /FVC ratio, mean ± SD	82.1 ± 9.8	75.9 ± 10.6	64.6 ± 11.1	<.0001
Inhaled steroid (n)	0	19	31	
BDP equivalent (μg), median (IQR)	0	400 (0-800)	1600 (1200-1600)‡‡	<.0001
Maintenance systemic steroids (n)	0	0	15 (median dose prednisolone, 5 mg [0-10 mg] plus 1 subject maintenance intramuscular triamcinolone)	
Theophylline (n)	0	1	17	<.0001
Long-acting β ₂ -agonists (n)	0	14	31	<.0001
Prior smoking history (n [pack years; median [range]])	1 [4]	5 [1, 1-10]	13 [2.5, 1-20]	.004
Eosinophils (10 ⁹ /L), median (IQR)	0.1 (0.07-0.21)	0.26 (0.14-0.45)#	0.49 (0.25-0.74)§§	<.0001
IgE (kU/L), geometric mean (95% CI)	24.6 (13.3-45.8)	159.8 (89.8-284.6)**	238.5 (136.2-417.5)	<.0001

NA, Not applicable.

Comparison with the healthy volunteer group: †P < .001, ‡P < .001, §P < .001, ||P < .001, ¶P < .001, #P < .05, and **P < .001.

Comparison with mild-to-moderate asthma group: †P < .001, ††P = .007, ‡P < .001, §P < .001, ||P < .001, ‡‡P < .0001, §§P < .05, and |||P < .001.

*Calculated by using ANOVA with the Bonferroni multiple comparisons test (parametric) or Kruskal-Wallis test with the Dunn multiple comparison test (nonparametric), the t test when only the asthmatic groups were compared, or χ² tests for categorical variables (sex, atopy, theophylline, long-acting β₂-agonists, and smoking history).**TABLE II.** Demographic characteristics of the patients with mild asthma, nonasthmatic coughers, and healthy volunteers recruited for experiments on cultured PBECs

	Healthy volunteers (n = 4)	Patients with mild asthma (n = 4)	Nonasthmatic coughers (n = 4)	P value
Age (y), median (IQR)	35.5 (21.3-43.8)	46.0 (39.3-56.8)	58.0 (45.0-72.5)	.05
Sex (male/female)	1/3	0/4	0/4	.336
Duration of cough (mo), mean ± SD	NA	49.5 ± 30.0	57.5 ± 27.0	.950
FEV ₁ (L/min), mean ± SD	3.45 ± 0.65	2.52 ± 0.29	2.3 ± 0.34	.014
FEV ₁ (% predicted), mean ± SD	107.3 ± 10.59	105.6 ± 15.23	118.0 ± 21.28	.530
FEV ₁ /FVC ratio, mean ± SD	86.6 ± 10.6	82.73 ± 4.11	80.2 ± 3.2	.446
Atopy (n)	0	0	0	NA
Inhaled steroid (n)	0	4	0	NA
Prior smoking (n)	0	0	0	NA

Calculated by using ANOVA with the Bonferroni multiple comparisons test (parametric) or Kruskal-Wallis with the Dunn multiple comparison test (nonparametric), the t test when only cough groups were compared, or the χ² test for categorical variables (sex).

NA, Not applicable.

Devices, Union City, Calif) interfaced to Digidata 1322A by using pClamp 9 software (Molecular Devices), as previously described by ourselves²² and detailed in the [Methods](#) section in this article's Online Repository.

Data analysis

Data are presented as means ± SEMs. For statistical analysis, 1-way ANOVA was performed with the Bonferroni test for multiple comparisons. Kendall τ-b statistical analysis was used to examine the trend in biopsy expression by participant group. Patch-clamp data were analyzed and plotted with Origin 8 (OriginLab, Northampton, Mass). A P value of less than .05 was considered significant.

RESULTS

Confirmation of TRPV1 expression in cultured human bronchial epithelial cells

Gene expression for multiple members of the TRPV family, including *TRPV1* (but not *TRPV5*), was identified in PBECs from

healthy control subjects (n = 3; [Fig 1, A](#)). TRPV1 protein was detected at its expected molecular mass of 95 kDa by using Western blotting in the same samples ([Fig 1, B](#)).

Molecular identification and localization (immunohistochemical expression) of TRPV1 in epithelium from healthy subjects and asthmatic patients

Microarray. Using gene expression microarray analysis of bronchial brush samples from the Leicester cohort, we found significantly greater *TRPV1* expression in patients with severe asthma (median, 0.673; interquartile range [IQR], 0.452-0.761) compared with that seen in patients with mild-to-moderate asthma (median, 0.341; IQR, 0.167-0.538; P = .008), and healthy volunteers (median, 0.322; IQR, 0.155-0.410; P = .004; [Fig 1, C](#)).

