Mast cells and reactive oxygen species in citric acid-induced airway constriction

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Submitted 15 September 2003; accepted in final form 2 January 2004

Wu, Li-Ling, Fung-Jou Lu, and Yih-Loong Lai. Mast cells and reactive oxygen species in citric acid-induced airway constriction. J Appl Physiol 96: 1879–1885, 2004; 10.1152/japphysiol.00999.2003.—The noncholinergic airway constriction is mediated by tachykinins, mainly neurokinin A and substance P, and this bronchoconstriction is usually enhanced during inflammatory episodes. We demonstrated previously that reactive oxygen species play an important role in capsaicin-, hyperventilation-, and citric acid (CA) inhalation-induced noncholinergic airway constriction. For understanding cellular involvement, we further investigated the relationship between mast cells, bradykinin (BK), reactive oxygen species, and noncholinergic airway constriction. Sixty-five guinea pigs were divided into seven groups: saline control; CA; BK + CA; cromolyn sodium (CS) + CA; BK + CS + CA; compound 48/80 + CA; and compound 48/80 + BK + CA. CS was used to stabilize mast cells, whereas a secretagogue, compound 48/80, was for the depletion of mast cells. Each animal was anesthetized, cannulated, paralyzed, and ventilated artificially. In control animals, CA aerosol inhalation caused decreases in dynamic compliance and forced expiratory parameters, indicating CA-induced noncholinergic airway constriction. Either CS or compound 48/80 significantly attenuated the CA-induced airway constriction. Also, we detected a significant increase in lucigenin-initiated chemiluminescence counts of the bronchoalveolar lavage sample in the BK + CA group. Furthermore, CA exposure caused an increase in bronchoalveolar lavage substance P level. Either CS or compound 48/80 prevented the above CA-induced increases in chemiluminescence and substance P. These results suggest that mast cells play an important role in CA aerosol inhalation-induced airway constriction via perhaps releasing constricting factors.

Bradykinin is a nonapeptide formed from plasma and tissue precursors during inflammation (5). Bradykinin, a metabolite of the kallikrein-kinin system, has multiple effects on airway function in guinea pigs, including bronchoconstriction, vasodilation, plasma extravasation, mucus secretion, and cough. These effects are thought to be due, at least in part, to the activation of sensory nerves leading to both axonal and central reflex activity (2, 14, 19, 21, 34, 42). A favorable condition for bradykinin release from its precursor proteins is a low pH of the milieu (5).

Reactive oxygen species (ROS) play an important role in citric acid-induced airway constriction (28). There are several sources for ROS, including neutrophils, eosinophils, and mast cells. Compared with other cell types, mast cells have received less attention, although they have been demonstrated to be involved with noncholinergic airway constriction (23, 30). Therefore, the purpose of this study was to examine the role of mast cells in citric acid-induced noncholinergic airway constriction.

We postulated that the involvement of mast cells in citric acid-induced airway constriction may be mediated via ROS and augmented by bradykinin. In this study, the role of mast cells was investigated by using a degranulating agent, compound 48/80, and a stabilizer cromolyn sodium. We explored the role of bradykinin by adding exogenous bradykinin. Furthermore, for ROS, we determined chemiluminescence counts before and after citric acid inhalation.

MATERIALS AND METHODS

The study was conducted according to the Guiding Principles in the Care and Use of Animals of the American Physiological Society and was approved by the Animal Care and Use Committee of the National Taiwan University.

Animal preparations. Sixty-five young Hartley strain guinea pigs weighing 213 ± 2 g were divided into seven groups: saline + saline (SA + SA, n = 9); saline + citric acid (SA + CA, n = 9); bradykinin + citric acid (BK + CA, n = 9); cromolyn sodium + citric acid (CS + CA, n = 9); bradykinin + cromolyn sodium + citric acid (BK + CS + CA, n = 9); compound 48/80 + citric acid (48/80 + CA, n = 9); and bradykinin + compound 48/80 + citric acid (BK + 48/80 + CA, n = 11).

