

Eosinophil peroxidase induces the expression and function of acid-sensing ion channel-3 in allergic rhinitis: *in vitro* evidence in cultured epithelial cells

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Summary

Background Acid-sensing ion channels (ASIC) are a family of acid-activated ligand-gated cation channels. As tissue acidosis is a feature of inflammatory conditions, such as allergic rhinitis (AR), we investigated the expression and function of these channels in AR.

Objectives The aim of the study was to assess expression and function of ASIC channels in the nasal mucosa of control and AR subjects.

Methods Immunohistochemical localization of ASIC receptors and functional responses to lactic acid application were investigated. *In vitro* studies on cultured epithelial cells were performed to assess underlying mechanisms of ASIC function.

Results Lactic acid at pH 7.03 induced a significant rise in nasal fluid secretion that was inhibited by pre-treatment with the ASIC inhibitor amiloride in AR subjects ($n = 19$). Quantitative PCR on cDNA isolated from nasal biopsies from control and AR subjects demonstrated that ASIC-1 was equally expressed in both populations, but ASIC-3 was significantly more highly expressed in AR ($P < 0.02$). Immunohistochemistry confirmed significantly higher ASIC-3 protein expression on nasal epithelial cells in AR patients than controls ($P < 0.01$). Immunoreactivity for EPO+ eosinophils in both nasal epithelium and submucosa was more prominent in AR compared with controls. A mechanism of induction of ASIC-3 expression relevant to AR was suggested by the finding that eosinophil peroxidase (EPO), acting via ERK1/2, induced the expression of ASIC-3 in epithelial cells. Furthermore, using a quantitative functional measure of epithelial cell secretory function *in vitro*, EPO increased the air-surface liquid depth via an ASIC-dependent chloride secretory pathway.

Conclusions This data suggests a possible mechanism for the observed association of eosinophils and rhinorrhoea in AR and is manifested through enhanced ASIC-3 expression.

Keywords acid-activated ligand-gated cation channels, acid-sensing ion channels, allergic rhinitis, eosinophil

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Introduction

Acid-sensing ion channels (ASIC) are a family of ligand-gated cation channels, which belong to the amiloride-sensitive degenerin/epithelial Na⁺ channel superfamily. They are Na⁺-selective and Ca²⁺-per-

meant, and are activated by acid (pH 7.2–6.0) and blocked by amiloride [1]. There are four subtypes of ASIC channels, ASIC-1, 2, 3, 4; both ASIC-1 and ASIC-2 have two isoforms each [2]. ASIC-1a, ASIC-2a and ASIC-2b are expressed in both the peripheral and central nervous systems, whereas ASIC-1b and ASIC-3 are primarily expressed in sensory neurons and epithelial cells [3–6].

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A variety of functions have been attributed to the ASIC, such as mediation of the sensation of pain, in particular, the pain associated with acidosis that develops as part of ischaemia [7–12]. Recent studies have described the expression of ASIC by other cells including malignant glioma tumours as well as epithelial cells, including rat oesophageal epithelial and airway cells [13–15]. In epithelial cells, ASIC-3 has been co-immunoprecipitated with the cystic fibrosis transmembrane receptor (CFTR), where they have been suggested to play a role in chloride secretion [16, 17]. Hence, within the nose and airways, there are several possible ways that ASIC could mediate physiological responses, enhancing for example, the increase in epithelial chloride ion secretion or mediating neural sensations.

The condensate from the exhaled breath of asthma and rhinitis patients is more acidic than that of healthy controls [14, 18–20]. Hence, ASICs may be stimulated by tissue associated acidosis of a chronic inflammatory condition, such as allergic rhinitis (AR). Although ASIC mRNA, and in particular ASIC-3, has been described in the airways, their presence and physiological role in the upper airways and in particular in allergic conditions has not been studied. Therefore, we sought to identify where ASIC are expressed in the nasal mucosa through nasal biopsy and to investigate their function by using physiological tests of nasal secretion and *in vitro* tests of epithelial cell secretion. The central hypothesis of this work is that ASIC are expressed by nasal tissues in subjects with AR. As AR is marked by local accumulation and release of eosinophil granule proteins, we further hypothesized that ASIC expression arises as a result of this eosinophil granule protein function and that their function includes epithelial fluid secretion.

Materials and methods

Ethical approval was obtained from the Scientific Committee Ethics Department at Beaumont Hospital, where the study was undertaken, reference 07/65.

DMEM Plus Glutamax, fetal calf serum (FCS) and penicillin/streptomycin solutions were purchased from GIBCO® Invitrogen (Paisley, UK). CuFi-1 cells were kindly donated from Prof Zabner at the University of Iowa, IA, USA. The SV40-transformed human bronchial epithelial cell line 16HBE14o was a kind gift of Dr Dieter Grunert (University of Vermont). Lactic acid, amiloride and TRI reagent™ were obtained from Sigma (Poole, UK), as were all common buffer constituents unless otherwise stated. All primers were obtained from Eurofins/MWG-Biotech AG (Ebersberg, Germany). RNA-Later, RNA Stabilization Reagent, RNeasy mini-kit, Quantiscript reverse transcriptase kit and Quantitect SYBR Green PCR kits were obtained from Qiagen (Hilden, Germany). Goat anti-rabbit Alexa-488 and goat

anti-mouse Alexa-568 were purchased from Invitrogen (Renfrew, UK). Rabbit polyclonal ASIC-3 antibody was obtained from Abcam (Cambridge, UK). Bovine anti-rabbit IgG HRP conjugate and Western blotting chemiluminescence luminol reagent were obtained from Santa Cruz (Santa Cruz, CA, USA). Monoclonal antibodies to PGP 9.5 was obtained from Novocastra (Newcastle-upon Tyne, UK) and to Eosinophil Peroxidase from Lee Laboratories (Mayo Clinic, AZ, USA). IPeroxidase-labelled Envision anti-rabbit or anti-mouse secondary antibodies were obtained from Dako (Hamburg, Germany). 4,6-diamidino-2-phenylindole Vectashield was purchased from Vector Laboratories Inc (Dublin, Ireland). Wizard[®] PCR Preps DNA Purification System was obtained from Promega (Madison, WI, USA). Eosinophil proteins were prepared as previously described [21].

Patient selection

The experiments were conducted within Beaumont Hospital Dublin, and conducted with the approval of the Ethics Committee of the host hospital. Each participant signed an informed consent document. Participants were enrolled into a healthy control group ($n = 23$) and a study group ($n = 19$) comprising patients with persistent AR. Criteria for inclusion into the study group were based on the symptoms and diagnosis of persistent allergic rhinitis as per the “Allergic Rhinitis and Its Impact on Asthma” (ARIA) criteria [22], (age 18–44 years, 15 women). Skin prick tests were undertaken to confirm the allergic nature of each patient’s rhinitis (all had at least 3 mm response and a positive response to control, histamine). Recruitment of AR patients was undertaken primarily from the general and specialist rhinology Ear Nose and Throat clinics, and via posters alerting the hospital public about the study. Healthy controls (age 18–45 years, 17 women) had no prior history of nasal or respiratory disease and were not atopic. Patients on blood thinning agents, smokers, cognitively impaired or pregnant persons and persons unwilling to consent to the study were excluded.

