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A New Model of Laryngitis: Neuropeptide, Cyclooxygenase, and Cytokine Profile

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Objectives/Hypothesis: To develop and characterize a new model of laryngeal inflammation by analyzing the presence of neurogenic peptides and expression of cyclooxygenases (COX) and cytokines in the mucosa.

Study Design: Laryngitis induced by nasogastric intubation (NGI) was evaluated by histopathologic changes of the mucosa, alterations in calcitonin gene related peptide (CGRP) and substance P (SP) neuropeptides in sensory fibers, and COX-1,2, and cytokine (interleukin [IL]-1, IL-6, IL-10, tumor necrosis factor [TNF]-α) expression in the laryngeal mucosa.

Methods: Rats submitted to NGI for 1 to 5 weeks were compared with controls. Laryngeal sections were immunostained for stereologic analysis of SP and CGRP fiber density and number of mucosal cells expressing COX-2. Alterations in inflammatory mediators were evaluated by quantitative reverse-transcriptase polymerase chain reaction.

Results: NGI induced metaplasia of the epithelium and narrowing of the laryngeal lumen because of hypertrophy of laryngeal glandules and edema. An initial decrease in CGRP- and SP-immunoreactive fibers in the laryngeal mucosa (1–3 wk) was reverted with time (5 wk). COX-2 expression in mucosal cells increased progressively, reaching a maximum level at 5 weeks, and was observed in mononuclear immune cells, which is indicative of a chronic inflammatory process. In regard to mRNA expression levels of inflammatory mediators, TNF-α was increased during the 5 week NGI, and IL-10 decreased during the 5 weeks,whereas IL-1β, IL-6, and COX-2 increased in the first 1 to 2 weeks and returned to baseline at 5 weeks.

Conclusions: This NGI model results in laryngeal chronic inflammation without direct mechanical aggression of the mucosa and may contribute to the study of future therapeutic approaches to this pathology.

Key Words: Laryngeal inflammation, neuropeptides, cyclooxygenase-2, cytokines, nasogastric intubation, immunocytochemistry, mRNA expression, reverse-transcriptase polymerase chain reaction analysis, animal model.

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INTRODUCTION

Nasogastric intubation (NGI) is largely used in clinical practice.[1] However, several complications such as laryngitis[2] and increased mortality because of aspiration pneumonia can be induced by this procedure. The experimental studies determining the mechanisms of laryngeal injury associated with nasogastric tubes are scarce.[2] Neurogenic mechanisms are implicated in many inflammatory diseases, and three group of factors are involved: 1) hydrogen ions (H^+) and adenosine triphosphate released by damaged tissue; 2) inflammatory mediators, including prostaglandins (PGs) and pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and anti-inflammatory cytokines (IL-10); 3) and neurogenic peptides substance P (SP) and calcitonin gene related peptide (CGRP) present in the nerve fibers of the mucosa.[3] We previously demonstrated that neuropeptides SP and CGRP are present in the laryngeal sensory fibers that reach the epithelium surface and project to the respiratory lumen.[4] Because these fibers are accessible to direct stimulation, the rat laryngeal mucosa may constitute a good experimental model for studying the activation of primary afferents during the development of neurogenic inflammation.[4] SP and CGRP are important peptides in laryngeal sensory reflexes of cough and bronchial-pulmonary defense and can induce inflammatory responses in the respiratory tract.[5] As concerns the larynx, it is possible that these peptides can also induce laryngitis, but to the best of our knowledge, there are no experimental studies analyzing the expression of peptides and ILs such as IL-1β, IL-6, IL-10, and TNF-α in laryngeal inflammation.

Acute and chronic inflammation induce the expression of cyclooxygenase (COX)-2 and release of PGs from arachidonic acid, which is cleaved from cell membrane phospholipids of damaged tissue.[3] PGs activate receptors in fiber membranes, triggering the cyclic adenosine monophosphate protein kinase A transduction pathway that increases intracellular calcium and releases SP from inflamed peripheral nerves, which further activates fibers by stimulating neurokin-1 receptors. CGRP receptors are colocalized with PG receptors at thin nerve terminals, and CGRP-receptor activation potentiates the effect of SP by retarding its release. Pro-inflammatory cytokines (IL-1, TNF-α, and IL-6) produced after tissue damage induce COX-2 synthesis and cells to release PGs.[3] The expression of COX-2 and COX-1 (constitutive) and their relation to neurogenic peptides and cytokines in chronic laryngitis is unknown. We designed an experimental rat model of NGI to obtain chronic inflammation of the larynx in which we evaluated potential changes in the 1) normal histology of the mucosa, 2) SP- and CGRP-immunoreactivity in laryngeal sensory fibers, 3) COX-2 expression in inflammatory cells of the lamina propria, and 4) COX-1, COX-2, IL-1β, IL-6, IL-10, and TNF-α mRNA expression in the mucosa.

MATERIALS AND METHODS

Animals

This study was performed in 54 male rats weighing 350 to 450 g obtained from the Wistar Han colony of Charles Rivers Company (Barcelona, Spain). Animals were lightly anesthetized with inhaled isoflurane to allow swallowing reflex and were submitted to the NGI procedure. A 10 to 12 cm narrow-bore nasogastric aspiration tube used for NGI in premature newborns (cat. No 533.04; Vigon Laboratoires Pharmaceutiques, Ecouen, France) was inserted through the nasopharynx until it reached the stomach. The external tip was sutured to the nasal lateral cartilages. Animals recovered and returned to their cages, being submitted to NGI for 1 (n_{imuno} = 6; n_{PCR} = 7), 2 (n_{imuno} = 6; n_{PCR} = 6), 3 (n_{imuno} = 6), and 5 (n_{imuno} = 6; n_{PCR} = 6) weeks. Twelve (n_{imuno} = 6; n_{PCR} = 6) nonintubated rats were used as controls (CONT). At the end of the intubation period, animals for immunohistochemical experiments were perfused under anesthesia (isoflurane and 35% chloral hydrate intraperitoneally) through the ascending aorta with 4% paraformaldehyde in phosphate-buffered saline (PBS) 0.01 mol/L. After confirmation that the NGI tube was still inserted in the stomach, the larynx was removed and immersed in the same fixative followed by 30% sucrose in 0.1 mol/L PBS overnight. Animals used for real-time reverse-transcriptase polymerase chain reaction (RT-PCR) studies were anesthetized with isoflurane and sacrificed with 35% chloral hydrate intraperitoneally. Larynxes were excised immediately, macerated, immersed in 0.8 mL of TRIzol (Invitrogen, Carlsbad, CA), and stored at −80°C. The experiments were carried out in accordance with regulation of local authorities for handling laboratory animals (Veterinary General Directive Board, Ministry of Agriculture, Rural Development and Fishing) and European Community Council Directive 86/609/EEC.

pHmetry

Measurements of pH were performed using a MI-414–4 cm pH combination microelectrode probe (Microelectrodes, Inc., Bedford, NH) attached to an Inolab pH Level 1 potentiometer (Wissenschaftlich Technische Werkstätten, Weillem, Germany). First, the pharyngeal pH and then the esophageal pH were recorded for each animal immediately before sacrifice (5 s for each pH measurement).