On the day of the study, each animal was anesthetized with pentobarbital sodium (30–40 mg/kg), and its trachea, carotid artery, and jugular vein were cannulated. After being paralyzed with gallamine triethiodide (4 mg/kg), the animal was ventilated artificially. To ensure that the animal was anesthetized during paralysis, we administered gallamine according to the following plans. 1) Gallamine was only given when its active period (40 min) was within the...
effective duration of pentobarbital (1–2 h). If it was necessary to administer gallamine beyond this effective period of the anesthetic, supplemental doses of pentobarbital were given before any more gallamine treatment. 2) If an additional dose of gallamine was needed after a single dose, we first examined the level of anesthesia and made sure that the expected anesthesia could be maintained longer than the effective duration of gallamine. The second injection of gallamine was then given to the animal. All animals were pretreated with propranolol (1 mg/kg iv) and atropine (1 mg/kg iv) to block adrenergic and cholinergic neural effects, respectively. Also, 30 min before the study, all animals were pretreated with indomethacin (5 mg/kg iv) and captopril (1 mg/kg iv) to avoid the indirect effects of bradykinin by producing prostaglandins and the endogenous breakdown of bradykinin by angiotensin-converting enzyme, respectively. Propranolol and captopril were injected carefully and slowly with monitoring of the systemic blood pressure. All animals in the SA group received 50 breaths of 4-ml saline aerosol generated from saline. All animals in the CA groups received 50 breaths of 4-ml citric acid aerosol generated from 0.3 M citric acid using a nebulizer (Ultra-Neb99, DeVilbiss, Somerset, PA). Bradykinin (0.1 nmol/kg) and cromolyn sodium (10 mg/kg) were intravenously injected 15 min before the citric acid aerosol inhalation. Compound 48/80, a mast cell-degranulating agent, was given to the animals by subcutaneous injection for 3 days before the study. Three consecutive daily doses were 6, 9, and 10 mg/kg. Usually the daily dose was divided into two injections for each animal each day (41).

Evaluation of bronchial function. Each anesthetized-paralyzed and ventilated animal was placed supine inside a whole body plethysmograph. According to our previous method (28), dynamic respiratory compliance (Crs) and maximal flow-volume parameters were obtained before and after SA or CA inhalation. The general experimental procedure consisted of obtaining the values of Crs and forced expiratory volume in 0.1 s (FEV0.1) before and 1–20 min after inhalation of SA or CA aerosol.

Collection of BAL fluid. An additional 63 young guinea pigs were divided evenly into the same seven groups, as those mentioned above, of nine animals each. BAL fluid was collected 3 min after SA or CA aerosol inhalation. To obtain bronchoalveolar lavage (BAL) fluid, 4 ml of saline at 37°C were instilled via the trachea 3 min after the instillation. Then 1.5 ml of the obtained BAL fluid was placed in a tube immediately wrapped with aluminum foil and kept in the icebox until measurement of chemiluminescence, which was used usually within 1 h. Another 1 ml of BAL fluid was placed in a tube containing 10 μl thiorphan (5 × 10–6 M) (neutral endopeptidase inhibitor) and then centrifuged at 300 g at 4°C for 10 min. The supernatant was stored at –70°C for subsequent analysis of SP.

Determination of SP. BAL fluid SP levels were detected using enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to our laboratory’s previous method (29). This method has a sensitivity of 12.5 ± 1 fmol/ml with both intra- and interassay coefficients of variance <10%.

Measurements of lucigenin-initiated chemiluminescence. Determination of lucigenin-initiated BAL fluid chemiluminescence was performed with the method of Sun et al. (47) with some modifications. BAL fluid (0.2 ml) was in a stainless-steel cell (5 cm in diameter). The chemiluminescence was then measured in an absolutely dark chamber of the Chemiluminescence Analyzing System (Tohoku Electronic Industrial, Sendai, Japan). This system contains a photon detector (model CLD-110), chemiluminescence counter (model CLC-10), water circulator (model CLH-20), and 32-bit IBM personal computer system. A cooler circulator was connected to the model CLD-110 photon detector to keep the temperature at 6°C. The model CLD-110, according to the manufacturer’s specifications, is so sensitive it is able to detect as low as 10–15 W of radiant energy (47). Photon emission from the BAL fluid was counted at 10-s intervals at 37°C and atmospheric conditions. At the 100-s time point, 1.0 ml of lucigenin (0.01 mM) in physiological buffer solution was injected into the cell. The chemiluminescence in the BAL sample was continuously measured for a 300-s time period. The total amount of chemiluminescence was calculated by integrating the area under the curve and subtracting it from the background level, which was equivalent to the dark average. The assay was performed in duplicate for each sample and was expressed as chemiluminescence counts per 10 s.

