Effects of HCl-pepsin laryngeal instillations on upper airway patency-maintaining mechanisms

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Gastroesophageal reflux (GER) has been indicated as an etiopathological factor in disorders of the upper airway. Upper airway collapsing pressure stimulates pressure-responsive laryngeal receptors that reflexly increase the activity of upper airway adductor muscles. We studied, in anesthetized dogs, the effects of repeated laryngeal instillations of HCl-pepsin (HCl-P; pH 2) on the response of laryngeal afferent endings and the posterior cricoarytenoid muscle (PCA) to negative pressure. The effect of negative pressure on receptor discharge or PCA activity was evaluated by comparing their response to upper airway (UAO) and tracheal occlusions (TO). It is only during UAO, but not during TO, that the larynx is subjected to negative transmural pressure. HCl-P instillation decreased the rate of discharge during UAO of the 10 laryngeal receptors studied from 56.4 ± 10.9 (SE) to 38.2 ± 9.2 impulses/s (P < 0.05). With UAO, the peak PCA moving time average, normalized by dividing it by the peak values of esophageal pressure, decreased after six HCl-P trials from 4.29 ± 0.31 to 2.23 ± 0.18 (n = 6; P < 0.05). The responses to TO of either receptors or PCA remained unaltered. We conclude that exposure of the laryngeal mucosa to HCl-P solutions, similar in composition to gastric juice, on the activity of pressure-responsive laryngeal receptors and the related reflex changes in PCA activity.

METHODS

General Preparation

Experiments were performed on dogs sedated with 10 mg/kg im ketamine and anesthetized with a mixture of α-chloralose (50 mg/kg) and urethan (500 mg/kg iv). The trachea was exposed in the neck and cut longitudinally to insert a three-sidearm cannula (Fig. 1). To prevent the epiglottis from falling and occluding the upper airway, an oral cannula (OC in Fig. 1) was inserted through the mouth and positioned just under the tip of the epiglottis, facing the laryngeal opening.

Intrathoracic pressure was evaluated by measuring esophageal pressure (Pes) with a saline-filled polyethylene catheter connected to a transducer. Subglottic pressure (Psg) was measured with a transducer connected to sidearm S2 of the tracheal cannula (Fig. 1). Polyethylene catheters were inserted into the femoral artery and vein to measure arterial blood pressure and to inject supplemental doses of anesthetics, respectively.

Receptor Study

Eight dogs were used for this study. The internal branches of both superior laryngeal nerves (SLNs) were isolated and cut to avoid reflexes that could interfere with the activity of the receptors. The peripheral cut end of the right SLN was placed on a dissecting tray filled with paraffin oil and desheathed with iridectomy scissors and watchmaker forceps under a dissecting microscope. Single-unit action potentials were recorded from thin filaments separated from the right SLN.

Arterial blood pressure, action potentials, and Pes were displayed on a thermal array recorder (Gould TA5000) and stored by using a PC waveform-acquisition system (Windaq, DATAQ Instruments, Akron, OH) for off-line analysis.

Reflex Study

For the reflex study (6 dogs), the SLNs were left intact; hooked wire electrodes were implanted in the PCA by using a
hypodermic needle as an introducer. Data were recorded as described for the receptor study, but instead of action potentials from the SLN, the electromyographic activity of the PCA was recorded.

Protocol

Instillations were performed by introducing a polyethylene catheter into the larynx through sidearm $S_2$ (Fig. 1); this catheter has several small holes along its distal portion so that a fine spray could reach the laryngeal mucosa. During the instillations, the dog was breathing through sidearm $S_2$ (tracheostomy breathing).

Each trial consisted of three separate instillations of 4-ml solution at 1-min intervals; after 20 min, the larynx was suctioned, and a series of three tracheal and three upper airway occlusions, separated by at least five unoccluded breaths, were performed. Tracheal occlusions were obtained by inflating the balloon of a Foley catheter introduced into the trachea through $S_2$, and upper airway occlusions by occluding the sidearms of the tracheal cannula and the oral cannula; all occlusions were performed at end-expiratory volume. At least 30 min were allowed to elapse between each series of instillations.

Each experiment consisted of a trial of saline instillations (control) followed by HCl-pepsin (pH = 2.0; 300 mosmol; 37°C) instillation trials (test). For the receptor study, the number of test trials was limited to the minimum necessary to obtain a clear change in receptor discharge and never exceeded three; challenges with HCl-pepsin at a pH of 2 were usually followed by an instillation at a pH of 1.7 and/or 1.0. For the reflex study, the number of test trials performed was six to eight. Following the same protocol, three additional dogs were exposed to saline instillations only to check for possible deterioration of the preparation; eight to nine instillation trials were performed.