Histology and Immunocytochemistry

Laryngeal coronal frozen sections (20 μm) obtained with a cryostat were processed for immunohistochemistry. Alternate sections were incubated overnight at room temperature with rabbit antiCGRP (1:6,000; Bachem, Sao Carlos, CA) or rabbit antiSP (1:6,000; Bachem) antibodies in PBS with 0.3% triton X-100 (PBST). After rinsing in PBST, the sections were then incubated with biotinylated goat anti-rabbit (1:200; Vector Laboratories Inc, Burlingame, CA) in PBST for 1 hour, washed in PBST, and then incubated with avidin-biotin complex (1:200; Vector Laboratories Inc, Burlingame, CA) in PBS for 1 hour. After rinsing in PBS and 0.1 mol/L tris-HCl buffer (pH 7.4), the

TABLE I.
Sequences of Primers Used for Real-Time Polymerase Chain Reaction.

Target	Oligo	Sequence	Gene Bank ACC
β-Actin	Forward primer	5′ – GATTTGGCACCACACTTTCTACA – 3′	NM_031144
	Reverse primer	5′ – ATCTGGGTCATCTTTTCACGGTTGG – 3′	
IL-1β	Forward primer	5′ – GAAACAGCAATGGTCGGGAC – 3′	M98820
	Reverse primer	5′ – GAGACCTGACTTGGCAGAGG – 3′	
TNF-α	Forward primer	5′ – CCAACAAGGAGGAGAAGTTC – 3′	NM_012675
	Reverse primer	5′ – CCTGGTGGTTTGCTACGAC – 3′	
IL-6	Forward primer	5′ – CAAGAGACTTCCAGCCAG – 3′	NM_012589
	Reverse primer	5′ – CTCCGACTTGTGAAGTGGT – 3′	
IL-10	Forward primer	5′ – GCCAAGCCTTGTCAGAAATGA – 3′	NM_012854
	Reverse primer	5′ – TTTCTGGGCCATGGTTCTCT – 3′	
COX-1	Forward primer	5′ – GGCGTTGCTCATCCATCTACTC – 3′	S67721
	Reverse primer	5′ – AGCATCTGTGAGCAGTACCGG – 3′	
COX-2	Forward primer	5′ – TTTGTTGAGTCATTCACCAGACAGAT – 3′	S67722
	Reverse primer	5′ – ACGATGTGTAAGGTTTCAGGGAGAAG – 3′	

IL = interleukin; TNF = tumor necrosis factor; COX = cyclooxygenase.

antigen-antibody reaction was visualized with diaminobenzidine (DAB, Sigma, St. Louis, MO).

COX-2 immunohistochemistry was carried out according to the streptavidin-biotin-peroxidase complex technique (Ultravision Detection System Antipolyvalent, HRP, Lab Vision Corporation, Fremont, CA) using a primary antibody raised against COX-2 protein (1:400; rabbit monoclonal antibody, clone SP21; Neomarkers, Fremont, CA). Slides were sequentially washed in PBS/0.02% Tween 20 and incubated with 3% H_2O_2 in methanol for 10 minutes. This was followed by incubation with blocking solution for 10 minutes and the primary antibody solution for 2 hours at room temperature. Sections were then sequentially washed in PBS/0.02% Tween 20 and incubated with biotinylated goat antipolyvalent antibody for 10 minutes and streptavidin peroxidase for 10 minutes. DAB was used as chromogen. Slides were counterstained with Mayer hematoxylin (Merck, Darmstadt, Germany). Negative controls were performed by omission of the primary antibody. A colon carcinoma sample was used as positive control.

Stereologic Procedures

Sections were analyzed with an Axioskop 2 plus light microscope (Carl Zeiss, Göttingen, Germany), and images of laryngeal histologic data were taken using an Axiocam HRC camera and AxioVision 3.1 software (Carl Zeiss). Cell and fiber numbers were estimated using the optical fractionator method.[6] This consists of virtual 3–dimensional-box ($150 \times 150 \times 30$ μm), equally spaced grids that were superimposed on every eighth coronal laryngeal section after drawing the limits of the area under study in the laryngeal mucosa. The number of COX-2 immunoreactive cells and SP- and CGRP-immunoreactive fibers that crossed the gridlines in every randomized site (chosen by the software) was counted according to well-defined stereologic rules. The estimated numbers were calculated from the ratio between the total number of counted cells and fibers crossing the grid site and the number of grid sites per area. The coefficients of error were automatically computed by the software according to the formulas of Gundersen for cell numbers.[6]

mRNA Extraction and Real-Time RT-PCR

Total mRNA in the larynx was extracted by adding 160 μL $CHCl_3$ followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The supernatant was carefully collected, and total mRNA was precipitated using isopropanol (2-propanol) followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The mRNA pellet was washed using 70% ethanol recentrifuged at 9,000 rpm for 5 minutes at 4°C, dried, and suspended with 50 μL of DNAse/RNAse free distilled water (Gibco, Carlsbad, CA). Quantification of total mRNA was performed by spectrophotometry (NanoDrop, NanoDrop Technologies, Inc., Wilmington, NC). Samples of total mRNA with the same concentration (2 ng/10 μL) were then reverse transcribed in a thermocyclator My Cycler Thermal Cycler (Bio-Rad, Hercules, CA) using a Superscript Kit II (Invitrogen) and Oligo dT (Invitrogen), according to the manufacturer's

