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Chlorine inhalation produces nasal airflow limitation in allergic rhinitic subjects without evidence of neuropeptide release

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Abstract

Background Seasonal allergic rhinitic (SAR) subjects are more physiologically reactive to airborne irritants than non-rhinitic (NR) subjects; however the mechanism underlying this difference is unclear. *Objective* We sought to determine whether irritant-induced nasal airflow limitation involves neuropeptide release into nasal lining fluid, and if so, whether such release occurs differentially by rhinitic status. *Methods* Eight SAR and 8 NR subjects were exposed to 1.0 ppm chlorine and filtered air in random order during separate visits; exposures were via nasal mask and lasted 15 min. Rhinomanometry was performed before, immediately post-, and 15 min post-exposure. Following a minimum of 2 weeks' time, exposures and symptom reporting were repeated with nasal lavage pre- and post-exposure. Neuropeptides (substance P, cacitonin gene-related protein, vasoactive intestinal peptide, and neuropeptide Y) as well as markers of plasma leakage (albumin and urea) and glandular secretion (lysozyme and 7F10-mucin) were measured using standard methods. *Results* Cl_2 provocation significantly increased nasal airway resistance in SAR but not NR subjects ($p < 0.05$). Neuropeptide levels in nasal lavage fluid, on the other hand, were unaffected, with the exception of a paradoxical increase in vasoactive intestinal peptide in non-rhinitic controls post-Cl_2 provocation. *Conclusions* Irritant-induced nasal airflow limitation is more pronounced among SAR than NR subjects. We could not, however, demonstrate a role for neuropeptide release in the nasal congestive response of SAR subjects.
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1. Introduction

Upper respiratory tract symptoms, including mucous membrane (eye, nose and throat) irritation, subjective nasal congestion (sensation of airflow limitation) and rhinorrhea, are highly prevalent in so-called "problem buildings" (Apter et al., 1994; Fisk et al., 1993; Mendell, 1993; Kreiss, 1989; Hodgson, 2000). In indoor environments these symptoms have been linked to extremes of temperature and humidity, as well as to air pollutants

including environmental tobacco smoke (ETS), volatile organic compounds (VOCs), and reactive cleaning products (such as chlorine and ammonia). Interestingly, in a number of field studies symptom reporting is elevated among individuals with pre-existing allergic disorders, even in the absence of identified indoor aeroallergen problems (Brasche et al., 2001; Cummings et al., 1991; Hall et al., 1993; Lundin, 1999).

Despite both clinical similarities and epidemiologic links between irritant-associated upper respiratory tract symptoms and the manifestations of allergic rhinitis, evidence points to non-allergic mechanisms in their pathogenesis. Bascom and colleagues, for example, showed that subjects with a history of reactivity to ETS (and coincidentally with an increased prevalence of skin test reactivity) had greater increases in nasal airway resistance (NAR) after experimental ETS exposure than did controls. However, these same subjects did *not* show evidence of mast cell degranulation (histamine, kinins, TAME esterase) or plasma leak (albumin) in nasal lavage fluid. The authors concluded that non-allergic (possibly neurogenic) mechanisms may be operative in the congestive response to nasal irritant provocation (Bascom et al., 1991; Bascom, 1992).

We have previously utilized chlorine gas (0.5–1.0 ppm in air; administered by nasal mask) as a model irritant for the upper airway, and have documented objective nasal congestion (airflow limitation) using rhinomanometry. Using this experimental system, we showed that: (1) subjects with seasonal allergic rhinitis (SAR) have a greater increase in NAR after chlorine inhalation than non-rhinitic (NR) subjects; (2) this differential response was not affected by cholinergic blockade; and (3) no evidence of mast cell degranulation accompanied the objective congestive/obstructive response (Shusterman et al., 1998, 2002, 2003). In order to better understand the mechanism(s) involved in the nasal congestive response to chemical irritants, we examined the role of neuropeptides in this phenomenon. We exposed 16 subjects – evenly divided by gender and allergy status – to dilute Cl_2 gas, and irritant-related nasal airflow limitation was measured. The experiment was then repeated on the same subjects, and neuropeptides in nasal lavage fluids were measured as markers of afferent nociceptive nerve axon responses and/or activation of central nervous system reflexes.

