HIGHLIGHTED TOPIC | Reflexes from the Lungs and Airways

Neural and hydroxyl radical mechanisms underlying laryngeal airway hyperreactivity induced by laryngeal acid-pepsin insult in anesthetized rats

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Tsai, Tung-Lung, Shyue-Yih Chang, Chin-Yin Ho, and Yu Ru Kou. Neural and hydroxyl radical mechanisms underlying laryngeal airway hyperreactivity induced by laryngeal acid-pepsin insult in anesthetized rats. J Appl Physiol 101: 328–338, 2006; doi:10.1152/japplphysiol.00064.2006.—Laryngopharyngeal or gastroesophageal reflux is associated with laryngeal airway hyperreactivity (LAH), but neither the cause-effect relationship nor the underlying mechanism has been elucidated. Here we established a rat model with enhanced laryngeal reflex reactivity induced by laryngeal acid-pepsin insult and investigated the neural and hydroxyl radical (OH) mechanisms involved. The laryngeal segments of 103 anesthetized rats were functionally isolated while animals breathed spontaneously. Ammonia vapor was delivered into the laryngeal segment to measure laryngeal reflex reactivity. We found that the laryngeal pH 5-pepsin treatment doubled the reflex apneic response to ammonia, whereas laryngeal pH 7.4-pepsin, pH 2-pepsin, and pH 5-denatured pepsin treatment had no effect. Histological examination revealed limited laryngeal inflammation and epithelial damage after pH 5-pepsin treatment and more severe damage after pH 2-pepsin treatment. In rats that had received the laryngeal pH 5-pepsin treatment, the apneic response to ammonia was abolished by either denervation or perineural capsacin treatment (PCT; a procedure that selectively blocks capsacin-sensitive afferent fibers) of the superior laryngeal nerves, but was unaffected by perineural sham treatment. LAH was prevented by laryngeal application of either dimethylthiourea (DMTU; a -OH scavenger) or deferoxamine (DEF; an antioxidant for -OH), but was unaltered by the DMTU vehicle or iron-saturated DEF (ineffective DEF). LAH reappeared after recovery from PCT, DMTU, or DEF (ineffective scavenger) or deferoxamine (DEF; an antioxidant for -OH mechanism).

enhanced reflex reactivity; laryngeal capsacin-sensitive afferent fibers; hydroxyl radicals

LARYNGOPHARYNGEAL OR GASTROESOPHAGEAL reflux, a syndrome caused by the backflow of gastric contents into the upper aerodigestive tract, is associated with the laryngeal airway hyperreactivity (LAH) (3, 39). LAH is manifested by increased sensitivity of laryngeal reflexes, such as cough, glottic-stop, or laryngeal adductor responses (4, 15, 41, 45, 60). Although refluxed acid and pepsin in the larynx have been postulated to be a major contributory factor (3, 39, 42, 63), neither the cause-effect relationship nor the underlying mechanism of LAH has been elucidated.

The superior laryngeal nerves (SLNs), a branch of the vagus nerve, provide the major sensory innervation to the larynx (50, 65). Both unmyelinated and myelinated afferents of the SLNs are involved in eliciting airway reflexes (50, 65) and thus play an important role in the regulation of laryngeal reactivity. Among them, a subpopulation of laryngeal afferents, mainly C fibers and some A-δ fibers, are sensitive to capsacin, a pungent active ingredient of hot pepper (6, 7, 35). The capsacin-sensitive afferent fibers in the airway are considered to be nociceptive-like free nerve endings (7) and have been implicated in the development of lower airway hyperreactivity (29, 62). It has been demonstrated that laryngeal application of capsacin can trigger several airway reflexes, including bronchoconstriction, cough, apnea, and glottic-stop reflex (9, 19, 30, 33, 36, 37, 45, 59). Thus LAH may develop when the sensitivity of these laryngeal afferents is enhanced under pathological conditions, such as inflammation (62). In fact, clinical studies (4, 15, 41, 45) have reported that patients with gastroesophageal reflux have an increase in cough reflex sensitivity to inhaled capsacin aerosol. However, whether the laryngeal acid-pepsin insult may sensitize these afferent fibers, resulting in enhanced laryngeal reflex reactivity, remains to be investigated.

Measurement of the pH values of acid reflux suggests that patients with laryngopharyngeal or gastroesophageal reflux can have their reflux subcategorized into acid (pH < 4), weakly acidic (pH = 4–7), and nonacid groups (54, 55). However, pepsin has a greater activity in a more acidic condition (12), and, therefore, laryngeal exposure to acid-pepsin solution at different pH causes different levels of laryngeal inflammation (1). It is known that excess production of reactive oxygen species (ROS) is one of the major consequences of tissue inflammation (14). The major ROS are the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical (-OH) (10). The superoxide anion radical dismutates to form hydrogen peroxide, which, in the presence of iron, can further react to form -OH, a more reactive oxygen radicals, via the Fenton reaction (10). Although ROS have been implicated in the pathogenesis of lower airway hyperreactivity (5, 8, 20), their role in LAH is still not known.