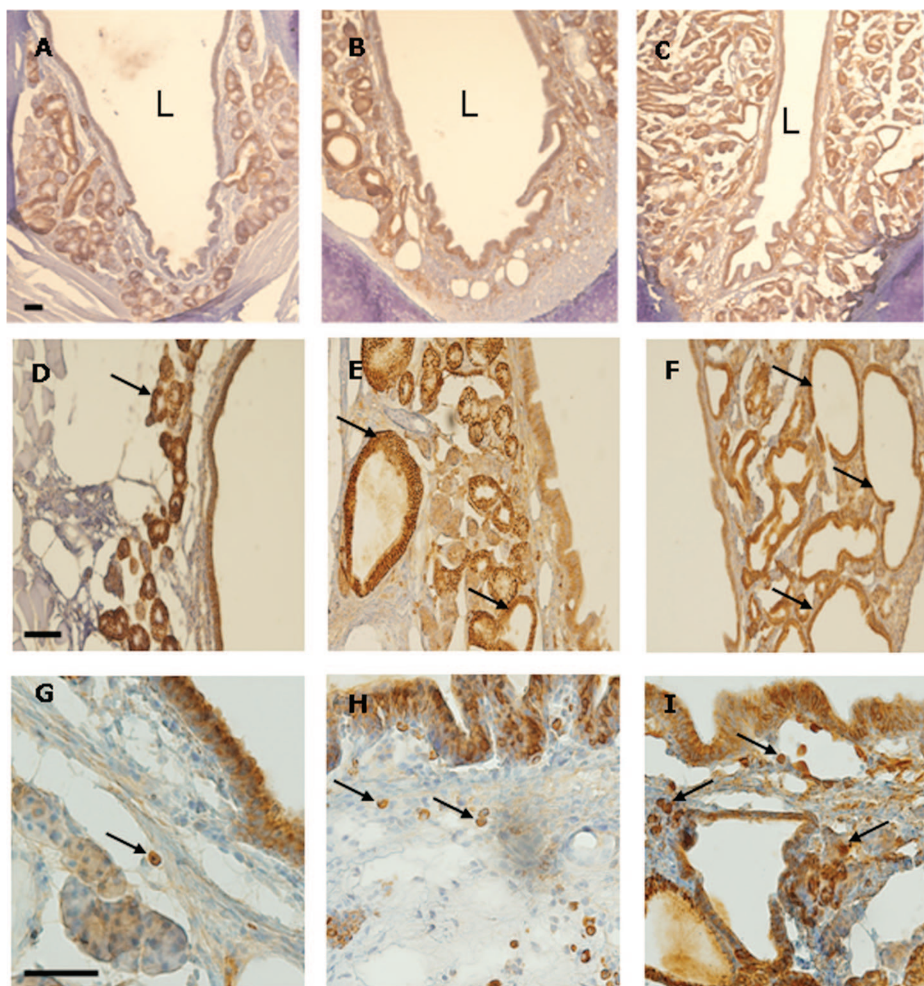


Fig. 1. Changes in histology and cyclooxygenase (COX)-2 expression of the laryngeal mucosa after no nasogastric intubation (NGI) (A, D, G) and 2 (B, E, H) and 5 (C, F, I) weeks of induction of the NGI model. A large increase of the glandular tissue was observed with increasing periods of laryngeal inflammation (A to C; arrows, D to F), with a consequent decrease in the laryngeal lumen (A to C). This was accompanied by large proliferation of mononuclear immune cells immunoreactive to COX-2 (arrows, G to I). L = laryngeal lumen. Magnification bar = 100 μm.

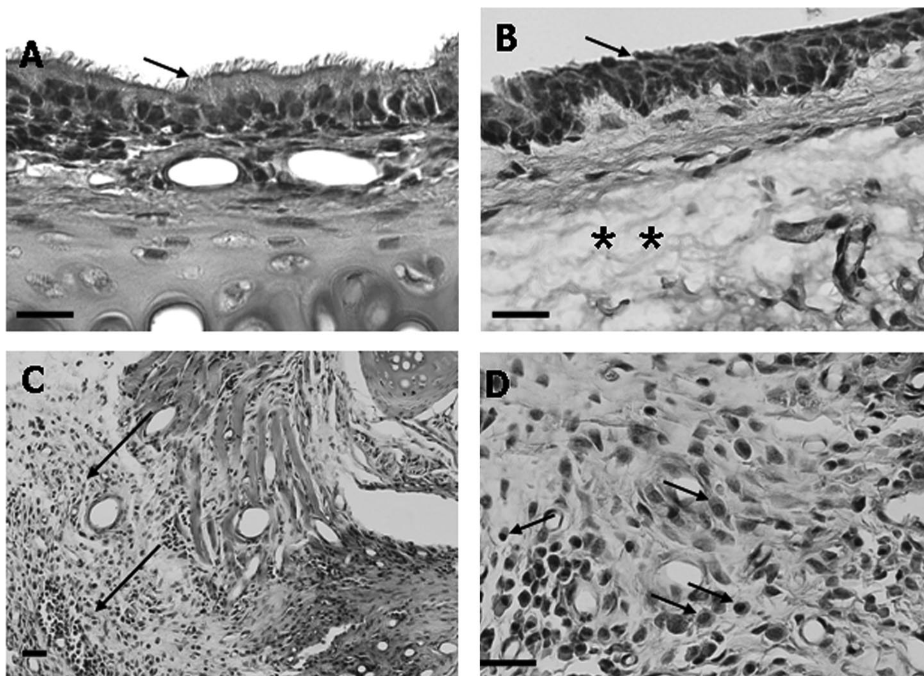


Fig. 2. Histologic changes in the connective tissue of the laryngeal mucosa induced by the nasogastric intubation (NGI) experimental model. Note replacement of typical ciliated pseudostratified respiratory epithelium (arrow, A) by another differentiated squamous stratified (nonkeratinized) epithelium (metaplasia) after 5 weeks of NGI (arrow, B). In addition, presence of chronic inflammation signs is evident, namely edema (asterisks, B) and large proliferation of mononuclear immune cells in the lamina propria (arrows, C), including plasmocytes (arrows, D). Magnification bar = 100 μm.

instructions. The cDNA was then subjected to real-time RT-PCR reactions for quantification of mRNA levels of β-Actin, IL-1β, TNF-α, IL-6, IL-10, COX-1, and COX-2, using the LightCycler (Roche, Basel, Switzerland), and an SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) was used according to the manufacturer instructions. Primer sequences used to amplify various cDNAs are shown in Table I. A typical real-time RT-PCR protocol was performed under the following conditions: 15 minutes at 95°C, followed by 40 cycles (94°C denaturing for 15 s; 58°C annealing for 20 s; 72°C extension for 15 s), melting at 60°C until 95°C for 90 seconds, and finally cooling to 35°C. The specificity of the SYBR Green assays was confirmed by melting point analysis. Gene expression of the housekeeping gene β-Actin was used for normalization of the results.

Data Analysis

Results were analyzed using Graph Pad Prism version 4.00 for Windows (Graph Pad Software, San Diego, CA). Means were compared using one-way analysis of variance (ANOVA) statistical evaluation followed by Tukey honestly significant difference post hoc test, and differences were considered to be significant at $P < .05$.

