Bronchoconstriction Induced by Citric Acid Inhalation in Guinea Pigs

Role of Tachykinins, Bradykinin, and Nitric Oxide

FABIO L. M. RICCIARDOLO, VANDA RADO, LEONARDO M. FABBRI, PETER J. STERK, GIUSEPPE U. DI MARIA, and PIERANGELO GEPPETTI

Institute of Respiratory Disease, University of Catania, Catania; Department of Clinical and Experimental Medicine, University of Ferrara, Ferrara, Italy; and Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands

Gastroesophageal acid reflux into the airways can trigger asthma attacks. Indeed, citric acid inhalation causes bronchoconstriction in guinea pigs, but the mechanism of this effect has not been fully clarified. We investigated the role of tachykinins, bradykinin, and nitric oxide (NO) on the citric acid–induced bronchoconstriction in anesthetized and artificially ventilated guinea pigs. Citric acid inhalation (2–20 breaths) caused a dose-dependent increase in total pulmonary resistance (R_L). R_L value obtained after 10 breaths of citric acid inhalation was not significantly different from the value obtained after 20 breaths ($p = 0.22$). The effect produced by a half-submaximum dose of citric acid (5 breaths) was halved by the bradykinin B_2 receptor antagonist HOE 140 (0.1 $\mu mol \cdot kg^{-1}$, intravenous) and abolished by the tachykinin NK_2 receptor antagonist SR 48968 (0.3 $\mu mol \cdot kg^{-1}$, intravenous). Bronchoconstriction induced by a submaximum dose of citric acid (10 breaths) was partially reduced by the administration of HOE 140, SR 48968, or the NK_1 receptor antagonist CP-99,994 (8 $\mu mol \cdot kg^{-1}$, intravenous) alone and completely abolished by the combination of SR 48968 and CP-99,994. Pretreatment with the NO synthase inhibitor, L-NMMA (1 mM, 10 breaths every 5 min for 30 min) increased in an L-arginine–dependent manner the effect of citric acid inhalation on R_L. HOE 140 and CP-99,994 markedly reduced the L-NMMA–potentiated bronchoconstriction to inhaled citric acid. We conclude that citric acid–induced bronchoconstriction is caused by tachykinin release from sensory nerves, which, in part, is mediated by endogenously released bradykinin. Simultaneous release of NO by citric acid inhalation counteracts tachykinin-mediated bronchoconstriction. Our study suggests a possible implication of these mechanisms in asthma associated with gastroesophageal acid reflux and a potential therapeutic role of tachykinin and bradykinin antagonists. Ricciardolo FLM, Rado V, Fabbri LM, Sterk PJ, Di Maria GU, Geppetti P. Bronchoconstriction induced by citric acid inhalation in guinea pigs: role of tachykinins, bradykinin, and nitric oxide.

AM J RESPIR CRIT CARE MED 1999;159:557–562.

Gastroesophageal acid reflux is more common in patients with asthma than in the general population, with an estimated prevalence of 34 to 80% (1). Furthermore, increasing data 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) (1–3). The latter theory of microaspiration as a cause of bronchospasm is supported by different evidence (3); for example, reflux of gastric juice into the lower airways during anesthesia has long been known to produce marked bronchoconstriction (4). The mechanism of such bronchomotor response is, however, unknown. There is evidence that protons can stimulate a subset of primary sensory neurons to release neuropeptides, namely the tachykinins substance P (SP) and neurokinin A (NKA) and the calcitonin gene-related peptide (CGRP) from their peripheral terminals in guinea pigs (5). Consistent with this finding is the observation that hydrochloric acid–induced airway mucosal protein extravasation in the rat is blocked by pretreatment with capsaicin (6), a drug that at high doses selectively destroys primary sensory neurons (7). Tachykinins, when released in the guinea pig airways, cause a series of inflammatory responses (8), including bronchoconstriction. Bronchoconstriction induced by tachykinin release in the guinea pig airways is mediated by activation of NK_2 and, to a lesser degree, of NK_1 receptors (9).

It has been demonstrated that citric acid–induced coughing and bronchoconstriction in conscious guinea pigs are abolished by pretreatment with capsaicin (10). Recently, Girard and coworkers (11) have also observed that pretreatment with capsaicin and SR 48968 (12), a selective NK_2 receptor antagonist, abolished and inhibited, respectively, citric acid–induced airway hyperresponsiveness in guinea pigs. Moreover, it has

(*Received in original form April 2, 1998 and in revised form August 13, 1998*)

Supported by a grant from Azienda Ospedale S. Anna, Ferrara, Italy.