In light of the existing knowledge and the unanswered questions described above, the present study was undertaken in anesthetized rats to investigate whether laryngeal exposure

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to an acid-pepsin insult may induce LAH; 2) whether capsaicin-sensitive laryngeal afferent fibers mediate LAH; 3) whether -OH participates in the development of LAH; and 4) whether LAH correlates with the extent of laryngeal inflammation on histological examination.

METHODS

All protocols were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health, and were approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University, Taiwan. All pharmacological agents used in this study were purchased from Sigma Chemical, St. Louis, MO.

Animal preparation. Male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of α-chloralose (100 mg/kg) and urethane (500 mg/kg) dissolved in a borax solution (2%). The right femoral artery and jugular vein were cannulated to record arterial blood pressure and for intravenous administration of pharmacological agents, respectively. During the experiment, the depth of anesthesia was regularly monitored at fixed intervals, and supplemental doses of chloralose (20 mg·kg⁻¹·h⁻¹) and urethane (100 mg·kg⁻¹·h⁻¹) were administered to maintain the absence of pain reflexes induced by pinching the animal’s tail. The animal was tethered in a supine position, the neck was opened in the midline, and the esophagus was ligated as rostrally as possible. The SLNs were isolated carefully for later experiments. Body temperature was maintained at 37°C during the experiment by means of a servo-controlled heating blanket.

Functionally isolated laryngeal preparation. The methods for the preparation of a functionally isolated larynx have been described in detail in previous studies (30, 31). In brief, after the trachea was exposed, a lower tracheal catheter (PE-260) was inserted caudally just above the thoracic inlet, while an upper tracheal catheter (PE-200) was inserted cranially with its tip placed slightly below the cricoid cartilage. A homemade trumpet-like glass tube (length = 55 mm) was introduced through the mouth with its tip placed at the vallecula by direct vision; the tip has an inner diameter of 4 mm, while the other end has an inner diameter of 10 mm. The position of this oral tube was fixed to the upper jaw of the rat. The position of the tube tip at the pharynx was confirmed by autopsy after animals had been euthanized at the end of the experiment. During the experiment, rats breathed spontaneously via the lower tracheal catheter. Respiratory flow was measured with a pneumotachograph (4/0; Fleisch, Richmond, VA) coupled with a differential pressure transducer (MP45–14; Validyne, Northridge, CA). The flow signal was integrated to give tidal volume. All physiological signals were recorded on a chart recorder (TA11; Gould, Cleveland, OH) and a tape recorder (DR-890; Neurocorroder, New York, NY) for later analysis.

Laryngeal challenge with ammonia. Laryngeal challenge with ammonia was used to measure laryngeal reflex reactivity (57) and used a method described previously (31). Briefly, ammonia vapor (0.2% in air) was continuously delivered at a constant flow rate of 1.4 ml/s by a syringe pump (367; Sage, Cambridge, MA) into a section of 6-ml Teflon tubing (8 mm inner diameter) connected to the proximal end of the upper airway catheter. The communication between the Teflon tubing and the upper airway catheter was quickly blocked by a three-way stopcock at the end of ammonia delivery. In each challenge, the total amount of ammonia delivered was 11 ml: 5 ml passed through the isolated larynx and flowed out to the environment via the oral tube, whereas the rest remained in the luminal space of the Teflon tubing. To avoid possible tachyphylaxis, at least 50 min were allowed to elapse between two ammonia challenges.

Laryngeal treatment with acid-pepsin, pH 7.4-pepsin, or acid-denatured pepsin solution. The original solution was prepared by adding 75-mg pepsin to 30-ml normal saline, which had a pH value of ~3.68. The pH of the original solution was then adjusted to either 5 (pH 5-pepsin) or 7.4 (pH 7.4-pepsin) by 1 N NaOH or adjusted to 2 (pH 2-pepsin) by 1 N HCl. The pH 5-denatured pepsin solution was prepared by adjusting the original solution to pH 12, maintaining at pH 12 for 30 min, and reacidifying to pH 5, as described in a previous study (22). A small piece of filter paper (~1 × 10 mm) was presoaked with one of these solutions, then carefully inserted into the laryngeal segment via the oral tube, and removed 40 s after its insertion. After laryngeal treatments, an elapsed time of at least 60 min was allowed before the ensuing ammonia challenge.