Physiological nasal secretion collection and nasal challenge with saline and amiloride

Lactic acid has been previously used to demonstrate a functional response of ASIC [7, 23, 24]. Measurement of secretory response was performed as previously described [25], using control and AR subjects. One nostril, randomly selected, was administered three sprays of either buffered saline or on another day the ASIC inhibitor amiloride, (1×10^{-3} M, diluted in buffered saline). These pre-treatments were allowed to absorb into the nasal mucosa. Two minutes later pre-weighed filter paper discs (Shandon Inc, Pittsburgh, PA, USA)

were placed in both nostrils, and then removed after 2 min; this gave the baseline measurements of nasal secretion. Amiloride, alone did not effect nasal secretion. Three sprays of a solution of 15 mM lactic acid solution (pH of 7.03) were then applied to that nostril. Two minutes after the lactic acid challenge, two fresh filter discs were placed in each nostril and nasal secretions were collected over 2 and 5 min. The calculated nasal secretions were the weight of secretion obtained at the 2 and 5 min time-point less the initial baseline nasal secretion weight, see Fig. 1.

Inferior turbinate nasal biopsies

Nasal mucosal biopsies were taken from both control, ($n = 6$) and AR ($n = 12$) subjects (seven women and five men, age 22–39 years). Three biopsy specimens were obtained from each subject from the anterior portion of the inferior turbinate of one randomly selected nasal cavity under local anaesthesia. Co-phenylcaine (lignocaine hydrochloride 5% and phenylephrine) was used as a local anaesthetic and local vasoconstrictor, as per previously described methods [26]. Biopsy material was stored in RNA-stabilizing agent RNALater (Qiagen, UK), according to the manufacturer's instructions, or in formaldehyde for subsequent paraffin embedding.

Immunohistochemistry and quantification

Immunohistochemistry was performed on 5 µm formalin-fixed, paraffin-embedded tissue sections using polyclonal antibody to ASIC-3 (1 : 50), and mouse

monoclonal antibodies to PGP 9.5 (1 : 50) to stain nerves and to eosinophil peroxidase (1 : 100) to highlight eosinophils. Primary antibodies were incubated on sections overnight at 4°C and detected using peroxidase-labelled Envision anti-rabbit or anti-mouse secondary antibodies with DAB as peroxidase substrate. As a control, the samples were processed in the same manner, but using isotype rabbit or mouse IgG, in place of the primary antibodies. For dual-immunofluorescence studies, sections were subject to antigen retrieval and then incubated overnight at 4°C in a mix of anti-ASIC-3 and anti-PGP 9.5. Immunostained sections were evaluated in a blinded manner without knowledge of the clinical status of the patients. For quantification of ASIC-3 expression in nasal epithelium, slides were available for three allergic patients and four control patients. Each slide was subsequently scanned using an Aperio ScanScope CS scanner (Aperio Technologies Inc. San Diego, CA, USA) at 40× magnification which is the resolution of 0.25 µm/pixel. After scanning, each digital slide was compressed using standard JPEG method at the quality of 70. These digital slides are ultra-large in size, up to 12 000 × 30 000 pixels representing a tissue region of 3 mm × 7.5 mm. Using the state-of-the-art Aperio ImageScope software (Aperio Technologies Inc. San Diego, CA, USA) and its accompanied Positive Pixel Counting algorithm, the nasal epithelium was selected for each slide at a magnification of 8 × -20 × using ImageScope's interactive pen tool. Consecutive sections of epithelium were digitally selected and annotated along the entire length of the basement membrane for each slide. The Positive Pixel Counting algorithm was then performed on all selected epithelium regions to give the total count of positively and negatively stained epithelium. The Positivity scores $P = \frac{N_{\text{positive}}}{N}$ were finally obtained, where N_{positive} is total number of positively stained epithelium pixels, and N is the total number of epithelium pixels (both positive and negative) in a region.

Quantitative PCR for ASICs from nasal tissue

Cell wall and plasma membrane disruption to release all RNA contained within the biopsy sample in RNALater was carried out. Total RNA was extracted using the RNeasy mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -20°C for cDNA preparation. Amplification of cDNA was carried out by quantitative PCR on a LightCycler using fast start Taq DNA polymerase containing the double-stranded DNA binding dye SYBR Green 1. The PCR mixtures contained 0.5 µM of primers specific to either ASIC-1, ASIC-2, ASIC-3 or β-actin; the sequences of primers used are shown in Table 1.

Laser capture micro-dissection was performed using a Pix-Cell II system. Nerve tissue stained with anti-S100

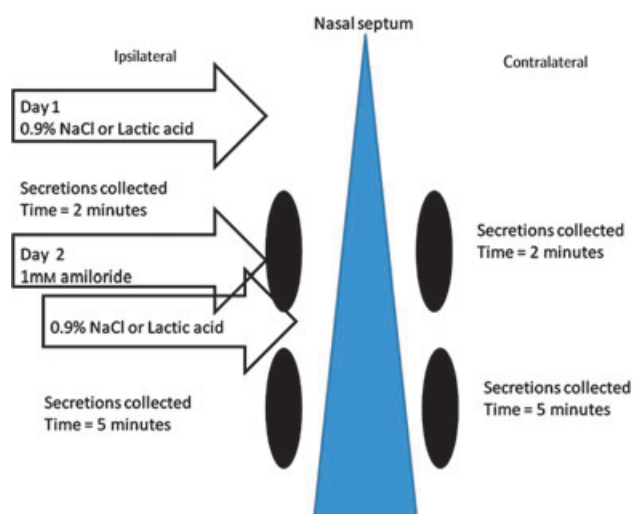


Fig. 1. The methodology for the *in vivo* lactic acid challenges is shown. The details are described in the Materials and methods section. Briefly, buffered saline, control or amiloride were applied and 2 min later the baseline sample secretions were obtained. Lactic acid was then applied and nasal secretions were obtained 2 and 5 min later.

Table 1. Primers used in this study

Gene	Primer sequence
β -actin	5'-GGACTTCGAGCAAGAGATGG3' (forward) 5'-AGGAAGGAAGGCTGGAAGAG-3' (reverse)
ASIC-1	5'-GCTATGGCAAAGAGCTGTCC-3' (forward) 5'-GTCCAGCACCAGGATGTTCT-3' (reverse)
ASIC-2	5'-ACAGGAGCAGAGGCTCACAT-3' (forward) 5'-CGGGTCTCACAGTCAATCCT-3' (reverse)
ASIC-3	5'-TGGAGGGACAATGAGGAGAC-3' (forward) 5'-CTGGCAAGAAACAAAGGTC-3' (reverse)

was captured using either the 7.5 μ m or the 15 μ m laser setting depending on the size of the nerve to be captured. The laser was set to a pulse of 100 mW. Images were collected using the PixCell II Image Archiving Workstation.

Cell culture: In vitro experiments with eosinophil peroxidase in co-culture with 16HBE140 cells

16HBE140- cells were cultured in minimal essential medium (MEM) supplemented with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin. Eosinophil peroxidase (EPO) was added at 1 μ g/mL for time points between 1 and 24 h. In experiments designed to investigate the role of MAP kinases, cells were pre-treated for 2 h with the ERK1/2 inhibitor PD98059 (50 μ M). After removal of media, total RNA was isolated from the cells using TRI reagentTM, according to the manufacturer's instructions. cDNA preparation from 1 μ g RNA and real-time PCR for ASIC-3 and β -actin from cDNA preparations were carried out as described above using the primer sets outlined in Table 1.