Correspondence and requests for reprints should be addressed to Fabio Ricciardolo, M.D., Lung Function Lab., C2-P, Leiden University Hospital, P.O. Box 9600, NL-2300 RC Leiden, The Netherlands. E-mail: fricciardo@pulmonology.azl.NL

been found that SR 48968 and the capsaicin antagonist capsazepine (13) reduced bronchoconstriction induced by citric acid inhalation in guinea pigs (14). A direct action of protons on sensory nerves may be the mechanism by which citric acid causes bronchoconstriction. However, additional possibilities may exist. For instance, plasma extravasation induced by hydrochloric acid instillation in the guinea pig conjunctiva (15) or the increase in blood flow caused by acid back diffusion in the rat stomach (16) are due to sensory nerve stimulation mediated by the release of bradykinin. Bradykinin, a nonapeptide formed from plasma and tissue precursors during inflammation (17), induces in guinea pigs a variety of inflammatory responses, including bronchoconstriction, mainly by releasing tachykinins from sensory nerve endings (18). A favorable condition for bradykinin release from its precursor proteins is a low pH of the milieu (17). However, there is also evidence that bradykinin causes protection of contracted guinea pig airways by releasing nitric oxide (NO) (19). NO seems to be a predominant mediator of relaxation of the airways by the nonadrenergic–noncholinergic (NANC) neurotransmission (20, 21). In the present study we postulated that not only tachykinins but also bradykinin and NO are involved in modulating citric acid–induced bronchoconstriction. To that end we investigated the role of tachykinins, bradykinin, and NO on the bronchoconstriction produced by different doses of citric acid in anesthetized and artificially ventilated guinea pigs. Finally, to determine a potential role of the cholinergic pathway in this bronchial response, we also studied the effect of a muscarinic receptor antagonist on citric acid–induced bronchoconstriction in guinea pigs.

METHODS

Animals

Male Hartley guinea pigs (Pampaloni Laboratories, Pisa, Italy), weighing 300–350 g at the time of housing, were used in this study. They were kept in a temperature-controlled environment with standard laboratory food and water freely available.

Measurements of Total Pulmonary Resistance (R_L)

Animals were anesthetized with sodium pentobarbital (45 mg · kg^{-1}, intraperitoneally; Antony Product Corp., Arcadia, CA) and then ventilated artificially through a tracheal cannula, using a costant-volume ventilator (model 683; Harvard Apparatus Co., Inc., South Natick, MA) at a frequency of 80 breaths · min^{-1}. The tidal volume was adjusted to maintain normal arterial blood gases as described previously (22). Airflow was monitored continuously with a pneumotachograph (A. Fleisch, Medical Inc., Richmond, VA) connected to a differential pressure transducer (model DP45; Validyne Engineering Corp., Northridge, CA). A fluid-filled polyethylene catheter was introduced into the esophagus to measure the esophageal pressure as an approximation of pleural pressure. Intratracheal pressure was measured with a polyethylene catheter inserted into a short tube connecting the trachea cannula to the pneumotachograph. The transpulmonary pressure (defined as the pressure difference between the intratracheal and the esophageal pressures) was measured with a differential pressure transducer (model DP7; Validyne Engineering Corp.). Output signals representing transpulmonary pressure and airflow were amplified with an amplifier (model CD19; Validyne Engineering Corp.) and recorded on a polygraph recorder (model 1508 B Visicorder; Honeywell Inc., Denver, CO). R_L was calculated as previously described (22). The right jugular vein and the left carotid artery were cannulated to permit administration of drugs and to withdraw a sample of blood for arterial blood gas measurement, respectively.

