Menthol suppresses laryngeal C-fiber hypersensitivity to cigarette smoke in a rat model of gastroesophageal reflux disease: the role of TRPM8

Bi-Yu Liu,1 Yu-Jung Lin,1 Hung-Fu Lee,2 Ching-Yin Ho,3,4 Ting Ruan,5* and Yu Ru Kou1*

1Institute of Physiology, School of Medicine, National Yang-Ming University, Taipei, Taiwan; 2Department of Neurosurgery, Cheng Hsin General Hospital, Taipei, Taiwan; 3Department of Otolaryngology, Taipei Veteran General Hospital, Taipei, Taiwan; 4Department of Otolaryngology, National Yang-Ming University, Taipei, Taiwan; and 5School of Medicine, Fu Jen Catholic University, New Taipei City, Taiwan

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GASTROESOPHAGEAL REFLUX DISEASE (GERD) is a common disorder of the gastrointestinal tract caused by the backflow of gastric contents into the upper digestive tract (39). GERD is associated with several respiratory manifestations including enhanced laryngeal reflex reactivity, which is characterized by increased sensitivity of laryngeal afferents to various stimuli; this leads to augmented vagally mediated reflexes such as cough, the glottic-stop reflex, and laryngeal adductor responses (4, 12, 22, 42, 43, 52). The superior laryngeal nerves (SLNs), a branch of the vagus nerve, provide the major sensory innervation to the larynx (46). Among these afferents, laryngeal C-fibers, a group of polymodal nociceptive-like free nerve endings (8), are sensitive to capsaicin, a pungent, active ingredient of hot pepper (7, 37). Stimulation of laryngeal C-fibers by capsaicin triggers several airway reflexes including apnea, bronchoconstriction, cough, and the glottic-stop reflex (10, 16, 33, 38, 51). Clinical studies have reported that patients with GERD show an increase in cough reflex sensitivity when treated with an inhaled capsaicin aerosol (4, 12, 42, 43). Additionally, other investigations using a rat model of GERD have shown that laryngeal C-fiber hypersensitivity is responsible for enhanced laryngeal reflex reactivity to ammonia induced by laryngeal treatment with pepsin in a pH 5 solution (53, 54). These findings suggest an important role for laryngeal C-fibers in the development of the enhanced laryngeal reflex reactivity as is observed with GERD. Thus pharmacological drugs that may suppress laryngeal C-fiber hypersensitivity would appear to be attractive potential therapies for the treatment of patients with GERD.

Cigarette smoke (CS) causes laryngeal sensory irritation in various species and this is known to be mediated through SLNs (6, 26, 27, 34). Particularly, stimulation of laryngeal C-fibers by CS in rats has been shown to be due to activation of both transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1) channels, which are located at the terminals of these laryngeal C-fibers. Our electrophysiological studies consistently revealed that laryngeal pH 5-5 pepsin treatment increased the sensitivity of laryngeal C-fibers to CS. Likewise, menthol suppressed this laryngeal C-fiber hypersensitivity and its effect could be reversed by pretreatment with AMTB. Our results suggest that laryngeal pH 5-5 pepsin treatment increases sensitivity to CS of both TRPV1 and TRPA1, which are presumably located at the terminals of laryngeal C-fibers. This sensory sensitization leads to enhanced laryngeal reflex reactivity and augmentation of the laryngeal C-fiber responses to CS, which can be suppressed by menthol acting via TRPM8.

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*Y Kou and T. Ruan contributed equally to this work.

Address for reprint requests and other correspondence: Y. R. Kou, Institute of Physiology, National Yang-Ming Univ., Shih-Pai, Taipei 112, Taiwan (e-mail: yrkou@ym.edu.tw or tingruan@hotmail.com).

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cyclohexanone (a TRPV1 agonist) or acrolein (a TRPA1 agonist) in mice (55). These observations suggest that menthol has the ability to regulate the sensitivity of TRPV1 and TRPA1 to laryngeal stimulation by agonists. However, whether menthol is able to suppress GERD-induced laryngeal C-fiber hypersensitivity to CS remains to be investigated.