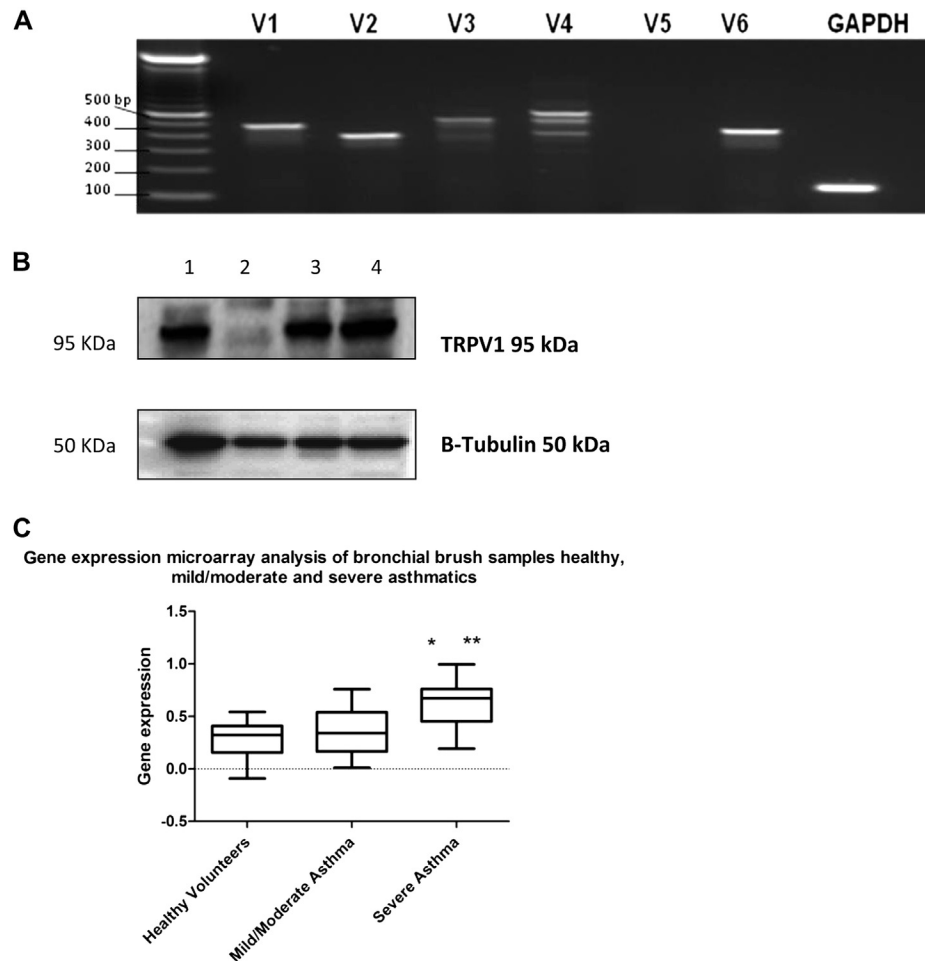


FIG 1. **A**, Molecular expression of TRPV channel subtypes in human PBECs. Conventional RT-PCR showing the expression of TRPV family subtypes, including *TRPV1*, in PBECs from healthy control subjects ($n = 3$). **B**, Expression of TRPV1 protein in PBECs. TRPV1-specific bands were observed at the molecular weight of 95 kDa in PBECs (lanes 2-4, upper panel). TRPV1 transfected HEK cells were used as a positive control (lane 1, upper panel). β -Tubulin (lower panel) was used to ascertain equal loading (Abcam 6046). Results are representative of 2 independent experiments. **C**, Gene expression microarray analysis of bronchial brush samples from healthy subjects, patients with mild-to-moderate asthma, and patients with severe asthma. Gene expression microarray analysis was performed on fresh bronchial brush samples from the Leicester cohort comprising patients with severe asthma ($n = 12$), patients with mild-to-moderate asthma ($n = 16$), and healthy subjects ($n = 8$). *TRPV1* expression was significantly increased in patients with severe asthma versus those with mild-to-moderate asthma (* $P = .008$) and healthy volunteers (** $P = .004$).

Quantitative PCR. *TRPV1* mRNA was detected in all of the epithelial brush samples. There was no difference in median relative fold expression of *TRPV1* mRNA calculated as $\Delta\Delta$ cycle threshold (C_T) values between groups: -1.262 (IQR, -3.598 to 2.518), -2.523 (IQR, -4.040 to 1.362), and -1.289 (IQR, -4.746 to 1.624) in healthy volunteers, patients with mild-to-moderate asthma, and patients with severe asthma, respectively (see Fig E1 in this article's Online Repository at www.jacionline.org).

IHC. IHC revealed bronchial epithelial expression of TRPV1 in healthy volunteers, patients with mild-to-moderate asthma, and patients with severe asthma (Fig 2). Dual-labeling immunofluorescence confirmed the expression of TRPV1 on cytokeratin-positive epithelial cells (Fig 3). We observed an intracytoplasmic distribution of the TRPV1, and to further characterize this, we

undertook additional experiments demonstrating IHC staining of bronchial epithelial cells positive for TRPV1 (Fig 4, A) and ER (Fig 4, B) markers. Colocalization was evident when the images were merged (Fig 4, C).

We observed greater TRPV1 expression in epithelium from asthmatic patients compared with that seen in epithelium from healthy subjects (Fig 5, $P = .001$).

Membrane current responses to TRPV1 agonist capsaicin

The mean membrane capacitance of PBECs was 30.8 ± 4.3 pico Farad ($n = 6$). In unstimulated cells only small background currents could be recorded in the whole range of membrane potentials tested, from -100 to $+140$ mV (Fig 6, A, left panel).

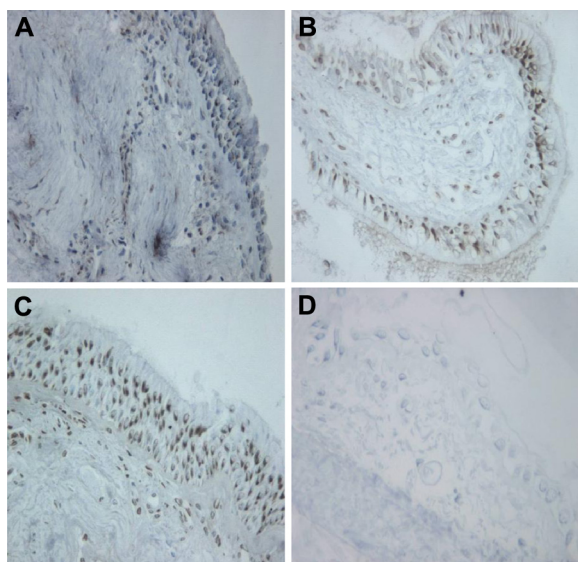


FIG 2. Digital photographic images of TRPV1 expression in JB4 sections from healthy control subjects (**A**; only patchy or focal areas of low-intensity epithelial expression), patients with moderate asthma (**B**; the majority of epithelial and submucosal cells express antigen), and patients with severe asthma (**C**; widespread and strong epithelial and submucosal cell expression). Sections incubated in the absence of antibodies showed no staining, and a representative image is shown (**D**). Representative IHC is shown at $\times 400$ magnification.