Visualization of ASIC-3 expression by epithelial cells

The NuLi-1 (normal lung) and CuFi-1 line, derived from a cystic fibrosis patient with Δ 508/ Δ 508 genotype were used for confocal and fluid secretion studies. The cell lines are able to form differentiated polarized monolayers that exhibit transepithelial resistance and maintain ion channel physiology for the genotypes. After 4–6 weeks of cell culture, the cells formed a polarized confluent monolayer with a high transepithelial resistance of $> 800 \Omega/\text{cm}^2$. Confluent monolayers of cells were exposed to EPO 1 μ g/mL applied to the basolateral surface for varying time points. The inserts were fixed with methanol and stained by incubating with the primary antibodies (rabbit polyclonal anti-ASIC-3 antibody from Neuromics diluted 1 : 50 in phosphate-buffered saline with 3% bovine serum albumin for 1 h on ice). The samples were rinsed in phosphate-buffered saline following blocking buffer incubation, and exposed to the secondary antibodies (1 : 1000; Molecu-

lar Probes, Eugene, OR, USA) for 2 h at room temperature. The monolayers were counterstained with rhodamine phalloidin (1 : 200; Molecular Probes) to highlight the actin cytoskeleton in cells for 2 h at room temperature. Control experiments were performed with rabbit IgG controls. The samples were mounted and imaged using a Zeiss LSM 710 laser scanning confocal microscope. Images were analysed using ZEN software.

Airway Surface Liquid (ASL) volume expansion studies

Airway Surface Liquid thickness was measured at nine predetermined points on the insert. To label the ASL, 8 μ L PBS containing 1 mg/mL Texas red-Dextran (40 kD; Molecular Probes) was added to the apical aspect of the confluent monolayer. The cells were labelled by addition of Calcein-AM (Molecular Probes) dissolved in medium culture for 20 min to the basolateral compartment on the insert. EPO (1 μ M) was added to the basolateral compartment of the hanging insert and ASL. Perflourocabon 72 (FC-72) was added to the apical compartment of the insert at a volume of 0.5 mL. The inert electronic fluid was used to prevent ASL evaporation on transferring the inserts from the incubator to the laser microscope during scanning. Perfluorocarbons are immiscible with the ASL, and they prevent ASL evaporation and serve as a vehicle for drug delivery. Values were measured at predetermined exposure times of 18 and 24 h. The images were scanned using a Zeiss LSM 510 Meta and analysed using Image J software (Rasband, W.S., Image J, National Institute of Health, Bethesda, MD, USA).

Statistical analysis

Data are presented as mean $\pm$ SEM unless otherwise indicated. Immunohistochemical, *in vivo* secretory responses and all *in vitro* data were normally distributed. ANOVA and independent *t*-tests were used as appropriate. Data analysis for immunohistochemical quantification was performed using Matlab R2011b (The MathWorks, Inc., Natick, MA, USA) and SPSS version 19 software (IBM) was used for all other analyses.

Results

Secretory responses to topical application of lactic acid

Functional experiments, with lactic acid, buffered to pH 7.03, were carried out to determine if nasal ASIC was activated by acidic solutions. Volunteers were recruited and tested as outlined in the Methods section (Fig. 1 illustrates the experimental design). After pre-treatment with placebo or amiloride (1×10^{-3} M) lactic acid challenges were performed, and at the specified time points

nasal secretion weights from the ipsilateral and contralateral sides of the challenge were recorded. There were 23 subjects in the control group and 19 subjects with a clinical diagnosis of persistent allergic rhinitis, as defined by the ARIA criteria [22]. Lactic acid administration caused no significant sensation, e.g. burning or irritation in either subgroup of subjects. In patients with AR, lactic acid significantly increased the nasal secretions on the ipsilateral side; 5 min after lactic acid the secretions had increased from a baseline 3.42 ± 0.80 mg to 10.17 ± 2.61 mg, $P = 0.01$. Lactic acid induced secretions were attenuated by pre-treatment with amiloride (1×10^{-3} M) see Fig. 2A. There was also a smaller, but significant increase in the secretions in the control subjects, from 3.76 ± 0.65 mg to 8.86 ± 2.23 mg, $P = 0.02$. However, amiloride did not prevent this rise; in the presence of amiloride pre-treatment, secretions still rose from 3.58 ± 0.55 mg to 7.79 ± 1.10 mg, ($P = 0.008$), suggesting that the response was not mediated through ASIC channels (Fig. 2B). Secretory responses were recorded on the contralateral side to which the lactic acid had been administered, to assess if there was a reflex response to the administered lactic acid [28]. These data showed that there were no changes in contralateral secretions in either the AR (Fig. 2C) or control groups (Fig. 2D), in response to lactic acid.

Real time PCR of inferior nasal turbinate biopsies

Real time PCR quantification of ASIC receptors in nasal biopsies demonstrated the presence of ASIC-1 in both control and AR subjects, but there was no difference in the level of its expression between the AR and control subjects (Fig. 3A). There was evidence of up-regulation of ASIC-3 receptors in patients with allergic rhinitis (AR) ($n = 12$) compared with healthy control subjects ($n = 4$) (Fig. 3B) ($P < 0.02$). There was no appreciable detection of ASIC 2 in control or AR subjects (data not shown).

Immunohistochemical localization of ASIC-3

As ASIC-1 transcriptional levels did not differ between AR and control subjects and because there was no appreciable detection of mRNA for ASIC-2 in the nasal biopsies, we proceeded only to localize the presence of ASIC-3, which was expressed in high levels in the AR subjects. There was immunostaining for ASIC-3 on the epithelial surface and epithelial glands in both AR (Fig. 4A) and control subjects (Fig. 4B). An isotype control antibody showed no staining (Fig. 4C). There was also strong staining in the nasal mucous glands (not shown). Expression of ASIC 3 protein was significantly greater in nasal epithelium from AR compared with