Laryngeal application of pharmacological agents. To study reflex responses resulting from stimulation of the capsaicin-sensitive laryngeal afferent fibers, a solution of capsaicin (0.2 μg/ml, 0.05 ml) was gently sprayed into the laryngeal segment via a spinal needle. To investigate the role of -OH, a piece of filter paper (~1 × 10 mm) containing dimethylthiourea (DMTU; 500 mg/ml; a -OH scavenger), vehicle of DMTU, deferoxamine (DEF; 250 mg/ml; an antioxidant for -OH), or iron-saturated DEF (250 mg/ml; ineffective DEF) was carefully inserted into the laryngeal segment and then removed 20 s after insertion. The stock solution of capsaicin (250 μg/ml) was prepared by dissolving the chemical in a solution of 98% ethanol, then returning 1% Tween 80, 1% ethanol, and 98% saline and was stored at ~20°C. A solution of capsaicin at the desired concentration was prepared daily by diluting with normal saline. Both DMTU and DEF were dissolved in saline. Iron-saturated DEF (DEF+Fe) was prepared by adding 98 mg FeCl₃·6H₂O to 1 ml DEF (250 mg/ml) for 1 h at room temperature, as described previously (56). Laryngeal pretreatments with DMTU, DMTU vehicle, DEF, and DEF+Fe were made 35 min before the laryngeal acid-pepsin treatment or ammonia challenge. The doses and treatment time of these agents were determined by a preliminary study, which indicated that the suppressive effect of DMTU and DEF on LAH could last for ~180 min.

Perineural capsaicin treatment and perineural sham treatment of the SLNs. Perineural capsaicin treatment (PCT) has been demonstrated to selectively block the reflex responses resulting from stimulation of capsaicin-sensitive airway afferent fibers (48), and the method has been described in detail previously (27). In brief, a segment (~2 mm) of each SLN was wrapped in a cotton strip, which had been presoaked in either capsaicin solution (30 μg/ml; capsaicin treatment) or capsaicin vehicle (sham treatment). After 5 min, the cotton strips were removed. The blocking effect of PCT was confirmed when the reflex response to laryngeal capsaicin was abolished, yet the reflex response to laryngeal mechanical stimulation by a nylon thread (diameter = 0.1 mm) is preserved (see RESULTS). Our preliminary study indicated that the blocking effect of PCT on the reflex responses to laryngeal capsaicin could last for ~45 min. Facilitation of the recovery from PCT was achieved by washing both SLNs with warm saline (see RESULTS).

Histological preparation and examination. After the animals were killed by intravenous injection of an overdose of the anesthetics, their larynxes were excised and fixed by immersion in a buffered neutral formalin solution for 48 h. Tissue specimens were embedded in paraffin and were cut transversely into 5-μm-thick sections, which were subsequently stained with hematoxylin and eosin. These sections were examined by a qualified pathologist in a blind fashion. The histological assessment was quantified by using a scoring system that assigned a value from 0 (none or normal) to 3 (severe or diffuse) for epithelial damage and infiltration of inflammatory cells of the laryngeal tissues. Total injury scores were obtained by adding the values assessed from these two injury variables. For each rat, three histological sections were examined. For each section, structures at three different areas were randomly selected, and their injury scores were averaged.

Experimental design and protocol. In this study, 103 rats (weight 380 ± 24 g) were randomly divided into 14 groups of animals to conduct four series of experiments: each of the groups 1–11 contained 8 rats, whereas each of the groups 12–14 contained 5 rats. In study 1, the reflex apanic responses to five repeated laryngeal challenges of
ammonia were studied in four groups of rats. In each group, the first challenge was made at 40 min before laryngeal treatment with pH 2-pepsin (group 1), pH 5-pepsin (group 2), pH 7.4-pepsin (group 3), or pH 5-denatured pepsin solution (group 4) to obtain the control response. One hour after these laryngeal treatments, four repeated challenges were made with any two challenges separated by 1 h. Denervation of the SLNs was performed at 30 min before the last challenge. In study 2, the reflex apneic responses to three repeated laryngeal challenges of ammonia were studied in two groups of rats. Furthermore, 5 min after each ammonia test, the reflex apneic response to mechanical probing of laryngeal segment by a nylon thread was also studied to confirm the viability of laryngeal afferent fibers. The first challenge was made at 40 min before laryngeal treatment with pH 5-pepsin to obtain the control response. A PCT (group 5) or a perineural sham treatment (group 6) of the SLNs was performed at 15 min before the second challenge, which was made at 2 h after laryngeal pH 5-pepsin treatment. The third challenge was made at 3 h after laryngeal pH 5-pepsin treatment. To assess the effectiveness of PCT, the reflex apneic responses to three repeated laryngeal challenges of capsaicin with any two separated by 1 h were investigated in the other group of rats (group 7). PCT of the SLNs was performed 15 min before the second challenge. In study 3, the reflex apneic responses to three repeated laryngeal challenges of ammonia were studied in four groups of rats. The laryngeal segment of these animals was locally pretreated with DMTU (group 8), vehicle of DMTU (group 9), DEF (group 10), or DEF+Fe (group 11). The first challenge was made at 35 min after these local pretreatments to obtain the control response. Subsequently, 40 min were allowed to elapse before laryngeal treatment with pH 5-pepsin. The second and third challenges were made at 2 and 3 h, respectively, after laryngeal pH 5-pepsin treatment. In study 4, laryngeal treatment with pH 2-pepsin (group 12), pH 5-pepsin (group 13), or pH 7.4-pepsin (group 14) was performed. Two hours later, the rat’s larynx was excised for pathohistological study.