Measurements of BAL histamine. Collected BAL fluid was cooled with ice and centrifuged (1,500×g, 30 min) at 4°C. The supernatant was obtained and stored at –70°C for later analysis of histamine. Histamine concentration was analyzed with a histamine enzyme immunoassay kit (KMI Diagnostics, Minneapolis, MN). The assay was based on a mouse monoclonal antibody directed against acetylhistamine and assumed that samples were acetylated before assay. The lower limit of histamine detection of the assay was 0.3 ng/ml.

BAL cell counts. An additional 126 animals were used for BAL cell counts. The animals in each group were further evenly divided into three subgroups: baseline (saline), 3 min recovery, and 20 min recovery. Procedures for animal treatments were the same as those described above: 2 ml warm (37°C) saline were instilled at an appropriate time after saline or citric acid aerosol and then withdrawn 30 s after the instillation. This was repeated 10 times. Pooled BAL fluid was centrifuged at 4°C for 10 min, and the cell pellet was resuspended in RPMI solution; 100 μl of Turk’s solution was added to the same volume of cell suspension. After mixing, 10 μl of the mixture were placed in a hemacytometer for total cell counts. Differential cell counts were determined from cytopsin preparations stained with Liu stain (29).

Statistical analysis. All values are reported as means ± SE. Analysis of variance was used to establish the statistical significance of differences among groups. For BAL cells, group and time effects were determined by two-way repeated measurements of analysis of variance. If significant differences among groups were obtained by the analysis of variance, Tukey’s multiple range test was used to differentiate differences between groups. Differences were considered significant if P < 0.05.

RESULTS

Body weight and baseline respiratory parameters in guinea pigs are listed in Table 1. Differences in body weight between groups were caused by pretreatment with various drugs. To account for individual differences, we compared the results by using percentage of baseline values for each animal.

Saline aerosol inhalation did not induce any significant change (expressed as percent baseline values) in Crs or FEV0.1. On the other hand, citric acid aerosol inhalation caused marked decreases in Crs and FEV0.1, indicating severe airway constriction (Fig. 1). This constriction was attenuated significantly either by cromolyn sodium or by compound 48/80, whereas the constriction was augmented slightly but not significantly by bradykinin.

In the BAL samples obtained ~3 min after inhalation of citric acid aerosol, a significant increase in lucigenin-initiated chemiluminescence count was observed only in the BK + CA group (Fig. 2). No significant difference between the BK + CA and BK + 48/80 + CA groups may suggest that bradykinin-induced ROS release was not derived from mast cells.

At 3 min after citric acid inhalation, there was a significant increase in BAL SP level in the SA + CA, BK + CA, BK + CS + CA, 48/80 + CA, and BK + 48/80 + CA groups (Fig. 3).

BAL histamine level was examined in four groups of animals. Compared with the saline control group, citric acid inhalation caused a significant increase in BAL histamine level.

J Appl Physiol • VOL 96 • MAY 2004 • www.jap.org
Table 1. Body weight and baseline respiratory parameters in guinea pigs