RESULTS

Histological and pHmetry Changes Induced by NGI

NGI induces a visible reduction of the laryngeal lumen (Fig. 1A–C), a consequence of a progressively increasing hyperplasia and hypertrophy of the glandular tissue (Fig. 1D–F). After 5 weeks of NGI, some areas of the epithelium showed a replacement of the differentiated pseudostratified respiratory epithelium of the larynx (Fig. 2A) by a squamous-stratified epithelium (metaplasia) and edema in the connective tissue (Fig. 2B). In addition, a large proliferation of plasmocytes and a few lymphocytes were present in the connective tissue of the mucosa (chronic inflammation) (Fig. 2C and D).

NGI resulted in significant changes in the pH recorded at the pharynx and esophagus (ANOVA, $P < .001$). Control rats showed a different pH between the pharyngeal and esophageal mucosa ($CONT_P \times CONT_E$, $P < .001$) (Fig. 3). No differences were observed after 1 week of NGI ($NGI_{P1w} \times NGI_{E1w}$, $P > .05$) because of a significant decrease in pharyngeal pH ($NGI_{P1w} \times CONT_P$, $P < .01$) (Fig. 3). After 2 weeks NGI, pH differences between

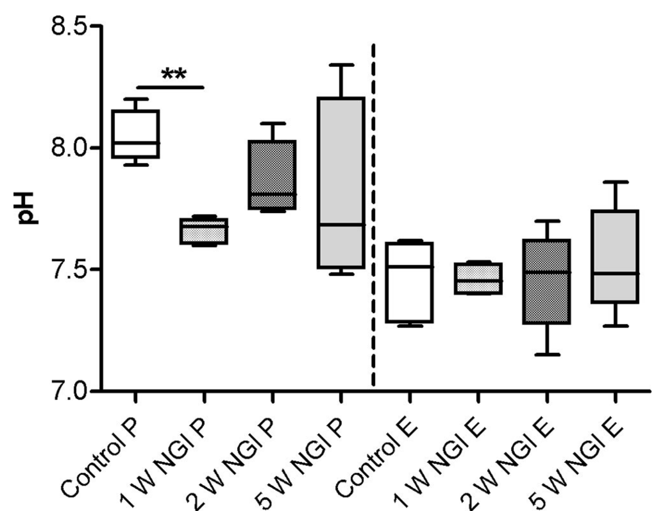


Fig. 3. Changes in the pH of the pharyngeal and esophageal mucosa measured at the end of the experimental period. Values for pharyngeal pH are placed at the left of the dashed line, whereas esophageal pH are recorded at the right. Control P and 1- to 5-week (W) nasogastric intubation (NGI) P = pharyngeal pH values for control and NGI animals; control E and 1 to 5 W NGI E = esophagic pH values for control and NGI animals. Only significant differences between pH values obtained in the same location are recorded in the graph. *$P < .05$, **$P < .01$, and ***$P < .001$.

mucosae were again significant ($NGI_{P2w} \times NGI_{E2w}$, $P <$ .001), a trend still observed after 5 weeks NGI (although not reaching significance) (Fig. 3). No differences were observed in the esophageal pH along the NGI period.

Changes in Laryngeal Peripheral Sensory Enervation

CGRP-immunoreactive fibers were present in the epithelium and connective tissue of the lamina propria of the laryngeal mucosa (Figs. 4A and 5A–C). Laryngeal immunostaining of SP depicted an intraepithelial nerve plexus with an overall location similar to that of CGRP fibers but with a lower density of fibers both in control and intubated animals (Figs. 4B and 5D–F). NGI induced changes in the expression of neurogenic peptides (ANOVA, $P < .05$), with the number of CGRP-and SP-immunoreactive fibers decreasing already during the first week of NGI (CGRP: $NGI_{1w} \times$ CONT, $P < .01$) and remaining decreased at the third week (CGRP: $NGI_{3w} \times$ CONT, $P < .001$; SP: $NGI_{3w} \times$ CONT, $P < .001$) (Figs. 4 and 5). Between 3 and 5 weeks, there was an increase in the number of fibers immunoreactive to CGRP ($NGI_{3-5w} \times$ CONT, $P \leq .05$) and SP ($NGI_{3-5w} \times$ CONT, $P \leq .01$) (Fig. 5), a recovery that reached control values in SP fiber density ($NGI_{5w} \times$ CONT, $P > .05$) (Fig. 4B) and was near control values in terms of CGRP fiber density ($NGI_{5w} \times$ CONT, $P < .05$) (Fig. 4A).

COX-2 Expression in Laryngeal Mucosa

The NGI model also induced changes in the expression of COX-2 in the laryngeal mucosa (ANOVA, $P < .05$). The number of cells expressing COX-2 enzyme in the laryngeal mucosa was higher after 2 weeks of NGI and increased significantly until the fifth week ($NGI_{5w} \times$ CONT, $P \leq .05$) (Fig. 6). This was caused by a progressive increase in the number of mononuclear inflammatory cells expressing COX-2 in the lamina propria (Fig. 1G–I), lymphocytes, and plasma cells (Fig. 2), which is characteristic of chronic inflammation. However, in regard to the relative expression of COX-2 mRNA levels in the laryngeal mucosa (ANOVA, $P = .01$), the progressive increase until the second week ($NGI_{2w} \times$ CONT, $P < .01$) was followed by a reduction to control values at 5 weeks ($NGI_{2w} \times NGI_{5w}$, $P < .05$) (Fig. 7A). On the contrary, no changes were observed in mRNA laryngeal levels of COX-1 constitutive enzyme (ANOVA, $P = .27$) (Fig. 7B). Thus, the ratio COX-2/COX-1 mRNA expression was changed after NGI (ANOVA, $P < .05$), with an increase until the second week (NGI2w $\times$ CONT, $P < .05$), followed by a return to control values at the end of the experimental period (Fig. 7C).

Laryngeal Levels of Pro-Inflammatory and Anti-Inflammatory Cytokines

The NGI model induced drastic changes of inflammatory mediators. The relative expression of IL-1β mRNA changed along the experimental period (ANOVA, $P <$ .0001), increasing continuously after 1 week ($NGI_{1w} \times$ CONT, $P < .01$) and 2 weeks ($NGI_{2w} \times$ CONT, $P < .001$; $NGI_{1w} \times NGI_{2w}$, $P < .01$) but returning to control values at 5 weeks NGI (NGI_{2-5w}, $P < .001$) (Fig. 8A). NGI affected the level of expression of TNF-α mRNA (ANOVA, $P < .001$), which increased after 1 week ($NGI_{1w} \times$ CONT, $P < .001$) and maintained a significant increased expression until the end of the experiment ($NGI_{2w} \times$ CONT, $P <$ .01; $NGI_{5w} \times$ CONT, $P < .01$) (Fig. 8B). IL-6 mRNA changed significantly (ANOVA, $P < .0001$) in the laryngeal mucosa, showing an increase 1 week after NGI ($NGI_{1w} \times$ CONT, $P < .001$) and then a progressive decrease until control values at 5 weeks ($NGI_{2w} \times$ CONT, $P < .01$; NGI_{1-5w}, $P < .001$; NGI_{2-5w}, $P < .05$) (Fig. 8C). Changes in mRNA levels of the anti-inflammatory cytokine IL-10 (ANOVA, $P < .001$) showed a strong decrease 1 week after NGI ($NGI_{1w} \times$ CONT, $P < .001$), which was still observed 5 weeks later ($NGI_{2W} \times$ CONT, $P < .001$; $NGI_{5w} \times$ CONT, $P < .001$) (Fig. 8D).