2. Materials and methods

2.1. Study subjects

Subjects were recruited through posters and newspaper advertisements. Inclusion criteria were: age 18–69 years. and "general good health." Exclusion criteria were: (1) a history of asthma, (2) cigarette smoking (active or within previous 6 months), (3) pregnancy or lactation, (4) a history of severe allergic reactions (anaphylaxis or angioedema), and (5) continuous therapy with medications having antihistaminic side effects (e.g., tricyclic antidepressants). Subjects read and signed an informed consent document approved by the Committee on Human Research of the University of California, San Francisco. Questionnaires were administered to each potential subject, and subjects were classified as having seasonal allergic rhinitis (SAR), no rhinitis (NR), or "other" (including perennial allergic rhinitis).

Allergy skin prick tests for 16 regionally common aeroallergens, plus saline and histamine controls were then administered. For purposes of this study, "seasonal allergic rhinitis" (SAR) was defined as: (1) a history of seasonally occurring sneezing, nasal pruritus, rhinorrhea, post-nasal drip, and/or subjective nasal congestion, with or without known precipitants; and (2) skin test reactivity to seasonally occurring agents from the panel that corroborated the history. "Skin test reactivity" was defined as one or more wheal reactions to skin-prick testing with a diameter $\geqslant$ the histamine control. Subjects with reactivity to perennial allergens (e.g., dust mites, animal danders) were included in the "rhinitic" group if allergen control methods rendered them asymptomatic during some portion of a typical day. "Non-rhinitics" were defined as subjects who report, at most, infrequent nasal symptoms, without identified seasonal variation or precipitants, with significant skin test reactivity to zero or one agent in the panel of 16 aeroallergens, and with normal findings on anterior rhinoscopy. Prior to skin testing, subjects were asked to refrain from taking antihistamines for 72 h (hydroxyzine for 3 weeks).

2.2. Provocation experiments

The study design was experimental, utilizing a semi-randomized cross-over design comparing the effect of dilute chlorine gas with that of air (Fig. 1). Two different endpoints, NAR by active posterior rhinomanometry and neuropeptide concentrations in nasal lavage fluid, were ascertained in separate subexperiments in order to avoid artifactual effects of lavage on NAR. Each Cl_2 provocation subexperiment involved the same 16 subjects – 8 SAR and 8 NR controls – who were tested on a total of four occasions each. The concentration and duration of Cl_2 exposure – 1.0 ppm times 15 min – is the US occupational short-term exposure limit, and hence is of both scientific and regulatory interest. SAR and NR subgroups were evenly divided by gender.

The two subexperiments were separated by at least two weeks. The first subexperiment, involving two exposures one week apart, used rhinomanometry to measure NAR at baseline, immediately after, and 15 min post-exposure. The second subexperiment, (again, two exposures, one
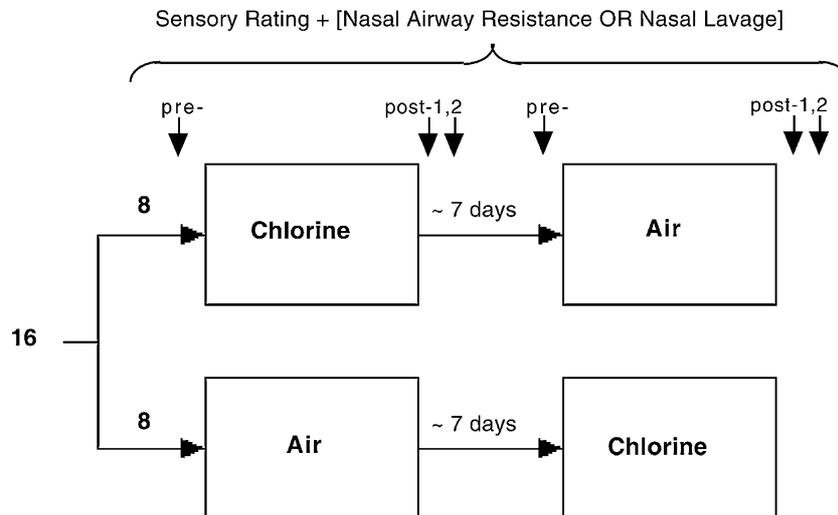
Sensory Rating + [Nasal Airway Resistance OR Nasal Lavage]

Fig. 1. Study design. For each sub-experiment (rhinomanometry and nasal lavage), subjects breathed either Cl_2 (1.0 ppm in medical grade air) or pure medical grade air by nasal CPAP mask in two separate sessions approximately one week apart. The order-of-exposure (i.e., Cl_2 or air first) was counterbalanced within each subgroup (stratified by gender/allergic rhinitic status).

week apart), involved nasal lavage, performed at baseline, immediately after, and 15 min post-exposure. On a given day, exposure was either to pure (medical grade) air or to chlorine diluted in air. The order of exposure within each pair of testing dates was determined by limited randomization so that equal numbers of subjects were exposed to air or Cl_2 first.