Experimental Design

Baseline R_L remained stable for at least 2 h, and no significant changes were produced by aerosol administration (2, 5, 10, and 20 breaths) or intravenous injection (1 ml · kg^{-1}) of saline (0.9% NaCl) after a stabilization period of 30 min. Aerosols of citric acid (0.4 M with pH 1.7, for 2, 5, 10, or 20 breaths) were generated from an ultrasonic nebulizer (Pulmo-Sonic model 25; DeVilbiss Co., Somerset, PA) and were delivered into the airways by the respirator via the tracheal cannula (aerosol delivery rate: 0.2 ml · min^{-1}; mass median aerodynamic diameter: 1.8 μm). The muscarinic receptor antagonist atropine (1.4 μmol · kg^{-1}, i.v.), the bradykinin B_2 receptor antagonist HOE 140 (0.1 μmol · kg^{-1}, i.v.), the tachykinin NK_2 receptor antagonist SR 48968 (0.3 μmol · kg^{-1}, i.v.), and the tachykinin NK_1 receptor antagonist CP-99,994 (8 μmol · kg^{-1}, i.v.) were administered 15 min before the aerosolized citric acid challenge. The doses of HOE 140 (18, 23), SR 48968 (9, 12), and CP-99,994 (15) used in the study had previously been shown to selectively block bradykinin B_2, and tachykinin NK_2 and NK_1 receptors, respectively, in guinea pigs *in vivo*. The dose of atropine used in the study was able to block the bronchoconstriction induced by acetylcholine (0.5 μmol · kg^{-1}, intravenous) in guinea pigs (F. L. M. Ricciardolo, personal observation).

To deliver NO synthase inhibitors, we adopted the validated protocol used previously (19): guinea pigs inhaled 10 breaths of an aerosol containing N^G-monomethyl-L-arginine (L-NMMA: 1 mM) or its inactive enantiomer, N^G-monomethyl-D-arginine (D-NMMA: 1 mM; control). This procedure was repeated every 5 min for 30 min (total, 60 breaths). Five minutes after the last inhalation of the NO synthase inhibitor or the inactive enantiomer a single aerosolized citric acid challenge (five breaths) was given to each animal. In other groups of animals the following pretreatment was used: 10 breaths of an aerosol containing L-arginine (L-Arg: 3 mM) or D-arginine (D-Arg: 3 mM) were administered every 5 min for 30 min to L-NMMA-pretreated animals. Five minutes after the last inhalation of L-Arg or the inactive enantiomer, a single aerosolized citric acid challenge (five breaths) was given to each animal. We already reported that administration of the present doses of L-NMMA, D-NMMA, L-Arg, or D-Arg by aerosol do not affect cardiovascular parameters (19).

Drugs

Citric acid was obtained from Merck (Darmstadt, Germany). Atropine, L-NMMA, D-NMMA, L-Arg, and D-Arg were obtained from Sigma Chemical (St. Louis, MO). HOE 140 (D-Arg^0-[Hyp^3,Thi^5, D-Tic^7,Oic^8]-bradykinin) (23), SR 48968 {(S)-*N*-methyl-*N*[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide} (12), and CP-99,994 (+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine) (24) were kindly provided by Dr. K. Wirth (Hoechst AG, Frankfurt, Germany), Dr. X. Emonds-Alt (Sanofi Recherche, Montpellier, France), and Dr. J. A. Lowe III (Pfizer Inc., Groton, CT), respectively. Drugs were dissolved in 0.9% saline or in dimethyl sulfoxide (HOE 140, SR 48968, and CP-99,994). Further dilutions were made in 0.9% saline. All drugs were freshly prepared each time.

Statistical Analysis

Values in the text and figures are mean ± standard error of the mean (SEM) from at least five experiments. Statistical comparisons were performed using a one-way analysis of variance and Dunnett's test or bilateral unpaired Student *t* tests, when appropriate. In all cases, a p value less than 0.05 was considered significant.

RESULTS

Dose–Response of Citric Acid–induced Bronchoconstriction

In naive guinea pigs baseline value R_L was 0.20 ± 0.02 cm H_2O/ml/s (n = 6). Aerosolization of 0.9% NaCl (2, 5, 10, or 20 breaths) did not change the baseline value of R_L (data not shown). Aerosolized citric acid (2, 5, 10, and 20 breaths) induced a dose-dependent increase in R_L (Figure 1), with the maximum increase in R_L (peak) for each dose within 2 min after the end of the inhalation. Two breaths of aerosolized citric acid significantly increased R_L above baseline value (obtained by subtracting the baseline R_L from the peak increase in R_L: 0.19 ± 0.03 cm H_2O/ml/s; $p < 0.05$). Five breaths of aerosolized citric acid caused a twofold increase above baseline value in R_L (0.42 ± 0.04 cm H_2O/ml/s; $p < 0.01$) (Figure 1). Ten and 20 breaths of aerosolized citric acid increased R_L by

