Hypertonic Saline Nasal Provocation Stimulates Nociceptive Nerves, Substance P Release, and Glandular Mucous Exocytosis in Normal Humans

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Hypertonic saline (HTS) induces bronchoconstriction. Potential mechanisms were evaluated in a human nasal provocation model. Aliquots of normal saline (1 × NS, 100 μl) and higher concentrations (3 × NS, 6 × NS, 12 × NS, 24 × NS) were sprayed into one nostril at 5-min intervals. Lavage fluids were collected from the ipsilateral and contralateral sides to determine the concentrations of specific mucus constituents. Nasal cavity air-space volume was assessed by acoustic rhinometry (AcRh). The distribution of substance-P-preferring neurokinin-1 (NK-1) receptor mRNA was assessed by *in situ* reverse transcriptase–polymerase chain reaction. Unilateral HTS induced unilateral dose-dependent increases in sensations of pain, blockage, and rhinorrhea, the weights of recovered lavage fluids, and concentrations of total protein, lactoferrin, mucoglycoprotein markers, and substance P. Contralateral, reflex-mediated effects were minor. There were no changes in IgG or AcRh measurements. NK-1 receptor mRNA was localized to submucosal glands. HTS caused pain with unilateral substance P release. The presumed nociceptive nerve efferent axon response led to glandular exocytosis, presumably through actions on submucosal gland NK-1 receptors. Vascular processes, including plasma exudation, filling of venous sinusoids, and mucosal edema were not induced in these normal subjects. Baraniuk JN, Ali M, Yuta A, Fang S-Y, Naranch K. Hypertonic saline nasal provocation stimulates nociceptive nerves, substance P release, and glandular mucous exocytosis in normal humans.

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Hypertonic saline (HTS) is an airway irritant that induces bronchospasm in asthmatics (1, 2). The mechanism is unclear, but it may involve neurogenic responses (1, 3). According to this hypothesis, hypertonic changes in the epithelial lining fluid lead to activation of nociceptive sensory nerves, with the release of neuropeptides such as substance P (SP) and others by the axon response mechanism in the airway mucosa and the peribronchial ganglia, as well as at the central nervous system terminations of the primary afferent axons. In the mucosa, neuropeptides may induce glandular secretion, vascular permeability, vasodilatation, smooth muscle contraction, cytokine production, and endothelial cell adhesion molecule upregulation with increased influx of inflammatory leukocytes (3–5). Although these functions have been demonstrated in animal models (6, 7) and *in vitro* (8), it has been difficult to determine if nociceptive nerve axon responses (9), neuropeptide release (10), or neurogenic inflammation (11, 12) occur in human airways. In part, this is because of the complexity of the tracheobronchial nociceptive and parasympathetic innervation and inaccessibility of these airways to intensive analysis of specific inflammatory mechanisms.

To overcome these obstacles, we investigated the effects of HTS in human nasal mucosa. The nasal mucosa shares many common anatomic features with the lower airways, but it differs in its mechanism for obstructing airflow: smooth muscle contraction predominates in the trachea and bronchi, whereas vasodilation of the nasal erectile tissues thickens the nasal mucosa (3, 13). HTS provokes pain (9, 14), an effect that is ablated by pretreatment with capsaicin (15). The newly cloned capsaicin receptor may be involved (16).

Unilateral HTS nasal provocations were performed in normal subjects to detect both the direct effects on the ipsilateral (IL) side and parasympathetic reflexes on the contralateral (CL) side (13). In order to determine if vascular or glandular secretory responses were induced, nasal lavages were performed, and the concentrations of total protein, IgG (vascular leak), lactoferrin (serous cells), Alcian blue staining mucin (ABSM, mucous cells), and 7F10-mucin (mucoglycoconjugate marker) were measured (13, 17–19). Symptoms of nasal pain intensity, pain duration, obstruction to airflow ("block"), and rhinorrhea ("drip") were assessed on the two sides by linear analog scales. Acoustic rhinometry (AcRh) was used to assess changes in nasal patency (15, 19, 20).

SP concentrations were measured (10), and the distribution of SP-preferring neurokinin type 1 (NK-1) receptors (3) was

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determined by *in situ* reverse transcriptase polymerase chain reaction (IS-RT-PCR) (21).

METHODS

Subjects for Nasal Provocations

Normal subjects gave informed consent to participate in this IRB approved study. Exclusion criteria included active rhinitis of any type, history of allergic rhinitis or nasal polyps, upper respiratory tract infection or sinusitis in the previous 6 wk, use of antihistamines, antidepressants with anticholinergic or antihistaminic properties, oral or nasal decongestants, any formulation of glucocorticoid drug, and any chronic disease that could interfere with airway mucosal and neural responses. Nasal lavage was performed in 29 subjects and acoustic rhinometry was performed in 10 additional subjects.