In light of existing knowledge and the unanswered questions described above, this study was carried out using an established rat model of GERD (53, 54) with the aim of investigating first whether laryngeal exposure to an acid-pepsin insult is able to induce enhanced laryngeal reflex reactivity to CS; second, whether this enhanced laryngeal reflex reactivity is due to laryngeal C-fiber hypersensitivity to CS; and third, whether local application of menthol is able to suppress laryngeal C-fiber hypersensitivity to CS via activation of TRPM8. To accomplish these objectives, the reflex afferent response to CS delivered into a functionally isolated larynx was measured to reflect laryngeal reflex reactivity in spontaneously breathing rats. In parallel, a study of the afferent responses to laryngeal CS was conducted by measuring the activity of single unit of laryngeal C-fiber in ventilator-assisted rats.

METHODS

Animal preparation. All experimental procedures described below were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University, Taiwan. Male Sprague-Dawley rats were initially anesthetized using an intraperitoneal injection of chloralose (100 mg/kg; Sigma Chemical, St. Louis, MO) and urethane (500 mg/kg; Sigma) dissolved in a borax solution (2%; Sigma). 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 abolition 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 carefully isolated for later experiments. In the study of laryngeal reflex reactivity, the rats were able to breath spontaneously via the lower tracheal catheter. Respiratory flow was measured with a pneumotachograph (Fleisch 4/0; Richmond, VA) coupled with a differential pressure transducer (MP45-14; Validyne, Northridge, CA). The flow signal was integrated to give the tidal volume (VT). In the studies that recorded C fiber action potentials, a PE-50 catheter was inserted into the carotid artery and advanced until the tip was in the left ventricle, which allowed intra-arterial administration of capsaicin. These rats received a midline thoracotomy and were ventilated via a rodent respirator (4/0; Richmond, VA) coupled with a differential pressure transducer (P300D; Validyne). To prevent air leakage. To perform laryngeal hyperinflation, the oral tube was occluded and the laryngeal segment was inflated by constant flow via the upper tracheal catheter until the intramural pressure reached 30 cmH₂O as measured by a pressure transducer (P300D; Validyne). To perform laryngeal mechanical stimulation, a nylon thread (diameter 0.1 mm) was used to gently probe the laryngeal mucosa to stimulate laryngeal afferents (6).

Generation and delivery of CS. The methods used for generation and delivery of CS have been reported previously (34). In brief, commercial cigarettes (10.8 mg nicotine and 10 mg tar per cigarette; Marlboro Red Label, Philip Morris, New York, NY) were used to generate CS. The smoke (5 ml) was then drawn into the syringe at a constant flow rate of 1 ml/s and was defined as 100% smoke. The 100% smoke was mixed with air in the syringe to generate 10% smoke. The diluted smoke was continuously delivered at a constant flow rate of 1.4 ml/s by a syringe pump (model 367; Sage, Cambridge, MA) into a section of 6-ml Teflon tubing (8 mm ID) connected to the proximal end of the upper airway catheter. For each delivery, 7 ml of smoke was allowed to pass through the isolated larynx and to flow out into the environment via the oral tube. To avoid possible tachyphylaxis, at least 60 min was allowed to elapse between two smoke challenges.

Perineural capsaicin treatment and perineural sham treatment of SLNs. Perineural capsaicin treatment of SLNs has been demonstrated to selectively block the reflex responses resulting from stimulation of laryngeal C-fibers, and the method has been described in detail previously (33). In brief, a segment (~2 mm) of each SLN was wrapped in a cotton strip that had been presoaked in either capsaicin solution (30 μg/ml) or capsaicin vehicle (perineural sham treatment). After 5 min the cotton strips were removed. The blocking effect of perineural capsaicin treatment was confirmed when the reflex response to laryngeal capsaicin was abolished, yet the reflex response to laryngeal mechanical stimulation by a nylon thread is preserved.

Recording of laryngeal C-fiber activity. Neural activities arising from laryngeal C-fibers were recorded using techniques described elsewhere with some modifications (37). Briefly, the peripheral end of the left SLN was placed on a dissecting platform. A fine afferent filament was split and placed on a platinum-iridium unipolar recording electrode to record afferent nerve activity. Action potentials were amplified (P511K; Grass, Quincy, MA), monitored by an audio monitor (AM10; Grass), and displayed on an oscilloscope (TDS 2002B; Tektronix, Beaverton, OR). To search for these afferent fibers, the laryngeal segment was hyperinflated to an intramural pressure of 30 cmH₂O. Suspected C fibers were identified as those with their evoked activities that did not adapt either rapidly or slowly to this laryngeal hyperinflation (37). The thin filament was further split until the afferent activity arising from a single unit was electrically isolated. Once the presence of a suspected single unit was detected, capsaicin (2 μg/kg) was injected as a bolus into the left ventricle. Only afferent fibers that showed stimulation within 2 s after injection were studied. Before the end of each experiment, the general locations of all fibers was identified by their responses to gentle probing of the laryngeal mucosa by a nylon thread. The conduction velocities of the afferent fibers identified by this method have been reported to be within the range of C fibers (37).