Data analysis and statistics. Respiratory flow, tidal volume, and expiratory duration (Ti) were analyzed on a breath-by-breath basis. At least 10 breaths before and 30 breaths after the challenge with ammonia, capsaicin, or mechanical stimulation were measured. Baseline data for Ti were calculated as the mean over 10 breaths immediately before challenge. Mean arterial blood pressure and heart rate were measured at 1-s intervals. These physiological parameters were analyzed using a computer equipped with an analog-to-digital converter (DASA 4600, Gould) and software (1.0; BioCybermetics, Taipei, Taiwan). To compare the responses evoked by the various experimental conditions and to minimize the influence caused by different breathing patterns among the animals, we normalized the apneic response in each rat to give a percentage apneic index. For this purpose, the longest Ti occurring during the first 5 s after laryngeal challenge with stimulant was divided by the baseline Ti, and the value was then multiplied by 100. Data for the cardiopulmonary parameters were compared using one-way repeated-measures ANOVA followed by Fisher’s least significant difference procedure, where appropriate. The total injury score data were evaluated by a Kruskal-Wallis nonparametric test followed by Mann-Whitney test, when appropriate. A value of $P < 0.05$ was considered significant. All data are presented as means ± SE.

RESULTS

LAH induced by laryngeal acid-pepsin treatment. To establish the LAH model, the laryngeal segments were treated with pepsin or denatured pepsin at various pH in four study groups. Before any laryngeal treatment, an apneic response (apneic index = $592 \pm 58\%$) was elicited within 1 s after laryngeal ammonia challenge (Figs. 1A and 2). After laryngeal treatment with pH 5-pepsin solution, the same ammonia challenge elicited a similar magnitude of apneic response (apneic index = $787 \pm 107\%$, $P = 0.22$) at the 1st h (Fig. 2), but evoked a significantly augmented apneic response at the 2nd h (apneic index = $1,227 \pm 142\%$, $P < 0.05$) and the 3rd h (apneic index = $1,193 \pm 136\%$, $P < 0.05$) after laryngeal treatment (Figs. 1A and 2), compared with the control response. In contrast, the apneic response to repeated ammonia challenges did not significantly alter with time after laryngeal treatment with pH 7.4-pepsin, pH 2-pepsin, or pH 5-denatured pepsin solution, compared with the control (Figs. 1B and 2). A subsequent denervation of the SLNs totally abolished the apneic response to ammonia challenge (Figs. 1 and 2), indicating that the response was a reflex mediated through the SLNs. The pH 5-pepsin solution was then chosen as the standard laryngeal treatment for the subsequent studies.

Role of capsaicin-sensitive laryngeal afferent fibers in the development of LAH. The technique of selective blockade of capsaicin-sensitive laryngeal afferent fibers by PCT was verified in one study group. Before PCT, either laryngeal capsaicin challenge or laryngeal mechanical stimulation evoked an apneic response in the same animals (Figs. 3 and 4A). PCT of the SLNs selectively abolished the apneic response to capsaicin challenge, whereas it failed to significantly affect the apneic response to mechanical stimulation (Figs. 3 and 4A). After a few washes of the SLNs by warm saline and 1 h of recovery time, the reflex apneic response to capsaicin challenge reappeared, while the apneic response to mechanical stimulation still persisted (Figs. 3A and 4A). Using this technique, PCT and perineural sham treatment were applied to the SLNs of the other two study groups to investigate the role of these afferent fibers in the LAH. Two hours after laryngeal pH 5-pepsin treatment, a time during which LAH was supposed to occur, PCT nearly abolished the reflex apneic response to ammonia challenge (Figs. 4B and 5A), whereas it did not significantly affect the apneic response to mechanical stimulation in the same animals (Figs. 4B and 5B). After a few washes of the SLNs by warm saline and 1 h of recovery time, the reflex apneic response to ammonia challenge measured at the 3rd h after laryngeal pH 5-pepsin treatment not only reappeared, but also was significantly greater than the control response (Figs. 4B and 5B). In contrast, perineural sham treatment did not affect the enhanced laryngeal responsiveness to ammonia challenge occurring at the 2nd and 3rd h after laryngeal pH 5-pepsin treatment (Fig. 4C).

