

Capsaicin treatment reduces nasal hyperreactivity and transient receptor potential cation channel subfamily V, receptor 1 (TRPV1) overexpression in patients with idiopathic rhinitis

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Background: Idiopathic rhinitis (IR) is a prevalent condition for which capsaicin nasal spray is the most effective treatment. However, the mechanisms underlying IR and the therapeutic action of capsaicin remain unknown.

Objective: We sought to investigate the molecular and cellular bases of IR and the therapeutic action of capsaicin.

Methods: Fourteen patients with IR and 12 healthy control subjects (HCs) were treated with intranasal capsaicin. The therapeutic effect was assessed in patients with IR by using visual analog scale and therapeutic response evaluation scores, and nasal hyperreactivity was evaluated by means of cold dry air provocation. Nasal samples served to measure the levels of neuromediators and expression of chemosensory cation channels, protein gene product 9.5 (PGP 9.5), and the mast cell marker c-kit. The effects of capsaicin were also tested *in vitro* on human nasal epithelial cells and mast cells.

Results: Patients with IR had higher baseline transient receptor potential cation channel subfamily V, receptor 1 (TRPV1) expression in the nasal mucosa and higher concentrations of substance P (SP) in nasal secretions than HCs. Symptomatic

relief was observed in 11 of 14 patients with IR after capsaicin treatment. Expression of TRPV1; transient receptor potential cation channel subfamily M, receptor 8 (TRPM8); and PGP 9.5 was only reduced in patients with IR after capsaicin treatment. Capsaicin did not alter c-KIT expression or nasal epithelial morphology in patients with IR and HCs nor did it induce apoptosis or necrosis in cultured human nasal epithelial cells and mast cells.

Conclusion: IR features an overexpression of TRPV1 in the nasal mucosa and increased SP levels in nasal secretions. Capsaicin exerts its therapeutic action by ablating the TRPV1-SP nociceptive signaling pathway in the nasal mucosa. (*J Allergy Clin Immunol* 2013;■■■■:■■■-■■■.)

Key words: Capsaicin treatment, idiopathic rhinitis, nasal hyperactivity, TRPV1, afferent nerves, TRPV1-SP signaling pathway

Nonallergic noninfectious rhinitis (NANIR) is a highly prevalent condition (5% to 10% worldwide) comprising a heterogeneous group of patients.¹ After exclusion of hormonal, medication-induced, local allergic rhinitis or so-called “entopy”² and conditions mimicking rhinitis, a substantial subgroup of patients with NANIR do not have a defined underlying cause. Patients with so-called idiopathic rhinitis (IR) most often have nasal hyperreactivity (NHR). NHR is defined as the induction of nasal symptoms by means of contact with aspecific stimuli, such as smoke, temperature changes, strong odors, and other irritants.^{3,4} It is generally assumed that IR is related to a dysfunction of sensory nerves in the nasal mucosa, giving rise to NHR. Activation of nasal afferent nerves by aspecific environmental factors is known to trigger sensations of pain and itch; defensive efferent responses, such as sneezing; and glandular and/or vascular activation, leading to rhinorrhea and nasal congestion.⁵ Sensory nerve endings can also locally release proinflammatory neuromediators (antidromic pathway), such as substance P (SP) and calcitonin gene-related peptide (CGRP), which can rapidly lead to a variety of nasal symptoms.⁵

Patients with IR benefit from intranasal treatment with capsaicin (ie, the main pungent agent in hot chili peppers). This was first described in 1991 by Lacroix et al⁶ and later confirmed by other groups.⁷⁻¹² In 1997, Blom et al⁸ published the first placebo-controlled trial showing therapeutic efficacy, and Van Rijswijk et al¹⁰ demonstrated that 5 nasal applications in 1 day were as effective as 5 applications in 2 weeks, which makes the treatment protocol more attractive for clinical practice.

Capsaicin is known to activate the transient receptor potential cation channel subfamily V, receptor 1 (TRPV1), a nonselective

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Abbreviations used

CDA:	Cold dry air
CGRP:	Calcitonin gene-related peptide
FTIC:	Fluorescein isothiocyanate
HC:	Healthy control subject
IR:	Idiopathic rhinitis
NANIR:	Nonallergic noninfectious rhinitis
NEC:	Nasal epithelial cell
NHR:	Nasal hyperreactivity
NKA:	Neurokinin A
PI:	Propidium iodide
PGP 9.5:	Protein gene product 9.5
PNIF:	Peak nasal inspiratory flow
SP:	Substance P
SPT:	Skin prick test
TRE:	Therapeutic response evaluation
TRPA1:	Transient receptor potential cation channel subfamily A, receptor 1
TRPM8:	Transient receptor potential cation channel subfamily M, receptor 8
TRPV1:	Transient receptor potential cation channel subfamily V, receptor 1
TUNEL:	Terminal deoxynucleotide transferase dUTP nick end labeling
VAS:	Visual analog scale

cation channel functioning as a polymodal receptor in C-type fibers innervating the human nasal mucosa.¹³ TRPV1 activation leads to a cationic influx in the nerve terminals, resulting in neuronal excitation, an increase in intracellular Ca^{2+} concentration, and antidromic release of neuropeptides, which triggers a local inflammatory response.³ The therapeutic effect of intranasal capsaicin application is thought to be induced by a strong excitation of the afferent C-fibers, leading to desensitization, “dysfunctionalization,” and/or degeneration of nerve terminals by the massive Ca^{2+} influx.^{14,15} For unclear reasons, most patients with IR only experience the recurrence of nasal symptoms after several months and subsequently seek the reapplication of capsaicin. Although capsaicin is often the only effective treatment for IR,¹⁶ it is rarely applied because the mechanisms underlying its beneficial effects remain speculative.

This article re-evaluates the therapeutic action of capsaicin by using visual analog scale (VAS) and therapeutic response evaluation (TRE) scores and provides an objective assessment by measuring NHR with the recently validated short cold dry air (CDA) provocation.¹⁷ Furthermore, we investigate the mechanisms underlying IR and the effects of capsaicin treatment by comparing patients with IR and healthy control subjects (HCs) regarding multiple anatomic features of the nasal mucosa and the presence of neuropeptides in nasal secretions. In addition, we perform *in vitro* experiments to determine whether capsaicin affects other cell types of the human nasal mucosa, namely mast cells and nasal epithelial cells (NECs).