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>BW, g</th>
<th>TLC, ml</th>
<th>FRC, ml</th>
<th>Crs, ml/cmH2O</th>
<th>FEV0.1, ml</th>
<th>Vmax50, m/s</th>
<th>BP, mmHg</th>
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<tbody>
<tr>
<td>SA + SA</td>
<td>9</td>
<td>250.6±5.8</td>
<td>11.7±0.3</td>
<td>3.27±0.3</td>
<td>0.28±0.01</td>
<td>9.3±0.3</td>
<td>80.6±2.0</td>
<td>62.8±4.3</td>
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<tr>
<td>SA + CA</td>
<td>9</td>
<td>261.7±7.9</td>
<td>9.8±0.5</td>
<td>2.74±0.2</td>
<td>0.28±0.01</td>
<td>8.2±0.4</td>
<td>77.2±8.1</td>
<td>49.8±3.4</td>
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<tr>
<td>BK + CA</td>
<td>9</td>
<td>255.6±9.1</td>
<td>10.0±0.6</td>
<td>2.75±0.2</td>
<td>0.28±0.01</td>
<td>8.4±0.4</td>
<td>80.6±4.0</td>
<td>43.7±3.2</td>
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<tr>
<td>CS + CA</td>
<td>9</td>
<td>250.0±6.9</td>
<td>9.5±0.3*</td>
<td>2.60±0.1</td>
<td>0.27±0.01</td>
<td>8.5±0.3</td>
<td>72.8±5.6</td>
<td>52.6±3.1</td>
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<tr>
<td>BK + CS + CA</td>
<td>9</td>
<td>255.6±6.6</td>
<td>9.3±0.6*</td>
<td>2.59±0.1</td>
<td>0.26±0.01</td>
<td>8.1±0.3</td>
<td>67.8±7.4</td>
<td>51.7±2.8</td>
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<td>Compound 48/80 + CA</td>
<td>9</td>
<td>241.7±7.6</td>
<td>10.4±0.2</td>
<td>2.74±0.1</td>
<td>0.29±0.01</td>
<td>8.5±0.2</td>
<td>81.7±2.4</td>
<td>54.8±3.0</td>
</tr>
<tr>
<td>BK + compound 48/80 + CA</td>
<td>11</td>
<td>236.4±3.6</td>
<td>10.7±0.3</td>
<td>2.65±0.1</td>
<td>0.27±0.01</td>
<td>9.3±0.3</td>
<td>84.6±2.4</td>
<td>48.1±1.9</td>
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</tbody>
</table>

Values are the means ± SE; n, number of animals; BW, body weight; TLC, total lung capacity; FRC, functional residual capacity; Crs, respiratory compliance; FEV0.1, forced expiratory volume in 0.1 s; Vmax50, maximal expiratory flow rate at 50% of baseline vital capacity; BP, blood pressure; SA, saline; CA, citric acid; BK, bradykinin; CS, cromolyn sodium. Statistical differences (P < 0.05) between groups: *compared with the SA + SA group; †compared with the SA + CA group; # compared with the BK + CA group; **compared with the CS + CA group. 

Fig. 1. Citric acid-induced alterations in dynamic respiratory compliance (Crs) (A) and forced expiratory volume in 0.1 s (FEV0.1) (B), expressed as percentage of baseline values, in 7 groups of guinea pigs. Statistical differences (P < 0.05) compared with the baseline or between groups: *compared with the baseline values; †compared with the saline + saline (SA + SA), cromolyn sodium + citric acid (CS + CA), bradykinin + cromolyn sodium + citric acid (BK + CS + CA), compound 48/80 + citric acid (48/80 + CA), and bradykinin + compound 48/80 + citric acid (BK + 48/80 + CA) groups; †compared with the SA + SA, BK + CS + CA, 48/80 + CA, and BK + 48/80 + CA groups; *compared with the 48/80 + CA and BK + 48/80 + CA groups.

Fig. 2. Lucigenin-initiated chemiluminescence counts (oxidative stress) obtained from bronchoalveolar lavage fluid in seven groups of guinea pigs. SA + CA, saline + citric acid group; BK + CA, bradykinin + citric acid group. n, Number of animals in each group. Statistical differences (P < 0.05) between groups: †compared with the SA + SA group; †compared with the SA + CA group; †compared with the CS + CA group. 

DISCUSSION

We confirmed that citric acid inhalation caused marked decreases in Crs and FEV0.1, indicating moderate to severe airway constriction. Cromolyn sodium and compound 48/80 pretreatment attenuated significantly citric acid-induced bronchoconstriction. We detected significant increase in lucigenin-initiated chemiluminescence counts of the BAL sample in only the BK + CA group. On the contrary, BAL SP levels, the index of tachykinin release, increased significantly 3 min into the recovery period in the SA + CA, BK + CA, BK + CS + CA, 48/80 + CA, and BK + 48/80 + CA groups. Citric acid inhalation caused an increase in BAL histamine level, which was attenuated by compound 48/80 pretreatment. In addition, (Fig. 4). However, this increase was significantly attenuated by the pretreatment of compound 48/80 in the BK + 48/80 + CA and 48/80 + CA groups.

All cell numbers in the BAL samples were time-dependently increased after citric acid aerosol inhalation (Fig. 5). For most cases, cromolyn sodium and/or compound 48/80 significantly attenuated these citric acid-induced increases in cell counts (Fig. 5).
citric acid induced time-dependent increase in BAL total cell numbers. Differential cell counts revealed significant increases in macrophage, neutrophil, and lymphocyte infiltration after citric acid aerosol inhalation. Several features of these results will be discussed below.