The test solutions were prepared by adding 200 mg of pepsin from porcine stomach mucosa (Sigma Chemical, St. Louis, MO) listed as 570 units/mg solid, 890 units/mg protein, to the appropriate amount of saline (0.9% NaCl) and 1 N HCl to obtain 100 ml of HCl-pepsin solution at pH 2.0, 1.7, and 1.0.

The animal use protocol was approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch.

Data Analysis

Receptor study. For each occlusion, the activity of the receptors was measured as the number of action potentials during inspiration divided by the inspiratory time (impulses/s); in each trial, the values of three tracheal and three upper airway occlusions were averaged. Comparisons were made among data obtained with tracheal and upper airway occlusions after the saline trial and the last HCl-pepsin (pH = 2) trial by using one-way repeated-measures analysis of variance; pairwise multiple comparisons were made with the Student-Newman-Keuls method. Peak Pes values were analyzed in the same manner. Changes were considered significant if $p < 0.05$. Data are means ± SE.

Reflex study. The moving time average was calculated off-line from the raw signal of the PCA. The peak value was measured for each occlusion and evaluated in arbitrary units. Because the activity of the PCA depends also on the value of Pes, the PCA activity was normalized by dividing it by Pes (PCA/Pes). For each trial, an average of PCA/Pes was calculated for three upper airway and three tracheal occlusions. The normalized PCA values were compared by using analysis of variance for a two-factor experiment with repeated measures on two factors. The two factors were the type of occlusion (upper airway and tracheal) and trial (saline and 6 HCl-pepsin instillations). Fisher's least significant difference procedure was used for multiple comparisons with Bonferroni adjustment for the number of comparisons.

For the experiments in which only the saline trials were performed, the PCA/Pes ratios for tracheal and upper airway occlusions were averaged for two trials at a time, so that four groups of data were obtained for each animal (saline 1 and 2, 3 and 4, 5 and 6, and 7 and 8). These values were compared with two-way repeated-measures analysis of variance. A P value < 0.05 was considered significant. Data are means ± SE.

Histology

The laryngeal tissue samples were obtained from two groups of dogs. In the first group ($n = 5$), the larynx in situ had been exposed to three to four instillation trials of HCl-pepsin at a pH of 2.0, followed by one to two trials at a pH of 1.0 or 1.7. The second group, the control group, received no acid treatment ($n = 4$).

![Fig. 2. Effect of HCl-pepsin on response to negative pressure of 1 of the laryngeal receptors tested. AP, action potentials; Psg, subglottic pressure; Pes, esophageal pressure. This receptor was stimulated by upper airway occlusion (top) and practically silent during tracheal occlusion (bottom). Instillations of HCl-pepsin greatly decreased response to negative pressure; middle panels represent responses of the receptor to occlusions (occl.) after 3rd HCl-pepsin instillation trial at a pH of 2.0; panels at right represent receptor responses after a successive trial at pH 1.7.](http://jap.physiology.org/Downloaded_from)
At the end of the experiment, the entire larynx was removed and immersed in a fixative containing 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer, pH 7.2, for several weeks. Tissue sampling was done at three different locations of the larynx along the posterior midline. It included: 1) the aryepiglottic fold near the posterior midline; 2) false vocal folds; and 3) true vocal folds.

Due to the presence of calcified cartilages, only the epithelium and lamina propria layers of each specimen were removed from the underlying cartilages and processed for light microscopy. Tissues were dehydrated through ascending concentrations of ethanol, cleared with xylene, and embedded in paraffin. Tissues were then sectioned at 10 µm in thickness and mounted on gelatin-coated slides. Sections were stained by routine hematoxyline and eosin, cover-slipped with permount, and examined with a compound light microscope.