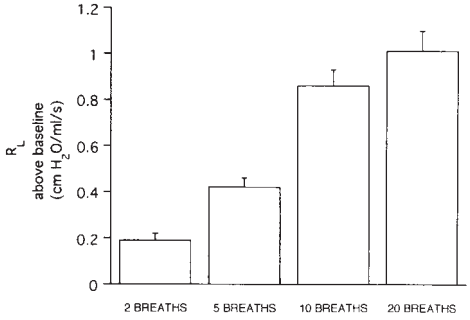

Figure 1. Effect of 2, 5, 10, and 20 breaths of aerosolized citric acid (0.4 M; pH: 1.7) on the maximum increase in total pulmonary resistance (R_L) above baseline value in anesthetized and artificially ventilated guinea pigs. Each column is the mean ± SEM of five experiments.

four- and fivefold above baseline, respectively ($p < 0.01$) (Figure 1). However, R_L value obtained after 10 breaths of citric acid inhalation did not differ significantly from the value obtained after 20 breaths ($p = 0.22$).

Effect of Atropine on Citric Acid–induced Bronchoconstriction

In guinea pigs pretreated with atropine (1.4 $\mu mol \cdot kg^{-1}$, intravenous, 15 min before the stimulus), the bronchoconstriction induced by 5, 10, and 20 breaths of citric acid was slightly, but not significantly, reduced in comparison to the bronchial response in vehicle-treated guinea pigs (Figure 2).

Effect of HOE 140 on Citric Acid–induced Bronchoconstriction

Vehicle of HOE 140 did not significantly change baseline value of R_L and did not affect R_L values after administration of aerosolized citric acid at the different doses (5, 10, and 20 breaths) compared with the naive group (Figures 1 and 3). Increases in R_L by the three doses of citric acid were reduced by approximately 50% after pretreatment with the bradykinin B_2 receptor antagonist HOE 140 (0.1 $\mu mol \cdot kg^{-1}$, intravenous, 15 min before the stimulus) (Figure 3).

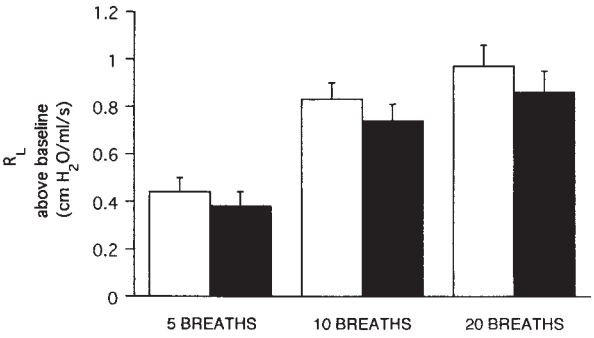

Figure 2. Effect of the muscarinic receptor antagonist (atropine; 1.4 $\mu mol \cdot kg^{-1}$, given intravenously, 15 min before the stimulus) (*filled columns*) on the maximum increase in total pulmonary resistance (R_L) above baseline value induced by three doses of aerosolized citric acid (0.4 M; pH: 1.7; 5 breaths, 10 breaths, and 20 breaths). *Open columns* indicate animals pretreated with the vehicle of atropine (control: 0.9% saline) 15 min before the stimulus. Each column is the mean ± SEM of at least five experiments.

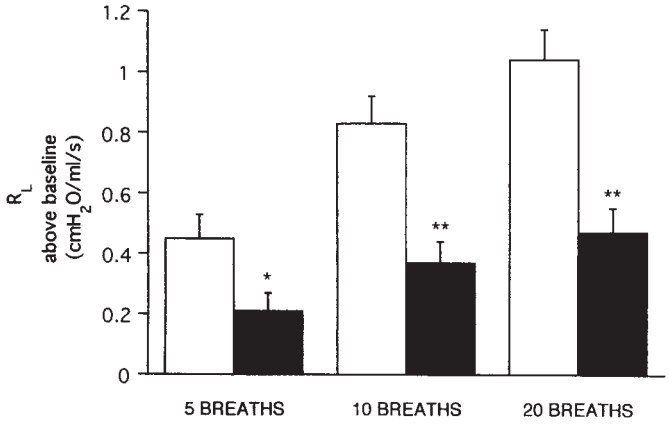

Figure 3. Effect of the bradykinin B_2 receptor antagonist HOE 140 (0.1 $\mu mol \cdot kg^{-1}$, given intravenously, 15 min before the stimulus) (*filled columns*) on the maximum increase in total pulmonary resistance (R_L) above baseline value induced by three doses of aerosolized citric acid (0.4 M; pH: 1.7; 5 breaths, 10 breaths, and 20 breaths). *Open columns* indicate animals pretreated with the vehicle of HOE 140 (control: 10% dimethyl sulphoxide in 0.9% saline) 15 min before the stimulus. Each column is the mean ± SEM of at least five experiments. *$p < 0.05$ versus control; **$p < 0.01$ versus control.