Preparation of pharmacological agents. The pH 5-pepsin and pH 5-denatured pepsin solutions were prepared by a method described in...
a previous study (53). The stock solution of capsaicin (a stimulant for laryngeal C-fibers) (53) was prepared by dissolving the chemical in a solution containing 1% Tween 80, 1% ethanol, and 98% saline. The stock solutions of HC030031 (a TRPA1 antagonist) (34), capsazepine (a TRPV1 antagonist) (34), SB-366791 (a TRPV1 antagonist) (15), and AMTB hydrate (a TRPM8 antagonist) (55) were prepared by dissolving the chemicals in DMSO. The stock solution of L-menthol (a TRPM8 agonist) (19) was prepared by dissolving the chemicals in ethanol. The working solutions of HC030031 (0.056 M), capsazepine (0.013 M), and SB-366791 (0.015 M) at concentrations for laryngeal application were prepared daily by further dilution of stock solutions with a solution containing 1% Tween 80, 1% ethanol, and 98% saline. The working solutions of capsaicin (0.164 μM), AMTB (0.12 mM), and menthol (10 mM) were prepared daily by further dilution of stock solutions with saline. The doses and treatment times of the drugs used in this study to be effective were adopted from previous studies (34, 53) or determined in our preliminary study. Except for HC030031 and AMTB, which were obtained from Tocris Cookson (Ellisville, MO), all other drugs were purchased from Sigma.

**Laryngeal treatment or application with pharmacological agents.** In the study of laryngeal reflex reactivity, a small piece of filter paper (~1 × 10 mm) containing a solution of pH 5-pepsin or pH 5-denatured pepsin was carefully inserted into the larynx via the oral tube to induce the enhanced laryngeal reflex reactivity, and then removed 40 s after insertion. To locally apply drugs, the solution (in a volume of 5 μl) of HC030031, capsazepine, SB-366791, menthol, AMTB, or their vehicles was carefully instilled into the laryngeal segment via a spinal needle. In the electrophysiological study, the recording of action potentials could be easily ruined by any slight movement of the animals and, therefore, different methods were employed to perform the laryngeal treatment and the application of drugs. Specifically, to examine the afferent responses of laryngeal C-fibers, an aerosol of a solution containing pH 5-pepsin or pH 5-denatured pepsin was generated by a nebulizer (Aeroneb Lab Nebulizer System; Aerogen, Galway, Ireland), and this was continuously delivered at a constant flow rate of 3 ml/s into the laryngeal segment for 40 s to induce laryngeal C-fiber hypersensitivity. To locally apply drugs, an aerosol of a solution containing menthol, the vehicle of menthol, AMTB, or the vehicle of AMTB was continuously delivered at a constant flow rate of 3 ml/s into the laryngeal segment for 10, 10, 20, and 20 s, respectively. After the laryngeal treatment with pH 5-pepsin or pH 5-denatured pepsin, an elapsed time of at least 60 min was allowed before CS stimulation.

**Experimental design and protocols.** In this study, 160 rats (weighing 350–400 g) were divided into 21 groups to conduct five series of experiments (groups 1–17 each n = 8; groups 18–21 each n = 6). Figure 1 depicts the experimental protocols. In all groups, the first CS challenge occurred 40 min before laryngeal treatment using pH 5-pepsin or pH 5-denatured pepsin solution to obtain the control response. To investigate the enhanced laryngeal reflex reactivity to CS (study 1, Fig. 1A), the reflex apneic responses to four repeated laryngeal CS challenges were studied in two groups of rats. Two hours after laryngeal treatment with pH 5-pepsin (group 1) or pH 5-denatured pepsin (group 2), three repeated CS challenges were made with any two challenges separated by 1 h. Denervation of the SLNs was performed 30 min prior to the last challenge. To investigate the role of laryngeal C-fibers in the enhanced laryngeal reflex reactivity (study 2, Fig. 1B), the reflex apneic responses to three repeated laryngeal CS