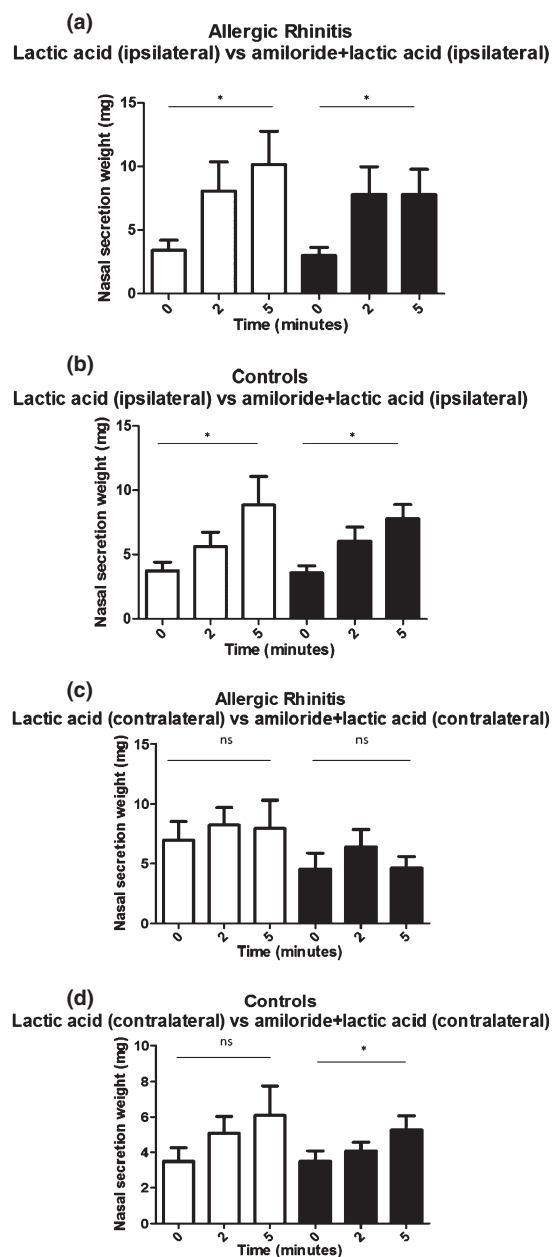


Fig. 2. (A-D) Lactic acid induced nasal secretory responses by the ipsilateral and contralateral nasal mucosa. Graphs show the changes in ipsilateral nasal secretion weights for subjects with allergic rhinitis (A) and control subjects (B) who were pre-treated with saline and then challenged with lactic acid (pH = 7.03), (open boxes) or pre-treated with amiloride and then challenged with lactic acid (closed boxes), as described in the Materials and methods section. In subjects with allergic rhinitis (shown in panel A) at both 2 and 5 min, lactic acid induced a significant increase in secretion weights on the ipsilateral nasal mucosa ($P = 0.002$); which was attenuated by pre-treatment of the ipsilateral nasal mucosa with amiloride (1×10^{-3} M), $P = 0.05$. In control subjects (B), on the ipsilateral side, there was a small rise in lactic acid induced secretions, which was not inhibited by amiloride pre-treatment. There were no changes in secretions obtained on the contralateral side to the challenge in either the allergic rhinitis (C) or control groups (D), in response to lactic acid. Values are mean $\pm$ SEM of nasal secretion weights obtained at the specified times * $P = 0.05$.

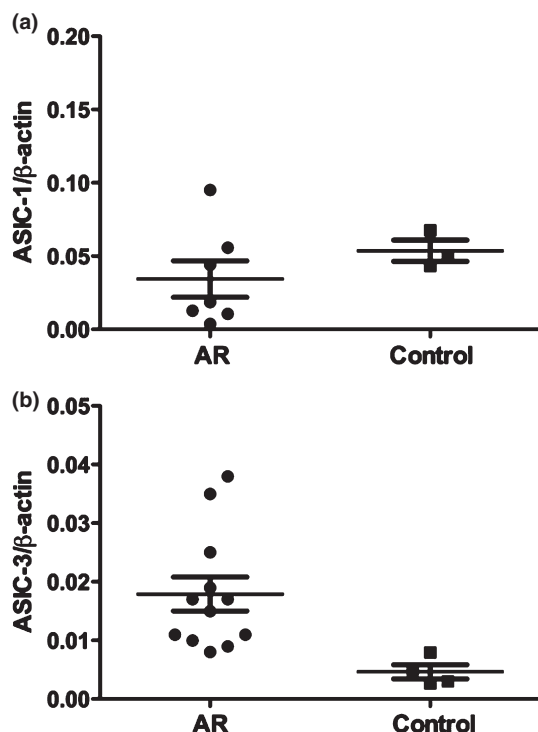


Fig. 3. (A–B) ASIC-3 mRNA expression is higher in nasal biopsies from allergic rhinitis patients than in control subjects. Airway expression of the mRNA for ASIC-1 and -3 quantified by real time PCR is shown. Nasal biopsies from the inferior turbinate of allergic rhinitis (AR) ($n = 12$) and control subjects ($n = 4$) were obtained as described in the Methods section. The DNA was extracted, reverse transcribed and subjected to real time PCR. The graphs show levels of ASIC-1 (A) and ASIC-3 (B) normalized to β -actin expression (mean $\pm$ SEM). ASIC-3 expression was increased in AR subjects compared with control subjects ($P < 0.02$), whereas ASIC-1 levels were not significantly different between the two groups (A). No appreciable ASIC-2 expression was detected in any subjects (data not shown). ASIC, acid-sensing ion channels.

control subjects [mean (SD) positivity score, $P = 0.62$ (0.03) in AR vs. $P = 0.26$ (0.02) in controls, $P < 0.01$] (Fig. 4D).

Immunohistochemical localization of ASIC-3 to nerves

Staining of nerve cells was performed with an antibody to the nerve cell marker PGP 9.5 and the sections were then co-stained with the antibody to ASIC-3 (Fig. 5). Several areas of co-localization are clearly present in major nerve bundles and in small fibres in the airway epithelium, but overall the neural staining was not as strong as that seen in the epithelium. There was no appreciable difference in the neural staining of PGP 9.5 immunoreactive nerves between controls and AR subjects (Fig. 5A–C). Laser capture microdissection of PGP stained sections was performed to enrich the neural tissue to specifically quantify neural mRNA for ASIC, but there was insufficient neural tissue obtained from the

nasal biopsies to extract sufficient mRNA to provide a meaningful result (data not shown).

Immunohistochemical localization of eosinophils

EPO+ eosinophils were evident in the nasal epithelium of AR subjects whereas no such staining was present in control biopsies (see Fig. 6A–D). The density of EPO+ eosinophils was increased in the nasal submucosa from AR patients compared with controls (7.10 ± 1.74 vs. 0.03 ± 0.33 , $P < 0.001$).

Induction of ASIC-3 expression by eosinophil peroxidase

Eosinophils and their released granule proteins are found in association with airway epithelial cells, mucous glands and nerve cells in patients with persistent AR compared with control subjects [29 and Thornton MA et al., unpublished]. As these are the sites at which ASIC-3 expression is also distributed, we investigated the effect of eosinophils and in particular their granule proteins on the expression of ASIC-3, *in vitro*. In these, *in vitro*, studies EPO (1 $\mu\text{g/mL}$) induced a significant up-regulation of ASIC-3 at both membrane protein level (Fig. 7A) and at an mRNA (transcriptional) level, Fig. 7B. The increased transcriptional expression of ASIC-3 was abolished by pre-treatment with the MEK inhibitor PD98059, indicating dependence of ASIC-3 expression on ERK activation (Fig. 7B). Similarly, in CuFi-1 epithelial cells (Fig. 8) there was evidence of apical induction of membrane bound ASIC-3 by eosinophil peroxidase at 18 and 24 h, ($n = 4$).