As concerns the analysis of the ratio between pro-inflammatory and anti-inflammatory cytokines, pro-inflammatory changes prevailed over anti-inflammatory

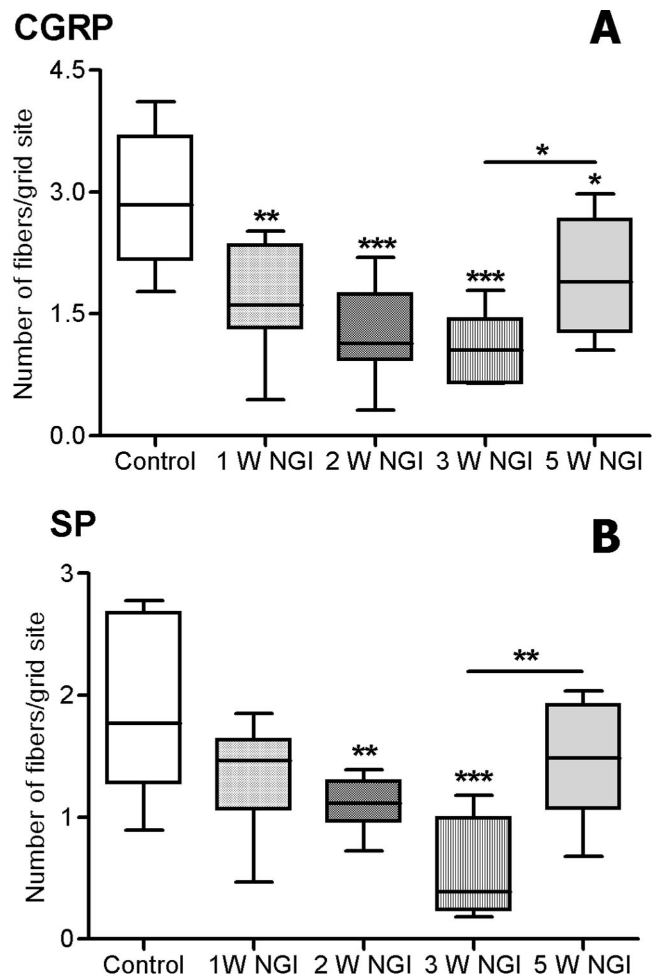


Fig. 4. Density of calcitonin gene-related peptide (CGRP)- (A) and substance P (SP)- (B) immunoreactive fibers in the laryngeal mucosa of animals without nasogastric tube (control group) and after different periods of nasogastric intubation (NGI). Note the significant decrease in the presence of these neurogenic peptides in laryngeal sensory fibers until the third week of NGI and the total (SP) or partial (CGRP) recovery observed at the end of the experimental period (5 wk). Asterisks over each week are comparisons with control values, whereas asterisks over each bar represent comparisons between values of groups submitted to NGI. *$P < .05$, **$P < .01$, and ***$P < .001$.

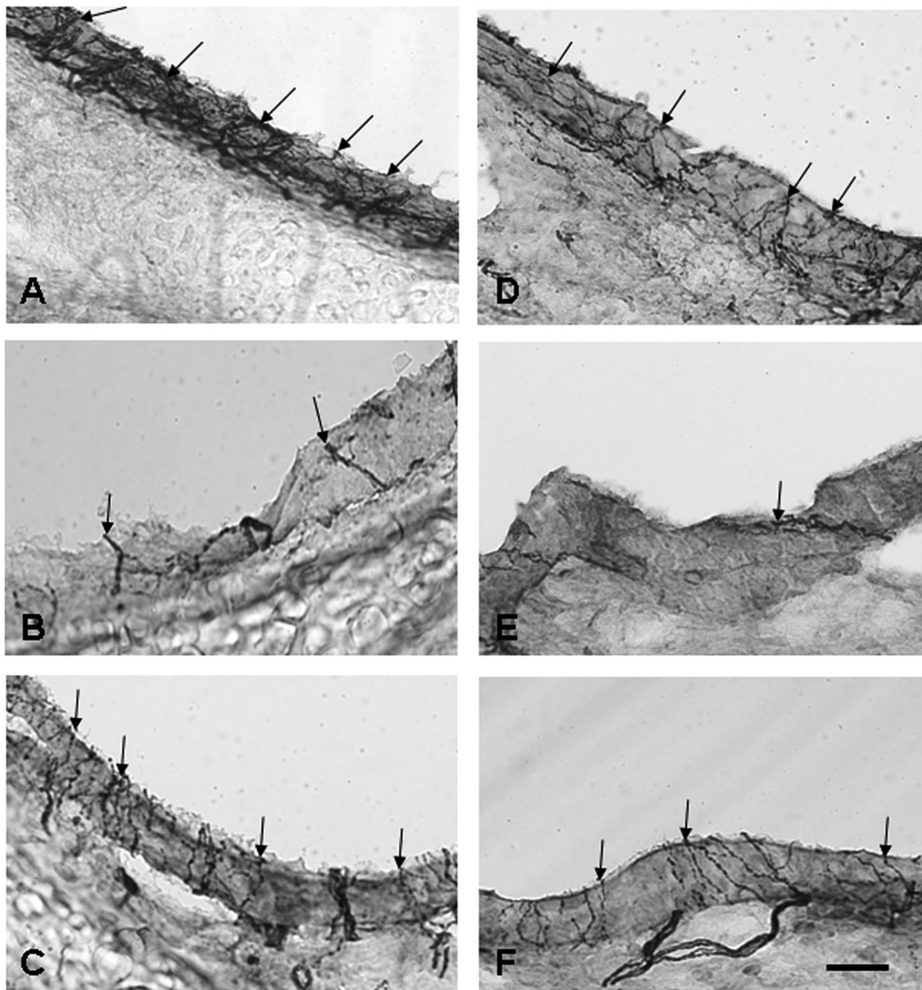


Fig. 5. Photomicrographs of calcitonin gene-related peptide (CGRP)- (A to C) and substance P (SP)- (D to F) immunoreactive fibers in the laryngeal epithelium of animals without nasogastric tube (controls, A and D) and after 3 (B and E) and 5 (C and F) weeks of nasogastric intubation (NGI). Note the strong decrease in CGRP and SP immunoreactive fibers (arrows) from nonintubation (A and D) to 3 weeks of laryngeal inflammation (B and E) and the recovery in the density of nociceptive primary afferent fibers containing CGRP and SP after 5 weeks of NGI (C and F). Magnification bar = 50 μm.

alterations. Accordingly, the ratio between IL-1β and IL-10 mRNA expression changed with time after NGI (ANOVA, $P < .0001$), with a progressive increase until the second week ($NGI_{1w} \times CONT$, $P < .05$; $NGI_{2w} \times CONT$, $P < .001$), followed by a return to control values (Fig. 8E). Interestingly, no changes were obtained for the ratio between TNF-α and IL-10 (Fig. 8F).