The current-voltage (I-V) relationship of the mean background current normalized by the membrane capacitance to account for some variations in cell size is shown in Fig 6, *B*. It should be noted that the composition of the external and pipette solutions was such that this facilitated isolation of cation currents but precluded activation of any potassium- or calcium-dependent conductances. Capsaicin application (50 $\mu\text{mol/L}$) caused rapid activation of large, outwardly rectifying currents (Fig 6, *A*, middle panel), which is the characteristic “biophysical” signature of TRPV1 channels. This effect was completely reversible on capsaicin washout (Fig 6, *A*, right panel). Corresponding I-V relationships are shown in Fig 6, *C*. The capsaicin-induced currents reversed close to 0 mV, indicating their nonselective cation nature.

Voltage ramps over the same range of potentials were applied at 10-second intervals to investigate the time course of these responses. Fig 7, *A*, shows a typical response to 50 $\mu\text{mol/L}$ capsaicin application consisting of rapid activation and desensitization in the continuous presence of the agonist, which developed over 2 to 3 minutes. The I-V relationships, which were measured in this experiment in control, at the peak response, and after agonist washout, are shown in Fig 7, *B* (compare with I-V curves in Fig 6, *C*, measured by voltage steps), whereas the mean results are summarized in panel Fig 7, *C*. The increase in current amplitude in response to capsaicin was significant ($P = .007$).

Membrane responses to reduction of external pH

Reduction of external pH from 7.4 to 6.4 activated inward and outward currents in cells from healthy patients, which were partially inhibited by the addition of the selective TRPV1 antagonist capsazepine (5 $\mu\text{mol/L}$; Fig 8, *A*). The

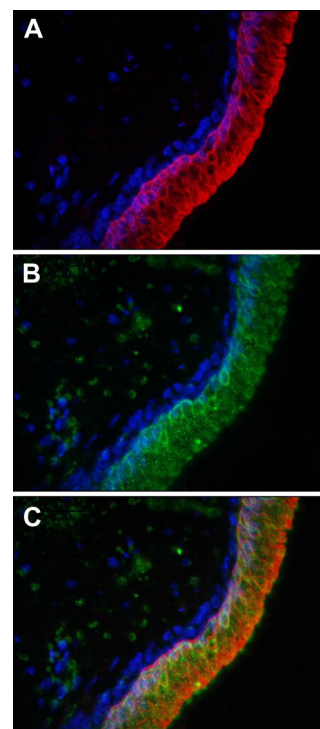


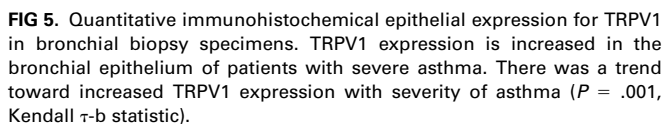
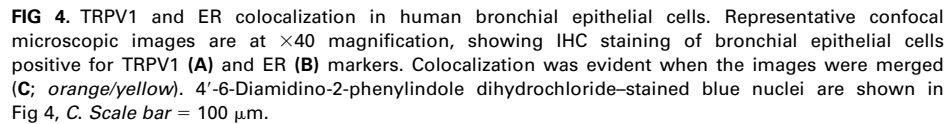
FIG 3. Confocal microscopic images of double-staining immunocytochemistry demonstrating localization of TRPV1 channels in bronchial epithelium (healthy subjects). Bronchial epithelial cells identified by means of positive red staining with the cytokeratin antibody AE1/AE3 (**A**) were also positive for TRPV1 (green; **B**), and colocalization was evident when the images were merged (yellow; **C**). Representative IHC is shown at $\times 400$ magnification.

mean capsazepine-sensitive I-V relation (Fig 8, *B*; $n = 4$) was similar in profile to the capsaicin-dependent currents, exhibiting strong outward rectification and reversal close to 0 mV.

Functional expression of TRPV1 in cultured human PBECS

Dose-dependent induction of IL-8 release by capsaicin was observed within each study group, although there was no significant difference in the fold increase in IL-8 release between the asthmatic and nonasthmatic groups, which might relate to sample size (data not shown). The dose-dependent increase in IL-8 release when data from all 3 groups were pooled is presented in Fig 9, *A*. When compared with baseline, a marginal 1.25-fold increase in IL-8 release was observed after incubation with 10 $\mu\text{mol/L}$ capsaicin, with significant increases of 1.76-fold ($P < .01$) and 2.15-fold ($P < .001$) compared with baseline observed after incubation with 25 and 50 $\mu\text{mol/L}$ doses of capsaicin, respectively.

To further test the specificity of the capsaicin effect, we used the selective TRPV1 blocker capsazepine. Capsazepine significantly inhibited capsaicin-induced IL-8 release compared with 25 $\mu\text{mol/L}$ capsaicin alone ($P < .001$) and compared with 50 $\mu\text{mol/L}$ capsaicin alone ($P < .001$). The data for 9 individual experiments is shown in Fig 9, *B*.



In this study, for the first time, we have shown that TRPV1, which is primarily recognized as the major neuronal hot receptor,^{23,24} is expressed and functional in human bronchial epithelium. In PBEC cultures we have shown that pharmacologic activation of the TRPV1 channel with capsaicin induces IL-8 release, which can be attenuated by pretreatment with the selective TRPV1 antagonist capsazepine.