METHODS**Patients and HCs**

Fourteen patients with IR and 12 HCs were recruited through the outpatient clinic of the Otorhinolaryngology Department of the University Hospitals of Leuven. Patients with IR were defined as nonsmokers with a minimum of 2 nasal complaints (ie, nasal obstruction, rhinorrhea, and sneezing) for more than

1 hour a day and for more than 1 year, with negative skin prick test (SPT) responses and no clinical signs of infection (discolored secretions) and without anatomic nasal abnormalities responsible for the nasal symptoms. Inclusion criteria were as follows: patients with IR between 18 and 60 years of age with reported lack of efficacy of nasal corticosteroid treatment at the recommended dose for at least 4 weeks who signed the informed consent form. Exclusion criteria were a positive SPT response for the 18 most frequent inhalant allergens in Belgium (see the [Methods](#) section in this article's Online Repository at www.jacionline.org), pregnancy or lactation, systemic disorders or malignancies, use of medication affecting nasal function (eg, β -blockers), use of local and/or systemic corticosteroids 4 weeks before the study, or a history of prolonged use or abuse of decongestant nasal spray, such as xylomethazoline. Patients with discolored secretions, inflammation, or both at the level of the osteomeatal complex were excluded after nasal endoscopy. Nasal secretions were collected at the screening visit and analyzed for total IgE by using ImmunoCAP (Thermo Scientific, Waltham, Mass) to exclude local allergic rhinitis.² Patients with IR were only included if the total IgE levels in the nasal fluid were less than 5.745 kU/L (P90 in HCs). The detection limit of total IgE was 2 kU/L.

HCs were age-matched nonsmokers; they denied any nasal symptom and had negative SPT responses. HCs did not have anatomic nasal deformities or signs of infection, as evaluated by means of nasal endoscopy.

During the entire study, no other nasal medication was allowed.

Study design

For more details on study design, see the [Methods](#) section and [Fig E1](#) in this article's Online Repository at www.jacionline.org. The study was registered at ClinicalTrials.gov (NCT 01223820) and approved by the Medical Ethical Committee on Clinical Investigations at the University Hospitals of Leuven. Informed consent was obtained from all subjects.

The capsaicin solution was prepared by the Pharmacy of the University Hospitals Leuven by using the formula previously reported by Van Rijswijk et al.¹⁰ On the day of treatment, 5 consecutive applications of capsaicin were administered intranasally (0.1 mmol/L solution, 2 puffs in each nostril, 0.4 mL per puff), with 1 hour between each application. The nasal mucosa was anesthetized before the first 2 applications with cocaine 5% nasal spray (same volume/spray as mentioned above). An interval of 15 minutes was maintained between the cocaine and capsaicin spray application to ensure effective local anesthesia.

Evaluation of nasal symptoms

All participants were asked to mark the typical nasal symptoms of IR (ie, rhinorrhea, nasal obstruction, and sneezing) on a VAS (score of 0–10) at the screening visit (visit 1), baseline visit (visit 2), immediately after capsaicin treatment (visit 3), and at 4 and 12 weeks after treatment (visits 4 and 5). Only if the VAS score was greater than 2 for one of the parameters was the symptom considered relevant. The major nasal symptom was selected based on the highest VAS score at screening. At visits 4 and 5, a TRE was performed. Patients were asked to score the overall improvement of their symptoms compared with baseline (0, total reduction or symptom free; 1, major reduction; 2, moderate reduction; 3, minor reduction; and 4, no reduction of symptoms).

CDA nasal provocation

NHR was assessed by using a CDA nasal provocation at visits 2 and 4 in line with the recently validated methodology¹⁷ and as described in the [Methods](#) section in this article's Online Repository.

Collection of nasal secretions and nasal biopsy specimens and measurement of SP, neurokinin A, CGRP, and mast cell degranulation in nasal secretions

See the [Methods](#) section in this article's Online Repository for a detailed description of nasal fluid collection, nasal biopsies, and SP, neurokinin A (NKA), and CGRP measurements in nasal fluid.

RT-PCR for *TRPV1*, *TRPA1*, *TRPM8*, *PGP 9.5*, and *c-KIT* on nasal biopsy specimens

The nasal biopsy specimens were homogenized in lysis buffer from the Qiagen Mini RNeasy kit (Germantown, Md). RNA isolation, cDNA synthesis, and RT-PCR for *TRPV1*; transient receptor potential cation channel subfamily A, receptor 1 (*TRPA1*); transient receptor potential cation channel subfamily M, receptor 8 (*TRPM8*); *PGP 9.5*; *c-KIT*; and the housekeeping genes β -actin (*ACT2B*) and *TRPM7* and the development of cDNA plasmid standards were performed, as described previously¹⁸ and in the [Methods](#) section in this article's Online Repository.

Immunohistochemistry for hematoxylin and eosin, TUNEL, *PGP 9.5*, and *c-KIT* on nasal biopsy specimens

See the [Methods](#) section in this article's Online Repository for a description of staining procedures.

In vitro cell isolation of human mast cells and human NECs

The LAD2 human mast cell line was a kind gift from Dr Arnold S. Kirshenbaum.¹⁹ RPMI 2650 human nasal septal squamous cell carcinoma cells were obtained from American Type Culture Collection (CCL-30; ATCC, Manassas, Va). As an additional control, freshly isolated NECs were isolated from resection specimens (concha), as described previously.²⁰ More details are shown in the [Methods](#) section in this article's Online Repository.

RT-PCR for *TRPV1* in NECs and LAD2 cells and intracellular Ca^{2+} imaging experiments

The different cell types were checked for *TRPV1* expression by using RT-PCR, as described in the [Methods](#) section in this article's Online Repository. Additionally, the functionality of *TRPV1* was investigated by using calcium measurements, as described previously.²¹

Determination of capsaicin-induced apoptosis by means of flow cytometry

Apoptosis was detected by means of flow cytometry with Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining (Detection Kit I; BD PharMingen, Erembodegem, Belgium), as described in the [Methods](#) section in this article's Online Repository.

Mast cell degranulation assay in LAD2 cells and IL-6, IL-8, and IL-33 analysis of the supernatants of NECs

These measurements are described in the [Methods](#) section in this article's Online Repository.

Statistical methodology

The measurements were compared by using a paired *t* test if the distribution was normal and a Wilcoxon signed-rank test if it was not. A difference was considered significant when the *P* value was less than .05. Each *in vitro* experiment was performed in triplicate. Values displayed represent means $\pm$ SEMs.

Analyses were performed with the GraphPad Prism program (Prism 4, version 4.03, 1992-2005; GraphPad Software, San Diego, Calif).

RESULTS

Clinical characteristics of patients with IR and HCs

Patients with IR and HCs showed no significant differences with respect to age (IR group: 44 years [22-60 years]; HC group:

36 years [20-56 years]) or race (all white). The female/male ratio was 6/8 in patients with IR and 8/4 in HCs. All subjects had negative SPT responses for the 18 most frequent inhalant allergens in Belgium, showed no IgE in nasal secretions (IgE levels, <5.745 kU/L; P90 in HCs), and showed no endonasal anatomic or mucosal signs of rhinosinusitis. HCs were symptom free at baseline and remained so throughout the study (data not shown).