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**Fig. 1.** Schematic illustration showing the experimental protocols used in this study. A–E depict protocols for studies 1–5 described in Methods. Pepsin in a pH 5 solution (pH 5-pepsin) represents laryngeal treatment for induction of laryngeal airway hyperreactivity, whereas denatured pepsin in a pH 5 solution represents the negative control treatment. Cigarette smoke (CS) and mechanical stimulation represent two different types of laryngeal stimulation with a 10-min elapsed time. SLN cut represents denervation of superior laryngeal nerves. B: perineural capsaicin treatment (PCT) of SLNs was applied 20 min before the second CS stimulation, whereas perineural sham treatment (PST) serves as the negative control treatment. C: capsazepine (CPZ), SB-366791, HC030031, a combination of CPZ and HC030031 (CPZ + HC030031), a combination of SB-366791 and HC030031 (SB-366791 + HC030031), or their vehicles (vehicles 1, 2, and 3) were locally applied 30 min before the second CS stimulation. D: menthol or its vehicle (vehicle 4) was locally applied 20 min before the application of menthol. E: menthol and AMTB were locally applied at time points identical to those in D.
challenges, each 10 min later followed by a mechanical stimulation, were studied in three groups of rats. Two hours after laryngeal treatment with pH 5-pepsin, two repeated CS challenges were made with an elapsed time of 2 h. No neural treatment (group 3), perineural capsaicin treatment (group 4), or perineural sham treatment (group 5) was performed 20 min before the second CS challenge. Denervation of the SLNs was performed 30 min prior to the last challenge. To investigate the involvement of TRPV1 and TRPA1 in the enhanced laryngeal reflex reactivity (study 3, Fig. 1C), the reflex apneic responses to three repeated laryngeal CS challenges were studied in eight groups of rats. Two hours after the laryngeal treatment with pH 5-pepsin, two repeated CS challenges were made with an elapsed time of 1 h. Pretreatment with capsazepine (group 6), SB-366791 (group 7), HC030031 (group 8), a combination of capsazepine and HC030031 (group 9), a combination of SB-366791 and HC030031 (group 10), or their vehicles [vehicles 1–3 (groups 11–13)] were made 30 min prior to the second CS challenge. To investigate the suppressive effect of menthol on the enhanced laryngeal reflex reactivity (study 4, Fig. 1D), the reflex apneic responses to three repeated laryngeal CS challenges were studied in four groups of rats. According to the pretreatments, the study groups were defined as follows: the vehicle of menthol (vehicle 4, group 14), menthol alone (group 15), a combination of the vehicle of AMTB (vehicle 5), and menthol (group 16), and a combination of AMTB and menthol (group 17). Two hours after the laryngeal treatment with pH 5-pepsin, two repeated CS challenges were made with an elapsed time of 1 h. Pretreatment with menthol or its vehicle was made 2 min prior to laryngeal treatment with pH 5-pepsin. Pretreatment with AMTB or its vehicle occurred 20 min prior to the application of L-menthol. To investigate the suppressive effect of menthol on laryngeal C-fiber hypersensitivity (study 5, Fig. 1E), the afferent responses of laryngeal C-fibers to two repeated laryngeal CS challenges were studied in four groups of rats; one C fiber being recorded from each rat. According to the interventions, the study groups were defined as follows: pH 5-pepsin with pretreatment with vehicles 1–4 (group 18), pH 5-denatured pepsin with pretreatment with vehicles 1–4 and 5 (group 19), pH 5-pepsin with pretreatment with menthol (group 20), and pH 5-pepsin with a combined pretreatment with AMTB and menthol (group 21). A second CS challenge occurred 2 h after laryngeal treatment with pH 5-pepsin or pH 5-denatured pepsin. The times used for pretreatment with vehicles, menthol, and AMTB were identical to those in study 4.

Data analysis and statistics. Respiratory flow, VT, and expiratory duration (TE) were analyzed on a breath-by-breath basis. At least 10 breaths before and 30 breaths after laryngeal stimulation were measured. Baseline data for TE were calculated as the mean over 10 breaths immediately before challenge. To compare the responses evoked by 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 ratio. For this purpose, the longest TE occurring during the first 10 s after laryngeal stimulation was divided by the baseline TE, and the value was then multiplied by 100. C fiber activity was continuously analyzed at 1-s intervals over an interval of at least 10 s before and 30 s after laryngeal stimulation. Baseline fiber activity (FA) was calculated as the average value over the 10-s period immediately preceding a challenge. The peak response of FA was the average over 2-s intervals after laryngeal stimulation. 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 appropriate software (1.0; BioCybermatics, Taipei, Taiwan). The normality of the data was checked by the Kolmogorov-Smirnov test. Comparisons of two sets of data from the same study groups were made by paired t-test. Data from different study groups were compared by two-way mixed factorial ANOVA followed by the Fisher’s test when appropriate; the time factor was used for within-subject comparisons, whereas the drug factor was used for between-subject comparisons. A value of $P < 0.05$ was considered significant. All data are presented as means ± SE.

