Pulmonary responses to tracheal or esophageal acidification in guinea pigs with airway inflammation

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Lopes, Fernanda D. T. Q. S., Glauco S. Alvarenga, Raquel Quiles, Mayra B. Dorna, Joaquim E. Vieira, Marisa Dolhnikoff, and Milton A. Martins. Pulmonary responses to tracheal or esophageal acidification in guinea pigs with airway inflammation. J Appl Physiol 93: 842–847, 2002.—The association between asthma and gastroesophageal reflux has been attributed to microaspiration of gastric contents and/or vagally mediated reflex bronchoconstriction. In previous experimental studies concerning the pulmonary effects of esophageal or tracheal acidification, only animals without airway inflammation have been studied. We assessed the effects of esophageal and tracheal administration of hydrochloric acid (HCl) on normal guinea pigs (GP) and GP with airway inflammation induced by repeated ovalbumin exposures. These GP were anesthetized (pentobarbital sodium) and received 1) 20 μl of either 0.2 N HCl or saline into the trachea, or 2) 1 ml of either 1 N HCl or saline into the esophagus. Intratracheal HCl resulted in a significant increase in both respiratory system elastance and resistance (P < 0.001). There were no significant changes in respiratory mechanics when HCl was infused into the esophagus. In conclusion, we observed that infusion of large volumes of HCl into the esophagus did not change pulmonary mechanics significantly, even in guinea pigs with chronic allergen-induced airway inflammation. In contrast, intratracheal administration of normal amounts of acid had substantial effects in normal GP and GP with airway inflammation.

Experimental asthma; gastroesophageal reflux; respiratory mechanics; airway inflammation

Two major mechanisms have been proposed to explain the association between gastroesophageal reflux (GER) and asthma symptoms: 1) microaspiration of refluxed gastric content into the airways, resulting in an inflammatory response and bronchospasm (the “reflux” hypothesis); and 2) activation of sensory nerve terminals in the lower esophagus, triggering a vagal reflex, with resulting bronchoconstriction and airway inflammation (the “reflex” hypothesis) (1, 3, 24, 27, 29).

Mansfield and Stein (20) observed that intraseophageal acid infusion could increase airway resistance in asthmatic patients and that antacid therapy would reverse these changes. Davis et al. (6) showed that the presence of acid in the lower esophageal portion can trigger bronchoconstriction in asthmatic children with a positive Bernstein test.

However, results of studies involving distal esophageal acid perfusion and bronchospasm are conflicting. Tan et al. (30), studying 15 sleeping asthmatic subjects, showed that intraseophageal acid (either spontaneous GER or induced) did not induce significant acute or sustained changes in airflow (V˙) resistance. Ekström and Tibbling (7) did not find any relationship between reflux, either at the proximal or the distal esophagus, and bronchial symptoms or peak expiratory flow changes in moderate and severe asthmatic patients with GER.

Some studies in experimental animals have been performed to clarify the mechanism of the worsening of asthma induced by GER but also with conflicting results. Mansfield et al. (19) induced esophagitis in dogs and observed a fall in respiratory function after intraseophageal acid infusion, whereas this response did not occur in dogs after bilateral cervical vagotomy. Recently, Hamamoto and co-workers (11) infused hydrochloric acid (HCl) into the esophagus of guinea pigs (GP) and observed the presence of airway plasma extravasation. In contrast, there are some studies that suggest that microaspiration is the major mechanism of bronchoconstriction induced by GER. Tuchman et al. (32) showed in cats that the pulmonary response to airway acid infusion is much greater than the response to intraseophageal acid infusion. However, all of these studies were performed in experimental animals without airway inflammation. Because bronchospasm induced by GER is observed more often in asthmatic than in nonasthmatic people, we reasoned that the use of an experimental model of chronic airway inflammation would be more relevant to elucidate the mechanisms of bronchospasm induced by GER.