We believe that bronchial epithelial TRPV1 channels are ideally positioned to detect chemical and physical irritants (eg, inflammatory mediators, environmental pollutants, acid pH, and changes in temperature) in the airway lumen. To date, most of the focus has been on neuronal TRPV1 in the human airway.^{14,25} However, TRPV1 expression has been reported in a variety of nonneuronal tissues, and therefore we hypothesized that the airway responses to environmental stimuli might in part be regulated by the bronchial epithelial TRPV1 receptors. Recent studies in immortalized airway epithelial cell lines provide some support for this,^{17,26} although evidence in human

We observed an intracytoplasmic distribution of the receptor in bronchial epithelial cells, as observed in other cell types.^{27,28} Our finding of TRPV1 staining in the endoplasmic reticulum (ER) is consistent with its functional role as a Ca^{2+} store release channel regulating intracellular Ca^{2+} homeostasis. Robust membrane current responses to the TRPV1 agonist capsaicin (Figs 5 and 6) suggest strongly that plasmalemmal TRPV1 is also expressed and functional in PBEcs. We also observed TRPV1 staining on a number of submucosal cells, although the focus of the present study is confined to bronchial epithelial expression. Previous studies from chronic coughers¹⁴ and healthy subjects¹⁵ have reported only sparse TRPV1 expression in the airway epithelium, although greater expression has been found in the upper airway.²⁹ Differences in tissue processing, antibodies used, and clinical phenotype might explain the discrepancy. In our studies to evaluate the functional properties of TRPV1 on the bronchial epithelium, we saw that PBEcs responded to the TRPV1 agonist capsaicin with the release of proinflammatory mediators, which was blocked by capsazepine, a TRPV1 selective antagonist. Capsaicin-induced IL-8 release has been previously observed in both human esophageal³⁰ and human corneal³¹ epithelial cells but not before in cultured PBEcs. Because IL-8 is known to induce and maintain neural sensitization,^{32,33} our data provide a mechanistic link, whereby activation of bronchial epithelial TRPV1 by environmental irritants or changes in airway pH or temperature (during exacerbations) might sensitize airway sensory neurons and contribute to the airway and cough hypersensitivity that characterizes asthma.

Studies in immortalized respiratory cell lines suggest airway epithelial TRPV1 regulates cell apoptosis in response to environmental irritants.³⁴ Here we provide the first evidence of a functional airway epithelial TRPV1 channel in primary cells. In the whole-cell patch-clamp experiments on PBECS, our results provide direct evidence for the presence of functional TRPV1 in the plasma membrane that can mediate membrane depolarization and calcium influx, steps that are necessary for release of proinflammatory mediators.³⁵ Both the kinetics and voltage-dependent properties of capsaicin-induced currents in PBECS are consistent with TRPV1

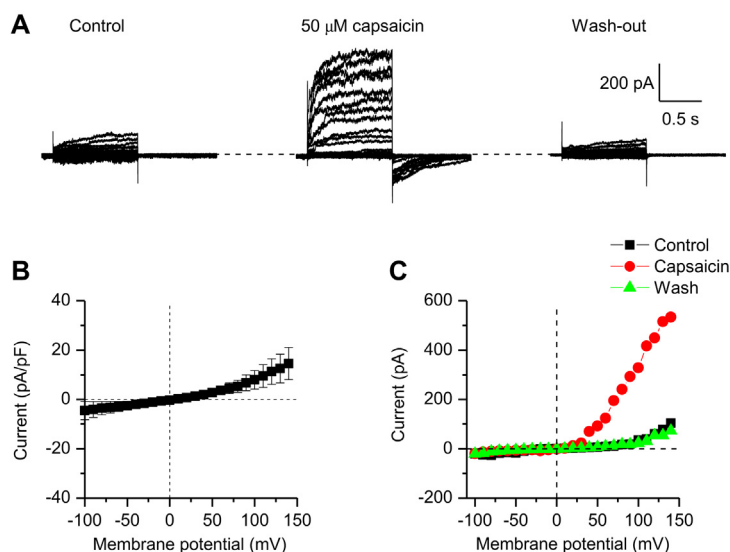


FIG 6. Whole-cell PBEC (healthy subject) membrane current responses to capsaicin application (50 μ mol/L) measured by using voltage steps. **A**, Superimposed current traces evoked by 1-second voltage steps before, in the presence of, and after capsaicin washout, as indicated. The dotted line indicates zero-level current. **B**, Mean normalized I-V relationship of background current (n = 4). **C**, I-V relationships for the current amplitude measured at the end of each voltage step in the experiment illustrated in Fig 6, A.

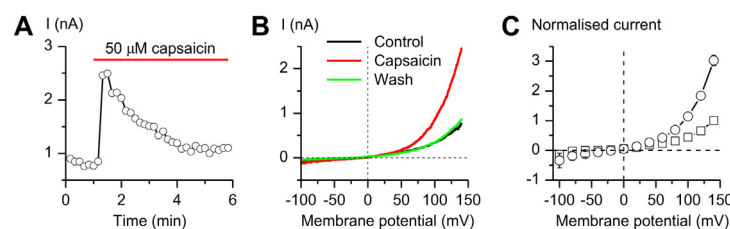


FIG 7. Time course of the capsaicin membrane current response. **A**, Current amplitude was monitored by applying voltage ramps from -100 to 140 mV at 10-second intervals. Circles show the current amplitude at 140 mV. **B**, The I-V relationships were derived from the ramps applied before, at the peak response, and 3 minutes after washout of the drug, as indicated. **C**, Mean normalized I-V relationships measured before (squares) and after (circles) capsaicin application (n = 3). In each cell current amplitude at 140 mV in control subjects was normalized as 1.0.

biophysical features, such as slow activation/deactivation and strong outward rectification.