Clinical improvement of patients with IR after capsaicin treatment

At baseline, nasal obstruction was reported as major symptom by 7 patients, rhinorrhea by 6 patients, and sneezing by 1 patient. The mean VAS score for major symptoms before treatment was 9.1 ± 2.2 cm ([Fig 1, A](#)).

Compared with baseline, VAS scores for nasal obstruction and sneezing decreased significantly at 4 and 12 weeks after capsaicin treatment, whereas rhinorrhea was significantly reduced after 12 weeks ([Fig 1, A](#)). Average VAS scores for major symptom were also significantly reduced at 4 and 12 weeks ([Fig 1, A and B](#)). The follow-up of each subject revealed that 13 of 14 patients reported a decrease in VAS score for the major symptom at 4 weeks after the treatment and that only 3 patients showed a reversion of this trend at 12 weeks ([Fig 1, B](#)).

The TRE score yielded clear changes in the distribution of patients toward reduction of the symptoms ([Fig 1, C](#)). Sixty-four percent and 78% of patients with IR reported improvement (score, 0-3) at 4 and 12 weeks, respectively. Major improvement (score, 0-2) was reported by 50% and 64%, respectively.

Abrogation of NHR to CDA after capsaicin treatment

To objectively assess the effects of capsaicin, we evaluated NHR using measurements of peak nasal inspiratory flow (PNIF) before and after short-term exposure to CDA, as previously reported.¹⁷ CDA induced a significant decrease in PNIF in patients with IR (from 98.2 ± 9.6 L/min to 72.5 ± 8.3 L/min, *P* = .002; [Fig 2, A](#)), which correlated with the VAS scores for major symptoms (*R* = 0.54, *P* = .049; [Fig 2, B](#)).

The CDA provocation did not alter PNIF in HCs (125.0 ± 10.3 L/min vs 125.8 ± 10.8 L/min, *P* = 1.0). Notably, CDA provocation did not induce any significant change in PNIF in 13 of 14 patients with IR 4 weeks after capsaicin treatment ([Fig 2, A](#)).

Capsaicin treatment induced specific anatomic changes in patients with IR

To gain insight into the mechanisms of IR and the effects of capsaicin, we compared HCs and patients with IR regarding multiple anatomic features of the nasal mucosa before and after treatment.

Sections of the hematoxylin and eosin staining were evaluated by a blinded experienced pathologist and showed no significant differences between patients with IR and HCs concerning tissue inflammation, glandular structures (no atrophy/hypertrophy), and integrity of the epithelial layer either before or after capsaicin treatment (data not shown). Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) staining did not show any sign of apoptosis either 5 hours after the first capsaicin application or at 12 weeks after treatment (data not shown).

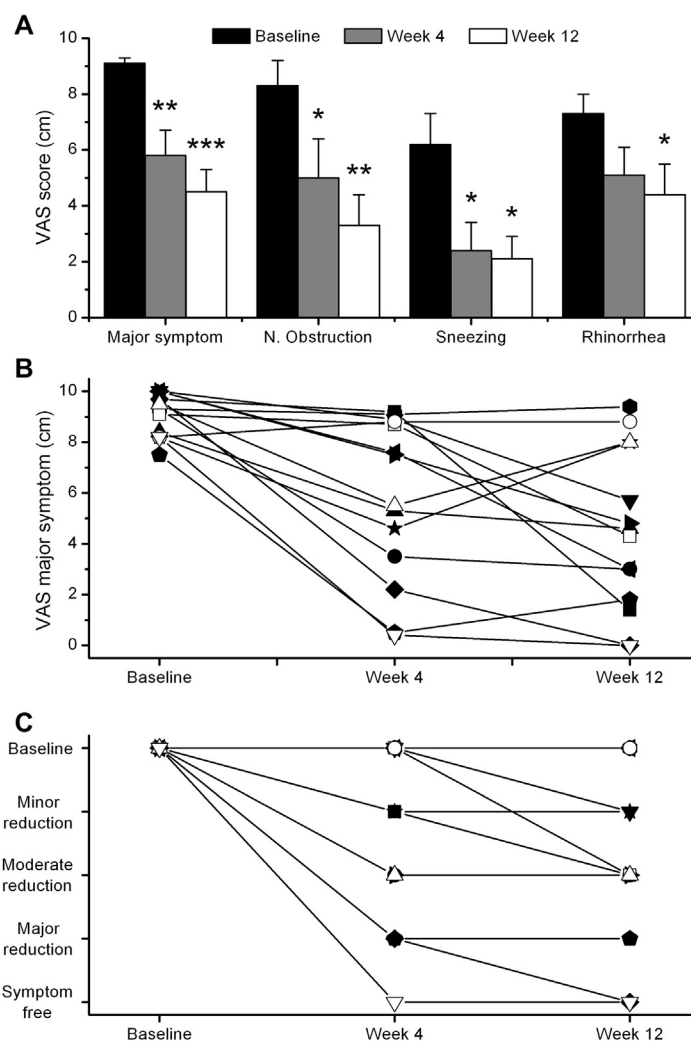


FIG 1. VAS and TRE scores at baseline and 4 and 12 weeks after capsaicin treatment in patients with IR ($n = 14$). **A**, Mean VAS scores for the major symptom, nasal obstruction, sneezing, and rhinorrhea at 3 time points of evaluation. **B**, Individual VAS scores for the major symptom in relation to treatment. **C**, The TRE score is presented at 4 and 12 weeks after capsaicin treatment in relation to baseline. * $P < .05$, ** $P < .01$, and *** $P < .001$.

Expression levels of the pan-neuronal marker PGP 9.5 (Fig 3, A) were not different between patients with IR and HCs at baseline ($P = .18$) but decreased significantly in patients with IR at 12 weeks after capsaicin treatment ($P = .008$; Fig 3, A). These findings were confirmed by PGP 9.5 immunostaining of the nasal mucosa, showing almost no PGP 9.5 in patients with IR treated with capsaicin (Fig 3, B). No changes in PGP 9.5 expression were found in HCs after capsaicin (data not shown).