RESULTS

Laryngeal acid-pepsin treatment induces enhanced laryngeal reflex reactivity to CS. On the basis of concentration-response relationship reported previously (34), 10% CS was chosen as the standard laryngeal challenge for all studies. Before any laryngeal treatment, an apneic response, characterized by a prolongation of the TE, was elicited within 1 or 2 s after laryngeal CS challenge (Figs. 2A and 3A). The same CS challenge evoked a significant augmentation of the apneic response at the second hour (351.7 ± 55.3% of control) and the third hour (191.8 ± 41.9% of control) after laryngeal treatment with pH 5-pepsin compared with the control response (Figs. 2A and 3A). The response measured at the third hour after laryngeal treatment was significantly smaller than that at the second hour (Figs. 2A and 3A), indicating that there was recovery of the laryngeal hypersensitivity. In contrast, the apneic response to laryngeal CS challenge did not significantly alter at the second hour and the third hour after laryngeal treatment with denatured pH 5-pepsin compared with the control response (Figs. 2B and 3A). Additionally, the apneic response to laryngeal mechanical stimulation did not significantly alter at the second hour after laryngeal pH 5-pepsin treatment compared with the control response (Fig. 3B), suggesting that pH 5-pepsin treatment did not induce laryngeal hypersensitivity to mechanical stimulation. A subsequent denervation of the SLNs totally abolished the apneic responses to both types of stimulus (Fig. 2, and Fig. 3, A and B), indicating that these responses were reflexes mediated through the SLNs.

Laryngeal C-fiber hypersensitivity is involved in the enhanced laryngeal reflex reactivity to CS. Because perineural capsaicin treatment of SLNs is able to selectively block the reflex responses resulting from stimulation of laryngeal C-fibers (33, 53), we employed this technique to investigate the role of these afferents. Two hours after laryngeal pH 5-pepsin treatment, a time during which the enhanced laryngeal reflex reactivity would be expected to occur, perineural capsaicin treatment entirely abolished the reflex apneic response to CS (Fig. 3C), whereas perineural sham treatment did not affect the augmented apneic response to CS (384.5 ± 88.5% of control) (Fig. 3D). In the same animals, both perineural capsaicin treatment and perineural sham treatment did not significantly affect the reflex apneic response to mechanical stimulation occurring at the second hour after laryngeal pH 5-pepsin treatment (Fig. 3, C and D). Again, a subsequent denervation of the SLNs totally abolished the apneic responses to both types of stimuli (Fig. 3, C and D).

Both TRPV1 and TRPA1 are important to induction of enhanced laryngeal reflex reactivity to CS. Because both TRPV1 and TRPA1 mediate the stimulation of laryngeal C-fibers by CS (34), we further investigated the role of these two types of receptors by laryngeal applications of a TRPV1 antagonist (capsazepine or SB-366791) or a TRPA1 antagonist (HC030031). Two hours after laryngeal pH 5-pepsin treatment, pretreatment with either capsazepine alone (Fig. 4A), SB-366791 alone (Fig. 4A), or HC030031 alone (Fig. 4B) greatly attenuated the augmented apneic response to CS. At the same time point, pretreatment with a combination of capsazepine...
and HC030031 or a combination of SB-366791 and HC030031 almost completely abolished the reflex apneic response to CS (Fig. 4C). In contrast, pretreatment with the vehicle of capsazipine or SB-366791 (vehicle 1), the vehicle of HC030031 (vehicle 2), or their combination (vehicle 3) failed to produce these effects. Three hours after laryngeal pH 5-pepsin treatment, the blocking effects of these antagonists vanished because the evoked apneic responses to CS in antagonist-treated groups were similar to those in the vehicle groups (Fig. 4).