TRP channels have the capacity to sense a vast range of stimuli, and their role in airways disease is currently under intense investigation.¹ In our immunohistochemical studies we have shown that TRPV1 is overexpressed in the bronchial epithelium of asthmatic patients compared with that seen in healthy subjects, and patients with refractory asthma express more epithelial TRPV1 than those with mild-to-moderate asthma. This was supported by our microarray analysis, confirming increased expression of *TRPV1* genes in patients with more severe asthma. Although mRNA expression was not concordant with protein abundance on quantitative PCR, it has become increasingly clear that at the genomic level, mRNA expression does not consistently predict corresponding protein levels.^{36,37} This might be because of complex posttranscriptional and translational modification, or that different biological mRNA and protein degradation rates may affect correlation. Posttranslational modification of TRPV1 has been described in experimentally induced tissue

injury³⁸ and therefore can occur in the inflamed asthmatic airway.

Although the vast majority of asthmatic patients respond to standard doses of current treatments (in particular corticosteroids), a proportion remain symptomatic despite treatment at GINA step 4 or 5 and are considered to have difficult or treatment-refractory asthma. Our finding that bronchial epithelial TRPV1 is overexpressed in patients with refractory (steroid-resistant) asthma suggests its activation might represent a novel proinflammatory pathway. Clinical trials of TRP receptor antagonists have been undertaken in other abnormal hypersensitive states, including neuropathic pain, and these drugs can prove effective in improving symptom control in asthmatic patients as well.

Our findings suggest that the bronchial epithelium might sense and respond to environmental stimuli through TRPV1 activation. However, it is not clear how bronchial epithelial cells signal to afferent sensory nerves. We suggest that release of sensitizing mediators, such as IL-8, might be one such mechanism. Such mediators might enhance and augment neurogenic inflammation

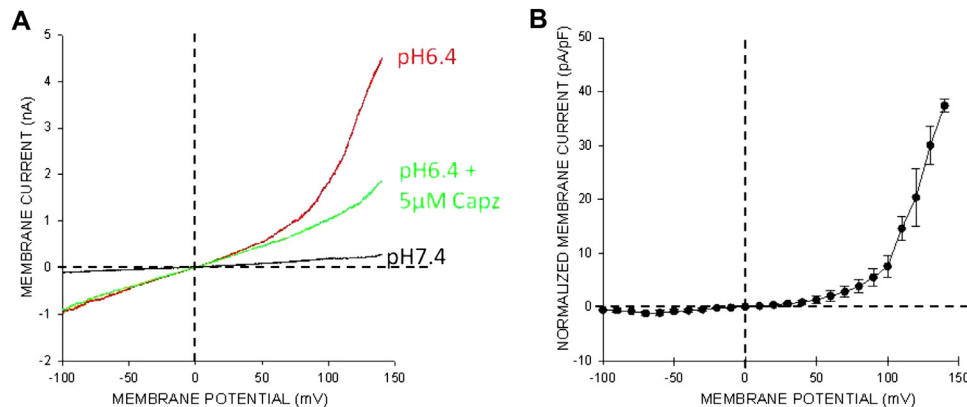


FIG 8. Whole-cell PBEC (healthy subject) membrane current responses to reduced external pH measured by using voltage ramps. **A**, Superimposed current traces evoked by voltage ramps from -100 to 140 mV at 10-second intervals in pH 7.4 and pH 6.4 ($\pm$ capsazepine; $5 \mu\text{mol/L}$) external solutions, as indicated. The dotted line indicates zero-level current. **B**, Mean $\pm$ SEM I-V relationship (normalized for cell capacitance) of capsazepine-sensitive current ($n = 4$).

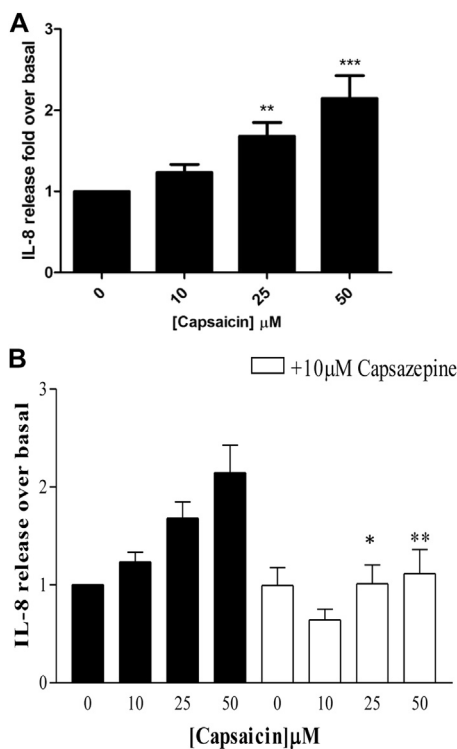


FIG 9. Capsaicin induces IL-8 release from human PBECs (asthmatic and nonasthmatic subjects). PBECs treated (24 hours) with 10, 25, or $50 \mu\text{mol/L}$ capsaicin or control medium. Values are expressed as the mean release over baseline $\pm$ SEM. **A**, Capsaicin-induced IL-8 release. $**P < .01$, $***P < .001$ compared with control, Kruskal-Wallis test with Dunn multiple comparison ($n = 12$ individual experiments). **B**, Capsazepine inhibits capsaicin-induced IL-8 release from human PBECs. PBECs were pretreated for 20 minutes with $10 \mu\text{mol/L}$ capsazepine and then with 10, 25, or $50 \mu\text{mol/L}$ capsaicin or control medium with or without capsazepine. Values are expressed as mean release over baseline $\pm$ SEM. Capsazepine significantly inhibited capsaicin-induced IL-8 release compared with $25 \mu\text{mol/L}$ ($*P < .001$) and $50 \mu\text{mol/L}$ ($**P < .001$) capsaicin alone (1-way ANOVA with the Bonferroni multiple comparison test, $n = 9$ individual experiments).

and nociceptive signaling. Therefore pharmacologic modulation of this receptor could be useful in the treatment of uncontrolled asthma.