Importance of ·OH in the development of LAH. The importance of ·OH in the LAH was investigated in four study groups initially pretreated with DMTU, vehicle of DMTU, DEF, or DEF+Fe. Two hours after laryngeal pH 5-pepsin treatment, a time during which LAH was supposed to occur, laryngeal ammonia challenge evoked an apneic response that did not significantly differ from the control response in animals pretreated with DMTU or DEF (Figs. 6A, 7A, and 8). Subsequently, after the effect of DMTU and DEF had been worn off, the same ammonia challenge elicited a significantly greater apneic response at the 3rd h after laryngeal pH 5-pepsin treatment, compared with the control responses (Figs. 6A, 7A, and 8). In contrast, animals pretreated with vehicle of DMTU or DEF+Fe displayed a significantly augmented apneic response to ammonia challenge at the 2nd and 3rd h after laryngeal pH 5-pepsin treatment (Figs. 6B, 7B, and 8), compared with the control responses.
Responses of arterial blood pressure to laryngeal ammonia.

In the controls, laryngeal ammonia challenge slightly but significantly increased mean arterial blood pressure from a baseline of 115 ± 1 mmHg to a peak of 125 ± 1 mmHg (n = 80) and significantly decreased heart rate from a baseline of 377 ± 2 beats/min to a minimum of 348 ± 7 beats/min (n = 80) measured at the first 10-s period following the challenge. These ammonia-induced cardiovascular changes were unaltered by the various laryngeal acid-pepsin insults (Table 1); were not influenced by perineural sham treatment, DMTU, vehicle of DMTU, DEF, or DEF+Fe (Table 2) but were abolished by denervation of the SLNs (Table 1) or PCT (Table 2).

Laryngeal inflammation induced by laryngeal acid-pepsin treatment. Histological examinations revealed that laryngeal pH 5-pepsin treatment produced limited epithelial damage and/or observable infiltration of inflammatory cells (Fig. 9B), while laryngeal pH 2-pepsin treatment produced observable epithelial damage and severe infiltration of inflammatory cells into laryngeal tissues (Fig. 9C). Such injurious signs were seldom seen in laryngeal tissues that had received the laryngeal pH 7.4-pepsin treatment (Fig. 9A). Overall, the total injury

Fig. 1. Immediate responses to laryngeal ammonia challenges in 2 anesthetized rats. Responses were obtained 40 min before and up to 4 h after laryngeal treatment with pepsin in pH 5 solution (pH 5-PS; A) or denatured pepsin in pH 5 solution (pH 5-DPS; B). Responses after denervation of superior laryngeal nerves (SLN cut) were obtained at the 4th h after laryngeal treatment. Vr, respiratory flow; Vt, tidal volume; ABP, arterial blood pressure. Arrows, onset of ammonia challenge. See text for detailed explanations of laryngeal treatment and ammonia challenge.

Fig. 2. Mean apneic responses to laryngeal ammonia challenges before and after laryngeal treatment with pH 2-pepsin, pH 5-pepsin, pH 7.4-pepsin, or pH 5-DPS in 4 study groups. Control responses were obtained 40 min before laryngeal treatments, whereas responses after denervation of SLN were obtained at the 4th h after laryngeal treatment. Baseline expiratory duration (Ti) was calculated as the mean over 10 breaths immediately before challenge, whereas the apnic duration was defined as the longest Ti occurring during the first 5 s after laryngeal ammonia challenge. Data in each group are the means ± SE from 8 rats. *Significantly different from the control response in the same group, P < 0.05.
score in the pH 2-pepsin group (5.8 ± 0.5) was significantly higher than that in the pH 5-pepsin group (3.5 ± 0.3), which was significantly higher than that in the pH 7.4-pepsin group (1.3 ± 0.7).

**DISCUSSION**

The results of the first part of this study demonstrate that laryngeal treatment with pH 5-pepsin solution augmented the apneic response to laryngeal ammonia challenge, whereas laryngeal treatment with pH 7.4-pepsin or pH 2-pepsin solution failed to produce this augmented effect. Additionally, the acidified pepsin seems to be the determining factor, because laryngeal treatment with pH 5-denatured pepsin was ineffective at producing this LAH. Furthermore, the apneic response to laryngeal ammonia challenge was totally abolished by denervation of the SLNs, indicating its reflex nature. Our histo-

**Fig. 3.** Immediate responses to laryngeal capsaicin challenge (A) and laryngeal mechanical stimulation (B) before, during, and after recovery from perineural capsaicin treatment (PCT) of SLNs in 1 anesthetized rat. PCT was performed at 15 min before the second capsaicin challenge by wrapping a segment of each SLN in a cotton strip containing capsaicin solution (30 μg/ml) for 5 min. After obtaining the responses during PCT, both SLNs were washed by warm saline to facilitate the recovery from PCT. Any 2 of the 3 repeated capsaicin challenges or mechanical stimulation were separated by 1 h. The elapsed time between capsaicin challenge and mechanical stimulation was 5 min. Arrows, onset of ammonia challenge or mechanical stimulation. See text for detailed explanations of capsaicin challenge and mechanical stimulation.