In the present study, we evaluated the pulmonary responses after acid infusion into the trachea or esoph-
agus of GP with chronic airway inflammation induced by repeated exposures to ovalbumin and also of normal GP. Our results support the hypothesis that microaspiration is a major mechanism of bronchospasm associated with GER.

**METHODS**

We used male Hartley GP weighing 300–450 g. All GP received humane care in compliance with the “Principles of Laboratory Animal Care” published by the National Institutes of Health (NIH publication 86-23, revised 1985). Our study was approved by the Institutional Review Board of the School of Medicine of the University of Sao Paulo.

**Study design.** GP were anesthetized with pentobarbital sodium (50 mg/kg ip), tracheostomized, and ventilated at 60 breaths/min with a tidal volume of 8 ml/kg by using a Harvard 683 ventilator (Harvard Apparatus, South Natick, MA).

In the first protocol, we studied five groups of normal GP (n = 6 for each group) that received, respectively, 1) tracheal infusion of 20 μl of 0.2 N HCl; 2) tracheal infusion of 20 μl of saline (0.9% NaCl); 3) esophageal infusion of 1 ml of 0.2 N HCl; 4) esophageal infusion of 1 ml of 1 N HCl; and 5) esophageal infusion of 1 ml of saline. Esophageal instillation of either saline or HCl was performed over 1 min with a polyethylene catheter inserted into the mouth and placed in the lower esophagus. The exact localization of the tube was ensured after laparotomy and distal esophagus visualization.

In the second protocol, four groups of GP with airway inflammation induced by repeated exposures to ovalbumin aerosol were studied (n = 6 for each group) and received, respectively, 1) tracheal infusion of 20 μl of 0.2 N HCl; 2) tracheal infusion of 20 μl of saline (NaCl 0.9%); 3) esophageal infusion of 1 ml of 0.2 N HCl; and 4) esophageal infusion of 1 ml of saline.

Tracheal pressure (Ptr), V, and lung volume (V) changes were obtained before; 30 s after; and 1, 2, 5, and 10 min after either saline or HCl instillation. Respiratory system resistance (Rrs) and elastance (Ers) were then computed. Ten minutes after the infusion of saline or HCl, animals were killed, and the lungs were removed for morphometric analysis of peribronchial edema formation.

**Measurement of respiratory system mechanics.** Ptr was measured with a differential pressure transducer (DP 45–28–2114; Validyne, Northridge, CA) connected to a side tap in the tracheal cannula. V was measured with a pneumotachograph (Fleisch no. 4–0) attached to the tracheal cannula and to a differential pressure transducer (Validyne DP 45–16–2114). V changes were obtained by electronic integration of V. Ptr and V signals were registered in a Gould RS-3400 recorder (Gould Instruments, Cleveland, OH), sampled at 200 Hz with an analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA), and stored in a microcomputer. Nine to ten respiratory cycles were averaged to provide one data point (23). Measurements of Ptr and V were obtained before; 30 s after; and 1, 2, 5, and 10 min after saline or HCl instillation.

Rrs and Ers were obtained by using the equation of motion of the respiratory system, as follows

\[ \text{Ptr}(t) = \text{Ers} \cdot V + \text{Rrs} \cdot V(t) \]

where \( t \) is time.

**Chronic airway inflammation protocol.** GP were submitted to a protocol of chronic airway inflammation as previously described (31). The animals were placed in a Plexiglas box (30 × 15 × 20 cm) coupled to an ultrasonic nebulizer (US-1000; ICEL, São Paulo, Brazil), and an aerosol of ovalbumin (Sigma Chemical, St. Louis, MO) diluted in 0.9% NaCl was generated for 15 min or until respiratory distress occurred. Respiratory distress was defined as the onset of sneezing, coryza, cough, and/or in-drawing of the thoracic wall. This protocol was repeated seven times, three times a week, and the last inhalation was 72 h before the experiments. We used increasing concentrations of ovalbumin (1–5 mg/ml) to overcome the effects of tolerance.