Hypertonic Saline Nasal Provocation

Subjects' nasal cavities were prewashed with 24 sprays (100 μl each) of sterile normal saline (1 × NS, 0.9% NaCl) using a Beconase AQ pump aspirator spray device (Glaxo-Wellcome, Research Triangle Park, NC) (17, 18). Subjects gently exhaled through their noses to expel the lavage fluid from both nostrils into a 5-ounce Dixie wax-paper cup (James River Corp., Norwalk, CT). This was repeated 10 min later using 12 sprays of 1 × NS. Immediately after this, unilateral hypertonic saline (HTS) nasal provocations were performed. 1 × NS (100 μl) was administered into the right, ipsilateral (IL) nostril, and 1 × NS (100 μl) was administered into the left, contralateral (CL) nostril as a control. Five minutes later, the subjects pressed their left nostrils closed, and then spritzed 12 sprays of 1 × NS into their right nostrils. Lavage fluid was gently blown out into a cup. The left nostril was then separately lavaged. This cycle was immediately repeated with 3 × NS (2.7% NaCl), then with 6 × NS (5.4% NaCl), and 12 × NS (10.8% NaCl), and then with 24 × NS (21.6%) into the IL nostril, and 1 × NS into the CL control nostril. Lavage fluids were gently shaken to disperse mucous globules, pipetted into preweighed Eppendorf tubes, and the weights of the right and left lavage fluid effluents were determined. Samples were frozen at −20° C until assays were performed.

The pH of HTS solutions freshly prepared with double distilled deionized water were 1 × NS at pH 5.79, 3 × NS at pH 5.69, 6 × NS at pH 5.78, 12 × NS at pH 5.70, and 24 × NS at pH 6.07.

Symptoms. Five minutes after each provocation, subjects scored the severity of their nasal pain using 10-cm linear analog scales and assessed the duration of these sensations. Sensations of nasal blockage and drip on the IL and CL sides were separately scored using 10-cm linear analog scales.

Lavage Fluid Assays

Total protein was measured by an adaptation of the Lowry method using 10-μl samples, 96-well microtitre plates, and an ELISA plate reader (17–19). IgG, lactoferrin, and 7F10-mucin were measured by previously published ELISA methods (8, 17–19). Alcian blue staining material was slot-blotted onto nylon membranes, stained with Alcian blue, and detected by colorimetric means (17).

Hypertonic Saline Nasal Provocations for SP Collection

Prewash, provocation, and lavage methods were the same as above except that 1 × NS, 6 × NS, and 24 × NS were applied unilaterally, and lavage was performed after 3 min. Immediately after collection, lavage fluids were gently shaken to disperse mucous globules, and a 50-μl aliquot was rapidly removed and stored at −20° C for total protein assay.

Radioimmunoassay

Specimens were thawed at 4° C, then 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, then three washes of 1% trifluoroacetic acid. Peptides were eluted with 1% trifluoroacetic acid, 60% acetonitrile, concentrated by Speed Vac (Savant Instruments, Farmingdale, NY) and reconstituted in radioimmunoassay (RIA) buffer (10). SP concentrations were measured by RIA (Peninsula Laboratories, Belmont, CA). The ratio of SP to total protein was determined for each specimen. SP immunoreactive material has been previously characterized by HPLC in this tissue (8).

Hypertonic Saline Nasal Provocations for Acoustic Rhinometry

The Eccovision Acoustic Rhinometry System (E. Benson Hood Laboratories, Pembroke, MA) was used to assess the geometry of the nasal cavity. Subjects were seated in a straight-backed chair and looked forward keeping their head level while the acoustic rhinometry (AcRh) tests were conducted (19, 20). Subjects stopped breathing for approximately 12 s for each AcRh measurement. The acoustic wave tube with a disposable nose tip was placed over the exterior nares to form an acoustic seal. The wave tube was activated, and the acoustic wave pattern was recorded once it had stabilized for at least 5 s.

The unilateral hypertonic saline provocations for AcRh were the same as for the secretion analysis with the following exceptions. The lavages were omitted. An initial dose of 100 μl 1 × NS was applied bilaterally. Baseline AcRh measurements were made for the IL and CL nostrils every 5 min during over the next 30-min period. AcRh was measured before each HTS provocation dose ("Pre"), and then 1-, 3-, and 5-min later. Subjects then blew any secretions from their nostrils and repeated the AcRh 10 to 20 s later ("Blow"). Immediately after AcRh they received their next dose of HTS.

Wave patterns were graphically displayed as the cross-sectional area of the nasal cavity as a function of distance from the end of the wave tube. These data were analyzed to show changes in the minimum cross-sectional area (Amin), which corresponds to the site of maximal resistance to nasal airflow near the anterior tip of the inferior turbinate, and the volume of the cavity from the site of Amin to a point 6 cm posterior (V6). HTS dose-dependent effects were assessed by calculating changes in V6 and Amin between different time points. "Pre – 5 min" assessed the overall changes caused by the HTS provocation. The timing of changes within this 5-min period were assessed for the first 3 min ("Pre – 3 min") and for the fourth and fifth minutes ("5 min – 3 min"). Changes caused by blowing the nose, including clearance of secretions, were assessed by the "Blow – 5 min" calculation.

In order to determine if blowing altered AcRh measurements, six subjects followed the same provocation protocol but received challenges with 1 × NS only. Blowing had no effect on AcRh measurements (data not shown). Therefore, changes in volume or Amin were the result of secretion or swelling of the nasal cavity wall.

IS-RT-PCR

IS-RT-PCR permits direct incorporation of digoxigenin-dUTP into reversely transcribed, PCR amplified transcripts in tissue sections on microscope slides (21).

NK-1 Receptor Primers

Unique NK1 receptor mRNA (22) primers that did not share sequence homology with NK2, NK3, or other receptor gene sequences (23, 24) were chosen: sense primer 5′GAGCATCCGAACAAGATTTATG from *exon 2* (amino acids 186-194), and RT/antisense primer 5′CTTCAGGTAGAGATCTGGGTT from *exon 4* (amino acids 276-282) (Lombardi Cancer Center Core Facility, Georgetown University, Washington, DC).