The baseline mRNA expression of TRPV1 was significantly higher in the nasal mucosa of patients with IR than that seen in HCs ($P = .030$; Fig 4, A). Notably, TRPV1 expression in patients with IR was significantly lower immediately and 12 weeks after capsaicin treatment ($P = .047$ and $P = .008$, respectively). In contrast, no changes were observed in HCs ($P = .94$ and $P = .78$, respectively; Fig 4, A). Capsaicin treatment also induced specific changes in the expression of TRPM8, another member of the TRP channel family. Admittedly, although the TRPM8 expression was not significantly different between patients with IR and HCs at baseline (4.7 ± 1.7 and 4.1 ± 1.4 in HCs and patients with IR, respectively; $P = .64$), it was significantly

reduced in the IR group at 12 weeks after capsaicin treatment (4.3 ± 1.1 and 0.5 ± 0.4 in HCs and patients with IR, respectively; $P = .003$). Remarkably, the changes in TRPM8 and TRPV1 expression observed in patients with IR 12 weeks after capsaicin were highly correlated ($R = 0.72$, $P = .005$; Fig 4, B). The decrease in TRPV1 expression 12 weeks after treatment had borderline significant correlation with the decrease in VAS scores for the major symptom ($R = 0.54$, $P = .0595$).

The mRNA levels of TRPA1 were negative or very low in both the IR and HC groups (data not shown).

Nasal biopsy specimens of patients with IR and HCs had the same expression of c-KIT at the mRNA and protein levels before and after treatment (data not shown).

Nasal secretions of patients with IR have higher baseline SP levels

Next, we determined whether the alterations in mRNA levels of TRPV1 observed in patients with IR were paralleled by changes in the concentration of neuropeptides in nasal secretions. Baseline

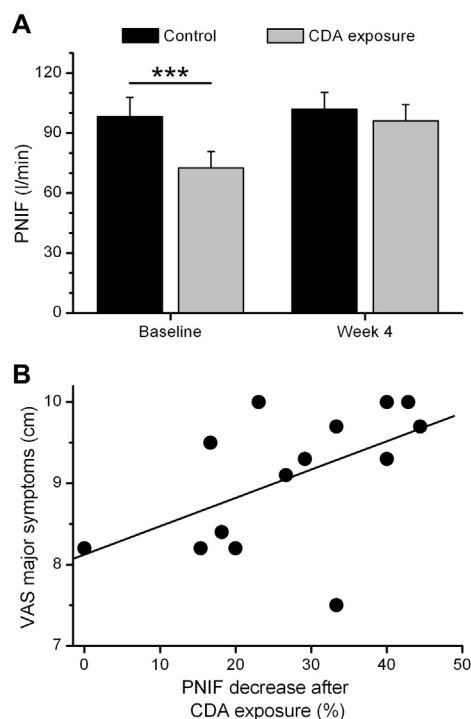


FIG 2. NHR in patients with IR before and after capsaicin treatment and in correlation with symptom severity. **A**, CDA challenge in patients with IR demonstrating a decrease in PNIF at baseline without changes in PNIF at 4 weeks after capsaicin treatment ($n = 14$). *** $P < .005$. **B**, Correlation between the degree of NHR and VAS scores for the major symptom ($P = .049$, $R = 0.54$, $R^2 = 0.29$ [at baseline]).

SP levels were significantly higher in nasal secretions of patients with IR than in HCs (8.02 ± 1.01 pg/mL in the IR group vs 6.09 ± 0.41 pg/mL in the HC group, $P = .012$), yet were reduced to the level of HCs after treatment.

NKA levels were not significantly different between patients and HCs at baseline (64.9 ± 14.3 and 46.8 ± 10.8 pg/mL, respectively), and CGRP levels were less than the detection limit in all samples.

Lack of effects of capsaicin on NECs and human mast cells

Finally, we investigated whether capsaicin exerted any effect on nonneuronal cells of the nasal mucosa. Although we could detect TRPV1 by using RT-PCR in NECs, intracellular Ca^{2+} imaging experiments revealed that there was no functional expression of TRPV1 channels in these cells (data not shown).

Human NECs of the RPMI 2650 cell line and freshly isolated from HCs were exposed to capsaicin at different concentrations (0.1, 0.25, and 0.5 mmol/L) and analyzed for apoptosis by means of flow cytometry (Annexin-V-PI) and TUNEL staining. After 1 hour of exposure, none of the capsaicin concentrations (or medium or vehicle) induced apoptosis of the NECs. Apoptosis, which was absent in the control conditions, could only be induced by the highest capsaicin concentration (0.5 mmol/L; ie, 5 times higher than the concentration used in patients with IR) at 4 hours' incubation in 98.6% of NECs. This effect was still observed after preblocking with capsazepine/AMG9810, suggesting a TRPV1-independent pathway. Counting after TUNEL staining yielded similar results (ie, no influence of 0.1,

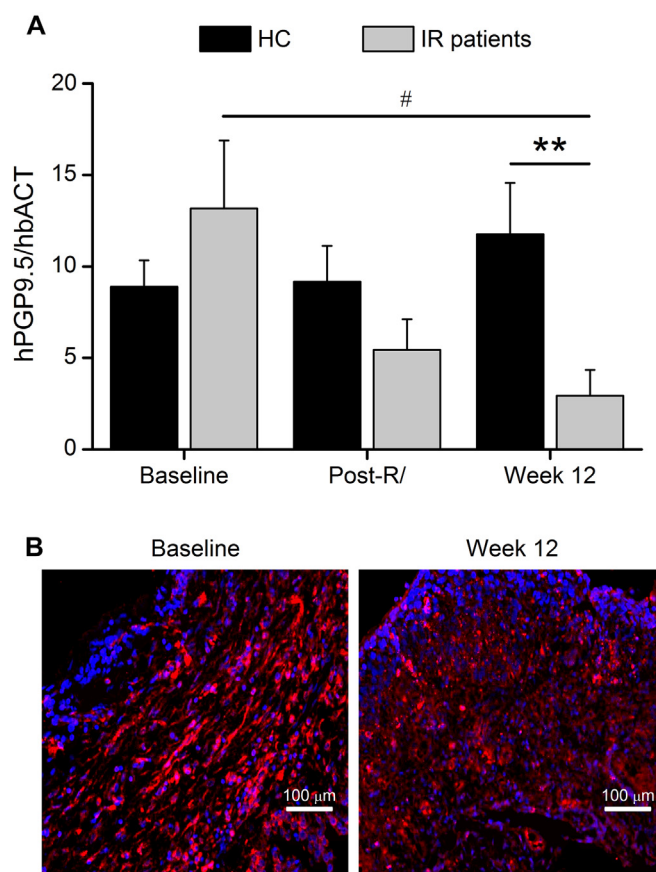


FIG 3. PGP 9.5 expression in HCs and patients with IR at different time points in relation to capsaicin treatment. **A**, PGP 9.5 expression (RT-PCR) in homogenates of nasal biopsy specimens in HCs and patients with IR at baseline, immediately after, and 12 weeks after treatment ($n = 13$). *hbACT*, Human beta-actin. **B**, Representative sample of immunostaining for PGP 9.5 on protein levels before and 12 weeks after treatment. Blue, 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI); red, PGP 9.5. ** $P < .01$, difference between groups; # $P < .01$, difference within group.