**Mast cells in citric acid-induced bronchoconstriction.** Compound 48/80 is widely used as a mast cell degranulating agent leading to acute release of histamine and other mast cell constituents (36), and thus chronic treatment of compound 48/80 depletes mast cells. This study confirmed this point via the finding that BAL histamine level was reduced by compound 48/80 pretreatment (Fig. 4). Compound 48/80 can also activate afferent C fibers and release tachykinins in the lungs (31). Mapp et al. (31) found also that pretreatment with compound 48/80 attenuated significantly toluene disocyanate-induced airway constriction. In addition, a tachykinin NK$_2$-receptor antagonist, MEN-10207, blocked significantly this attenuation induced by compound 48/80. Saria et al. (41) showed that compound 48/80-induced vascular protein leakage could be inhibited by capsaicin pretreatment and by a SP receptor antagonist. According to the above studies, it is apparent that compound 48/80 acutely activates afferent C fibers, which release tachykinins, in the lungs. Subsequently, tachykinins cause the release of inflammatory mediators from various cell types and the stimulation of inflammatory cellular activities, including lymphocyte proliferation, mast cells degranulation, and modulation of polymorphonuclear neutrophil activity (12). Lai and Lin (30) found also that hyperpnea-induced airway constriction was prevented in animals pretreated with compound 48/80, and this prevention could be explained by depletion of mediators (such as leukotrienes) in mast cells and/or depletion of tachykinins in afferent C fibers. Compound 48/80 pretreatment attenuated citric acid-induced airway constriction of all groups in this study, showing the important role of mast cells in inducing noncholinergic airway constriction.

Degranulation of mast cells releases inflammatory mediators such as histamine, prostaglandins, and various enzymes, including kinin-activating trypase, and, hence, has always been associated with the pathogenesis of inflammatory and allergic diseases (50). Morphological studies provided visual evidence that confirms the close anatomic association between mast cells and nerve fibers, especially those containing SP (6). Priming of mast cells to subsequent stimulus by picomolar of SP had been previously suggested by patch-clamp studies (22). Mast cell degranulation has also been documented among various in vivo models of neurogenic inflammation in the airways (26). In rats pretreated with compound 48/80 to deplete mast cells, neurokinin A and SP no longer caused significant bronchoconstriction (23). Similarly, in this study, high plasma SP could not produce significant airway constriction after compound 48/80 or CS treatment (Figs. 1 and 3). Therefore, it is possible that the bronchoconstrictor effect of tachykinins may be mediated via mediators of mast cells (24). The slight difference in citric acid-induced decreases in Crs and FEV$_{0.1}$ in the cromolyn sodium-treated group (Fig. 1) could be due to cromolyn sodium acts heterogeneously on different portions of airways.

Pretreatment with sodium cromoglycate and nedocromil sodium is very effective in inhibiting the airway response to bradykinin (10). Sodium cromoglycate inhibits unmyelinated afferent vagal fibers in dogs (11) and in some other species. We also found in this study that sodium cromoglycate attenuated citric acid-induced airway constriction. Furthermore, mast cell tryptase has also been identified as a kininogen; thus mast cell degranulation would result in increased kinin generation in airways.

**Bradykinin in citric acid-induced bronchoconstriction.** Bradykinin, a nonapeptide formed from plasma and tissue precursors during inflammation (5), induces in guinea pigs a variety of inflammatory responses including noncholinergic bronchoconstriction (39). Kaufman et al. (25) showed also that bradykinin causes airway constriction by activation of C-fiber sensory nerve endings. A favorable condition for bradykinin release from its precursor proteins is a low pH of the milieu (5). Ichinose and coworkers (20) have demonstrated that intravenous bradykinin causes bronchoconstriction predominantly via the release of cyclooxygenase products and a cholinergic reflex in guinea pigs in vivo. Recent pharmacological findings suggest the existence of further bradykinin-receptor types. Receptor-independent mechanisms for bradykinin have also been

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**Fig. 3.** Substance P levels in bronchoalveolar lavage at 3 min after citric acid aerosol inhalation in 7 groups of guinea pigs. *n.* Number of animals in each group. *Statistical difference (P < 0.05) between groups: *compared with the SA + SA group; †compared with the CS + CA group.