Tissue

Human inferior turbinates were obtained from three nonatopic subjects undergoing sphenopalatine surgery. Human main-stem bronchi were obtained at the time of surgical resection for neoplasia. Tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline for 16 h and then embedded in paraffin. Sections (6 μm) were cut onto silinated slides.

Pepsinogen and DNAse Step

Pairs of slides from the same block were used for parallel positive and negative studies. Sections were dewaxed in xylene for 20 min, placed in 100% ethanol for 5 min, and then air-dried. Pepsinogen (1 mg/ml in

0.01 N HCl; Sigma Chemical, St. Louis, MO) was placed on each section (12.5 μl per section) for 8 min at room temperature in a humidified chamber (SciMedx). Proteolysis was stopped by neutralizing the solution with 20 μl 10× DNase buffer (1 M sodium acetate, 0.5 mM $MgSO_4$, at pH 5.0), DEPC water (90 μl), 20 μl DNase stock solution (10^4 U/ml; Boehringer Mannheim, Indianapolis, IN), and 1 μl RNase Inhibitor (20 U/L) were added to the fluid above the tissue section, and the slide was incubated in a humidified chamber for 60 min at 37° C. The DNase was denatured by heating for 5 min at 99° C on a preheated Perkin-Elmer Thermocycler block (Perkin-Elmer Medical Instruments, Pamona, CA). Slides were washed in 0.1 M TRIS and 0.1 M NaCl at pH 7.5 for 3 min, then dehydrated by passage through 70%, then 100% ethanol, before air-drying.

Reverse Transcriptase Step

Reagents (Perkin-Elmer) were mixed in the following proportions at 4° C: 3 μl DEPC water, 4 μl 25 mM $MgCl_2$, 2 μl 10× PCR buffer, 2 μl each of 10 mM ATP, TTP GTP, and CTP (200 μM final concentrations), 1 μl 15 μM antisense NK1 primer, 1 μl RNase inhibitor (20 U/ml), and 1 μl RT (50 U/μl). Slides were preheated to 42° C for 30 to 60 s on the Perkin-Elmer Thermocycler, and 20 μl of RT solution were added per tissue section. Solutions were covered with HybriSlips (Research Products International, Mount Prospect, IL), then the slides were flooded with mineral oil and incubated at 42° C for 60 min. Selective pairing of NK1 selective primers and NK1 receptor mRNA was promoted by this approach. At the end of 60 min, the reverse transcriptase (RT) was denatured at 99° C for 5 min, the oil-covered slides were cooled to 60° C, and the oil was washed off in xylene twice, acetone, 100% ethanol, and air-dried. Negative control slides were prepared without RT.

First Polymerase Chain Reaction Step

Reagents were mixed at 4° C in the following proportions: 2 μl 10 × buffer, 4 μl 25 mM $MgCl_2$ (final concentration, 4.5 mM), 2 μl each of 10 mM ATP, GTP, CTP, and TTP, 2 μl of 15 μM antisense/RT primer, 2 μl 15 μM sense primer, and 1 μl DEPC water. The solution was heated to 55° C before adding 1 μl (5 U/L) *taq* (Perkin-Elmer). The slides were heated to 55° C on the thermocycler, and then 20 μl of the preheated *taq* solution were added ("Hot-Start") (21) to prevent nonspecific primer binding. A HybriSlip was added, and the slide was flooded with mineral oil and heated to 94° C for 2 min to denature the cDNAs before starting step-cycles of 94° C for 1 min/55° C for 1 min/72° C for 1 min (25). After 15 cycles, the slides were washed in xylene, acetone, and ethanol, placed in 2× SSC for 5 min, then 100% ethanol and air-dried as before.

Second Polymerase Chain Reaction Step

Reagents were mixed at 4° C: 2 μl 10 × buffer, 4 μl 25 mM $MgCl_2$, 1 μl each of 10 mM dATP, dGTP, and dCTP, 0.65 μl of 1 mM dTTP, 0.35 μl 1 mM digoxigenin-11-dUTP (Boehringer Mannheim), 2 μl each of 15 μM sense and antisense primers, and 5 μl DEPC water. The solution was heated to 55° C, 1 μl *taq* was added, and the solution was pipetted onto the preheated slides. HybriSlips and mineral oil were applied, the solutions and tissue cDNAs were denatured at 94° C for 2 min, and PCR was performed with step-cycles of 94° C 1 min/55° C 1 min/72° C 1 min. After 15 cycles, the slides were again washed sequentially with xylene, acetone, and ethanol and air-dried.

Immunodetection and Visualization

Nonspecific binding sites were blocked by adding 100 μl TRIS-NaCl at pH 7.4, 2% Boehringer Mannheim Blocking Reagent, 2% Amersham ECL Blocking Reagent (Arlington Heights, IL), and 2% BSA for 60 min at room temperature in a humidified chamber. Alkaline-phosphatase-labeled sheep antidigoxigenin Fab′ was added (2 μl per section of a 1:50 dilution), and incubated for 60 min at room temperature. The solutions were washed twice in TRIS-NaCl for 15 min each, then the pH was adjusted by incubation in freshly prepared pH 9.5, 100 mM TRIS, 150 mM NaCl, and 50 mM $MgCl_2$ at pH 9.5 ("Buffer C") for 2 min at room temperature. Alkaline phosphate substrate was prepared by mixing 4.5 μl NBT plus 3.5 μl XPhos in 1,000 μl "Buffer C." Pairs of positive and negative (no RT control) slides were always incubated side by side, and color development was followed by microscopy. The reactions were stopped by dipping the slides into TRIS-NaCl at pH 7.4. Slides were coverslipped with Crystal mount. No counterstaining was used.