DISCUSSION

In the present study, we developed an NGI experimental model to induce laryngeal inflammation without direct mechanical injury to preserve the epithelium and lamina propria of the larynx. For model characterization, we showed that CGRP and SP present in nociceptive peripheral fibers decreased initially and then returned to control values. The inducible COX-2 enzyme shows the inverse pattern, increasing initially and then returning to baseline. However, the number of cells expressing COX-2 increased in inflammatory cells with increasing periods of NGI. Finally, IL-1β and IL-6 cytokines showed a pattern of mRNA expression along the 5 week NGI that was similar to COX-2 and inverse to CGRP and SP, whereas TNF-α was always increased and levels of IL-10 were always decreased along NGI.

Neurogenic inflammation is a well-defined process by which inflammation is triggered by the nervous system. As a pathway distinct from antigen-driven immune-mediated inflammation, it may play a key role in the understanding of a broad class of environmental health problems. It is a common pathway for disease in many organs and systems,[7–9] and a growing amount of evidence implicates the involvement of neurogenic etiology in disorders such as asthma, rhinitis, contact dermatitis, migraine headache, and rheumatoid arthritis.[10,11] In the respiratory tract, recent progresses in understanding the morphology of nerve fibers showed that intraepithelial nociceptors containing SP and CGRP neurogenic peptides project into the lumen of the larynx and can be directly stimulated by irritant substances.[4] The role of primary sensory neurons in arteriolar vasodilatation, increased vascular permeability, and leukocyte infiltration is mediated by peripheral release of peptides such as SP and CGRP from nerve terminals of depolarized peripheral nerve fibers.[12] SP binds to the neurokinin-1 receptor on target cells such as immune cells and vascular endothelial cells[11,13] and is the main mediator of vascular permeability and leukocyte infiltration, whereas CGRP is the prime mediator of neurogenic vasodilatation.[8,9] Moreover, SP

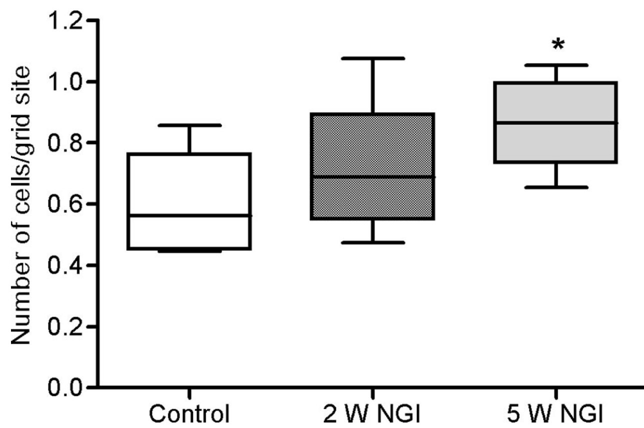
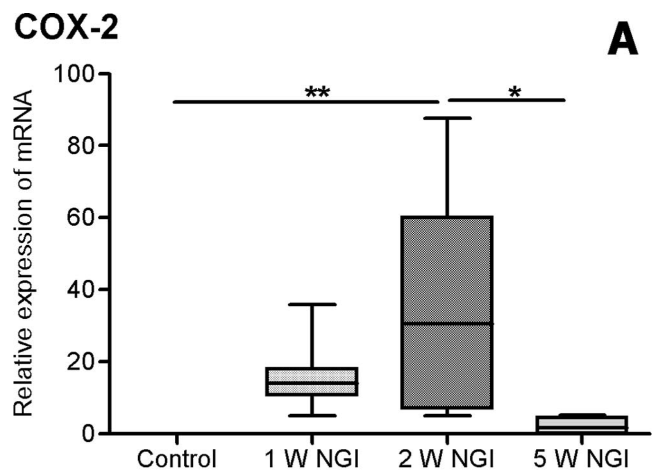


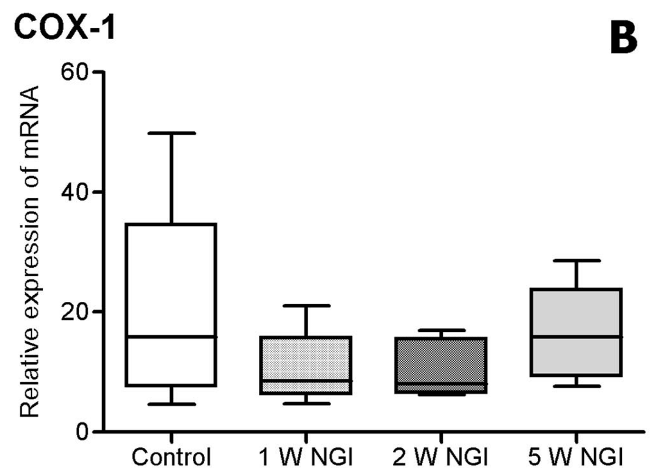
Fig. 6. Density of cyclooxygenase (COX)-2 immunoreactive cells in the laryngeal mucosa of animals without nasogastric tube and after 2 and 5 weeks of nasogastric intubation (NGI). Note the continuous increase in COX-2 containing cells in the laryngeal mucosa, which reaches significance at 5 weeks. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

can increase production and release of PGE2[14] and release of IL-1.[15] Thus, the here observed initial decrease in CGRP and SP content in laryngeal sensory fibers (first 3 wk) probably resulted from the release of these peptides from mucosal nociceptors and induction of neurogenic inflammation. This is supported by the fact that PGE2, one important member of the E series of PGs, facilitates the release of neurogenic peptides SP and CGRP from primary sensory neurons.[16]