Clinical implications: TRPV1 is a functional channel activated by physical and chemical irritants. We report its overexpression in the airway epithelium of asthmatic patients, most notably patients with severe disease, suggesting a role in patients with uncontrolled asthma.

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METHODS

Study subjects

The studies were approved by the research ethics committees of each institution (Belfast: Office of Research and Ethics Committee of Northern Ireland reference 06/NIR02/114 and 09/NIR02/51; Leicester: Leicestershire, Northamptonshire, & Rutland Research Ethics Committee reference 04/Q2502/74). Written informed consent was gained from all participants before their involvement.

Bronchial biopsy specimens

Belfast cohort. Participants with refractory asthma were recruited from the Belfast City Hospital, where systematic evaluation protocols ensure patients have well-characterized refractory asthma. All participants had persisting symptoms despite treatment at step 4 or 5 of the GINA guidelines.^{E1} Patients with mild-to-moderate asthma and healthy subjects were recruited by advertisement and had a clinical diagnosis of asthma with a current history of recurrent wheezing and response to asthma medication. Healthy volunteers had no history of asthma or persistent respiratory symptoms and normal lung function. All asthmatic participants were clinically stable on their usual medication at the time of bronchoscopy, and all participants had an FEV₁ of greater than 60% of predicted value. Demographic details are shown in Table I.

Leicester cohort. For the gene expression studies, asthmatic subjects (n = 36) and healthy volunteers (n = 12) were recruited from respiratory clinics, including the Leicester Difficult Asthma Clinic, staff at Glenfield Hospital, and the general population through advertisement (Leicester cohort). Asthmatic subjects provided a suggestive history and had objective evidence of variable airflow obstruction, as indicated by 1 or more of the following: (1) methacholine airway hyperresponsiveness (PC₂₀ FEV₁ <8 mg/mL), (2) greater than 15% improvement in FEV₁ 10 minutes after 200 µg of inhaled salbutamol, and (3) peak expiratory flow of greater than 20% of the maximum within-day amplitude from twice daily peak expiratory flow measurements over a period of 14 days. Subjects underwent spirometry; allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat, grass pollen, and *Aspergillus fumigatus*; a methacholine inhalation test using the tidal breathing method; and sputum induction using incremental concentrations of nebulized hypertonic saline (ie, 3%, 4%, and 5%, each for 5 minutes).^{E2} Asthmatic patients also kept a diary card for 2 weeks before bronchoscopy, recording daytime and nighttime symptoms, daily short-acting β₂-agonist use, and twice daily peak expiratory flow. Asthma severity was defined by British Guidelines on the Management of Asthma treatment steps (mild = step 1, β₂-agonist only; moderate = steps 2 and 3, inhaled corticosteroid [≤800 mg of beclomethasone equivalent per day] ± long-acting β₂-agonist; and severe = step 4 and 5).^{E3} Of the 16 patients with severe asthma at step 4 or 5, 13 met the American Thoracic Society criteria for refractory asthma.^{E4}

In both cohorts all participants were current nonsmokers with no upper or lower respiratory tract infections in the 6 weeks before bronchoscopy. All asthmatic participants were clinically stable on their usual medication at the time of bronchoscopy. Demographic details for each cohort are shown in Table I.

PBEC cultures. To investigate the functional role of TRPV1, we performed a series of experiments on PBEC cultures from bronchial brushings obtained from patients with mild asthma (n = 4) and nonasthmatic patients with chronic cough (n = 4) referred to the Belfast City Hospital specialist cough clinic.^{E5} Healthy volunteers (n = 4) were recruited by local advertisement. Demographic details for this cohort are shown in Table II.

Bronchoscopy and samples

Bronchoscopy was performed by using a standard technique. In brief, after intravenous sedation and local anesthesia with topical lignocaine, bronchial biopsy specimens and bronchial brushings were obtained from the lobar/segmental airways. One biopsy specimen from each participant was immediately placed in RNA preservative (RNAlater, Ambion) and submitted for microarray analysis and qRT-PCR, and 3 biopsy specimens were placed immediately into dry acetone containing protease inhibitors (iodoacetamide and phenylmethylsulfonyl fluoride) and fixed overnight at −20°C. Brushings

were placed in RNAlater or PromoCell medium containing penicillin/streptomycin and Primocin for cell culture (PromoCell UK, Heidelberg, Germany.). PBECs were cultured with methods developed in our laboratory, which resulted in homogenous cultures of basal epithelial cells.^{E6}

For IHC, biopsy samples were processed by using local protocols. Twenty-eight biopsy specimens (10 from healthy volunteers, 8 from patients with mild asthma, and 10 from patients with severe asthma) were available for IHC. Infiltration and embedding with JB4 resin was performed, as previously described.^{E7} Two-micrometer sections were cut from each biopsy specimen. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide. All sections were pressure cooked in 0.01 mol/L Tris-EDTA, pH 9.0, on full steam for 3 minutes for antigen retrieval. Sections were incubated with TRPV1 polyclonal antibody (1:200, Abcam) overnight at 4°C, and then primary antibodies were detected by means of incubation for 30 minutes at room temperature in peroxidase-labeled EnVision anti-rabbit secondary antibody (Dako, Ely, United Kingdom) with 3,3'-diaminobenzidine (Dako) as chromogen. On selected tissue sections from this series, immunofluorescence was carried out to permit high-resolution analysis of TRPV1 antigen distribution. Sections were treated as for IHC and after incubation in a mix of TRPV1 and an mAb to the epithelial specific antibody AE1AE3 (1:50, Dako). Antigen-binding sites were detected with a mixture of anti-mouse Alexa Fluor 568 and anti-rabbit Alexa Fluor 488 (Invitrogen, Paisley, United Kingdom). Sections were counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride HardSet mounting medium (Vector Laboratories, Peterborough, United Kingdom). All fluorescently stained slides were assessed, and digital fluorescent images were acquired with a Leica DFC350 FX digital camera and processed with Leica FW4000 software.