**Fig. 4.** Histamine levels in bronchoalveolar lavage in 4 groups of guinea pigs. *n.* Number of animals in each group. *Statistical difference (P < 0.05) compared with the SA + CA group.
proposed, including histamine release from mast cells. This activity has been attributed to a direct interaction of bradykinin with G proteins (8). This so-called peptidergic pathway of mast cell activation, carried out by peptides and polyamines, is unrelated to antigen contact (35). Although supplemental bradykinin induced higher ROS production (Fig. 2), it did not lead to a significant increase in noncholinergic airway constriction (Fig. 1).

**ROS and SP in citric acid-induced bronchoconstriction.** Free radicals enhance the exocytotic secretion of histamine behaving as calcium ionophores by translocating extracellular calcium into mast cell cytosol. ROS have also been shown to enhance the release of histamine from mast cells (17, 51). Histamine can also trigger the release of tachykinins (43). Lai et al. (28) found that oxygen radicals may be the main contributing factor to elicit tachykinin release after inhalation of...
citric acid aerosol. Goldman et al. (16) found that acid aspiration caused increases in both ROS and lung permeability. Lucigenin-initiated chemiluminescence has been shown to be an effective monitor of mitochondrial superoxide generation (38). In agreement with our laboratory’s previous results (28), we found in this study that citric acid inhalation induced an increase in airway constriction. However, citric acid alone did not produce a significant increase in chemiluminescence counts in this study (Fig. 2). The difference from the previous study (28) might be due to different treatments in animals. Pretreatment with propanolol, atropine, indomethacin, and captopril were carried out in this study but not in the previous study (28).

Increased generation of ROS has been demonstrated in BAL cells from asthmatic patients compared with nonasthmatic individuals (9). Several inflammatory cell types, including macrophages and neutrophils, are capable of generating ROS. These oxidants, including superoxide anion, hydroxyl radicals, hydrogen peroxide, singlet oxygen, and hypochlorite radicals, may cause cellular injury and alteration in airway function (1, 13).

Leukocytes in citric acid-induced bronchoconstriction. Because there was a time-dependent increase in most leukocytes (macrophages, neutrophils, and lymphocytes) after citric acid inhalation, the increased leukocytes may act to maintain airway constriction via releases of constricting agents and ROS. Thus the citric acid-induced airway constriction may be sustained for prolonged period of time. Macrophages have been shown to be the source of tachykinins (27). Thus increased macrophages could contribute, partly at least, to the observed citric acid-induced increase in SP level (Fig. 5). However, the time-dependent increase in BAL cell counts was opposite to the gradual decrease in airway constriction severity (Fig. 1) after citric acid inhalation. BAL cells might thus not be the major contributing factor for the initial citric acid-induced airway constriction. In addition, our data are compatible with the fact that neutrophils are an important source of ROS (40) because the BK + CA group had highest neutrophil counts and ROS levels. Nearly all lymphocytes in BAL fluid are T lymphocytes (3, 37, 48). Activated T cells have been related to disease severity assessed by decreased forced expiratory volume in 1 s and increased bronchial hyperreactivity (49).

Significance. Gastroesophageal acid reflux is more common in patients with asthma than in the general population, with an estimated prevalence of 34–80% (44). Furthermore, several reports suggest that gastroesophageal acid reflux may well represent a trigger of bronchoconstriction either by vagal reflex (the reflex theory) or microaspiration of refluxed acid gastric contents into the respiratory tract (the reflux theory) (7, 44, 46). The latter theory of microaspiration as a cause of bronchospasm is supported by the fact that reflux of gastric juice into the lower airways during anesthesia has long been known to produce marked bronchoconstriction (33). The mechanism of such bronchomotor response is, however, unknown. This study provided more detailed mechanism for acid-induced airway constriction.

In summary, on the basis of the above data, it can be concluded that mast cells play an important role in citric acid aerosol inhalation-induced airway constriction via perhaps releasing constricting factors. Even the increased SP seems to act on mast cells before its induction of noncholinergic airway constriction. A schematic figure (Fig. 6) was used to explain possible sequences for citric acid-induced airway constriction according to our results.

GRANTS

The investigation was supported by National Science Council Grant NSC91-8002-07M59 and National Health Research Institutes Grant NHRI-EX92-913ONN.

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