Menthol, acting via TRPM8, suppresses the enhanced laryngeal reflex reactivity to CS. To investigate the suppressive effect of menthol, the drug was topically applied to the laryngeal segment. Two hours after laryngeal pH 5-pepsin treatment, a time during which the enhanced laryngeal reflex reactivity would be expected to occur, pretreatment with menthol largely attenuated the augmented apneic response to CS (Figs. 5A and 6A), whereas pretreatment with its vehicle (vehicle 4) failed to produce this effect (Figs. 5B and 6A). Analysis of group data revealed that menthol eliminated only the part of the apneic response that was augmented by laryngeal pH 5-pepsin treatment; the apneic response to CS after menthol (apneic ratio = 346.5 ± 104.5%) did not significantly differ from that before menthol (apneic ratio = 387.8 ± 96.7%, P > 0.05, n = 8) (Fig. 6A). Three hours after laryngeal pH 5-pepsin treatment, the suppressive effect of menthol vanished because the evoked apneic response to CS in the menthol-treated group was similar to that in the vehicle groups (Fig. 6A). Furthermore, this suppressive effect of menthol was reversed by pretreatment with AMTB, a TRPM8 antagonist, but was unaffected by pretreatment with its vehicle (Fig. 6B).

Menthol, acting via TRPM8, suppresses laryngeal C-fiber hypersensitivity to CS. To investigate the suppressive effect of menthol on laryngeal C-fiber hypersensitivity, we recorded the neural activity arising from these afferents. In this study, 24 laryngeal C-fibers recorded from 24 rats had sparse or no activity during the baseline period, and their mean baseline activity was 0.03 ± 0.03 impulses/s (n = 24). These laryngeal C-fibers were stimulated by laryngeal hyperinflation to 30
cmH2O (Fig. 7A) and by left ventricular injection of capsaicin (Fig. 7B), which led to a peak evoked activity of 7.50 ± 1.09 impulses/s (n = 24). All these afferent fibers were localized within the laryngeal segment. Before any laryngeal treatment, each of these afferent fibers was mildly stimulated by laryngeal CS challenge (Fig. 7C), which resulted in a peak evoked activity of 0.94 ± 0.21 impulses/s (n = 24). Two hours after laryngeal pH 5-pepsin treatment, the same CS challenge evoked a significantly augmented afferent response compared with the control response (Fig. 7D). The increases in FA (ΔFA = peak evoked activity − baseline activity) induced by CS before and after laryngeal treatment were 0.65 ± 0.41 and 6.80 ± 1.84 impulses/s, respectively (P < 0.05, n = 6) (Fig. 8). In contrast, laryngeal denatured pH 5-pepsin treatment did not produce an augmented effect on the afferent response to laryngeal CS challenge (Fig. 8). Analysis of the group data revealed that pretreatment with menthol eliminated only the part of the afferent response that was augmented by the laryngeal pH 5-pepsin treatment; the ΔFA evoked by CS after menthol (ΔFA = 1.06 ± 0.32 impulses/s) did not significantly differ from that before menthol (ΔFA = 0.89 ± 0.23 impulses/s, P > 0.05, n = 6) (Fig. 8). Furthermore, this suppressive effect of menthol was entirely reversed by pretreatment with AMTB, a TRPM8 antagonist (Fig. 8).

DISCUSSION

The results of the first part of our study demonstrate that laryngeal treatment with pH 5-pepsin solution induced an enhanced laryngeal reflex reactivity to CS in a rat model of GERD. Laryngeal C-fibers apparently were involved in this event because the apneic response to CS was completely...
abolished by perineural capsaicin treatment and by denervation of SLNs. Both TRPV1 and TRPA1 appear to be important for this event because the augmented apneic response to laryngeal CS was partially attenuated by pretreatment with either a TRPV1 or a TRPA1 antagonist, and was entirely prevented by pretreatment with a combination of these two antagonists. The importance of laryngeal C-fibers is further supported by our results from the electrophysiological studies showing that laryngeal pH 5-pepsin treatment indeed could sensitize these afferent fibers and induced their hypersensitivity to CS. Thus our findings provide the first experimental evidence to support the notion that patients with GERD should avoid exposure to CS (18).