SAR subjects were tested out of their relevant pollen/symptom season. All subjects were asked to avoid exercising, consumption of spicy foods, and use of scented cosmetics on the day of testing. In addition to antihistamine preclusion (as specified for skin prick testing), subjects were asked to avoid using nasal steroids for at least 2 weeks, and nasal decongestants for at least 48 h prior to testing. Upon arrival at the laboratory, subjects entered a climate-controlled chamber (22 ± 1 °C; $40 \pm 3\%$ relative humidity) with filtered air (activated charcoal and high-efficiency particulate).

For both subexperiments, Cl_2 or air was administered on a single-blinded basis for a period of 15 min. The dilution apparatus has previously been described in detail (Shusterman et al., 1998). Chlorine concentrations were measured in real-time using an electrochemical monitor (Model 1340; Interscan Corp., Chatsworth, CA), which was re-calibrated on a daily basis using the certified contents of the chlorine cylinder as the standard. Exposures were via nasal CPAP mask (Series 3121; Respironics, Inc., Murraysville, PA).

Immediately prior to exposure, at five-minute intervals during exposure, and again 15 min post-exposure, symptoms were rated using computer-based visual analog scales (VAS) using a mouse pointing device, as previously detailed (Shusterman et al., 1998). Values were recorded directly to computer file using digital data acquisition software (LabView, National Instruments, Austin, TX).

2.3. Rhinomanometry subexperiment

NAR for each testing condition was taken as the mean of three values, as ascertained by active posterior rhinomanometry using a commercial instrument (Model NR6-2, GM Instruments, Kilwinnig, UK). NAR was calculated using the pressure-cutoff method (75 Pa), and was obtained at baseline, immediately post-exposure, and 15 min post-exposure on both Cl_2 and air days. Calibration procedures were employed as previously detailed (Shusterman et al., 1998). The hypothesis to be tested was that SAR subjects would show a significantly greater increase in NAR over baseline than would NR controls, comparing chlorine- vs. air-exposure days.

2.4. Nasal lavage subexperiment

To obtain nasal lavage specimens, each nostril was slowly instilled with 2.5 mL of 37 °C 0.9%, sterile, pyrogen-free, nonbacteriostatic saline (5 mL total). After a ten second retention time, fluid was expelled into a cup. Two baseline samples were obtained on each subject, one before and one after a "cleansing" lavage (a total of three 10-mL boluses). Two additional samples were obtained, one immediately after, and the other 15 min after exposure. Samples were weighed, pipetted to homogenize the gel and sol phases, then centrifuged at 960g for 15 min, and the supernatant aliquoted for analysis of neuropeptides and biochemical markers. Specimens for biochemical markers were immediately frozen at −80 °C. Specimens for neuropeptide analysis had an equal volume of 50% ethanol, 50% 0.2 N acetic acid, 0.2% sodium bisulfite added, were stored overnight at −20 °C, then were centrifuged at 960g for 30 min to

remove precipitated proteins (presumably including proteases) and frozen at -80 °C. Once complete, both sets of specimens were transported by overnight courier in a frozen state to the Division of Rheumatology, Immunology and Allergy, Georgetown University, for neuropeptide and biochemical marker analysis.

Neuropeptide specimens were thawed at 4 °C. An equal volume of 1% trifluoroacetic acid was added. Samples were centrifuged and the supernatant poured over Sep-Pak C-18 cartridges that had been prewashed with 100% acetonitrile, followed by three washes of 1% trifluoroacetic acid. Peptides were eluted with 1% trifluoroacetic acid, 60% acetonitrile. The eluates were dried by Speed Vac (Savant) and reconstituted in radioimmunoassay (RIA) buffer. RIA for substance P (SP), calcitonin gene-related protein (CGRP), vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY) were conducted using kits according to manufacturer's instructions (Peninsula Laboratories, Belmont, CA) (Baraniuk et al., 1990a,b,c, 1991, 1999; Mosimman et al., 1993).