Effect of SR 48968 and CP-99,994 on Citric Acid–induced Bronchoconstriction

The vehicle of SR 48968 and CP-99,994 did neither significantly change baseline value of R_L nor affect bronchoconstriction caused by 5, 10, and 20 breaths of citric acid (Figure 4). The tachykinin NK_2 receptor antagonist SR 48968 (0.3 $\mu mol \cdot kg^{-1}$, intravenous, 15 min before the stimulus) virtually abolished bronchoconstriction induced by five breaths of citric acid inhalation and decreased by more than 50% the increase in R_L induced by inhalation of 10 and 20 breaths of citric acid (Figure 4). The tachykinin NK_1 receptor antagonist CP-99,994

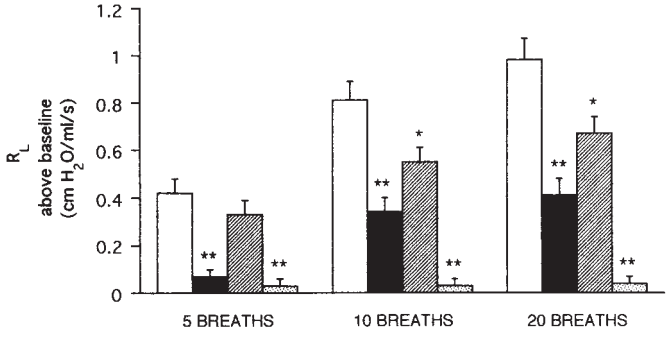

Figure 4. Effect of the tachykinin NK_2 receptor antagonist SR 48968 (0.3 $\mu mol \cdot kg^{-1}$, given intravenously, 15 min before the stimulus) (*filled columns*), the tachykinin NK_1 receptor antagonist CP-99,994 (8 $\mu mol \cdot kg^{-1}$, given intravenously, 15 min before the stimulus) (*hatched columns*), or the combination of the tachykinin NK_2 (SR 48968) and NK_1 (CP-99,994) receptor antagonists (*dotted columns*) on the maximum increase in total pulmonary resistance (R_L) above baseline value induced by three doses of aerosolized citric acid (0.4 M; pH: 1.7; 5 breaths, 10 breaths, and 20 breaths). *Open columns* indicate animals pretreated with the vehicle of SR 48968 and CP-99,994 (control: 10% dimethyl sulphoxide in 0.9% saline) 15 min before the stimulus. Each column is the mean ± SEM of at least five experiments. *$p < 0.05$ versus control; **$p < 0.01$ versus control.