**Morphometric analysis.** To study the effects of acid infusion on the formation of bronchial edema, 10 min after the infusion of either saline or HCl, the airways were occluded at end expiration, and the GP were killed by exsanguination. The lungs were rapidly removed and quickly frozen by immersion in liquid nitrogen. Lungs were then fixed in Carnoy’s solution (ethanol-chloroform-acetic acid, 60:30:10 by volume) at −70°C. After 24 h, the concentration of ethanol was progressively increased (70, 80, 90%, respectively, 1 h for each solution, at −20°C). The lungs were kept in 100% ethanol for 24 h at 4°C and then allowed to reach and remain at room temperature (33). After fixation, sagittal slices obtained from the right lung were embedded in paraffin. Five-micrometer-thick histological sections were obtained and stained with hematoxylin and eosin.

Morphometric analysis was performed with an optical microscope provided with an integrating eyepiece with 100 points and 50 lines. We studied only transversely sectioned airways and measured the amount of peribronchial edema accumulation by using the point-counting technique described below. Airways were considered to be transversely cut when the relation between maximal diameter of the airway and the diameter at the widest point perpendicular to this axis was ≥0.6. The diameters of the airways were assessed by using the lines of the grid, with known length.

The number of points of the integrating eyepiece (NP) falling on areas of peribronchial edema was counted, as well as the number of the intercepts (NI) of the lines with epithelial basal membrane of bronchi. The magnitude of peribronchial edema [edema index (EI)] was computed as follows

\[ \text{EI} = \sqrt{\frac{\text{NP}}{\text{NI}}} \]

To determine EI, we studied five to seven randomly selected, noncartilaginous airways per lung.

**Statistical analysis.** Statistical analysis was performed by using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Values of Rrs and Ers were compared by using two-way repeated-measures analysis of variance for one factor repeated followed by Tukey’s test for multiple comparisons. Peribronchial edema values were evaluated by using two-way analysis of variance. A P value of <0.05 was considered statistically significant.

**RESULTS**

Figures 1 and 2 show, respectively, Rrs and Ers values observed before and after infusion of acid or saline into either the trachea or the esophagus of normal GP. Infusion of 20 μl of 0.2 N HCl into the trachea resulted in an immediate and substantial increase in Ers compared with baseline values (P < 0.001). In contrast, infusion of 1 ml of 0.2 N or 1 N HCl into the esophagus did not result in significant changes in Ers. Maximal values of Ers observed in the group of GP that received intratracheal administration of HCl...
(4.72 ± 0.27 cmH2O/ml) were significantly greater than those observed in the other four groups (P < 0.001). Similar results were observed for Rrs values. In fact, Rrs increased significantly only in the group of GP that received intratracheal HCl (maximal values of 0.53 ± 0.04 cmH2O⋅ml⁻¹⋅s, P < 0.001, compared with baseline values and with peak values of the other four experimental groups).

Figures 3 and 4 show, respectively, the mean values of Rrs and Ers observed in the experiments performed with GP previously submitted to a protocol of repeated exposures to ovalbumin aerosol. We did not observe significant differences in baseline values of either Rrs or Ers when the four groups were compared. Infusion of 20 μl of 0.2 N HCl into the trachea of GP with chronic airway inflammation resulted in a significant increase in values of both Rrs and Ers (P < 0.001). Infusion of 1 ml of 1 N HCl into the lower esophagus of these animals did not result in any significant change in both Ers and Rrs. Interestingly, infusion of 20 μl of normal saline into the trachea resulted in a small but statistically significant increase in Rrs (P = 0.041).

Peak values of Ers and Rrs observed in the group of GP that received acid into the trachea were significantly greater than the values observed in the other groups of GP (P < 0.001 for Ers and P < 0.005 for Rrs). Figure 5 shows the results of the morphometric evaluation of peribronchial edema. GP with chronic airway inflammation had more peribronchial edema than GP without chronic inflammation (P < 0.004). There were...
no significant differences among the five groups of normal GP studied. Although the GP with induced chronic airway inflammation that received intratracheal acid showed a tendency to higher values of edema index compared with the other groups with airway inflammation, this difference did not reach statistical significance (P = 0.274).