0.25, and 0.5 mmol/L capsaicin after 1 hour exposure on the NECs; data not shown).

LAD2 cells exposed to the different capsaicin concentrations for 1 or 4 hours showed results similar to those seen with NECs. Even after a 4-hour incubation at the highest capsaicin concentration, no apoptosis was detected by using flow cytometry (data not shown). TUNEL staining could not be performed to confirm the findings because the LAD2 cells do not attach to the surface, as stated previously.¹⁹

Supernatants of LAD2 cells with or without incubation with capsaicin at different concentrations (0.1, 0.25, and 0.5 mmol/L) were analyzed for β -hexosaminidase to check mast cell degranulation by capsaicin. The supernatant collected at 30 minutes and 2 hours showed no differences in β -hexosaminidase release (release identical to release in medium condition). The positive control samples (stimulation with 2.5 μ g/mL SP and 0.5 μ g/mL Compound 48/80) showed clear degranulation ($97.0\% \pm 0.6\%$ and $90.5\% \pm 1.5\%$, respectively), whereas supernatants of NECs stimulated with capsaicin did not induce degranulation of LAD2 cells *in vitro* (data not shown).

IL-8 was detectable in the supernatants of all conditions of the freshly isolated NECs but not in those of RPMI 2650. Comparable levels were found in samples exposed to control and capsaicin.

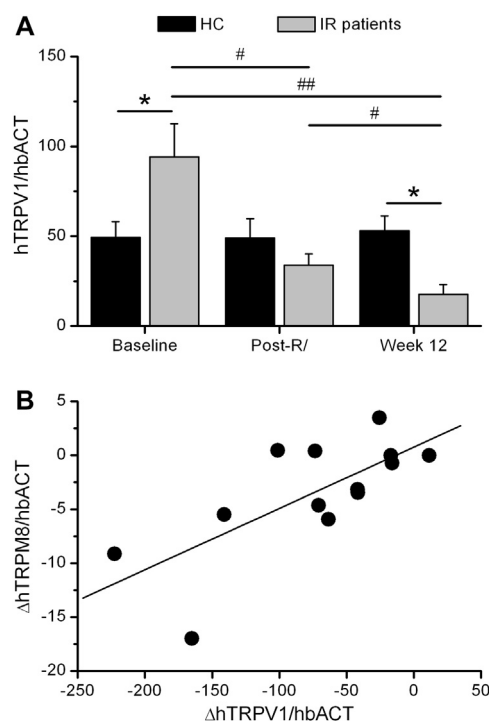


FIG 4. TRPV1 expression in HCs and patients with IR at different time points in relation to capsaicin treatment. **A**, TRPV1 expression (RT-PCR) in homogenates of nasal biopsy specimens in HCs and patients with IR at baseline, immediately after, and 12 weeks after treatment ($n = 13$). **B**, Correlation between changes in mRNA for TRPV1 and TRPM8 after capsaicin treatment in homogenates of nasal biopsy specimens in patients with IR ($P = .005$, $R = 0.72$, $R^2 = 0.52$). * $P < .05$, difference between groups; # $P < .05$, difference within group; ## $P < .01$, difference within group.

IL-6 was less than the detection limit in all conditions (10-10,000 pg/mL). IL-33 was only detectable after 4 hours of exposure to 0.5 mmol/L capsaicin and to the positive control.

DISCUSSION

The beneficial effects of capsaicin treatment in patients with IR were first described by Lacroix et al⁶ and later confirmed by others.⁷⁻¹² Despite the reported benefits of capsaicin nasal treatment on symptoms and good tolerability, the mechanisms of action have remained speculative. For the first time, we provide insight into the beneficial effects of capsaicin treatment in patients with IR. Our data on the combined reduction of nasal symptoms, NHR, and expression of SP, PGP 9.5, TRPV1, and TRPM8 by nasal capsaicin treatment strongly suggest the involvement of the TRPV1-SP nociceptive signaling pathway in patients with IR.

The mean strength of our study is the homogeneous population of patients with IR who were included. The homogeneous nature of the population was achieved by excluding those patients with rhinitis with reported benefit of intranasal corticosteroids, as well as those with increased nasal IgE levels or those with nasal endoscopic signs of rhinosinusitis. Indeed, histologic analysis of the patients with IR studied here confirmed the absence of a granulocytic influx in the nasal mucosa. The homogeneous population of patients with IR allowed us to evaluate the therapeutic action of capsaicin on NHR and the TRPV1-SP nociceptive signaling pathway.

After the recent validation of a short-term CDA exposure protocol,¹⁷ this CDA tool was used for objective evaluation of the

effects of capsaicin on NHR. The CDA provocation technique was reported to be superior to histamine provocation.²² The relevance of NHR measurements in this study was further supported by the fact that the degree of NHR correlated with the severity of the VAS scores for major symptom in patients with IR. More importantly, these measurements served to confirm the effectiveness of capsaicin because 13 of 14 patients with IR had abrogation of NHR after treatment.

In a condition like IR, with no detectable systemic or local IgE or mucosal influx of leukocytes, neural dysfunction emerges as the most probable cause for the development of symptoms and NHR, as is also suggested for irritable bowel disease²³ and some phenotypes of asthma.²⁴ Before capsaicin treatment, expression of the pan-neuronal marker PGP 9.5 was not significantly different between patients with IR and HCs, although TRPV1 expression and SP levels were significantly higher in patients with IR. These results strongly suggest that a specific upregulation of the nociceptive signaling pathway involving TRPV1 activation (by endogenous stimuli, exogenous stimuli, or both) and the consequent release of SP contribute to the IR symptoms without structural alterations of the nasal mucosa innervation. A similar association has been found in patients with irritable bowel syndrome, showing enhanced TRPV1 expression and hypersensitivity of the bowel.²³ The importance of the TRPV1 overexpression in patients with IR as part of the underlying pathophysiology is also stressed by the trend toward a positive correlation between the decrease in TRPV1 expression 12 weeks after capsaicin treatment and the decrease in VAS scores for the major symptom ($R = 0.54$, $P = .0595$).

In contrast to TRPV1 and SP, patients with IR did not show higher expression of TRPM8 (see also Keh et al²⁵) or higher NKA levels in nasal secretions.

Regarding the therapeutic action of capsaicin, it was interesting to note that capsaicin treatment of patients with IR decreased not only the expression of TRPV1 and SP levels but also the expression of the pan-neuronal marker PGP 9.5 and TRPM8. The simplest explanation for this finding is that TRPV1 is expressed in the majority of nasal cells expressing PGP 9.5 and TRPM8. In that case a massive Ca^{2+} influx associated with TRPV1 activation by capsaicin would lead to a widespread degeneration of nerve endings. Alternatively, it is possible that TRPV1 is not expressed in all PGP 9.5- and TRPM8-positive neurons and that capsaicin induces extended nerve ending degeneration through an additional TRPV1-independent mechanism by means of direct inhibition of mitochondrial respiration.¹⁵ The lack of specific antibodies for human TRPV1 and human TRPM8 hinders the ability to test the pattern of (co)expression of this channel.