RESULTS

Receptor Study
We studied 10 receptors in 8 dogs. All receptors were activated to a greater extent during upper airway than during tracheal occlusions after both saline (56.4 ± 10.9 and 16.4 ± 8.8 impulses/s, respectively) and HCl-P instillations (38.2 ± 9.2 and 16.8 ± 8.1 impulses/s, respectively; Figs. 2-3). Because the inspiratory efforts were similar, as indicated by similar values of Pes (upper airway occlusion 2.54 ± 0.40 kPa, tracheal occlusion 2.61 ± 0.40 kPa), the higher discharge of the receptors during upper airway occlusions compared with tracheal occlusions discloses the stimulatory effect of negative pressure. With one exception, repeated HCl-P instillations reduced the response of the receptors to upper airway occlusion; overall, the average discharge of the endings after HCl-P was 67.7% of that present after saline instillation (P < 0.05; Fig. 3). The responses to tracheal occlusions were not significantly affected. As inferred from the amplitude of the Pes swings, the inspiratory efforts were not significantly altered by the acidic solutions (upper airway occlusion 2.41 ± 0.33, tracheal occlusion 2.60 ± 0.39 kPa; P = 0.54).

Reflex Study
After the saline instillation trial (control), the PCA/Pes was 2.22 ± 0.36 during tracheal occlusion and 4.29 ± 0.31 during upper airway occlusion; the difference between the values for the two types of occlusion was significantly different. After the second HCl-pepsin trial, PCA activation during upper airway occlusion decreased progressively; at the fourth, fifth, and sixth trial, the PCA/Pes values were significantly different from control (3.11 ± 0.39, 2.61 ± 0.36, and 2.23 ± 0.18, respectively) as shown in Figs. 4 and 5. After the fourth trial, the difference between upper airway and tracheal occlusion was no longer statistically significant. The small decrease of the response to tracheal occlusions did not reach statistical significance (Fig. 5).

In the dogs exposed only to saline, the response to upper airway and tracheal occlusions did not change in the course of the experiment. The averages of the PCA/Pes ratios for the first two saline trials were 5.63 ± 1.32 for upper airway occlusion and 4.05 ± 1.27 for tracheal occlusion. The averages for the last two trials (7th and 8th) were 5.59 ± 0.71 for upper airway occlusion and 3.69 ± 0.11 for tracheal occlusion. The values for tracheal occlusion remained significantly different from those of upper airway occlusion.

Fig. 3. Bar diagram summarizing effects of HCl-pepsin instillations for receptor study (n = 10). Bars represent means ± SE. Pressure-responsive laryngeal receptors are more stimulated by upper airway than by tracheal occlusions (*P < 0.05), but the former response is greatly reduced (**P < 0.05) after HCl-pepsin instillations of pH 2.0.

Fig. 4. Effect of successive laryngeal instillations of HCl-pepsin on response to pressure of posterior cricoarytenoid muscle (PCA) in 1 experiment. Traces represent arterial blood pressure (BP); raw activity of PCA (PCA raw); PCA moving time average (PCAMTA), and Psg and Pes. Effect of laryngeal negative pressure on activity of PCA is indicated by its higher discharge during upper airway occlusion (UAO; negative swing in Psg tracing) compared with tracheal occlusion (TO; no changes in Psg). Response of PCA to negative pressure is not much affected after 2nd instillation trials but is markedly decreased after repeated HCl-pepsin instillation (6th trial).
Histology

In control animals, the epithelial lining and lamina propria of the three regions of the larynx were intact. The epithelium of the mucous membrane is a stratified squamous type (Fig. 6, left) with different thickness depending on locations. The lamina propria is a loose connective tissue containing many blood vessels, mucous and serous glands, and nerve bundles in the aryepiglottic fold (Fig. 6, left) and in the vestibular fold. In some specimens, a piece of cartilage and/or some skeletal muscles were found deeper into the lamina propria.

In the experimental group treated with HCl-pepsin, the epithelial lining of the aryepiglottic fold was damaged extensively (Fig. 6, right). In the severely damaged regions, the entire epithelial layer was stripped off completely, and lamina propria was disrupted, with many red and white blood cells scattered in the interstitial space. In the regions where damage was moderate, epithelial lining was removed partially (Fig. 6, right), thus leaving only a thin layer of epithelium superficial to the lamina propria. In these cases, most of the features of the lamina propria were maintained as normal, with an occasional sign of clusters of inflammatory cells. Despite the extensive damage to the aryepiglottic folds, both vestibular folds and vocal cords maintained their intact epithelial lining.

DISCUSSION

The results of this study demonstrate that instillations of HCl-pepsin solutions into the laryngeal lumen lessen the response of laryngeal receptors, and the associated reflex augmentation of PCA activity, to negative pressure, suggesting an impairment of the upper airway patency-maintaining mechanisms.

GER, defined by Johnson (13) as a drop in esophageal pH to $<4.0$, is a frequent occurrence. Pathological
conditions related to reflux occur in a significant portion of the population (16, 35); elderly, obese, asthmatic people, and pregnant women are particularly at risk (2, 6, 9, 23).