Increased ASL in response to eosinophil peroxidase

To determine if ASIC were functional *in vitro* measurements of the ASL were carried out. These experiments (Fig. 9A and B) were performed as described previously [30]. Briefly, over a period of 40 h the ASL was intermittently measured to determine the mean plateau value of the stabilized ASL height. In these experiments, the plateau levels were reached at 12 h with an average height of $7.97 \pm 0.21 \mu\text{m}$ ($n = 27$). Basolateral treatment with EPO (1 $\mu\text{g/mL}$) increased the baseline ASL to $19.51 \pm 0.66 \mu\text{m}$ ($n = 6$) at 18 h and to $14.56 \pm 0.42 \mu\text{m}$ ($n = 5$) at 24 h, $P = 0.0001$. The observed increase was abolished using apical amiloride (1 μM , $n = 4$) dissolved in the FC-72 layer, and in further experiments basolateral bumetanide (10 μM , $n = 4$) also completely reduced the EPO induced increase in ASL height. We were unable to dissolve the CFTR inhibitor CFTR-172 in the perfluorocarbon and the ASIC-3 inhibitor APETx2 was ineffective at inhibiting the EPO induced increase in ASL ($n = 3$), presumably because of the residual presence of ASIC-1.

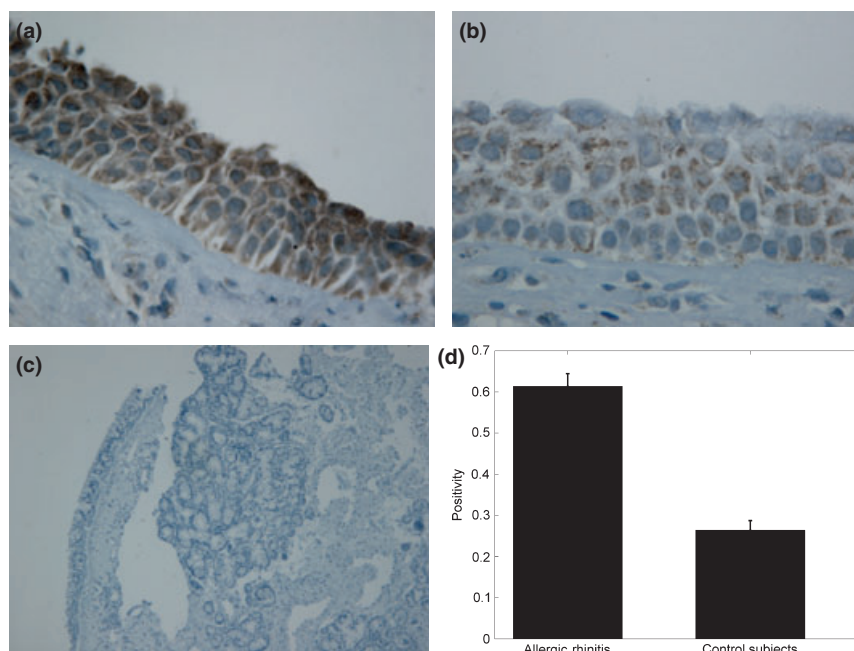


Fig. 4. (A-D) ASIC-3 is mainly expressed on epithelial cells. Immunohistochemical localization of ASIC-3 on biopsies of the inferior turbinate of AR (A) and control subjects is shown (B). The specificity of staining was shown by use of normal rabbit serum in place of the rabbit polyclonal anti-ASIC-3 antibody (C). The predominant staining of ASIC-3 was by epithelial cells and mucous glands although there was also some patchy staining on mononuclear cells in the submucosa. Expression of ASIC 3 protein was significantly greater in nasal epithelium from AR compared with control subjects [Mean (SD) Positivity score $P = 0.62(0.03)$ in AR vs. $P = 0.26(0.02)$ in controls, $P < 0.01$] (D). ASIC, acid-sensing ion channels; AR, allergic rhinitis.

Discussion

In this study, we have shown that among the ASIC family both ASIC-1 and ASIC-3 were detected in nasal epithelial cells. Immunohistochemical studies indicated that ASIC-3 expression was localized to airway epithelial cells, glands and nerves. ASIC-3 but not ASIC-1 expression was increased in the nose of subjects with AR compared with control subjects. *In vivo*, studies showed that application of lactic acid, pH 7.03, induced a significant rise in nasal secretions and this secretion was inhibited by pre-treatment with the ASIC inhibitor amiloride, only in AR subjects, indicating that the ASIC were functional. As control subjects had a similar level of expression of ASIC-1 to AR subjects but substantially and significantly less ASIC-3, we suggest that the functional ASIC in AR subjects corresponds to ASIC-3. Our histological findings confirm the presence of eosinophils in the nasal epithelium of patients with allergic rhinitis. A mechanism of induction of ASIC-3 expression relevant to AR was suggested by our *in vitro* findings that the eosinophil granule protein EPO induced the expression of ASIC-3 via ERK 1/2 in cultured epithelial cells. Furthermore, *in vitro*, EPO acting via ASIC increased the ASL height, a measure of airway hydration, via a chloride mediated secretion. These data suggest that rhinorrhea may be because of EPO induced expression of ASIC-3.

We undertook these experiments to investigate which ASIC were expressed in the nose of patients with AR. Having demonstrated that only ASIC-3 mRNA was increased in AR subjects compared with control subjects, we investigated the sites of expression of ASIC-3. In both immunohistochemical and functional studies, there was a clear difference in the level of expression of ASIC-3 by epithelial cells between control and AR subjects. ASIC-3 was expressed on the apical surface of epithelial cells in both biopsy samples of AR subjects and cell culture studies after exposure to EPO. As many reports have also described neural expression of ASIC, we co-localized the channel to nerves by dual immunostaining nerves with the general neural marker, PGP 9.5 and then probed for ASIC-3. In these studies, it was shown that there was evidence of staining by small nerve fibres within the epithelium, where they may be positioned to detect luminal acidification. There was no definite increase in expression of the ASIC-3 on nerves in the AR subjects as judged by immunohistochemistry. Even using laser capture microdissection of PGP stained sections there was insufficient neural tissue obtained from the nasal biopsies to extract sufficient mRNA to perform quantitative analysis. Hence, we cannot state definitely if there are differences in the level of expression of ASIC-3 between AR and control subjects.

As little is known about the regulation of ASIC channels in the setting of allergic inflammation we