Total protein was measured by an adaptation of the Lowry method using 10-µl samples, 96-well microtitre plates, and an enzyme-linked immunosorbent assay (ELISA) plate reader (Ali et al., 1996; Baraniuk et al., 1994, 1998; Naranch et al., 2002). Urea (blood urea nitrogen kit; Sigma, St. Louis, MO), lysozyme (Micrococcus lysodicticus cell wall degradation), albumin (immunomicroaggglutination assay, and 7F10-mucin (ELISA)) were measured by previously published methods (Baraniuk et al., 1991, 1994). Concentrations of these four analytes were examined as both crude values and divided by total protein concentrations in order to normalize across specimens.

The hypotheses tested included: (1) concentrations of vasodilatory neuropeptides (SP, CGRP, and VIP) in NL fluid will be significantly increased after Cl_2, but not air, exposure, and this effect will be more pronounced among allergic rhinitic than control subjects; (2) the vasoconstrictor neuropeptide, NPY, will be significantly *decreased* after Cl_2, but not air, exposure, and this effect will be more pronounced among allergic rhinitic than control subjects; (3) markers of plasma leakage (albumin), transepithelial flux of interstitial fluid (urea), and glandular secretion (lysozyme, 7F10-mucin) will be increased after Cl_2, but not air, exposure.

2.5. Analysis

Based upon our earlier work, we determined that a sample size of 8 SAR and 8 NR subjects provided 80% power to demonstrate a 20% difference in exposure-related objective nasal congestion (i.e., increase in NAR) between subgroups (Shusterman et al., 1998). In order to normalize different subjects' baseline NAR values (and to allow for day-to-day changes in baseline

NAR for a given subject), proportional changes in NAR were studied throughout. This metric took the form of "*proportional change in NAR*" (from daily baseline). To show a treatment effect, the proprotional change in NAR on the air day was subtracted from that on the chlorine day, yielding "*net proportional change in NAR*". This latter later metric was then compared, on a group basis, between SAR and NR subjects. For statistical purposes, biochemical results that were below the level-of-detection were treated as one-half of the level-of-detection.

For each statistical comparison, data were first examined for normality. Testing then proceeded using either ANOVA or a non-parametric (i.e., Wilcoxon rank sum) test, as indicated. All tests were 2-tailed, with statistical significance defined as $p < 0.05$. Calculations were performed on a MacIntosh G4 computer (Apple Computers, Cupertino, CA) using JMP statistical software (SAS Institute, Carey, NC).

3. Results

3.1. Subject demographics

Sixteen subjects completed the two chlorine provocation substudies; their demographic characteristics are summarized in Table 1.

3.2. Irritant provocation

3.2.1. Subjective symptoms/sensations

Symptoms are reported here for the rhinomanometry sub-experiment only. Subjectively rated nasal irritation, congestion, and odor were all quite modest during and after Cl_2 exposure. The mean delta [from baseline] VAS ratings for odor and irritation at the end of the 15-min. exposure, for example, were less than "slight" (i.e., <1 on a scale of 0–5), and subjective nasal congestion actually appeared to decrease with time (Fig. 2). Of these apparent trends, however, only odor ratings differed significantly from zero ($p < 0.05$). There was no significant differential symp-

Table 1
Characteristics of participants

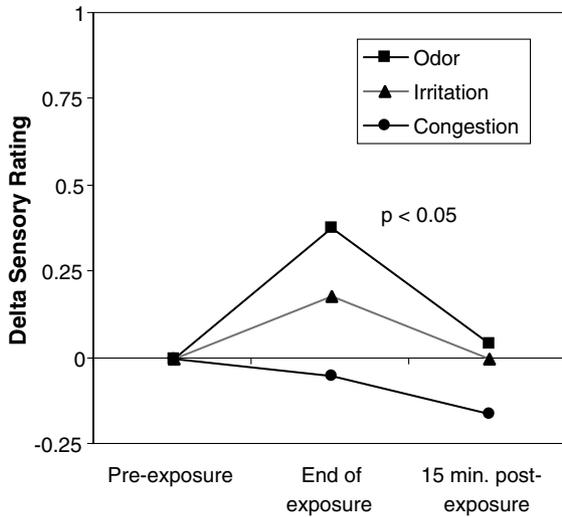|	No.	Mean age (range)
Males		
Rhinitic	4	30.3 (21–39)
Non-rhinitic	4	33.5 (23–51)
Females		
Rhinitic	4	31.0 (26–43)
Non-rhinitic	4	26.3 (21–38)