Our results strongly indicate that targeting the TRPV1-SP signaling pathway is beneficial in patients with IR. We have previously reported that SP can induce airway hyperreactivity *in vivo*.²⁶ The development of airway hyperreactivity after nasal allergen provocation was associated with bronchial release of SP, suggesting a causative link between SP and airway hyperreactivity in experimental asthma. In contrast to SP and NKA levels, CGRP levels were less than the detection limit in our patients with IR, which is in line with previous studies in allergic patients.²⁷

Interestingly, the CDA-induced NHR was also reduced after capsaicin treatment in patients with IR. This could have resulted from a reduction in expression of TRPM8, a channel functioning as a cold sensor.^{28,29} Alternatively, this could be explained by a reduction in nerve endings expressing TRPA1, another cold

sensor, which typically coexpresses with TRPV1 in nociceptive neurons.^{30,31} However, we did not detect significant TRPA1 expression in the nasal samples, which is in line with previous research showing almost no TRPA1 immunoreactivity in HCs and patients with NANIR.²⁵

Another important and intriguing aspect of our results is that, in contrast to patients with IR, HCs did not show any changes in expression after capsaicin treatment. Hence we tentatively suggest that the higher expression of TRPV1 in patients with IR compared with HCs renders the nasal mucosa of the former group more sensitive to capsaicin and therefore more prone to defunctionalization of nerve endings.

Finally, it was important to determine which cells of the nasal mucosa were targeted by capsaicin. As discussed above, PGP 9.5 staining demonstrates that the number of nasal mucosal nerve endings underwent a dramatic decrease after capsaicin treatment. Our data indicate that the process or processes underlying this effect occurred within a few hours after capsaicin administration because apoptosis was not detectable by means of TUNEL staining 5 hours after treatment. Taking biopsy specimens at earlier stages was not feasible because this would interfere with the success of therapy.

It has been suggested that other cell types are involved in the therapeutic action of capsaicin as well.³² Reilly et al³³ described epithelial cell death by capsaicinoids in the airways, and Ständer et al³⁴ reported on a functional TRPV1 receptor on epithelial cells (keratinocytes) and mast cells in the skin.

We have addressed this issue by testing the effects of capsaicin in a mast cell line (LAD2) and an epithelial cell line (RPMI 2650). LAD2 cells closely resemble native human mast cells regarding the ultrastructural features; expression of CD4, CD9, CD13, CD14, CD22, cytoplasmic histamine, tryptase, and chymase; and β -hexosaminidase release after Fc ϵ RI or Fc γ RI aggregation.¹⁹ Similarly, we used RPMI 2650 cells because they closely resemble native human NECs with respect to the karyotype, cytokeratin polypeptide expression patterns, and the presence of mucous material on the cell surface.³⁵ All experiments were repeated on freshly isolated NECs to compensate for the limited differentiation state of RPMI 2650 cells. The effects of capsaicin on human trigeminal neurons could not be investigated *in vitro* because of the lack of a human trigeminal cell line or freshly isolated trigeminal neurons.

We found that LAD2 cells were not activated by capsaicin or underwent apoptosis on capsaicin exposure but did degranulate on stimulation with SP and Compound 48/80, as described by Kulka et al.³⁶ In addition, we observed no differences in c-kit expression between patients with IR and HCs at the protein and mRNA levels, confirming the findings of Van Rijswijk et al.³⁷

Capsaicin also did not induce activation or apoptosis/cell death in RPMI 2650 cells and freshly isolated NECs, even after 1 hour of exposure, which is much longer than the time taken by the mucociliary mechanisms to clear the surface of the intact epithelium *in vivo*. Furthermore, the nasal epithelial layer of patients with IR remained intact after capsaicin treatment. Finally, application of 0.1 mmol/L capsaicin did not induce the release of IL-33 or IL-8 and IL-6, which is known to be triggered on epithelial cell damage³⁸ or by exposure to inflammatory cytokines,²⁰ respectively. Taken together, these results indicate that mast cells and NECs do not play a significant role in the mechanisms underlying IR and the therapeutic action of capsaicin.

In conclusion, we have shown that IR is associated with an increased expression of TRPV1 in the nasal mucosa and SP levels in nasal secretions. We documented that the scores of IR symptoms, as well as the NHR triggered by CDA, are reduced after capsaicin treatment. These improvements are paralleled by a decrease in nasal mucosal innervation and a downregulation of the TRPV1-SP nociceptive signaling pathway. In contrast, the capsaicin treatment does not seem to affect the integrity and function of NECs or mast cells. These findings add substantially to our understanding of the beneficial effects of capsaicin treatment in patients with IR.

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Key messages

- The nociceptive TRPV1-SP signaling pathway is upregulated in patients with IR.
- IR symptoms and NHR are significantly reduced after capsaicin treatment.
- Capsaicin reduces the density of the innervation of the nasal mucosa and the TRPV1-SP signaling pathway without affecting the integrity and function of NECs or mast cells.

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METHODS

Patients and HCs

A positive SPT response for the 18 most frequent aeroallergens in Belgium (house dust mite; timothy grass, smooth meadow grass, orchard grass, nettle, plantago, oxeye daisy, mugwort, alder, birch, and hazel pollen; horse; cat; dog; rabbit; and spores of *Alternaria*, *Aspergillus*, and *Cladosporium* species; HAL Allergy, Leiden, The Netherlands) was an exclusion criterion.

Study design

The patients with IR and HCs were invited for an outpatient visit at the Department of Otorhinolaryngology of the University Hospitals of Leuven on 5 occasions. Fig E1 shows the list of registrations and sample collections that were performed at each visit.

The capsaicin solution was prepared by the Pharmacy of the University Hospitals Leuven by using the formula previously reported.^{E1} In brief, the solution contained capsaicin at 0.1 mmol/L with 30.3 mg of pelargonic acid vanillylamide (Sigma-Aldrich, Bornem, Belgium) dissolved in 3 mL of alcohol (96%) and diluted in 1 L of NaCl solution (0.9%). On the day of treatment, a total volume of 3.5 to 4 mL of the solution was sprayed into both nostrils.

All patients with IR and HCs were treated with capsaicin nasal spray 5 times on a single day at 1-hour intervals, as reported previously.^{E1} The nasal mucosa was anesthetized before the first 2 applications with cocaine 5% nasal spray. An interval of 15 minutes was maintained between the cocaine and capsaicin application to ensure effective local anesthesia.