Stimulation and sensitization are two different pathophysiological features of airway C-fibers; stimulation triggers reflex responses to stimuli, whereas sensitization leads to an en...
enhanced reflex reactivity to stimuli. Our previous study (34) showed that CS may stimulate laryngeal C-fibers via activation of both TRPV1 and TRPA1, whereas this study demonstrated that the laryngeal pH 5-pepsin insult may induce laryngeal C-fiber hypersensitivity to CS, and this would seem to be due to increases in the sensitivity of both TRPV1 and TRPA1 to CS stimulation. Thus different from our previous findings, this study shows that the laryngeal effect of CS can actually be augmented after sensitization of laryngeal C-fibers, which is another pathophysiological feature of these afferents. The precise mechanism underlying this sensitization remains unclear. However, it is known that various chemical mediators released during tissue inflammation may regulate the sensitivity of TRPV1 and TRPA1 and that this can lead to sensitization of C-fibers in the lower airway (5, 29, 45). For example, laryngeal acid-pepsin treatment causes laryngeal inflammation and produces excess reactive oxygen species (ROS) (41, 53, 54), which are vital to the development of laryngeal C-fiber hypersensitivity to ammonia (53, 54). Additionally, ROS have been shown to be able to increase the sensitivity of TRPV1 and TRPA1 to agonists (9, 45, 48). Thus it is plausible that the same ROS mechanism is working in our model. Whatever the mechanism(s) involved, laryngeal pH 5-pepsin treatment does not seem to affect the sensitivity of laryngeal myelinated afferents to mechanical stimulation. This is because laryngeal pH 5-pepsin treatment did not alter the apneic response to laryngeal mechanical stimulation; this reflex is unaffected by perineural capsaicin treatment and is believed to be mediated by myelinated afferents (31).

The results of the second part of our study demonstrate that topical application of menthol largely attenuated the pH 5-pepsin-induced increases in laryngeal reflex reactivity and laryngeal C-fiber sensitivity to CS. The suppressive effect of menthol is unlikely to be due to possible damage to the laryngeal

![Fig. 6. Mean apneic responses to laryngeal stimulation with CS before and after laryngeal pH 5-pepsin treatment in our study groups. A: two study groups received local application of menthol (a TRPM8 agonist) or its vehicle (vehicle 4) 2 min before the laryngeal treatment. See Fig. 1D for protocol details. B: two additional study groups received AMTB (a TRPM8 antagonist) or its vehicle (vehicle 5) 20 min before local application of menthol. See Fig. 1D for protocol details. *P < 0.05 vs. the response at the same time point in the vehicle group; **P < 0.05 vs. the control response in the same group; ***P < 0.05 vs. the response at 2 h after laryngeal treatment in the same group. Data in each group are means ± SE from eight rats.

![Fig. 7. Afferent responses of a single unit of laryngeal C-fiber to laryngeal hyperinflation (A), left ventricular injection of capsaicin (B), and laryngeal CS (C and D) in one rat. A: the laryngeal segment was hyperinflated to a constant pressure of 30 cmH2O. B: capsaicin (2 μg/kg) was injected into the left ventricle as indicated by the arrow. C and D: 10% CS (7 ml) was delivered into the laryngeal segment by a syringe pump as indicated by horizontal bars. Laryngeal treatment with pH 5-pepsin was performed 40 min after the first CS challenge and 2 h before the second CS challenge. The elapsed time intervals between hyperinflation and capsaicin injection, and capsaicin injection and the first CS challenge were 5 min and 15 min, respectively. AP, action potential; Pua, upper airway pressure; Ptr, tracheal pressure.

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menthol may possess anti-inflammatory activity (20) and reduce the production of inflammatory mediators that are responsible for the sensitization of TRPV1 or TRPA1 in laryngeal C-fibers. However, a species difference between rats and mice has been proposed (13) and, for example, the coexpression of TRPM8 with TRPV1 or TRPA1 has been demonstrated in visceral nociceptive fibers in rats (17). If coexpression does occur in rats, TRPM8 may directly or indirectly interact with TRPV1 or TRPA1 to desensitize their sensitivity to stimuli (17), thus preventing laryngeal C-fiber hypersensitivity to CS.

In this study, laryngeal treatment with pepsin in a weakly acidic solution (pH 5) was employed as the insult to the larynx; this is an intervention that has been adopted from our previous studies (53, 54). As a result, the profiles of the development of the enhanced laryngeal reflex reactivity in this and previous studies (53, 54) are very similar, although our previous studies employed ammonia, another C fiber stimulant, as the laryngeal challenge. Laryngeal treatment with pepsin in a more acidic (pH 2) solution is ineffective at producing the enhanced laryngeal reflex reactivity, possibly because it causes a greater degree of laryngeal tissue damage and thus affects the functioning of laryngeal C-fibers (53). Because pepsin exhibits activity when the pH is greater than 4 (2), the acidified pepsin seems to be the determining factor. In this context it should be noted that the presence of both acid and pepsin in the larynx is not uncommon in patients with GERD (23, 39). Several clinical studies have shown that a significant portion of these patients exhibit acid reflux with a pH greater than 4, and it has been suggest that a new category of weakly acidic reflux with a pH ranging from 4 to 7 should be defined (44, 49).