Fig. 2. Subjective rating by visual analog scale of Cl_2-related odor, nasal irritation, and nasal congestion: pre-exposure, at the conclusion of exposure, and 15 min post-exposure (all subjects combined). Values represent the mean change from baseline ("delta" value). 0–5 scale, where 0, none; 1, slight; 2, moderate; 3, strong; 4, very strong; 5, overpowering.
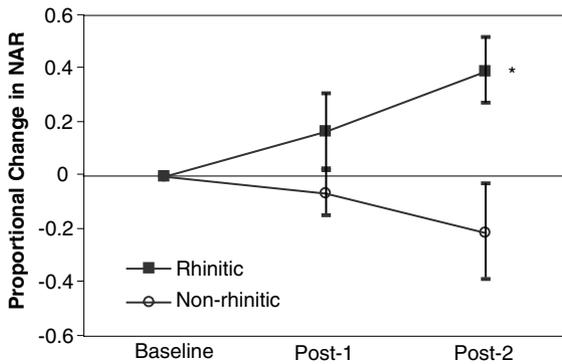

Fig. 3. Net proportional change in nasal airway resistance (NAR), pre- to post-exposure, Cl_2 minus air session. At 15 min post-exposure, seasonal allergic rhinitics congested significantly more to Cl_2 than did non-rhinitic controls.

tom rating by rhinitis status or gender (data not shown).

3.3. Objective endpoints: NAR and nasal lavage biochemical markers

As a group, SAR subjects showed greater exposure-related increases in NAR than did NR controls. The controls tended to decongest after Cl_2 exposure (Fig. 3). This difference reached statistical significance at 15 min post-exposure ($p < 0.05$). Cl_2 inhalation, in general, produced no significant changes in neuropeptide concentrations in nasal lavage fluid (see Fig. 4 for representative results). The single exception was VIP, which increased post-Cl_2 in non-rhinitic controls only ($p < 0.05$ immediately after and 15 min after provocation; Fig. 5). The biochemical markers albumin, urea, lysozyme, and 7F10-mucin, did not vary systematically by exposure or rhinitic status (data not shown); this finding did not change when data were normalized for total protein concentration in NL specimens (data not shown).

4. Discussion

As in our earlier studies, SAR subjects showed an augmented tendency to congest nasally in response to irritant provocation (Cl_2 vs. air) relative to control (NR) subjects (Shusterman et al., 1998, 2002, 2003). Repetition of the provocation protocol after a suitable interval failed to show consistent evidence of Cl_2-induced neuropeptide release. The exception was the elevation in VIP in the 15 min after Cl_2 exposure in the non-rhinitic controls only. VIP would be expected to produce vasodilation and consequently increase NAR (Baraniuk, 1998), a parameter that actually decreased in controls. The reason for this discrepancy is unknown,
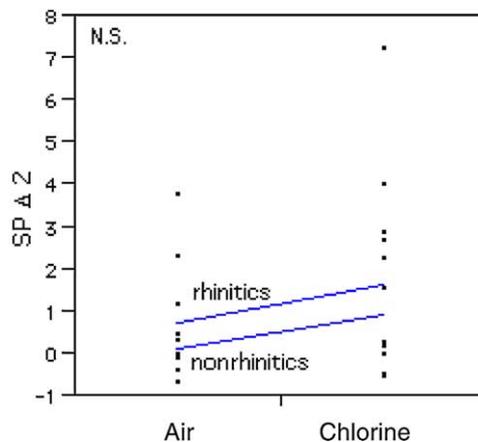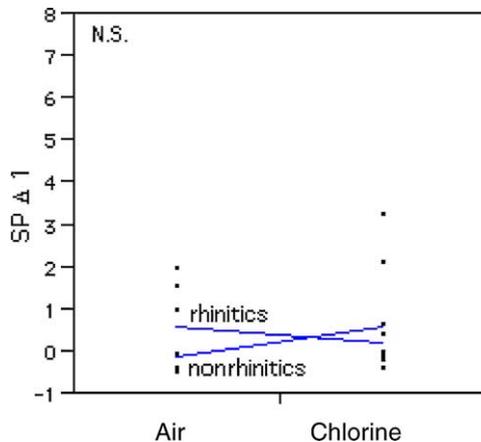