**Fig. 4.** Mean apneic responses to laryngeal capsaicin challenge, laryngeal mechanical stimulation, and laryngeal ammonia challenge before, during, and after recovery from PCT (A and B) or perineural sham treatment (PST; C) of SLNs in 3 study groups. A: responses to capsaicin challenge and mechanical stimulation in 1 group without any laryngeal treatment. B and C: responses to ammonia challenge and mechanical stimulation at 40 min before, 2 h after, and 3 h after laryngeal treatment with pH 5-PS in the other 2 groups. Data in each group are the mean ± SE from 8 rats. *Significantly different from the response before PCT or PST, P < 0.05.
Fig. 5. Immediate responses to laryngeal ammonia challenge (A) and laryngeal mechanical stimulation (B) before, during, and after recovery from PCT of SLNs. The first, second, and third ammonia challenge or mechanical stimulation was made 40 min before, 2 h after, and 3 h after laryngeal treatment with pH 5-PS. PCT was performed at 15 min before the second ammonia challenge. The elapsed time between ammonia challenge and mechanical stimulation was 5 min. Arrows, onset of ammonia challenge or mechanical stimulation.

Fig. 6. Immediate responses to laryngeal ammonia challenge before and after laryngeal treatment with pH 5-PS in two anesthetized rats pretreated with laryngeal application of dimethylthiourea (DMTU; A) or its vehicle (B). Laryngeal application was performed at 35 min before the first ammonia challenge. Responses were obtained 40 min before, 2 h after, and 3 h after laryngeal pH 5-PS treatment. Arrows, onset of ammonia challenge. See text for detailed explanations of laryngeal application of DMTU or its vehicle.
logical study reveals that laryngeal pH 7.4-pepsin treatment did not cause detectable histological change in the laryngeal tissues. This is not surprising because pepsin at pH 7.4 has no activity (12). However, we further found that pepsin at pH 2 resulted in more severe laryngeal inflammation compared with pepsin at pH 5, a result that is consistent with previous findings in dogs (1). Thus it appears that the development of LAH is dissociated from the severity of laryngeal inflammation in our model. The exact reason why pH 2-pepsin treatment is ineffective at producing LAH is not clear, but perhaps the greater severity of laryngeal tissue damage affects the function of laryngeal sensory nerve endings, as has been suggested by other investigators (49). These investigators (49) showed that instillations of pH 2-pepsin solutions into the laryngeal lumen of the dog caused laryngeal injury and actually lessened the laryngeal afferent responses and reflex responses to negative pressure. Similarly, laryngopharyngeal infusions of pH 1.2 HCl solution have been shown to impair laryngopharyngeal mechanosensitivity in patients with chronic cough and gastroesophageal reflux, a result that was also attributed to the deleterious effect of acid on laryngeal sensory receptors (44). Taken together, our results suggest that the laryngeal exposure to pepsin at a weakly acidic pH (pH 5), but not at an acidic pH (pH 2), is important in the development to the LAH, and that this enhanced laryngeal reflex reactivity is probably mediated through sensitization of the SLN afferent fibers by the laryngeal acid-pepsin insult.

Fig. 7. Immediate responses to laryngeal ammonia challenge before and after laryngeal treatment with pH 5-PS in two anesthetized rats pretreated with laryngeal application of deferoxamine (DEF; A) or iron-saturated DEF (DEF+Fe; B). Laryngeal application was performed at 35 min before the first ammonia challenge. Responses were obtained 40 min before, 2 h after, and 3 h after laryngeal pH 5-PS treatment. Arrows, onset of ammonia challenge. See text for detailed explanations of laryngeal application of DEF or DEF+Fe.

Fig. 8. Mean apneic responses to laryngeal ammonia challenges before and after laryngeal treatment with pH 5-PS in 4 study groups. A: animals pretreated with DMTU or its vehicle in 2 groups. B: animals pretreated with DEF or DEF+Fe in the other 2 groups. Data in each group are the mean ± SE from 8 rats. *Significantly different from the response before pH 5-PS in the same group, P < 0.05.
The presence of acid and pepsin in the larynx is not uncommon in patients with laryngopharyngeal or gastroesophageal reflux (3, 26, 39). Measurements of pH and pepsin in the reflux fluid provide information helpful to understanding the pathophysiology of these patients (17, 23, 39). Traditionally, acid and nonacid reflexes are defined as having a pH value lower and greater than 4, respectively. However, recent clinical studies reveal that a significant portion of these patients has acidic reflux with a pH of >4 (46, 54, 55). Furthermore, pepsin is believed to exhibit activity when the pH is >4 (2). Moreover, the larynx may be easily assaulted by a pH of >4, since the larynx has weaker protection mechanisms against acid than the esophagus (52). These notions promote the emerging concept that a new category of weakly acidic reflux with a pH ranging from 4 to 7 should be defined (46, 54, 55). Indeed, weakly acidic reflux has been demonstrated to be associated with extra-esophageal symptoms, such as chronic cough or other respiratory symptoms (53). Our finding that the laryngeal pH 5-pepsin insult can cause LAH is in good agreement with this concept.