Indeed, weakly acidic reflux is associated with extraesophageal symptoms such as chronic coughing or other respiratory symptoms (50). Our finding that the laryngeal pH 5-pectsin insult is able to increase laryngeal reflex reactivity to CS is in good agreement with these clinical observations. However, caution should be taken before interpreting our findings. The larynx of patients with GERD is chronically exposed to acid-pepsin insult (39), whereas the larynx of our animals received only one insult with short duration. Our experimental model is more akin to immediate hypersensitivity that could result from an acute exposure. Perhaps a more powerful model for GERD may be a rat model in which chronic acid-pepsin exposure is used in future studies.

There are at least three other important issues that should be discussed. First, acid-sensing ion channels (ASICs), another type of channel located at the terminals of airway C fibers, are known to be stimulated by acid or under tissue inflammation (30). Because our experimental model involves acid-pepsin as the insult and laryngeal inflammation as the consequence (53), it is natural to consider the involvement of ASICs. However, because laryngeal treatment with pH 5-denatured pepsin was ineffective at producing laryngeal C-fiber hypersensitivity [(53) and the present study], the involvement of acid alone and ASICs in our model seems to be unlikely. Second, it has been reported that capsazepine, in addition to its blocking effect on TRPV1, has a nonspecific blocking action on ASICs in our model (53) and the present study), the involvement of acid alone and ASICs in our model seems to be unlikely.

In addition, we also found that the suppressive effect of menthol could be reversed by pretreatment with AMTB, suggesting that it is mediated through TRPM8 and that it is unlikely to be a consequence of nonspecific analgesic effects. Indeed, menthol is known to be a ligand of TRPM8 (19, 55), which is also expressed at the terminals of laryngeal afferents in rats (47). There are at least two possibilities to explain the suppressive effect of menthol on pH 5-pepsin-induced laryngeal C-fiber hypersensitivity to CS. It has been reported previously that TRPM8 is not coexpressed with TRPV1 or TRPA1 in airway vagal afferent nerves in mice (40). In this case, because it vanishes within 3 h after application. Interestingly, menthol eliminated only that part of the reflex and afferent responses to CS that is augmented by laryngeal pH 5-pepsin treatment. This indicates that it prevents the sensitization of laryngeal C-fibers but does not affect the stimulation of laryngeal C-fibers by CS. Up to this point, the ability of menthol in CS to suppress the sensory irritation of the upper airway in smokers has been controversial (1, 24). Our results are consistent with other studies indicating that inhaled menthol fails to affect the airway reflex responses to inhaled capsaicin and citric acid in normal subjects (21, 35), but it does suppress the enhanced airway reflex reactivity to capsaicin in patients with chronic cough (35). Our results are not in line with those indicating that inhaled menthol is able to suppress the airway reflex responses to inhaled citric acid in normal subjects (36) and conscious guinea pigs (25) or to inhaled TRPA1 and TRPV1 agonists in mice (55). Any inconsistency of the findings in this and previous studies could be due to differences in concentrations of menthol used, the species of experimental animal used, the sites within the airway where agonist stimulation and menthol action took place, or a combination of these.
toration of capsazepine used was 13 mM and the suppressive effects of capsazepine on the sensitization of laryngeal C-fibers vanished at 3 h after laryngeal pH 5-pepsin treatment, suggesting that the blocking effect of capsazepine was reversible. Additionally, because SB-366791, another selective TRPV1 antagonist, had the same effect as did capsazepine, the involvement of voltage-activated Ca\textsuperscript{2+} channels in our model is unlikely. Third, laryngeal CS challenge caused a slight increase in mean arterial blood pressure coinciding with the reflex apnea in our model (Figs. 2 and 5). Although the pH 5-pepsin insult augmented the apneic response to CS, it did not affect this pressor response. These results are consistent with those reported previously in the study of ammonia as the laryngeal insult (53). Because stimulation of arterial baroreceptors may induce prolongation of TE (3), the possibility that reflex apnea could be in part caused by arterial baroreceptor stimulation should be considered.

In summary, laryngeal treatment with pH 5-pepsin solution seems to augment both C fiber-mediated laryngeal reflex reactivity and laryngeal C-fiber responses to CS in rats, possibly through sensitization of TRPV1 and TRPA1, which are located at the terminals of these afferents. Topical application of through sensitization of TRPV1 and TRPA1, which are located

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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