In regard to the partial recovery of CGRP and SP innervation between the third and fifth week of NGI, this may reflect a progressive refilling of laryngeal nociceptors with these peptides after their synthesis in perikarya of primary sensory neurons. Accordingly, the decreased expression of COX-2 mRNA to control values may account for a decrease in the release of SP and CGRP from primary afferents, which results in an increase of SP and CGRP immunoreactivity in peripheral fibers after 5 weeks NGI. On the other hand, there was an apparent disagreement between the continuous increase of COX-2 positive cells present in the laryngeal mucosa along the 5 weeks of NGI and the peak of COX-2 mRNA expression after 2 weeks NGI followed by a return to control values at 5 weeks. The progressive increase of the number of COX-2 immunoreactive cells until the fifth week of laryngeal inflammation should reflect not mRNA expression for de novo production of the enzyme but rather the recruitment of immune cells containing COX-2 to the inflamed larynx. Interestingly, prolonged over-production of PGs by COX-2 can increase SP and CGRP immunoreactivities in primary sensory neurons.[17] Thus, the increased number of COX-2 immunoreactive cells in the laryngeal mucosa after 5 weeks (but not 3 wk) of laryngeal inflammation may also contribute to the recovery of laryngeal SP and CGRP content after 5 weeks of NGI.

In the present study, the inflammation observed with NGI may have two origins. First is the presence of the nasogastric tube passing through the pharynx to the esophagus, which can indirectly have a mechanical effect





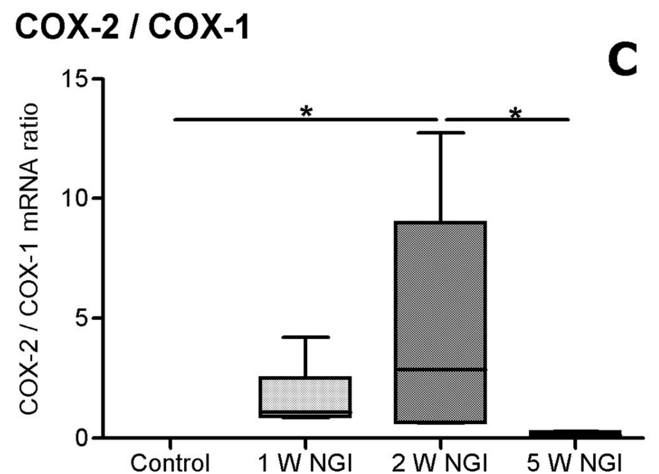


Fig. 7. Relative expression of cyclooxygenase (COX)-2 and COX-1 mRNA levels obtained by reverse-transcription polymerase chain reaction in the laryngeal mucosa of animals with or without induction of the nasogastric intubation (NGI) model. Note the rise of COX-2 mRNA expression until the second week and the return to basal values at the end of the experimental period (A). On the contrary, no changes were observed on the levels of the constitutive COX-1 enzyme (B). In regard to COX-2/COX-1 ratio of mRNA expression, the sequence of alterations was similar to that described for COX-2 (C). $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

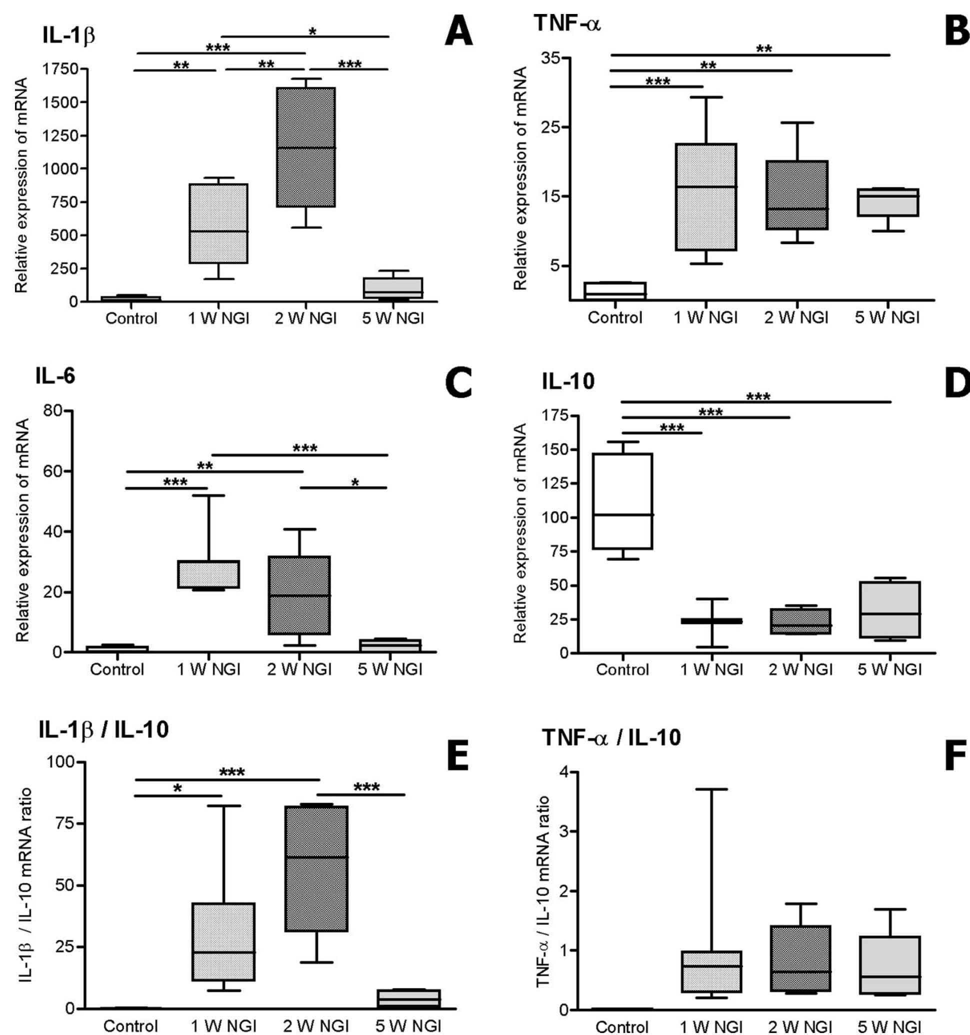


Fig. 8. Relative expression of mRNA levels obtained for pro-inflammatory and anti-inflammatory cytokines in the laryngeal mucosa of animals with or without induction of the laryngitis inflammatory model. Note the different evolution of expression of interleukin (IL)-1β (A), tumor necrosis factor (TNF)-α (B), IL-6 (C), and IL-10 (D). Although IL-1β and IL-6 showed a peak of expression (1–2 wk nasogastric intubation [NGI]) followed by a return to baseline values (5 wk NGI, A and C), TNF-α was increased along the entire period of NGI (B), and IL-10 was decreased along the 5 weeks of NGI (D). In regard to the IL-1β/IL-10 (E) and TNF-α/IL-10 (F) ratios of mRNA expression comparing pro- and anti-inflammatory cytokines, the sequence of alterations on the former was similar to that described for Il-1β, whereas the latter showed no alterations during experimental period. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