There is a general consensus on the association between GER and airway pathology of various origin (10, 11, 15–17, 24, 28, 34). However, there is disagreement among investigators concerning the origin of these conditions attributed by some to a direct exposure of the airway to the refluxate but considered by others as a reflex response from the esophagus (10). Whereas the presence of gastric refluxate in the upper airway and the trachea has been documented (4, 5, 12, 16), the hypothesis of an esophageal reflex does not have much support. In fact, when acid exposures of the esophageal and tracheal mucosa were compared, only minor effects were reported for the esophagus, compared with consistent effects observed with tracheal exposure (3, 12, 32).

In this study, HCl and pepsin solutions rather than acid alone have been used, since they simulate more closely the gastric refluxate. Furthermore, it has been demonstrated that either in the larynx (16) or in the esophagus (14, 18) acid with pepsin cause more extensive damage than acid alone. Because pepsin is most active at a pH of ~2 (8, 25), we used this level of acidity. Indeed, a refluxate of such a pH was reported even in the lowermost portion of the trachea in a patient with severe nocturnal asthma and symptomatic GER (5).

The pressure receptors studied differed for their vulnerability to the HCl-pepsin solutions: whereas some reduced their response to upper airway occlusion after the first test trial, others required two to three HCl-pepsin trials to show a clearly reduced response to negative pressure. These differences are possibly due to a diverse location within the laryngeal mucosa as it was found for similar receptors challenged with topical anesthetics (1).

Studies in which topical application of anesthetics was used suggest that most of the laryngeal receptors have a superficial location. This conclusion was based on the fact that 80% of the receptors studied ceased their activity within 50 s from the application of the anesthetic (1) and that the increase of genioglossus muscle activity in response to negative pressure was abolished (21). However, it must be considered that Mathew et al. (21) used a very high concentration of local anesthetics, and in the experiments of Anderson et al. (1) some of the receptors could not be blocked even after 30 min of exposure. This indicates that some of the receptors may be less vulnerable than others; in fact, in this study, one of ten receptors remained unaffected by the instillation of acid-pepsin. The impairment of the receptors to respond to negative pressure is likely due to the marked structural alterations caused by HCl-pepsin solutions on the laryngeal mucosa that we and others (7, 16) have found. These lesions include extensive necrosis of the laryngeal epithelium and infiltration of inflammatory cells, which are expected to compromise the function of the pressure-responsive receptors superficially located. It is interesting to note an absence of any injury on the mucosa overlying the vocal folds; a finding also reported by Koufman (16) for the mucosa overlying the vocal processes. We do not have any explanation for these differential effects. Actually, several pathological processes seem to occur preferentially on the vocal folds; indeed, these structures seem to lack the protection provided by mucus glands and the ciliary escalator.

The progressive reduction in the response of the PCA to upper airway occlusion with repeated exposures of the laryngeal mucosa to HCl-pepsin cannot be attributed to a deterioration of the experimental preparation, since in dogs exposed only to repeated instillations of saline there was no decrease of the PCA response to upper airway collapsing pressure. This contention is also supported by the observation that the responses to tracheal occlusion were not significantly affected. The lack of a significant reduction of the laryngeal abductor activation during tracheal occlusion suggests that, whereas the reflex activation of the PCA during this type of occlusion is mainly due to withdrawal of lung volume negative feedback, with upper airway occlusion there is an additional reflex recruitment mechanism that is impaired by exposure of the laryngeal mucosa to HCl-pepsin. It seems, therefore, reasonable to assume that the impairment of the pressure-sensitive receptors due to the exposure to HCl-pepsin causes the reduction in PCA activity during upper airway occlusions.

A significant deterioration of the response of laryngeal receptors to pressure occurred after one to three test trials, whereas the PCA response was affected only at a later stage. This difference may reflect some degree of redundancy in the afferent activity that triggers this reflex; i.e., a large decrease in overall receptor discharge is needed to significantly decrease the reflex effects.

Considering that harmful effects are present with exposures lasting few hours, our results strongly suggest that patients with a history of GER episodes are at risk of having their patency-maintaining mechanisms severely compromised.

Airway protective mechanisms may be diminished during sleep (30, 31), a time at which GER is most likely to occur; thus the additional impairment of these mechanisms due to GER would increase upper airway resistance and the likelihood of snoring and obstructive sleep apnea.

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