Negative Controls

To ensure that genomic DNA was degraded and that there was no nonspecific PCR amplification, negative control slides were obtained by omitting RT, the antisense primer in the RT step, and *taq* in the first PCR step.

Confirmation of RT-PCR Product Size

After IS-RT-PCR, tissue sections on the slides were digested overnight using 1.25 mg/ml protease K, 0.1% SDS at 52° C. The digestate was precipitated with ethanol and 1 M sodium acetate, centrifuged, resuspended in 18 μl water, and 4 μl electrophoresis loading buffer (Novex) were added. Samples were loaded onto 4 to 20% gradient PAGE gels (Novex) along with a digoxigenin-labeled cDNA molecular weight ladder (Boehringer Mannheim), and run in TRIS-boric acid-EDTA (TBE) buffer for 2 h at 120 V. Gels were denatured in 50 mM NaOH for 5 min, neutralized in TBE for 5 min, and then electroblotted to nylon (30 V for 2 h) with TBE buffer on Novex apparatus. The DNA was fixed to the nylon under 1,200 j/cm^2 UV light (Stratalinker).

Nonspecific binding sites on the blots were blocked with 2% Boehringer Mannheim blocking reagent, 2% BSA, in 150 mM NaCl, 50 mM TRIS at pH 7.5 for 1 h at room temperature. Anti-digoxigenin sheep Fab′ (1:3,000 dilution in blocking reagent) was added for 1 h with constant agitation at room temperature. Blots were washed twice in TRIS-NaCl for 15 min each, and then washed for 3 min in Buffer C. In a darkroom, 1 ml of Lumi-Phos 530 (Boehringer Mannheim) was flooded over the blot and then encased in plastic wrap (Saran). The blot and chemiluminescent substrate were incubated for 30 min at 37° C, then for 2.5 h at room temperature, then exposed to ECL Hyperfilm X-ray film (Amersham) for 1 min or longer and developed by Kodak X-Omat. The size of the RT-PCR product was calculated from the sizes of the digoxigenin-labeled standards. Lumi-Phos and labeled probes can be washed from the blots so the blot can be rehybridized. After washing in water for 20 min at room temperature, blots were stored in 20× SSC at 4° C.

Statistics

Scores, concentrations, and volumes for each assay were tabulated in Excel and the mean ± standard error of the mean (SEM) was determined, then the data were transferred to SPSS for statistical analysis. Consequences of HTS provocations were first assessed by ANOVA for multiple comparisons, then individual sets of data were compared to results after 1 × NS using Student's paired *t* tests. One-tailed tests were used for comparison of symptoms and two-tailed tests for secretions and AcRh data. Differences between IL and CL results were compared by Student's two-tailed unpaired *t* tests. Significance was ascribed for $p < 0.05$. Parametric and nonparametric tests gave comparable levels of significance.

RESULTS

Pain

HTS induced a burning sensation within the first 10 s of application that faded with time. Pain intensity became significantly different from 1 × NS at 3 × NS ($p = 0.03$) and increased in a dose-dependent fashion to 24 × NS ($p < 1 \times 10^{-12}$) (Figure 1). Pain duration followed the same pattern with increases from 3 × NS ($p = 0.03$) to 24 × NS ($p < 1 \times 10^{-12}$).

Nasal Blockage

The sensation of blockage in the HTS-challenged nostrils was increased in a dose-response fashion beginning with a small but significant increase after 3 × NS ($p = 0.03$) (Figure 1). There was no contralateral blockage. IL responses were significantly greater than CL responses after 24 × NS ($p = 0.002$).

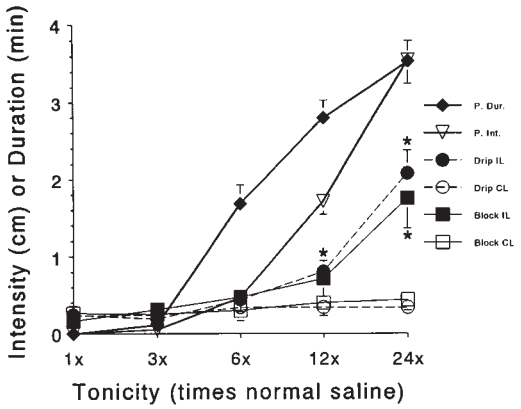

Figure 1. Symptoms. Pain intensity and duration were increased above 1 × NS values in dose-response fashion with significant changes from 3 × NS ($p = 0.03$) to 24 × NS ($p = 6 \times 10^{-14}$). On the IL side, changes in the sensation of obstruction began at 3 × NS ($p = 0.03$), whereas rhinorrhea was detectable at 6 × NS ($p = 0.02$). The *asterisk* indicates significant differences between IL and CL responses ($p < 0.008$) for block (24 × NS) and drip (12 × NS, 24 × NS).

Drip

The sensation of rhinorrhea began at 6 × NS on the IL side ($p = 0.02$), and increased in dose-dependent fashion (Figure 1). There was no significant contralateral drip. The differences in sensations between the two sides were significant for 12 × NS ($p = 0.008$) and 24 × NS ($p = 0.00001$).

Weight of Returned Lavage Fluid

Weights on the IL side increased significantly and in dose-dependent fashion beginning with the 6 × NS provocation (1.2-fold, $p = 1 \times 10^{-6}$) (Figure 2). There were no changes on the CL side. The net differences in the masses of lavage fluids between the IL and CL sides were 78 mg after 6 × NS, 82 mg after 12 × NS ($p = 0.048$), and 442 mg after 24 × NS ($p = 0.0003$).