The results from the second part of this study demonstrate that PCT abolished the reflex apneic response to laryngeal ammonia and prevented LAH induced by laryngeal pH 5-pepsin treatment, whereas perineural sham treatment failed to produce this suppressive effect. The same technique also abolished the reflex apneic response resulting from stimulation of capsaicin-sensitive laryngeal afferent fibers by laryngeal capsaicin. Thus our result is consistent with previous findings (37) that the reflex apneic response to laryngeal ammonia is mediated through this subtype of afferent fibers. The inability of laryngeal ammonia to trigger reflex apnea in these pH 5-pepsin-treated animals is unlikely to be due to the possible damaging effects of PCT on laryngeal afferents, because PCT did not affect the reflex apneic response to laryngeal mechanical stimulation. This notion also gains support from our observations that, after the recovery from PCT, the reflex apnea evoked by laryngeal ammonia and the LAH induced by laryngeal pH 5-pepsin treatment reappeared after having been abolished by PCT. Collectively, it appears that sensitization of capsaicin-sensitive laryngeal afferent fibers by laryngeal acid-pepsin insult is responsible for the development of LAH in our model.

Electrophysiological (35, 50) and immunohistochemical studies (24) have clearly indicated the presence of capsaicin-sensitive afferent fibers in the larynx. These afferent fibers are thought to be nociceptive-like free nerve endings (7), and their increase in sensitivity to stimuli is analogous to primary hyperalgesia, resulting from a decrease in the threshold for pain-producing stimuli. In fact, it is well established that the capsaicin-sensitive afferent fibers play an important role in the pathogenesis of lower airway hyperreactivity (29, 62). Stimulation of these afferent fibers by laryngeal application of capsaicin triggers various airway reflexes, including cough (9, 19, 36, 59), bronchoconstriction (33), and apnea (30, 37). It is thus conceivable that sensitization of these afferent fibers may result in augmentation of airway reflexes in response to laryngeal stimuli. To that end, it is known that patients with laryngopharyngeal or gastroesophageal reflux have increased sensitivity to cough reflexes with inhaled capsaicin (4, 15, 41, 45, 60) and are associated with chronic cough, asthma, the sudden infant death syndrome, and obstructive sleep apnea (13, 39, 43). Accordingly, the functional significance of LAH induced by

### Table 1. Immediate cardiovascular changes induced by laryngeal ammonia in four study groups with different laryngeal acid-pepsin insults

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<tr>
<th>Group</th>
<th>Control</th>
<th>2 h After Laryngeal Insult</th>
<th>After SLN Cut</th>
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<td>HR, beats/min</td>
<td>MABP, mmHg</td>
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</tbody>
</table>

Values in each group are the means ± SE of 8 animals. MABP, mean arterial blood pressure; HR, heart rate; SLN Cut, denervation of superior laryngeal nerves. Peak increase in MABP and maximal reduction of HR were measured during the first 10-s period following laryngeal ammonia challenge. Baseline data were calculated as the mean over 10 s immediately before laryngeal ammonia challenge. \( \Delta \) value = (peak or lowest value – baseline value). *Significantly different from the corresponding control, \( P < 0.05 \).

### Table 2. Immediate cardiovascular changes induced by laryngeal ammonia in six study groups with different experimental interventions

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>2 h After Laryngeal pH 5-Pepsin Insult</th>
<th>3 h After Laryngeal pH 5-Pepsin Insult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MABP, mmHg</td>
<td>HR, beats/min</td>
<td>MABP, mmHg</td>
</tr>
<tr>
<td>PCT</td>
<td>15.1±2.1</td>
<td>-19.1±2.3</td>
<td>0.8±0.4*</td>
</tr>
<tr>
<td>SNT</td>
<td>4.6±3.3</td>
<td>-14.1±9.6</td>
<td>3.8±2.5</td>
</tr>
<tr>
<td>DMTU</td>
<td>5.6±4.0</td>
<td>-47.8±15.9</td>
<td>3.4±3.0</td>
</tr>
<tr>
<td>Saline</td>
<td>1.4±3.5</td>
<td>-19.5±11.5</td>
<td>5.6±1.6</td>
</tr>
<tr>
<td>DEF</td>
<td>7.3±1.0</td>
<td>-6.9±4.3</td>
<td>5.1±1.4</td>
</tr>
<tr>
<td>DEF + Fe</td>
<td>10.5±1.4</td>
<td>-7.4±4.5</td>
<td>13.9±2.4</td>
</tr>
</tbody>
</table>

Data in each group are the mean ± SE of 8 animals. PCT, perivagal capsaicin treatment; SNT, sham nerve treatment; DMTU, dimethylthiourea; Saline, DMTU vehicle; DEF, deferoxamine; DEF + Fe, iron-saturated DEF. Peak increase in MABP and maximal reduction of HR were measured during the first 10-s period following laryngeal ammonia challenge. Baseline data were calculated as the mean over 10 s immediately before laryngeal ammonia challenge. \( \Delta \) value = (peak or lowest value – baseline value). *Significantly different from the corresponding control, \( P < 0.05 \).
laryngeal acid-pepsin insult in these clinical settings remains to be elucidated and warrants investigation.