VAS scores for rhinorrhea, nasal obstruction, and sneezing were recorded at 3 different time points during treatment (before and 5 minutes after the first and fifth capsaicin applications) in both HCs and patients with IR to check for general acceptability. In addition to nasal symptoms, nasal discomfort (defined as pain, itch, or a burning feeling) was scored.

CDA: Nasal provocation

NHR was assessed based on a CDA nasal provocation at the baseline visit (before treatment) and at visit 4 in line with the recently validated and reported methodology.^{E2} Before the exposure to CDA, subjects were asked to acclimatize to room temperature (20°C) for 15 minutes. All provocations were performed in the morning by a single experienced examiner (L.V.G.) to avoid diurnal variation and intertest error. Compressed dry air for medical use (Dräger, Lübeck, Germany) was delivered through a transparent anesthesia mask placed over the nose and mouth of the patient/control subject. Subjects were instructed to breathe through the nose only. Exposure to CDA lasted for 15 minutes. The flow, measured by a VT plus HF gas flow analyzer from Dräger, was set at 25 L/min. CDA was obtained by passing the compressed air through a coiled copper tube immersed in a glycol bath. The construction was placed in an industrial freezer, and the entire compartment was isolated. The temperature of the air reaching the nose was −10°C, with a relative humidity of less than 10% to 15%. Before starting each provocation, the temperature and humidity were measured with a Testo 610 device (Lenzkirch, Germany). Variations in temperature reaching the nostrils were allowed between −10°C and −20°C (start at −10°C and stop around −18°C). Nasal patency before and immediately after CDA exposure was evaluated with the PNIF device (Clement Clark International, Essex, United Kingdom).^{E3} A decrease in PNIF of 20% caused by CDA exposure was chosen as the pre-defined cutoff point for the presence of NHR.^{E2}

Collection of nasal secretions and nasal biopsy specimens

A nasal sponge (Post-Op Sinus Pack, K9; Ivalon Surgical Products, San Diego, Calif) was weighed and inserted in one of the nostrils before, immediately after, and 4 weeks after treatment to collect secretions.^{E3} After 5 minutes, the sponge was removed and weighed again. A volume of saline was added, depending on the weight of the collected sponge (1:5 dilution). The sponge was then squeezed and centrifuged at 1500g at 4°C for 5 minutes. Supernatants were stored at −20°C until analysis.

Before, immediately after, and 12 weeks after capsaicin treatment, 2 nasal biopsy specimens were taken from the inferior turbinate with the Fokkens

forceps. One biopsy specimen was stored at −80°C for RT-PCR, and the other was fixed for immunohistochemistry.

Measurement of SP, NKA, CGRP, and mast cell degranulation in nasal secretions

NKA, SP, and CGRP levels were measured with commercially available competitive ELISAs sensitive to 3.9 pg/mL (Cayman Chemical, Ann Arbor, Mich), 0.01 ng/mL (Bachem, Torrance, Calif), and 7.81 pg/mL (SPI-Bio, Montigny le Bretonneux, France), respectively. A β -hexosaminidase assay was performed to evaluate mast cell degranulation.

RT-PCR for *TRPV1*, *TRPA1*, *TRPM8*, *PGP 9.5*, and *c-KIT* on nasal biopsy specimens

The nasal biopsy specimens were homogenized in lysis buffer from the Qiagen Mini RNeasy kit. RNA was isolated with the Qiagen Mini RNeasy kit, and first-strand cDNA was reverse transcribed by using SuperScriptIII RT (Invitrogen, Carlsbad, Calif) with 1 μ g of total RNA, according to the manufacturer's instructions. Real-time quantitative PCR was performed for *TRPV1*, *TRPA1*, *TRPM8*, *PGP 9.5*, and *c-KIT* (CD117) and the housekeeping genes β -actin (*ACT2B*) and *TRPM7* in the ABI prism 7700 Sequence Detector System (Applied Biosystems, Foster City, Calif). cDNA plasmid standards, consisting of purified plasmid DNA specific for each target, were used to quantify the target gene in the unknown samples, as described before.^{E4} The primer and probe sequences were designed with Primer Express (Applied Biosystems).

Immunohistochemistry for hematoxylin and eosin, TUNEL, PGP 9.5, and c-kit on nasal biopsy specimens

Immediately after harvesting, nasal biopsy specimens were fixed in 6% formol and subsequently embedded in paraffin. Six-micrometer sections of embedded tissue were cut, placed on glass slides, deparaffinized, and stained with hematoxylin and eosin. A blinded experienced pathologist (E.H.) evaluated the sections.

For immunohistochemical detection of PGP 9.5 and c-kit (CD117), deparaffinized 6- μ m sections were incubated with antigen retrieval solution (Dako, Heverlee, Belgium) for 20 minutes at 95°C. After cooling down, sections were washed and subsequently blocked in 1:5 goat serum (X0907, Dako) and incubated with primary antibody (AB1761; Millipore, Overijse, Belgium, and MBL 566, Sanbio, Uden, The Netherlands, respectively, 1:1000 [both antibodies]; host species: rabbit) overnight. Negative control was incubated without primary antibody. After washing, slides were incubated with a secondary antibody (1:200 goat anti-rabbit Alexa Fluor 568; Invitrogen 997761, Merelbeke, Belgium). Slides were mounted by adding mount prolong gold and 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI; Life Technologies P36935).

For TUNEL staining, the apoptosis detection kit from Roche (Vilvoorde, Belgium) was used, according to the manufacturer's instructions. As a positive control, tissue was pretreated with DNase I, as instructed by the manual.

In vitro cell isolation of human mast cells and human NECs

The LAD2 human mast cell line was a kind gift of Dr Arnold S. Kirshenbaum.^{E5} Before use, the LAD2 cells were tested for expression of c-kit (eBioscience, Vienna, Austria) and Fc ϵ RI (eBioscience). The LAD2 cells were cultured in serum-free medium (StemPro-34 SFM, Life Technologies) supplemented with 2 mmol/L L-glutamine (Gibco, Merelbeke, Belgium), 100 U/mL penicillin (Gibco), 100 μ g/mL streptomycin (Gibco), and 100 ng/mL human SCF (Miltenyi Biotec, Leiden, The Netherlands) and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

RPMI 2650 human nasal septal squamous cell carcinoma cells were obtained from the American Type Culture Collection (ATCC CCL-30).^{E5} The epithelial cells were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% FCS (Sigma), 2 mmol/L L-glutamine (Gibco),

100 U/mL penicillin (Gibco), and 100 μ g/mL streptomycin (Gibco) and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Once the cells were 100% confluent, they were detached from the culture flask by incubating for 5 minutes with 0.25% trypsin-EDTA solution (Invitrogen). As an additional control, freshly isolated NECs were isolated from resection specimens (concha), as described previously,^{E6} and experiments were repeated to confirm the findings.