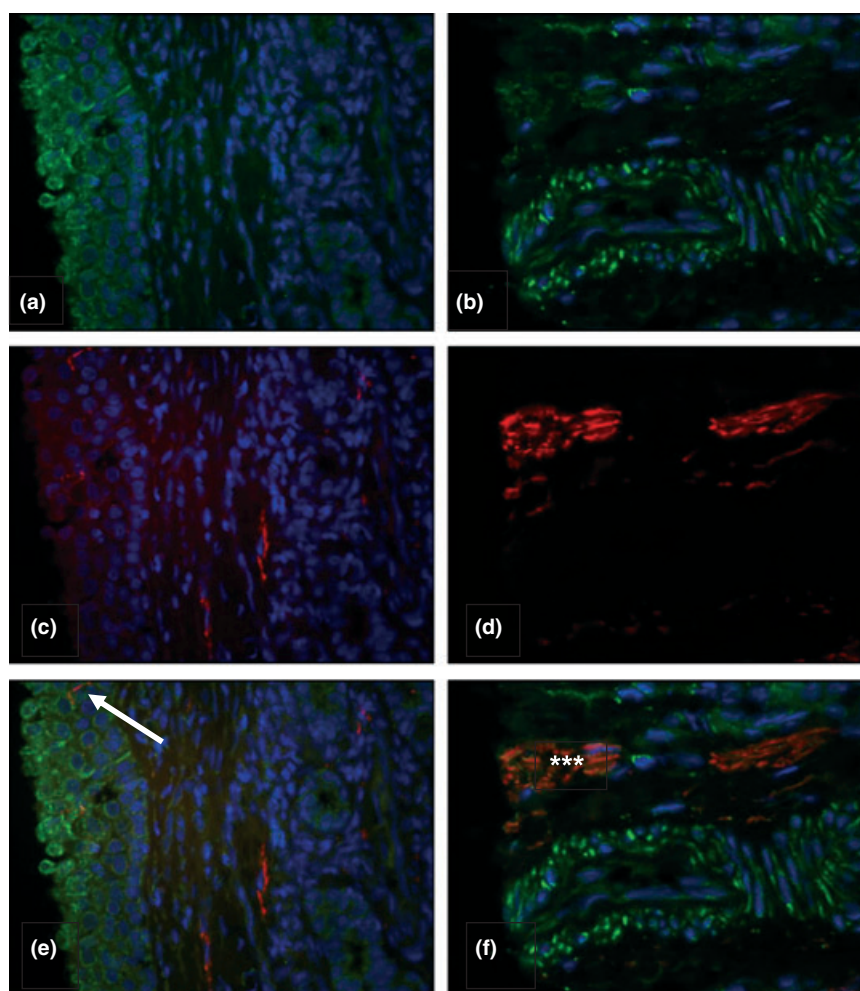


Fig. 5. (A-F) ASIC-3 is expressed by nasal nerves. Immunohistochemical localization of ASIC-3 on nerves on biopsies of the inferior turbinate of an allergic rhinitis (AR) subject is shown. ASIC-3, (green staining in A & B) and nerve tissue (red staining in C & D). Expression of ASIC-3 was seen on small nerve fibres, among the epithelial cells (arrow in E). The merged image (F) shows expression of ASIC-3 within a plexi of nerve fibres (***). There was also expression of ASIC-3 by nerves, in low levels in the control subjects (data not shown). ASIC, acid-sensing ion channels.

investigated a possible role for eosinophils in the induction of the ASIC-3. In this study, we observed immunohistochemical evidence of eosinophil infiltration into the nasal mucosa and epithelium of patients with allergic rhinitis. In prior immunohistochemical studies, it has been demonstrated that there is a marked association of eosinophils and released eosinophil granule proteins in association with nerves and epithelial cells in the subjects with AR, the sites at which ASIC-3 was most abundantly expressed. Hence, we speculated that induction of ASIC-3 may be mediated by eosinophils, and in particular their cationic granule proteins. We have previously used an *in vitro* model of granule proteins and IMR-32 cholinergic cells, to study eosinophil and nerve interactions. In these studies, we have shown that the eosinophil granule [27] proteins signal via the MAP kinase pathways leading to a variety of cellular

effects including the promotion of cell growth and survival. We studied this model and several epithelial cell lines alone and in the presence of eosinophil peroxidase over varying time points. In all of these cell lines there was a rapid increase in the transcriptional expression of ASIC-3 within 1 h of exposure to EPO. Protein expression of ASIC-3 was most pronounced at 18–24 h in all cell lines studied and furthermore ASIC-3 was localized to the cell membrane. In prior studies, we have shown that the eosinophil granule proteins major basic protein and eosinophil derived neurotoxin cause activation of the MAP kinase pathways, in particular the MEK ERK1/2 pathway [33]. In recent studies, we have shown that EPO activates the expression of the Her2 receptor and that this mediates a signalling cascade which leads to activation of ERK1/2 [Walsh et al. in review]. In this study, we have shown that EPO also induced

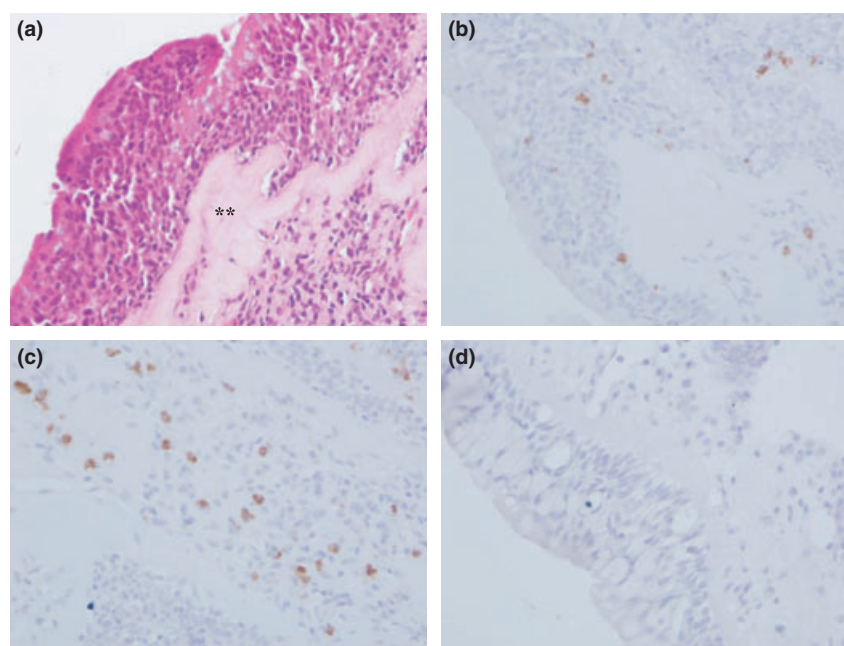


Fig. 6. (A-D) H&E and EPO expression in nasal biopsies (A) H&E showing thickened basement membrane (**). (B) EPO expressing eosinophils in epithelium and submucosa (AR subject) (C) foci of EPO expressing eosinophils in the submucosa (AR subject). (D) Absence of EPO expressing cells in epithelium or submucosa (control subject). ASIC, acid-sensing ion channels; AR, allergic rhinitis; EPO, eosinophil peroxidase.