Fig. 4. Change in nasal lavage substance P (SP) concentrations (in picomoles/litre), pre- to post-exposure, by exposure condition and rhinitis status, immediately post-exposure ("SP Δ 1") and 15 min post-exposure ("SP Δ 2"). Neither exposure condition nor rhinitis status significantly predicted changes in nasal lavage SP levels.
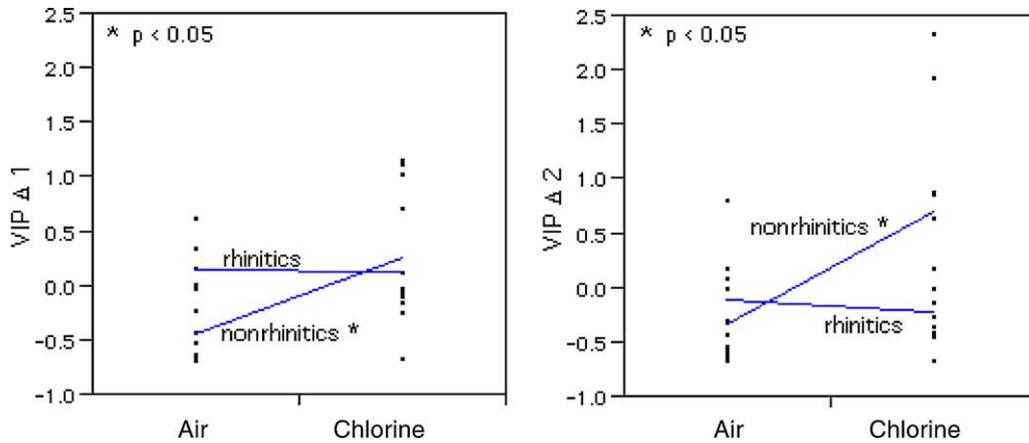
Fig. 5. Change in nasal vasoactive intestinal peptide (VIP) concentrations (in picomoles/litre), pre- to post-exposure, by exposure condition and rhinitis status, immediately post-exposure ("VIP Δ 1") and 15 min post-exposure ("VIP Δ 2"). For both sampling times, non-rhinitic controls alone showed a significant increase in nasal lavage VIP levels after Cl_2 provocation ($p < 0.05$).

but could imply the operation of a counter-regulatory reflex.

A potential limitation of our study was the lack of a positive internal control (e.g., demonstration of neuropeptide release after capsaicin or hypertonic saline challenge within the same subjects). However, the laboratory analyzing our specimens previously documented SP elevations in nasal lavage fluid after irritant [hypertonic saline] challenge with similar specimen collection and handling procedures, in essence constituting a positive external control (Baraniuk et al., 1999).

To put our results in context, a schematic diagram illustrating potential pathophysiologic mechanisms of irritant-induced nasal airflow obstruction appears in Fig. 6. Of the alternative response mechanisms listed,