Total Protein

The initial total protein (TP) was 451 ± 52 μg/ml on the IL and 424 ± 50 μg/ml on the CL sides. TP was significantly increased on the IL side after 24 × NS compared with 1 × NS

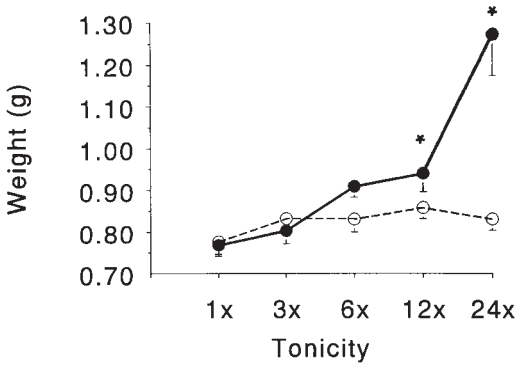

Figure 2. Weights of returned lavage fluid. Weights increased only on the IL, challenged side (*closed circles*, *solid line*). There was no contralateral reflex change in weight of secretions (*open circles*, *dashed line*). The *asterisk* indicates significant differences between IL and CL responses ($p < 0.05$).

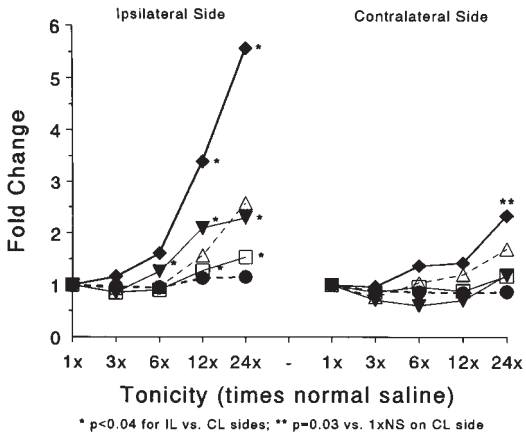

Figure 3. Lavage fluid. On the IL side, significant increases above 1 × NS were found for 7F10-mucin (*solid diamond*, *solid line*) beginning at 6 × NS ($p = 0.02$), lactoferrin (*solid triangles*, *solid line*) at 6 × NS ($p = 0.004$), ABSM (*open triangle*, *dashed line*) at 24 × NS ($p = 0.006$), and total protein (*open squares*, *solid line*) at 24 × NS ($p = 0.02$). IgG (*solid circles*, *dashed line*) did not change on either the IL or CL sides. Only 7F10-mucin was increased on the CL side (24 × NS, $p = 0.03$ versus 1 × NS). IL responses were significantly greater than CL for 7F10-mucin (12 × NS, 24 × NS), lactoferrin (6 × NS, 12 × NS, 24 × NS), and total protein (12 × NS, 24 × NS) (*$p < 0.04$).

($p = 0.02$) (Figure 3). There were no significant changes on the CL side. IL responses were larger than CL after 12 × NS ($p = 0.025$).

IgG

Concentrations of this plasma marker were not changed by any dose of NS on either the IL or CL sides (Figure 3). HTS had no effect on plasma exudation in these normal subjects.

Lactoferrin

Lactoferrin was significantly increased above 1 × NS (6.5 ± 1.4 μg/ml) in a dose-response fashion on the IL side beginning with 6 × NS ($p = 0.004$) (Figure 3). There were no significant changes from 1 × NS (6.9 ± 1.6 μg/ml) on the CL side. IL re-

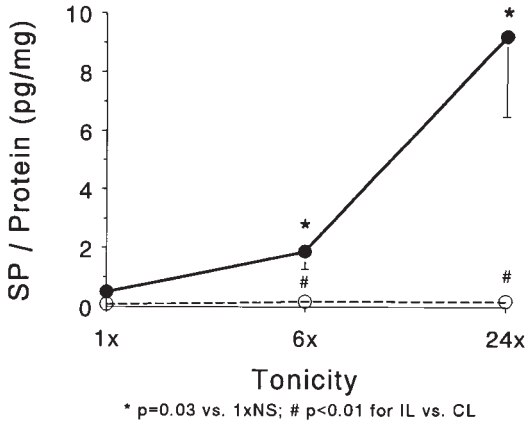

Figure 4. Substance P (SP). SP was increased after hypertonic saline in dose response fashion on the challenged side (*closed circles*) (*$p < 0.03$). There were no changes on the CL side (*open circles*). IL responses were greater than CL (#$p < 0.01$).

sponses were significantly greater than CL for 6 × NS ($p = 0.022$), 12 × NS ($p = 0.018$), and 24 × NS ($p = 0.04$).

Alcian Blue Staining Material

ABSM increased significantly from 1 × NS (155 ± 25 μg/ml) after 24 × NS ($p = 0.006$) on the IL side (Figure 4). The increase in ABSM on the CL side after 24 × NS was not significantly greater than 1 × NS (147 ± 21 μg/ml) ($p = 0.12$). IL and CL responses were not significantly different.

7F10-mucin

On the IL side, 7F10-mucin was significantly increased from 1 × NS (28.1 ± 4.4 mU/ml) beginning with 6 × NS ($p = 0.02$) (Figure 3). 7F10-mucin was also significantly increased from 1 × NS (34.3 ± 6.2 mU/ml) on the CL side after 24 × NS ($p = 0.03$), indicating that a small, but significant parasympathetic reflex had been stimulated. The IL responses were greater than the CL responses after 12 × NS ($p = 0.053$) and 24 × NS ($p = 0.039$).