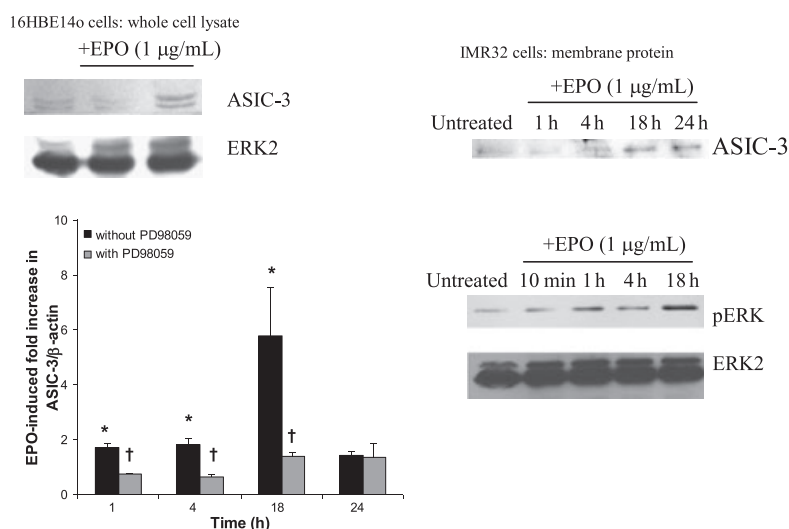


Fig. 7. Eosinophil peroxidase induces ASIC-3 expression *in vitro* in 16HBE14o- epithelial cells via an ERK1/2 dependent pathway. In (A) 16HBE14o- cells were stimulated with EPO (1 mg/mL) for times varying from 0 min to 24 h and whole cell lysates or membrane fractions were probed with an antibody to ASIC-3. There was a clear expression of ASIC-3 after 18–24 h of exposure to EPO. ASIC-3 expression assessed by real-time PCR is shown in (B), the graph shows EPO-induced fold increase over untreated cells for ASIC-3 normalized to the housekeeping gene β -actin. There was a significant increase in the mRNA expression of ASIC-3 as early as 1 h, which peaked at 18 h ($n = 4$, 5.79 ± 1.73 , $*P < 0.05$, significantly increased over untreated). Cells were pre-treated (grey bars) or not (black bars) for 2 h at 50 mM with the MEK/ERK inhibitor PD98059 prior to EPO stimulation. PD98059 pre-treatment abolished EPO-induced ASIC-3 mRNA up-regulation ($n = 4$, 1.39 ± 0.13 , $^{\dagger}P < 0.05$, significantly reduced compared with EPO treated only cells). ASIC, acid-sensing ion channels; EPO, eosinophil peroxidase.

phosphorylation of ERK. Furthermore, ASIC-3 expression was inhibited by the MEK/ERK inhibitor PD95089 suggesting that EPO induces ASIC-3 expression via ERK activation. Hence, eosinophil localization to and release

of EPO in patients with AR, may promote the expression of ASIC-3.

There have been prior reports of ASIC-3 expression by epithelial cells and prior *in vitro* studies suggest

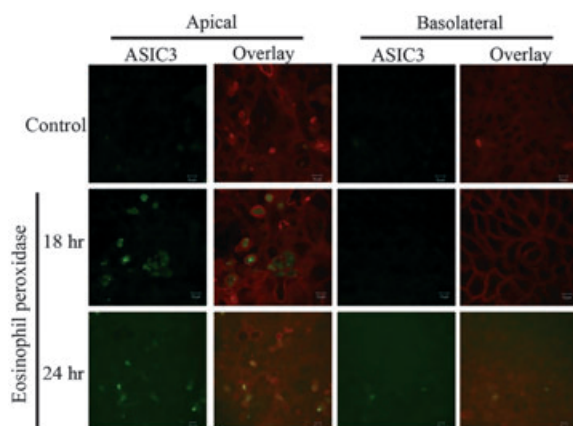


Fig. 8. Eosinophil peroxidase induces ASIC-3 expression *in vitro* in epithelial cells. Confluent monolayers of CuFi-1 cells were exposed to basolateral eosinophil peroxidase (1 $\mu\text{g/mL}$) for 18 and 24 h. Monolayers exposed to EPO showed increased apical expression of ASIC-3, detected with Alexa-488, when compared with nonstimulated cells ($n = 4$). ASIC, acid-sensing ion channels; EPO, eosinophil peroxidase.

that the CFTR and ASIC-3 are co-localized. Furthermore, *in vitro* studies suggest that there is a co-operative interaction of ASIC-3 with the CFTR leading to enhanced chloride ion transport [30–32]. In AR subjects, lactic acid induced an ipsilateral production of nasal secretions and pre-treatment with amiloride prevented this rise in secretions. To further investigate the hypothesis that the enhanced secretion seen in the AR subjects was mediated through ASIC-3 associated epithelial fluid secretion further *in vitro* studies were performed. Measurement of the airway surface liquid height was used as a model to investigate the functional effects of EPO and epithelial ASIC-3. Measurement of the airway surface liquid height is a functional quantitative assessment of epithelial cell ion transport. The rationale to these studies was that amiloride is both an epithelial sodium channel (ENaC) and

ASIC inhibitor. By inhibiting ENaC amiloride inhibits Na reabsorption, increasing the ASL height and that by inhibiting ASIC inhibits chloride secretion, reducing ASL height. In these studies EPO applied to the basal surface induced a significant ASL expansion at the time points that ASIC-3 protein expression was maximal, i.e. 18–24 h after exposure to EPO. That this EPO induced increase in ASL height was mediated via ASIC was suggested by the finding that amiloride as an ASIC-3 inhibitor resulted in attenuation of the ASL height, which would not have occurred if the prominent channel being inhibited was Na absorption. In the presence of the chloride channel inhibitor (bumetanide) there was a reduction in ASL when this chloride channel inhibitor was used. These data are in keeping with the previously described co-interaction of ASIC and CFTR [16, 17]. In conjunction with immunocytochemical analysis that demonstrated increased apical ASIC-3 in response to EPO treatment, this suggests involvement of ASIC-3 in the EPO induced hypersecretory response observed in airway epithelia.

We also investigated possible functional effects of these ASIC-3 expressing nerves, and we used a well characterized model of nasal reflex responses. In these experiments, a stimulus is applied on one side of the nose and nasal secretions on the contralateral side are collected and measured. Changes in contralateral secretions are mediated via a neural reflex as the nasal septae separate the two sides of the nose. In these studies, there was no evidence of induced secretions on the contralateral side in either the control or the AR subjects, suggesting that there is little if any secretory role to the neurally expressed ASIC-3 in the nose. Furthermore, it has been suggested that neurally expressed ASIC-3 channels in the skin mediate pain responses. In our studies, there was no evidence of pain, irritation, lacrimation or burning reported by any subjects after the administration of the lactic acid. Hence, although

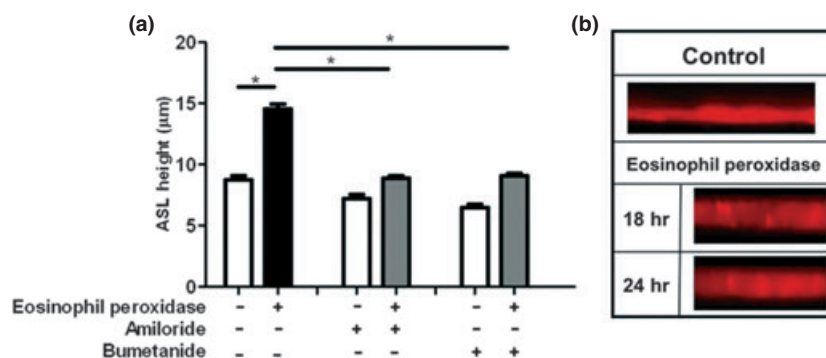


Fig. 9. (A–B) ASL Expansion is mediated via effect of eosinophil peroxidase acting via ASIC-3. In (A) NuLi-1 cells pretreated with EPO for 18 and 24 h, respectively, demonstrated a significant increase in ASL. The increase in ASL was attenuated by amiloride (1 μM) and the chloride inhibitor, bumetanide (10 μM) ($n = 4$, $P < 0.0001$). A representative diagram of the ASL stained with Dextran-Texas Red in response to EPO at baseline and 18 and 24 h after treatment is shown in (B). ASIC, acid-sensing ion channels; EPO, eosinophil peroxidase; ASL, airway surface liquid.

there is evidence of neural expression of ASIC-3 in the nose, we have not shown a definite function of these channels.