Immunostained sections were evaluated by 2 independent persons, after which results were discussed and consensus scoring was applied to any conflicting results. A 4-point scoring system was used: 0, no detectable expression in epithelium or submucosa; 1, patchy or focal areas of low-intensity epithelial expression, not all cells expressing positivity for protein, scattered positivity in submucosa; 2, moderate/majority of epithelial and submucosal cell expression; and 3, widespread strong epithelial and submucosal cell expression.

TRPV1 and ER colocalization in human bronchial epithelial cells

Human bronchial epithelial cells were grown on cover slips and fixed in 4% paraformaldehyde for 30 minutes at room temperature. Cells were briefly washed in PBS before being permeabilized with 0.1% Triton X-100 in PBS for 20 minutes at room temperature. Cells were blocked with 10% BSA in PBS for 1 hour at room temperature and incubated with primary antibodies, mouse monoclonal anti-KDEL (1:500), ER marker and rabbit polyclonal anti-VR1 (1:200), and TRPV1 marker (AB12223 and Ab63083 respectively, Abcam) overnight at 4°C, followed by incubation for 1 hour at room temperature with secondary antibodies, goat anti-mouse Alexa Fluor 488 (1:500, Invitrogen), and goat anti-rabbit Alexa Fluor 568 (1:500, Invitrogen). Cell nuclei were stained with Vectashield mounting medium containing 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories). Staining was visualized and images were captured with the Nikon EZ-C1 confocal system running C1 acquisition software (Nikon UK, Surrey, United Kingdom) at ×40 magnification.

Gene expression analyses

RNA was isolated from homogenized bronchial biopsy specimens, and qRT-PCR was performed, as described previously.^{E8} TaqMan Gene Expression Assays (Applied Biosystems) were purchased and conducted per the manufacturer's instructions for *TRPV1*. RNA was amplified (Ambion) for Agilent 2-color Whole Human Genome 4 × 44k gene expression microarray analysis. Universal Human Reference RNA (Stratagene, La Jolla, Calif) was used for the reference channel. Probe intensities were transformed as log₂ ratios of test and reference channels calculated with the Agilent Feature Extraction software, protocol GE2-v5_95 (Agilent). Flagged outliers were not included in any subsequent analyses. All gene expression analyses and

plotting were performed with the R Project software package, version 2.10.1 (refer to <http://www.R-project.org>).

qRT-PCR

Bronchial brushings. qRT-PCR analyses were performed with RNA from homogenized bronchial brushings (36 subjects; 10 healthy volunteers, 16 patients with mild asthma, and 15 patients with severe asthma) by using TaqMan Gene Expression Assays (Applied Biosystems), as previously described. The $\Delta\Delta C_T$ method was used to calculate relative fold expression with GAPDH as the housekeeping gene.

PBEC cultures. PBECs were trypsinized and cells were lysed by using Qiagen RLT buffer (Qiagen) before homogenization with QIAshredders (Qiagen). RNA was then extracted with the RNeasy Mini Kit (Qiagen). The RNA concentration was measured with the nanodrop spectrophotometer (Thermo Scientific), and 1 μ g of RNA was used to synthesize cDNA by using the high-capacity cDNA reverse transcription kit (Applied Biosystems). PBEC cDNA was mixed with Power SYBR Green PCR master mix (Applied Biosystems) and either *GAPDH* or *TRPV1* forward and reverse primers (Table E1). The following PCRs were performed in a 7300 Real Time PCR system (Applied Biosystems). Melting curves showed formation of only 1 gene product per primer pair. Normalizations and calibrations of the data from all studies were performed with the $2^{-\Delta\Delta C_T}$ method, as described in Applied Biosystems User Bulletin no. 2 (P/N 4303859). ΔC_T was calculated as target gene expression (C_T) minus internal housekeeping control *GAPDH* gene expression (C_T). Human Universal Reference RNA (P/N 639654; Clontech, Mountain View, Calif) was prepared as cDNA and used as a plate calibrator. $\Delta\Delta C_T$ was calculated as sample ΔC_T minus plate calibrator ΔC_T . *GAPDH* and *TRPV1* forward and reverse primers are detailed in Table E1.

Western blotting. PBECs were collected and snap-frozen, homogenized, and lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined by using the BCA protein assay kit (Thermo Scientific). Thirty micrograms of total protein was separated on the 8% SDS-polyacrylamide gel by means of electrophoresis and transferred to Hybond-P polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). The membrane was blocked for 30 minutes in 3% BSA/PBS and then probed overnight in 3% BSA/PBS with a rabbit polyclonal TRPV1 antibody (ab63083, Abcam) at a dilution of 1:3000, followed by incubation for 1 hour with the secondary antibody of horseradish peroxidase-labeled anti-rabbit (1:3000; Bio-Rad Laboratories, Hercules, Calif). Images were obtained by using the UVP bioimager (UVP, Cambridge, United Kingdom). Benchmark prestained protein standards (Invitrogen) were used to determine molecular mass.

Patch-clamp experiments. Whole-cell currents were recorded by using borosilicate heat-polished patch pipettes (2–3 M Ω resistance when filled with the pipette solution) and an Axopatch 200B amplifier (Molecular Devices) interfaced to Digidata 1322A with the pClamp 9 software (Molecular Devices). Series resistance was compensated by approximately 70%. Holding potential was -40 mV. Steady-state I–V relationships were measured by applying either voltage steps (test potentials from -100 to $+140$ mV with a 20-mV increment) or slow voltage ramps from -100 to $+140$ mV. The voltage ramps were applied at 10-second intervals to monitor the time course of TRPV1 activation by capsaicin. The I–V curves obtained by using the voltage ramp and step protocols were similar, confirming steady-state channel activity at each test potential when ramps were used.