RT-PCR for TRPV1 in NECs and LAD2 cells and intracellular Ca²⁺ imaging experiments

The different cell types were checked for *TRPV1* expression by using RT-PCR, as described in the article. Additionally, the functionality of TRPV1 was investigated by using calcium measurements.^{E2,E7} Cells used in calcium imaging experiments were seeded on 18-mm glass cover slips coated with poly-L-lysine (0.1 mg/mL) and grown in their specific medium at 37°C in a humidity-controlled incubator at 10% CO₂. Before the measurements, cells were incubated with 2 μ mol/L Fura-2AM ester (Biotium, Leuven, Belgium) for 30 minutes at 37°C. Intracellular Ca²⁺ concentrations were monitored by using the ratio of fluorescence measured on alternating illumination at 340 and 380 nm with an MT-10 illumination system and CellM software (Olympus). Experiments were performed with standard Krebs solution (150 mmol/L NaCl, 6 mmol/L KCl, 1.5 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4). Capsaicin, 1 μ mol/L, was used as a TRPV1 agonist.

Determination of capsaicin-induced apoptosis by means of flow cytometry

Apoptosis was detected by means of flow cytometry with Annexin-V-FITC and PI staining (Detection Kit I, BD PharMingen). RPMI 2650 and LAD2 cells were seeded in 24-well plates (1 $\times$ 10⁶ cells per well) and cultured for 48 hours. Cells were then exposed to different concentrations of capsaicin (0.1 mmol/L [ie, the concentration used in patients], 0.25 mmol/L, and 0.5 mmol/L). At the end of the 1- and 4-hour incubation/stimulation, cells were trypsinized, if necessary (RPMI 2650); centrifuged; and washed with PBS. Cells were subsequently incubated for 15 minutes at room temperature in the dark with PI/Annexin-V-FITC fluorescent dyes. Results were determined by using a flow cytometer equipped with an Argon laser (FACSort, BD). Ten thousand cells were analyzed for fluorescent intensity in FL1 (FITC) and FL2 (PI) for each assay, and assays were repeated 3 times. Living cells were Annexin-V-FITC and Annexin-V-PI double negative, whereas late apoptotic and necrotic cells were double positive. Early apoptotic cells were only Annexin-V-FITC positive. A parallel experiment was performed with the vehicle of the capsaicin solution. To investigate whether apoptosis was induced through activation of a functional TRPV1 channel, we pretreated cells with 2 different TRPV1 antagonists (capsazepine, Sigma, 5 and 10 μ mol/L, or AMG 9810, Sigma, 10 μ mol/L) before adding capsaicin and performing flow cytometry.

Mast cell degranulation assay in LAD2 cells

After exposure of LAD2 mast cells (0.8 $\times$ 10⁶ cells per well) to different concentrations of capsaicin (0.1, 0.25, and 0.5 mmol/L), SP (2.5 and 5 μ g/mL, Sigma) and Compound 48/80 (0.5 and 1 μ g/mL), the culture supernatant was collected at 30 minutes and 2 hours of exposure. The β -hexosaminidase release into the supernatant was quantified by means of hydrolysis of 1 mmol/L p-nitrophenyl N-acetyl- β -D-glucosamide (Sigma) in 0.1 mol/L sodium citrate buffer (pH 4.5) for 90 minutes at 37°C in the dark. The percentage

of β -hexosaminidase release was calculated as a percentage of total content. NECs were stimulated with capsaicin at different concentrations (0.1, 0.25, and 0.5 mmol/L) for 4 hours to investigate whether mediators released by epithelial cells exposed to capsaicin can activate mast cells. Supernatants were collected, 1 mL was incubated with 0.8 $\times$ 10⁶ LAD2 cells for 30 minutes and 2 hours, and the β -hexosaminidase release assay was performed on these samples.

IL-6, IL-8, and IL-33 analysis of the supernatants of NECs

RPMI 2650 cells (1 $\times$ 10⁶ cells/mL) and primary NECs (1 $\times$ 10⁶ cells/mL) were incubated with different concentrations of capsaicin (0.1, 0.25, and 0.5 mmol/L) for 1 and 4 hours. LPS (10 mmol/L, Sigma) was used as a positive control.

Supernatants were harvested and stored at -20°C until subsequent analysis. Human IL-6 and IL-8 levels were measured in the supernatants by using sandwich ELISA (capture antibody codes 554543 and 554716, respectively; biotinylated detection antibody codes 554546 and 554718, respectively; rhIL-6 and rhIL-8 as standards [550071 and 554609, respectively]; BD PharMingen). Human IL-33 was measured in the supernatants by using a sandwich ELISA (DY3625; R&D Systems, Abingdon, United Kingdom).

RESULTS

General acceptability of capsaicin treatment in patients with IR and HCs

Only rhinorrhea was significantly induced by intranasal capsaicin application (VAS scores of 0.0 $\pm$ 0.0, 1.5 $\pm$ 0.3, and 1.2 $\pm$ 0.3 at the 3 time points; P < .0001 and P = .0004, respectively) in HCs. Patients with IR had high baseline VAS scores for rhinorrhea, nasal obstruction, and sneezing without additional induction by capsaicin treatment (data not shown). The mean VAS for nasal discomfort was 1.1 $\pm$ 0.5 after treatment in HCs and 1.0 $\pm$ 0.7 in patients with IR.

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	Screening visit (Week -3)	Baseline visit (Week-1)	Capsaicin R/ (0)	4 w after R/	12 w after R/
Explanation of study rationale and protocol	+	-	-	-	-
Evaluation inclusion and exclusion criteria	+	+	-	-	-
Informed consent	+	-	-	-	-
Symptom evaluation (VAS)	+	+/#	+/-/+ [§]	+/#	+
Evaluation therapeutic response	-	-	-	+	+
Clinical ENT- examination (Endoscopy and RA)	+	+	-	+	+
Explanation treatment	-	-	+	-	-
Merocel collection	+	-/+ [#]	-/-/+ [§]	+/#	-
Biopsy collection	+	-	-/-/+ [§]	-	+
Inspection biopsy site	-	+	-	+	-
Measurements of PNIF values	+	+/#	+/-/+ [§]	+/#	-
Blood Analysis	+	-	-	-	-
Skin prick tests (if not performed yet)	+	-	-	-	-

= before and after CDA provocation

§ = before and 5' after the first and last capsaicin application

FIG E1. Overview table of registrations and sample collections that were performed at each visit. *ENT*, Ear, nose, and throat; *RA*, anterior rhinoscopy.