To rule out the influence of the anesthesia with barbiturates on our results, we did additional experiments in GP that were anesthetized with xylazine (30 mg/kg ip), tracheostomized, mechanically ventilated, and also received succinylcholine (13 mg/kg ip). Animals received infusion of 1 ml of either 0.9% NaCl or 1 N HCl into the lower esophagus, as described in Methods (n = 7 for each group). GP that received 1 N HCl presented maximal changes in Rrs and Ers of, respectively, 7.1 ± 4.9 and 6.3 ± 2.8% (means ± SE), whereas GP that received 0.9% NaCl presented maximal percent changes in Rrs and Ers of, respectively, 10.7 ± 3.1 and 6.2 ± 2.2% (P = not significant).

DISCUSSION

The main purpose of our study was to evaluate the effects of acid infusion in the lower esophagus and the trachea of experimental animals previously submitted to a protocol to induce airway inflammation. Our laboratory (31) has previously demonstrated that the protocol used in this study results in significant airway infiltration of lymphocytes and eosinophils. Some foci of inflammatory cells (mono- and polymorphonuclear) are also seen in alveoli and the pulmonary vascular bed. In a previous study with this experimental model, lung slices were stained with markers of total T lymphocytes (H159), CD4+ T cells (H155), and for eosinophil peroxidase activity. There was a significant recruitment of T lymphocytes, mainly of CD4+ subset, and eosinophils around airways in GP chronically exposed to ovalbumin compared with control animals (31). We reasoned that the evaluation of the effects of acid infusion into the esophagus and the trachea of experimental animals with chronic airway inflammation would add relevant information concerning the mechanisms of airway obstruction induced by GER.

We infused acid or saline into the trachea or the esophagus of GP 72 h after the last challenge of aerosolized ovalbumin because it was previously demonstrated that, at this time, there is airway hyperresponsiveness and bronchial inflammation corresponding to a late-phase airway response. Hutson et al. (14) studied the early and late bronchoconstrictor responses induced by antigen challenge in sensitized GP and observed an acute response that reached the lowest value of airway conductance 2 h after challenge and two late responses that peaked at 17 and 72 h, respectively. Bronchoalveolar lavage fluid was evaluated and showed an increase in the number of eosinophils at these two time points. In a previous work, our laboratory (31) observed an increase in the number of lymphocytes and eosinophils in bronchoalveolar lavage fluid obtained 2–3 days after the last challenge in the GP model of chronic airway inflammation used in the present study. We also observed the presence of peribronchial edema and pulmonary hyperresponsiveness to aerosolized methacholine 48–72 h after the last ovalbumin challenge (18, 31).

We were not able to demonstrate that infusion of high amounts of 1 N HCl (1 ml) into the lower esophagus of GP with airway inflammation results in either bronchoconstriction or airway edema formation. In contrast, very small amounts of HCl (20 μl) in the trachea resulted in substantial increases in Ers and Rrs. Only one difference was observed when the results obtained in normal GP were compared with those in GP with airway inflammation: infusion of a small amount of saline into the trachea of these animals resulted in a significant increase in Rrs, probably reflecting the airway hyperresponsiveness present in this experimental model (14, 18).

Hamamoto et al. (11) infused 1 N HCl (0.4 ml) into the esophagus of GP and measured the leakage of Evans blue dye in the airways. They observed a significant increase in plasma extravasation in the trachea but not in the main bronchi. However, the HCl-induced extravasation was potentiated in the trachea and main bronchi by the neutral endopeptidase inhibitor phosphoramidon. Because the neutral endopeptidase inhibitor is an epithelial enzyme that plays a major role in the metabolism of tachykinins, such as substance P and neurokinin A (21, 22), Hamamoto et al. (11) suggested that intraesophageal infusion of HCl results in a neural reflex that results in airway release of tachykinins. We also evaluated the formation of airway edema, but we did not observe a significant effect of the administration of acid into the esophagus, either in normal GP or in GP with airway inflammation (Fig. 5).