Incremental Changes

The yields of each mucus constituent on the IL and CL sides were estimated (mass of secretion × concentration/density of water), and the incremental changes between 1 × NS and 24 × NS were determined. The increments were much larger on the IL than on the CL sides for total protein (IL, 548 mg versus CL, 85 mg), IgG (24 mg versus 2 mg), lactoferrin (16.4 mg versus 1.4 mg), 7F10-mucin (179 mg versus 69 mg), and ABSM (396 mg versus 84 mg). It was proposed that the CL secretion was due to parasympathetic reflex-induced glandular secretion, and that the magnitude of the reflex component was similar on the IL and the CL sides. If so, then these reflexes may have contributed 15.5% of the total protein, 8.3% of the IgG, 8.4% of the lactoferrin, 37.4% of the 7F10-mucin, and 21.2% of the ABSM in the IL secretions (% = 100 × [CL]/[IL]). Therefore, bilateral parasympathetic reflexes may have been activated by HTS, but they represented a relatively minor contribution to secretions on the IL side after HTS provocation.

There were no significant changes in IgG.

Total Protein Ratios

The ratios of IgG (0.08), lactoferrin (0.0014), 7F10-mucin (0.071), and ABSM (0.35) to total protein were calculated for 1 × NS lavage fluids. On the IL side after 24 × NS, the ratio was modestly increased for lactoferrin (0.0022) and markedly increased for 7F10-mucin (0.22) and ABSM (0.57). There was

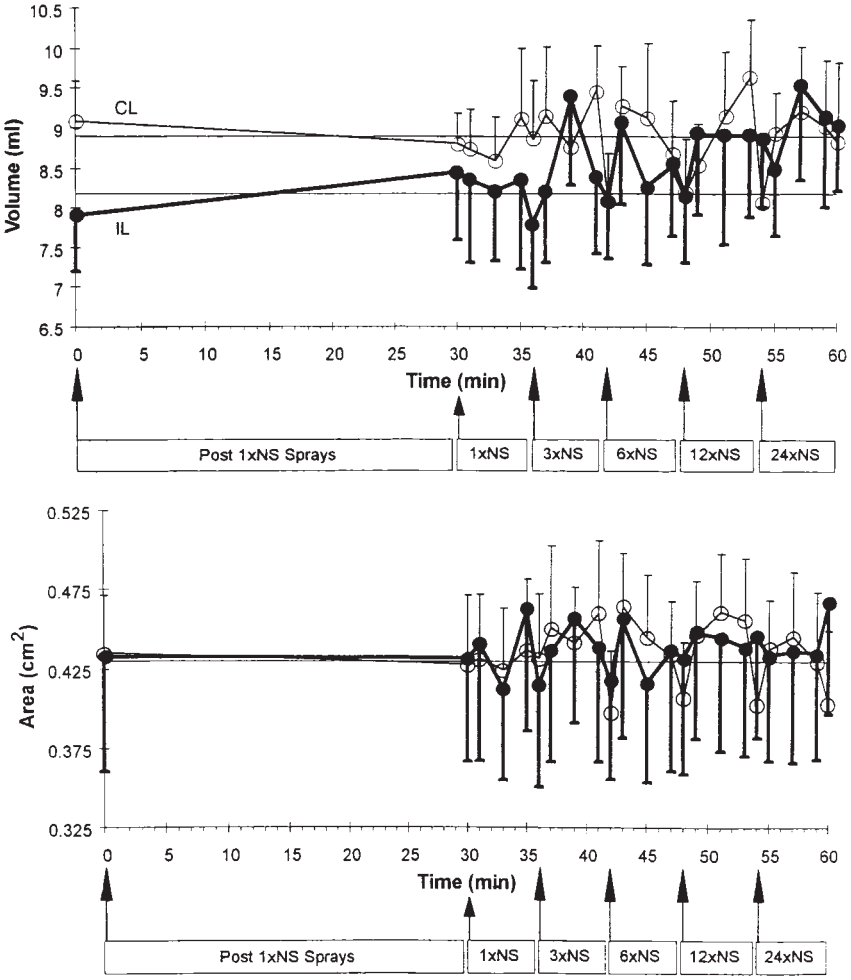

Figure 5. Acoustic rhinometry. Mean ± SEM measurements of (*top panel*) nasal air-space volumes and (*bottom panel*) minimum cross-sectional areas (anterior valve, Amin) are shown at 0, 30, and all "Pre," 1-, 3-, and 5-min time points for the HTS-challenged (*closed circles*) and contralateral (*open circles*) nostrils. *Horizontal lines* show the mean of the 0- and 30-min points. There were no significant changes in either parameter at any time point.

no change for IgG (0.05). On the CL side after 24 × NS, these ratios were increased for 7F10-mucin (0.16) and ABSM (0.50) and unchanged for lactoferrin (0.0016) and IgG (0.06), suggesting an enrichment of mucous macromolecules without changes in vascular leak as part of parasympathetic reflex effects.

Substance P

SP was almost undetectable after 1 × NS provocation; 6 × NS and 24 × NS induced a significant dose-response increase in the SP/total protein ratio, suggesting release of SP from nociceptive nerve endings after HTS provocation (Figure 4). There was no SP release on the CL side, indicating that HTS did not "leak" over to the CL side and that SP was not a neurotransmitter of parasympathetic reflexes.