mast cell degranulation was first evaluated using environmental tobacco smoke (Bascom et al., 1991), with the finding that histamine levels in nasal lavage fluid did not rise in subjects who congested after ETS exposure. Koskela et al. (2000) similarly found that dry mannitol powder, a noxious stimulus that produced nasal airflow obstruction in allergic rhinitic subjects, did not release tryptase, a mast cell marker. One of our Cl_2 studies (Shusterman et al., 2003) also showed a lack of involvement of mast cell degranulation based upon unchanged tryptase concentrations in nasal lavage fluid. Of note, this study included a positive (antigen challenge) control condition, demonstrating that the specimen collection procedures and tryptase assay were of sufficient sensitivity to detect a biological effect. Thus,
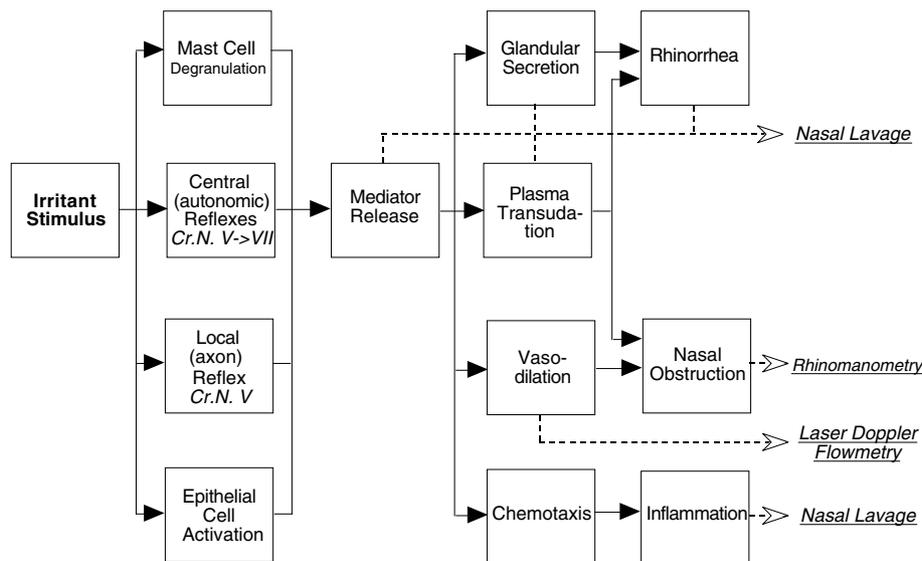

Fig. 6. Potential pathophysiologic mechanisms involved in the acute upper airway obstructive response to irritant inhalants, along with available study techniques.

a total of three studies using distinct irritant stimuli have exonerated the mast cell as a participant in the acute nasal obstructive response to irritant provocation.

In terms of potential cholinergic parasympathetic reflexes, our chlorine study using pretreatment with the cholinergic antagonist *ipratropium bromide* (IB) showed no evidence of parasympathetic involvement (Shusterman et al., 2002). By contrast, McLean et al. (1979) found that ammonia vapor-induced nasal congestion was prevented by topical administration of atropine. One possible reason for these discrepant results may be that atropine is readily absorbed into the systemic circulation, whereas IB is a charged molecule that is only active in the superficial tissues.

In terms of neuropeptide release, in this experiment Cl_2 caused nasal airflow obstruction in the AR group, but SP and CGRP were not increased, suggesting that local axon responses were not recruited. Additionally, AR subjects did not show an increase in parasympathetic peptidergic (VIP) or a decrease in sympathetic peptidergic (NPY) release. Our negative results with SP are consistent with those of Koskela et al. (2000) who, as noted above, examined mediators associated with mannitol-induced nasal obstruction. On the other hand, Baraniuk et al. (1999) did see a rise in nasal lavage SP levels after noxious hypertonic saline challenge, but no change in nasal caliber by acoustic rhinometry. However, Baraniuk's subjects were limited to non-rhinitics, who have shown no decrease in nasal caliber in our studies or those of Koskella.

In terms of secondary evidence of response mechanism, the lack of increase for markers for either plasma extravasation (albumin and urea) or glandular secretion (lysozyme or mucin) after Cl_2 provocation imply that observed changes in nasal patency may occur due to vascular engorgement rather than plasma extravasation or increased airway secretions. More direct evidence of stimulus-related blood flow changes was obtained by Rangi et al. (1990a,b), who observed changes in nasal mucosal blood flow using laser Doppler flowmetry after either CGRP or antigen challenge. However, attempts to correlate nasal mucosal blood flow with NAR have been variably unsuccessful, perhaps due to the complexity of the multicompartment vasculature of the nose (Kurita et al., 1988; Ohki et al., 1987; Wight and Cochrane, 1990; Witek et al., 1992). Thus, the proximate physiologic event(s) underlying irritant-induced nasal airflow obstruction remain in question.

If neither mast cell degranulation nor neurogenic reflexes (central or peripheral) are responsible for irritant-induced nasal airflow obstruction, then one alternative might be via epithelial cell activation. As noted above, Koskela et al. (2000) compared the response of allergic rhinitic and control subjects to dry mannitol powder, and found that the former group had augmented sensory and congestive responses. In

that study, the arachidonic acid metabolite 15-hydroxyeicosatetraenoic acid (15-HETE, a marker of epithelial cell activation) was elevated. These findings suggest that rapidly acting mediators derived from epithelial cells may be responsible for the acute congestive response caused by some nasal irritants. This hypothesis will provide a future focus for our research.

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