Our experimental model has some limitations: each GP received only one dose of acid, and these animals did not have esophagitis. In human GER disease,
flux occurs several times and is usually associated with inflammation of the esophageal mucosa. However, in a previous study, Tuchman and co-workers (32) infused HCl into the esophagus of cats with esophagitis. Esophagitis was induced by continuous infusion of 0.2 N HCl via an oroesophageal polyethylene tube, positioned in the midesophagus, over a 30-min period, on 2–3 successive days before the study, and the presence of esophagitis was confirmed by esophageal biopsy. The presence of esophagitis did not enhance the airway response to esophageal acidification. In the study of Tuchman et al., the increase in total lung resistance was substantially greater after infusion of small amounts of acid into the trachea than when larger amounts of HCl were infused into the esophagus.

Another limitation of our experimental model was the possible influence of anesthesia on the pulmonary reflexes. It was previously shown that intravenous anesthesia with barbiturates can inhibit the reflex bronchoconstriction induced by histamine aerosol (15, 16). However, a very small amount of HCl administered into the trachea resulted in a substantial bronchoconstriction in our experimental model, and, in GP with chronic airway inflammation, even intratracheal administration of only 20 μl of saline resulted in bronchoconstriction. Using anesthesia with xylazine, we obtained the same results: infusion of acid into the lower esophagus did not result in significant pulmonary effects.

The absence of a significant pulmonary response to infusion of acid in the lower esophagus was not due to an inhibition of acid with vagal reflexes. It has been previously demonstrated that vagal reflexes are increased in anesthetized GP after a single or multiple antigen challenges. Costello et al. (5) showed that, in sensitized and anesthetized GP, a single antigen challenge increases the bronchoconstriction induced by electrical stimulation of the vagus nerves. Perretti et al. (26) observed an increase in the pulmonary response to electrical stimulation of the vagus nerves in anesthetized GP that were previously submitted to a protocol of multiple antigen challenges.

In humans, the majority of studies that support the idea that the presence of an esophageal reflex is important to explain the association between bronchospasm and GER have been performed in asthmatic patients with esophagitis (6, 10, 12, 28). However, the changes observed in pulmonary function are usually modest and not observed in all subjects tested (24, 25). In addition, in some of these studies, one cannot totally exclude the possibility that a small quantity of acid may be aspirated (8). It has been shown that the presence of stimuli, such as methacholine or isocapnic hyperventilation, potentiates the esophageal reflex induced by the presence of acid in the lower esophagus (13).

Results of studies aimed to detect the presence of aspiration of isotopes from the stomach in asthmatic patients have been negative, although the studies were performed with a small group of asthmatic subjects (9). However, it has been suggested that methods used with isotopes are not sensitive enough to detect aspiration into the airways of very small amounts of liquid. Wynne et al. (35) showed in mice that aspiration of very small amounts of either HCl or gastric juice with low pH (~15 μl) results in a marked damage to the tracheal mucosa (desquamation of the superficial cell layer with loss of ciliated and nonciliated cells). It has been suggested that the microaspiration of gastric contents stimulates airway receptors and evokes a significant airway response (2). Studies in animals have showed that activation of the tracheal irritant receptors in the upper airway epithelium is associated with a vagally mediated reflex bronchoconstriction (4, 34, 36). Airway hyperresponsiveness in asthma has been related to epithelial damage (17).

In conclusion, we observed that infusion of large volumes of HCl into the esophagus did not change pulmonary mechanics significantly, even in GP with chronic allergen-induced airway inflammation. In contrast, intratracheal administration of small amounts of acid had substantial effects. Small amounts of intratracheal acid resulted in bronchoconstriction but not in airway edema. Our results support the view that, even in the presence of airway inflammation, microaspiration into the trachea is a more likely mechanism for the bronchoconstriction associated with GER than a reflex induced by the presence of acid in the lower esophagus.

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REFERENCES


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