For experiments involving the addition of capsaicin, the external solution contained the following: NaCl, 120 mmol/L; glucose, 12 mmol/L; HEPES, 10 mmol/L, pH 7.4 (adjusted with NaOH). Pipette solution contained the following: CsCl, 80 mmol/L; MgATP, 1 mmol/L; creatine, 5 mmol/L; GTP, 1 mmol/L; D-glucose, 5 mmol/L; HEPES, 10 mmol/L; BAPTA, 10 mmol/L;

CaCl₂, 4.6 mmol/L ($[Ca^{2+}]_i = 100$ nmol/L), pH 7.4 (adjusted with CsOH). For experiments involving altered pH, the external solution contained the following: NaCl, 150 mmol/L; CsCl, 6 mmol/L; MgCl₂, 1.3 mmol/L; CaCl₂, 2 mmol/L; glucose, 5 mmol/L; HEPES, 10 mmol/L, pH 7.4 or 6.4 with Tris. Pipette solution contained the following: CsOH, 130 mmol/L; D gluconic acid, 120 mmol/L; MgCl₂, 1 mmol/L; ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid, 0.5 mmol/L ($[Ca^{2+}]_i = 30$ nmol/L), pH 7.2 with Tris.

IL-8 ELISA. IL-8 concentrations were measured from aliquots of culture medium taken 24 hours after treatment with capsaicin (Enzo Life Sciences, Exeter, United Kingdom) by using a commercial ELISA kit from R&D Systems Europe.

Data analysis

Data are presented as means $\pm$ SEMs. For statistical analysis, 1-way ANOVA was performed with the Bonferroni test for multiple comparisons. Kendall τ -b statistical analysis was used to examine the trend in biopsy expression by participant group. Patch-clamp data were analyzed and plotted by using Origin 8. A *P* value of less than .05 was considered significant.

RESULTS

qRT-PCR analysis of fresh bronchial brush samples from the Leicester cohort comprising healthy subjects, patients with mild-to-moderate asthma, and patients with severe asthma

Analysis was performed on fresh bronchial brush samples from the Leicester cohort comprising patients with severe asthma ($n = 15$), patients with mild-to-moderate asthma ($n = 16$), and healthy subjects ($n = 10$). *TRPV1* mRNA was detected in all of the epithelial brush samples. There was no difference in median relative fold expression of *TRPV1* mRNA calculated as $\Delta\Delta C_T$ values between groups: -1.262 (IQR, -3.598 to 2.518), -2.523 (IQR, -4.040 to 1.362), and -1.289 (-4.746 to 1.624) in healthy volunteers, patients with mild-to-moderate asthma, and patients with severe asthma, respectively (Fig E1).

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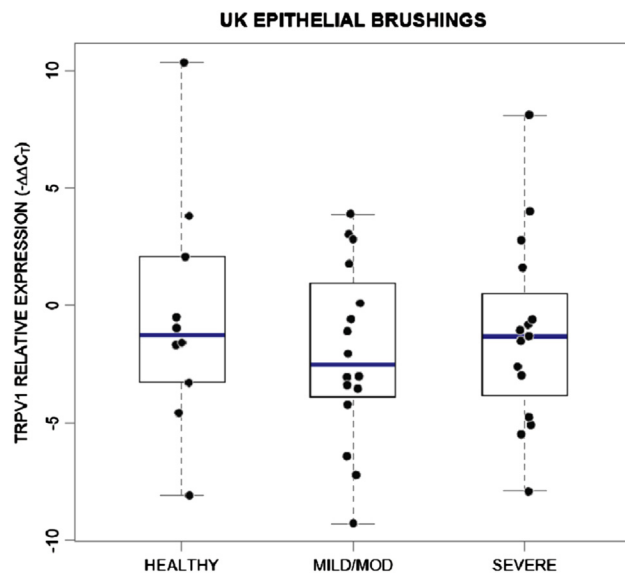


FIG E1. qRT-PCR expression of *TRPV1* in fresh bronchial epithelial brush samples from healthy volunteers, patients with mild-to-moderate asthma, and patients with severe asthma. Analysis of fresh bronchial brush samples from the Leicester cohort comprising healthy subjects ($n = 10$), patients with mild-to-moderate asthma ($n = 16$), and patients with severe asthma ($n = 15$) was performed. Data are presented as median (IQR) relative fold expression of *TRPV1* mRNA calculated as $\Delta\Delta C_T$ values.

TABLE E1. Forward and reverse primers for *TRPV1* and *GAPDH*

Primer name	Forward	Reverse	Size (bp)
<i>TRPV1</i>	GCCTGGAGCTGTTCAAGTTC	GCCTGAAACTCTGCTTGACC	452
<i>TRPV2</i>	CCAGCTCTCCAGTTTTCAGG	GTTCAGCACAGCCTTCATCA	365
<i>TRPV3</i>	TTCCTCATGCACAAGCTGAC	TGCGCTTCACAAAGTCATTC	490
<i>TRPV4</i>	CCCGTGAGAACACCAAGTTT	GAAGAGCGTAATGACCTCGC	374
<i>TRPV5</i>	TTGCTCAGTTGCTGTCAATCC	GCTCCTCTTTGTCTGAACGTC	307
<i>TRPV6</i>	TCACTTCGCTTCCTGGAAT	ACACGCTTCCACAAGCTCT	401
<i>GAPDH</i>	GCTCTCTGCTCCTCTGTTC	AAATCCGTTGACTCCGACC	100

Source: Invitrogen.