The results of the third part of this study demonstrate that pretreatment with DMTU or DEF prevented LAH induced by laryngeal pH 5-pepsin treatment, whereas pretreatment with DMTU vehicle or DEF+Fe failed to produce this suppressive effect. DMTU scavenges ·OH after their formation (16), whereas DEF inhibits the formation of ·OH derived from hydrogen peroxide by chelating the catalyzing iron (18). It is conceivable that this LAH was prevented by lowering the ·OH burden, but not by possible deleterious effects of DMTU or DEF on laryngeal sensory nerve endings, because laryngeal ammonia could still evoke similar magnitudes of reflex apneic responses compared with the control. Thus our observations suggest that ·OH plays a vital role in the development of LAH and the sensitization of capsaicin-sensitive laryngeal afferent fibers induced by laryngeal acid-pepsin insult. To that end, it is possible that, although pepsin is more active at pH 2, pH 2 may be less optimal for ·OH formation compared with the situation at pH 5. This possibility offers another reason to explain why pH 2-pepsin insult is ineffective at producing LAH.

It is known that ROS are generated in large amounts during tissue inflammation (14). ROS produced by inflammatory cells are involved in the damage to the esophageal mucosa induced by acid-pepsin solution in animals with esophagitis (21, 40). Abundant evidence suggests the involvement of ROS in the pathogenesis of lower airway hyperreactivity (5, 8, 20). ROS have also been shown to participate in the development of primary hyperalgesia or inflammatory pain (38, 58, 64). Here we report, for the first time, that ROS are involved in LAH of the inflamed larynx. The mechanisms by which ·OH are associated with the development of LAH induced by laryngeal acid-pepsin insult remain unclear. One plausible mechanism is that ·OH themselves act on capsaicin-sensitive laryngeal afferent fibers and increase their sensitivity to laryngeal stimuli. A direct action of ·OH on the capsaicin-sensitive afferent fibers located at the heart (51) and the lower airway (47, 48) has been postulated. In this study, the duration of laryngeal acid-pepsin treatment was only 40 s, and it took over 1 h to develop LAH. This long latency in the development caused us to doubt whether ROS are the only mediators involved. Therefore, alternatively, it is possible that ·OH may induce the release of other chemical mediators, such as ATP (11) or arachidonate metabolites (32), which subsequently increase the sensitivity of these laryngeal afferents to laryngeal stimuli. Indeed, both ATP (34, 61) and arachidonate metabolites, such as prostaglandin E2 (28), have been demonstrated to sensitize capsaicin-sensitive ion channels in primary sensory neurons or capsaicin-sensitive afferent fibers located in the lower airway.

In this study, laryngeal ammonia challenge caused a slight increase in mean arterial blood pressure and a slight decrease in heart rate. These ammonia-induced cardiovascular changes were eliminated by denervation of SLNs or PCT, suggesting that they are probably reflexes resulting from stimulation of capsaicin-sensitive laryngeal afferent fibers. While laryngeal pH 5-pepsin insult augmented the apneic response to ammonia, it did not affect these ammonia-induced cardiovascular changes. Similarly, DMTU or DEF prevented the LAH induced by laryngeal pH 5-pepsin insult, but it did not influence these ammonia-induced cardiovascular changes. It is possible that the input from these afferent fibers in the control condition already evoked a maximal cardiovascular response, so that no additional response could be evoked after sensitization.

In summary, a single laryngeal insult by pepsin at a weakly acidic pH value profoundly augmented the laryngeal reflect activity in anesthetized rats, and this is mediated, at least in part, through the sensitization of capsaicin-sensitive SLN afferent fibers by the action of ·OH. While the role of capsaicin-sensitive laryngeal afferent fibers in the development of LAH has been obscure, our study provides evidence to suggest their pathogenetic significance. The role of LAH in the extraesophageal symptoms in these patients is largely unknown and thus requires further investigation. Furthermore, due to the involvement of acid, proton pump inhibitors have been widely
used to treat these patients but are not always effective (25, 39). Accordingly, administration of antioxidants and improvement in the patient’s antioxidant capacity are possible target choices for potential therapeutic regimes to treat LAH in these patients.

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REFERENCES

44. Phua SY, McGarvey LP, Ng MC, and Ing AJ. Patients with gastrooesophageal reflux disease and cough have impaired laryngopharyngeal mechanosensitivity. *Thorax* 60: 488–491, 2005.