Acoustic Rhinometry

Pain intensity and duration and block and drip scores for these 10 subjects were similar to those of the larger group who had secretion analysis performed (data not shown). V6 and Amin on the IL and the CL sides were not significantly changed by the provocations (Figure 5). There was a nonsignificant trend for an increase in volume on the IL side (i.e., nasal patency). Analysis of minute-to-minute changes failed to detect any significant HTS-induced regulation of V6 or Amin (data not shown).

NK-1 Receptor IS-RT-PCR

NK-1 receptor mRNA was localized to submucosal glands in both nasal (Figure 6) and bronchial (Figure 7) mucosa. There was a tendency for the more distal seromucous cells to have the highest content, but lack of expression in mucous cells could not be confirmed. There was faint staining of some epithelial and endothelial cells. The size of the digoxigenin-containing PCR product was approximately 285 bases as predicted (data not shown). No PCR products were detected when RT, antisense primer in the RT step, or *taq* in the first PCR step were excluded.

DISCUSSION

Hypertonic saline induced sensations of pain, nasal blockage, and rhinorrhea, mucosal SP release, and exocytosis of glandular products. Significant changes that were limited to the side of the HTS provocation were present after 6 × NS and 12 × NS. After 24 × NS there were continued increases in all the

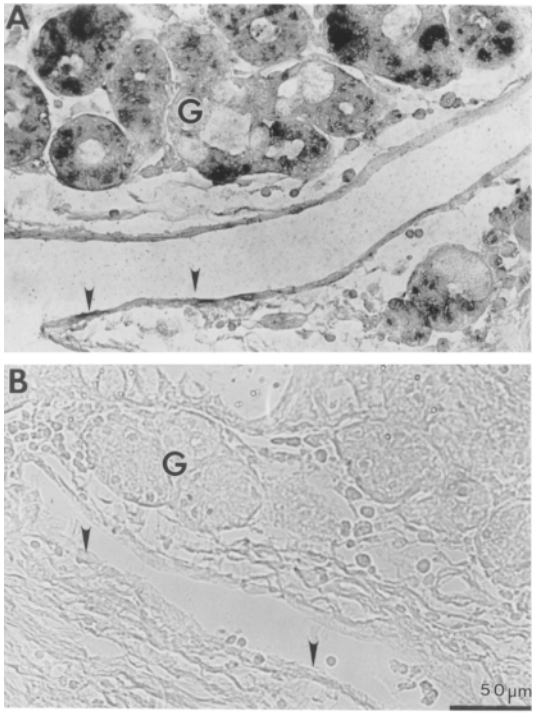

Figure 6. NK-1 receptor mRNA expression in human nasal mucosa. (*A*) IS-RT-PCR demonstrated NK-1 receptor mRNA in gland cells (G), endothelium of venous sinusoids (*arrowheads*), and occasional epithelial cells in the normal tissue. (*B*) Reverse transcriptase was excluded in this negative control study of a subsequent section of the same tissue.

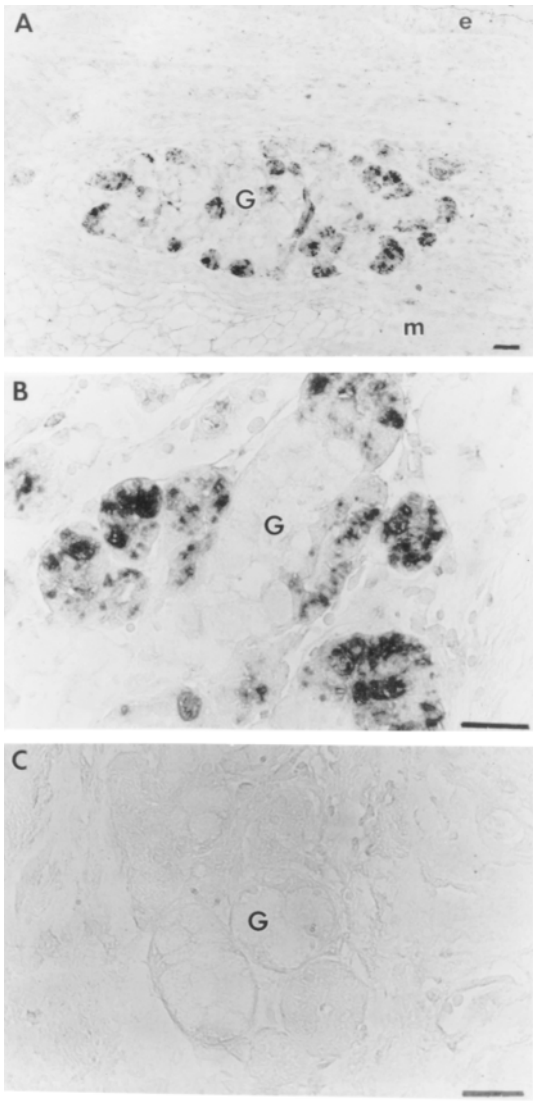

Figure 7. NK-1 Receptor *in situ* reverse transcription–polymerase chain reaction (IS-RT-PCR). NK-1 mRNA was identified by dark staining in bronchial mucosa. (*A*) NK-1 receptor mRNA was most prominent in submucosal glands. Epithelium (e) and bronchial smooth muscle (m) showed little staining. (*B*) NK-1 receptor mRNA was most dense in peripheral lobules of submucosal gland acini, suggesting a seromucous cell localization. (*C*) *Taq* was excluded in this negative control study of a subsequent section of the same tissue. The *bar* represents 100 μm.