on the mucosa of the adjacent larynx. Second, the presence of acid from gastroesophagic reflux can directly activate nociceptors because H+ ions have receptors in the cell membrane of these fibers.[7] Accordingly, protons can induce CGRP release after activation of their receptors,[16] as observed here during the first weeks of NGI. The activation of nociceptors by noxious stimulation induces release of SP and CGRP, which increase blood vessel permeability and plasma extravasation.[7] Resident mast cells and other immune cells attracted to the inflamed laryngeal mucosa release cytokines and contribute to recruitment of more leukocytes and macrophages that release PGs and additional cytokines.[18] Cytokines IL-1β, IL-6, and TNF-α are well-known mediators of inflammation[18] because they cause PG release after production of arachidonic acid. Thus, cytokines form a link between tissue damage and inflammatory responses.[7] Binding of IL-1β to receptors on cell membrane initiates signaling cascades that upregulate transcription of genes such as COX-2, TNF-α, and IL-6. Moreover, IL-1β facilitates release of CGRP from nociceptors, probably by direct sensitization of nociceptors, whereas TNF-α sensitizes and induces ectopic activity in these primary afferent fibers.[18] These data strongly support the here observed matching opposite evolution of mRNA expression levels of IL-1β, IL-6, and COX-2 when compared with neurogenic peptides CGRP and SP present in nociceptive laryngeal fibers (decreasing during the first weeks and returning to control values at 5 wk). In regard to pro-inflammatory TNF-α and anti-inflammatory IL-10, their mRNA levels of expression in the laryngeal mucosa was, respectively, increased or decreased along the 5 weeks of NGI. This matching opposite evolution between the two cytokines is supported by other studies showing a similar close interaction.[18]

Although many functions have been suggested for COX-2, and the anti-inflammatory COX-2 inhibitors are known to have less aerodigestive adverse side effects,[16] the precise distribution and role of this enzyme is still unclear, and few studies on its role in chronic inflammation have been performed in the respiratory tract. In our study, the large increase in COX–2-immunoreactive inflammatory cells in the laryngeal mucosa after chronic NGI was noticed in mononuclear immune cells. This is clearly suggestive of the involvement of COX-2 in this form of laryngitis, and the location in mononuclear cells demonstrates a chronic inflammatory process. Selective COX-2 nonsteroidal anti-inflammatory drugs (NSAIDs) have less side effects than traditional (both antiCOX-1

and COX-2) NSAIDs on the digestive and respiratory systems because protective PGs do not decrease, whereas pro-inflammatory leukotrienes do not increase.[16] Accordingly, COX-1 inhibitors such as ibuprofen are effective in acute respiratory diseases but not in chronic pathologies because of their adverse side effects. The present model can be used in the future for evaluating the potential therapeutic value of selective COX-2 drugs in the treatment of chronic laryngitis.

CONCLUSIONS

A new animal model of chronic laryngitis was described, which results from NGI, a common technical procedure used in clinical practice. The resulting laryngitis develops an inflammatory process that presents a neurogenic component. COX-2 is also implicated in the inflammatory mechanism because its expression increases with time of intubation in mononuclear immune cells. Different patterns of mRNA expression of pro-inflammatory and anti-inflammatory mediators further characterized the inflammatory changes along time. This experimental NGI model of chronic laryngitis may be useful for future studies 1) in analyzing the molecular development of chronic inflammatory syndromes of the larynx, 2) in determining the clinical long-term consequences for chronic NGI patients, and 3) in evaluating the effect of new therapeutic approaches for clinical treatment of prolonged laryngitis.

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BIBLIOGRAPHY

1. Apostolakis L, Funk G, Urdaneta L, et al. The nasogastric tube syndrome: two case reports and review of the literature. *Head Neck* 2001;23:59–63.
2. Friedman M, Baim H, Shelton V, et al. Laryngeal injuries secondary to nasogastric tubes. *Ann Otol Rhinol Laryngol* 1981;90:469–474.
3. Coutaux A, Adam F, Willer JC, Le Bars D. Hyperalgesia and allodynia: peripheral mechanisms. *Joint Bone Spine* 2005; 72:359–371.
4. Lima-Rodrigues M, Nunes R, Almeida A. Intraepithelial nerve fibers project into the lumen of the larynx. *Laryngoscope* 2004;114:1074–1077.
5. Kohrogi H, Hamamoto J, Kawano O, et al. The role of substance P release in lung with oesophageal acid. *Am J Med* 2001;111:25S–30S.
6. Gundersen H, Jensen E, Kieu K, et al. The efficiency of systematic sampling in stereology-reconsidered. *J Microsc* 1999;193:199–211.
7. Holzer P. Neurogenic vasodilatation and plasma leakage in the skin. *Gen Pharmac* 1988;30:5–11.
8. Meggs WJ. Neurogenic inflammation and sensitivity to environmental chemicals. *Environ Health Perspect* 1993;101: 234–238.
9. Lacroix J. Chronic rhinosinusitis and neuropeptides. *Swiss Med Wkly* 2003;133:560–562.
10. O'Connor T, O'Connell J, O'Brien, et al. The role of substance P in inflammatory disease. *J Cell Physiol* 2004;201: 167–180.
11. Schmelz M, Petersen L. Neurogenic inflammation in human and rodent skin. *News Physiol Sci* 2001;16:33–37.
12. Richardson J, Vasko M. Cellular mechanisms of neurogenic inflammation. *J Pharm Exp Therap* 2000;302:839–845.
13. Geppetti P, Capone JG, Trevisani M, et al. CGRP and migraine: neurogenic inflammation revisited. *J Headache Pain* 2005;6:61–70.
14. Hingtgen CM, Waite KJ, Vasko MR. Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3′,5′-cyclic monophosphate transduction cascade. *J Neurosci* 1995;15:5411–5419.
15. Lotz M, Vaughan JH, Carson DA. Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science* 1988;241:1218–1221.
16. Vanegas H, Schaible H-G. Prostaglandins and cyclooxygenases in the spinal cord. *Prog Neurobiol* 2001;64: 327–363.
17. Ma W, Eisenach JC. Intraplantar injection of a cyclooxygenase inhibitor ketorolac reduces immunoreactivities of substance P, calcitonin gene-related peptide, and dynorphin in the dorsal horn of rats with nerve injury or inflammation. *Neuroscience* 2003;121:681–690.
18. Moalem G, Tracey DJ. Immune and inflammatory mechanisms in neuropathic pain. *Brain Res Rev* 2006;51:240–264.