There are several novel findings in this study, which is the first description of a functional role for ASIC in the nose. For example, this is the first study to show how an eosinophil mediator of inflammation induces epithelial and neural expression of ASIC-3 and it describes a novel role for EPO, mediation of chloride secretion. This excess epithelial fluid secretion may be relevant to the pathogenesis of rhinitis, as it may contribute to the symptom of rhinorrhoea.

In summary, these studies indicate that epithelial cells in patients with AR expression express ASIC-3

receptors. *In vitro*, studies suggest that the increased expression in AR may be mediated through the eosinophil granule protein EPO. The function of nasal epithelial ASIC-3 channels appears to be that they promote nasal secretions and this may be a mechanism for rhinorrhea, one of the common and troublesome features of particular forms of rhinitis, such as non-allergic eosinophilic rhinitis.

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References

- Lingueglia E. Acid-sensing ion channels in sensory perception. *J Biol Chem* 2007; 282:17325–9.
- Krishtal O. The ASICs: signaling molecules? Modulators? *Trends Neurosci* 2003; 26:477–83.
- Dube GR, Elagoz A, Mangat H. Acid sensing ion channels and acid nociception. *Curr Pharm Des* 2009; 15:1750–66.
- Hobson AR, Aziz Q. Modulation of visceral nociceptive pathways. *Curr Opin Pharmacol* 2007; 7:593–7.
- Shimada S, Ueda T, Ishida Y, Yamamoto T, Ugawa S. Acid-sensing ion channels in taste buds. *Arch Histol Cytol* 2006; 69:227–31.
- Wemmie JA, Price MP, Welsh MJ. Acid-sensing ion channels: advances, questions and therapeutic opportunities. *Trends Neurosci* 2006; 29: 578–86.
- Immke DC, McCleskey EW. Lactate enhances the acid-sensing Na⁺ channel on ischemia-sensing neurons. *Nat Neurosci* 2001; 4:869–70.
- Chen CC, Zimmer A, Sun WH, Hall J, Brownstein MJ. A role for ASIC3 in the modulation of high-intensity pain stimuli. *Proc Natl Acad Sci U S A* 2002; 99:8992–7.
- Duan B, Wu LJ, Yu YQ *et al*. Upregulation of acid-sensing ion channel ASIC1a in spinal dorsal horn neurons contributes to inflammatory pain hypersensitivity. *J Neurosci* 2007; 27:11139–48.
- Zhang M, Gong N, Lu YG, Jia NL, Xu TL, Chen L. Functional characterization of acid-sensing ion channels in cultured neurons of rat inferior colliculus. *Neuroscience* 2008; 154:461–72.
- Hattori T, Chen J, Harding AM *et al*. Acid-sensing ion channels 2a and 3 heteromultimerize to form pH-sensitive channels in mouse cardiac dorsal root ganglia neurons. *Circ Res* 2009; 105:279–86.
- Lin YW, Cheng CM, Leduc PR, Chen CC. Understanding sensory nerve mechanotransduction through localized elastomeric matrix control. *PLoS ONE* 2009; 4:e4293.
- Akiba Y, Mizumori M, Kuo M *et al*. CO₂ chemosensing in rat oesophagus. *Gut* 2008; 57:1654–64.
- Faisy C, Planquette B, Naline E *et al*. Acid-induced modulation of airway basal tone and contractility: role of acid-sensing ion channels (ASICs) and TRPV1 receptor. *Life Sci* 2007; 81:1094–102.
- Berdiev BK, Xia J, McLean LA *et al*. Acid-sensing ion channels in malignant gliomas. *J Biol Chem* 2003; 278:15023–34.
- Su X, Li Q, Shrestha K *et al*. Interregulation of proton-gated Na(+) channel 3 and cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 2006; 281:36960–8.
- Ye JH, Gao J, Wu YN, Hu YJ, Zhang CP, Xu TL. Identification of acid-sensing ion channels in adenoid cystic carcinomas. *Biochem Biophys Res Commun* 2007; 355:986–92.
- Failla M, Crimi N, Vancheri C. Exhaled bronchial cysteinyl leukotrienes in allergic patients. *Curr Opin Allergy Clin Immunol* 2007; 7:25–31.
- Gratziau C, Rovina N, Makris M, Simoes DC, Papapetropoulos A, Roussos C. Breath markers of oxidative stress and airway inflammation in seasonal allergic rhinitis. *Int J Immunopathol Pharmacol* 2008; 21:949–57.
- Tai CF, Baraniuk JN. Upper airway neurogenic mechanisms. *Curr Opin Allergy Clin Immunol* 2002; 2:11–9.
- Ten RM, Pease LR, McKean DJ, Bell MP, Gleich GJ. Molecular cloning of the human eosinophil peroxidase. Evidence for the existence of a peroxidase multigene family. *J Exp Med* 1989; 169:1757–69.
- Bousquet J, Khaltayev N, Cruz AA *et al*. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008; 63 (Suppl. 86): 8–160. Review.
- Yagi J, Wenk HN, Naves LA, McCleskey EW. Sustained currents through ASIC3 ion channels at the modest pH changes that occur during myocardial ischemia. *Circ Res* 2006; 99:501–9.
- Light AR, Huguen RW, Zhang J, Rainier J, Liu Z, Lee J. Dorsal root ganglion neurons innervating skeletal muscle respond to physiological combinations of protons, ATP, and lactate mediated by ASIC, P2X, and TRPV1. *J Neurophysiol* 2008; 100:1184–201.
- Sheahan P, Walsh RM, Walsh MA, Costello RW. Induction of nasal hyper-responsiveness by allergen challenge in allergic rhinitis: the role of afferent and efferent nerves. *Clin Exp Allergy* 2005; 35:45–51.
- Thornton MA, Walshe P, Costello RW, McConn-Walsh R, Walsh MA. An alternative technique for nasal biopsy. *Laryngoscope* 2004; 114:1060–2.
- Walsh MT, Curran DR, Kingham PJ *et al*. Effect of eosinophil adhesion on

- intracellular signaling in cholinergic nerve cells. *Am J Respir Cell Mol Biol* 2004; **30**:333–41.
- 28 Riccio MM, Proud D. Evidence that enhanced nasal reactivity to bradykinin in patients with symptomatic allergy is mediated by neural reflexes. *J Allergy Clin Immunol* 1996; **97**:1252–63.
- 29 Godthelp T, Holm AF, Fokkens WJ *et al.* Dynamics of nasal eosinophils in response to a nonnatural allergen challenge in patients with allergic rhinitis and control subjects: a biopsy and brush study. *J Allergy Clin Immunol* 1996; **97**:800–11.
- 30 Coakley RD, Boucher RC. Regulation and functional significance of airway surface liquid pH. *JOP* 2001; **2**(Suppl. 4):294–300.
- 31 Tarran R. Regulation of airway surface liquid volume and mucus transport by active ion transport. *Proc Am Thorac Soc* 2004; **1**:42–6.
- 32 Boucher RC. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med* 2007; **58**:157–70.
- 33 Morgan RK, Costello RW, Durcan N *et al.* Diverse effects of eosinophil cationic granule proteins on IMR-32 nerve cell signaling and survival. *Am J Respir Cell Mol Biol* 2005; **33**:169–77.