IL responses, as well as the induction of small but significant CL reflex-mediated secretion of 7F10-mucin. There were no changes in vascular responses, including plasma extravasation with exudation, vasodilation with filling of venous sinusoids, and mucosal thickening. There was no reduction in nasal air-space volume. Because NK-1 receptor mRNA was most distinctly expressed in submucosal gland cells, we conclude that hypertonic saline leads to nociceptive nerve stimulation with axon response–mediated SP release and exocytosis of mucous and serous cell markers with no change in plasma extravasation or vascular tone.

An alternative explanation for the mucin secretion is the possibility that HTS directly induced goblet cell exocytosis of 7F10-mucin and ABSM by stimulating osmotically driven shifts in intracellular water concentrations. However, epithelial cells cannot account for the IL release of serous lactoferrin from submucosal gland serous cells seen at 6 × NS (13, 17, 18). Also the ratio of gland cells to goblet cells is approximately 40:1 in the nasal mucosa (26), suggesting that submucosal glands are more likely the source of mucus. SP released by axon responses may also activate epithelial cell NK1-receptors (7, 27). Mast cell activation by HTS is also possible (1, 28) and is currently under evaluation in this model. Parasympathetic reflexes were detected on the CL side after 24 × NS but involved 7F10-mucin and were too small in magnitude to account for the IL changes. Parasympathetic reflexes may have a larger contribution in active allergic rhinitis (18, 29).

These data are at odds with rodent models where vascular permeability has been the predominant pathophysiologic response (6, 7). Mucus secretion is largely limited to goblet cell exocytosis in these species. The absence of significant vascular swelling or leak in porcine (30) and human (31) nasal mucosal models has led to the perception that neurogenic responses are important in rodents, but perhaps irrelevant in larger mammals (12). This misconception is based upon the supposition that human airway function can be directly extrapolated from rodent models. Although neurogenic responses appear to be present in both groups, axon responses invoke vascular effects in rodents and glandular ones in normal humans.

HTS responses can be compared with those of capsaicin, another stimulant of nociceptive neurons. Capsaicin pretreatment blocks responses to HTS, suggesting that common nerve populations are activated (14, 15). Subjects with severe untreated allergic rhinitis have increased basal vascular permeability compared with normal subjects (11). SP (4) and capsaicin (9) augment the vascular leak in allergic subjects (11). Comparison of the linear analog pain scale results suggest greater pain in the allergic rhinitis/capsaicin study (9) than in ours, suggesting that more painful or prolonged nociceptive stimulation may have evoked vascular leak in normal subjects. Parasympathetic reflex effects may be expected to be more pronounced after a more painful nociceptive stimulus. Alternatively, neurogenic vascular leak may be an amplifying mechanism that is active only during airway inflammation.

NK-1 receptor mRNA was identified in submucosal glands. It was not possible to determine if staining was limited to seromucous cells or if both seromucous and mucous cells were labeled. ^{125}I SP receptor binding sites were more widespread in both nasal and bronchial mucosa (8, 26); differences may be due to mRNA and receptor protein concentrations and turnover. Bai and colleagues (32) have demonstrated an increase in NK-1 receptor mRNA expression in chronic bronchitis and asthma.

The finding that HTS stimulates SP release and glandular secretion has important implications for asthma, cystic fibrosis, and chronic bronchitis. Bronchial hyperreactivity to HTS in asthma has a steeper dose-response curve in asthmatics and does not appear to correlate well with methacholine and histamine provocations (1, 2). This may indicate that methacholine and histamine directly activate bronchial smooth muscle, whereas HTS effects are initiated in part by nociceptive nerves. Drugs such as cromolyn, nedocromil, and glucocorticoids have only small effects on bronchial responses to HTS, suggesting that neurogenically mediated bronchospasm may be relatively resistant to current therapies (1, 2).

HTS is commonly used to induce sputum (33, 34). The current findings suggest that inhalation of HTS may induce neurogenically mediated glandular exocytosis. If so, then the copious sputum production induced by HTS in asthmatic and chronic bronchitic subjects would imply upregulation of neurogenic mechanisms in these disease states. This is further supported by the high levels of sputum SP recovered after HTS inhalation by subjects with chronic bronchitis and asthma (35) and the presence of NK-1 receptors in bronchial glands (Figure 7). Parasympathetic reflexes may also be recruited and induce cholinergic glandular exocytosis in both the nasal and the bronchial airways (13, 17, 29, 30). It is of interest that HTS-induced sputum production was not altered by prednisone (32), indicating that this was a steroid-resistant pathophysiologic process. Drugs directed toward nociceptive neurogenic mechanisms may be effective for treatment of mucous hypersecretion.

These data may also have implications for the pathophysiology of cystic fibrosis. Elevations of epithelial lining fluid NaCl concentrations may lead to a state of perpetual hypertonicity and neurogenic inflammation that could contribute to their mucous hypersecretion.

The acoustic rhinometry measurements indicate that there was no correlation between the measured nasal air-space volume or minimum cross-sectional area of the anterior nasal valve and the sensation of nasal blockage. In contrast, sensations of blockage, drip, and pain rose in parallel, suggesting that the increases in epithelial lining fluid volume that follow exocytosis and/or nociceptive nerve stimulation may be interpreted by the subject as obstructed airflow even though the anterior nasal valve and nasal cavity remain patent. This model may permit investigation of the mucosal conditions that lead to perceptions of respiratory difficulty.

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