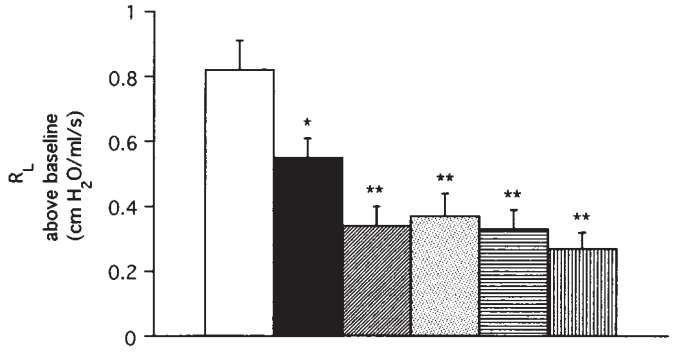

Figure 5. Effect of the tachykinin NK_1 receptor antagonist CP-99,994 (8 μmol · kg^{-1}, given intravenously, 15 min before the stimulus) (*filled columns*), the tachykinin NK_2 receptor antagonist SR 48968 (0.3 μmol · kg^{-1}, given intravenously, 15 min before the stimulus) (*hatched columns*), the bradykinin B_2 receptor antagonist HOE 140 (0.1 μmol · kg^{-1}, given intravenously, 15 min before the stimulus) (*dotted columns*), the combination of the bradykinin B_2 (HOE 140) and the tachykinin NK_1 (CP-99,994) receptor antagonists (*horizontal-lined columns*), or the combination of the bradykinin B_2 (HOE 140) and the tachykinin NK_2 (SR 48968) receptor antagonists (*vertical-lined columns*) on the maximum increase in total pulmonary resistance (R_L) above baseline value induced by 10 breaths of aerosolized citric acid (0.4 M; pH: 1.7). *Open columns* indicate animals pretreated with the vehicle of the antagonists (control: 10% dimethyl sulphoxide in 0.9% saline) 15 min before the stimulus. Each column is the mean ± SEM of at least five experiments. *$p < 0.05$ versus control; **$p < 0.01$ versus control.

(8 μmol · kg^{-1}, intravenous, 15 min before the stimulus) did not significantly affect the bronchoconstriction induced by five breaths of citric acid, whereas it partially reduced (30%) the bronchial response to 10 and 20 breaths of aerosolized citric acid (Figure 4). The combination of SR 48968 (0.3 μmol · kg^{-1}, intravenous, 15 min before the stimulus) and CP-99,994 (8 μmol · kg^{-1}, intravenous, 15 min before the stimulus) abolished bronchoconstriction induced by 5, 10, and 20 breaths of aerosolized citric acid (Figure 4). R_L values obtained after 10 and 20 breaths of citric acid inhalation in the SR 48968–pretreated group were significantly different ($p < 0.01$) from those obtained in the group pretreated with the combination of SR 48968 and CP-99,994.

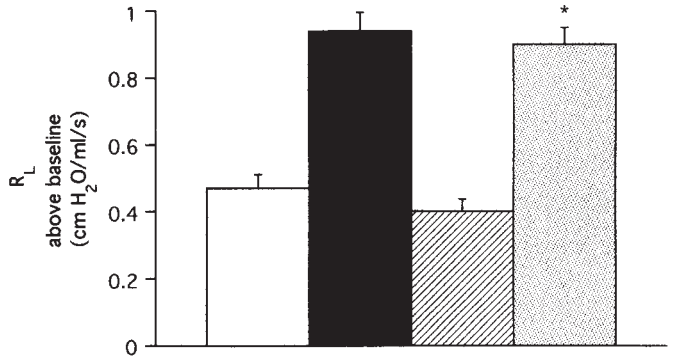

Figure 6. Effect of aerosolized N^G-monomethyl-D-arginine (D-NMMA: 1 mM) (*open column*), N^G-monomethyl-L-arginine (L-NMMA: 1 mM; *filled column*), L-NMMA plus L-arginine (L-Arg: 3 mM; *hatched column*), L-NMMA plus D-arginine (D-Arg: 3 mM) (*dotted column*) on the increase in total pulmonary resistance (R_L) above baseline value induced by five breaths of aerosolized citric acid (0.4 M; pH: 1.7) in anesthetized and artificially ventilated guinea pigs. D-NMMA and L-NMMA were administered by giving 10 breaths every 5 min for 30 min. L-Arg and D-Arg (10 breaths every 5 min for 30 min) were given 5 min after the last administration of L-NMMA. Each column is the mean ± SEM of at least five experiments. *$p < 0.05$ versus D-NMMA group.

Effect of HOE 140 Plus SR 48968 or CP-99,994 on Citric Acid–induced Bronchoconstriction

The combination of the bradykinin B_2 receptor antagonist HOE 140 (0.1 μmol · kg^{-1}, intravenous, 15 min before the stimulus) plus the tachykinin NK_2 (SR 48968, 0.3 μmol · kg^{-1}, intravenous, 15 min before the stimulus) or NK_1 (CP-99,994, 8 μmol · kg^{-1}, intravenous, 15 min before the stimulus) receptor antagonist did not additionally decrease the bronchoconstriction induced by 10 breaths of aerosolized citric acid in comparison to the group of guinea pigs pretreated with HOE 140 alone (Figure 5).

Effect of NO-Synthase Inhibitor on Citric Acid–induced Bronchoconstriction

Aerosolization of D-NMMA (1 mM) or L-NMMA (1 mM, all, 10 breaths every 5 min for 30 min) did not change the baseline values of R_L (data not shown). After administration of L-NMMA (1 mM, 10 breaths every 5 min for 30 min), the bronchoconstrictor response to five breaths of aerosolized citric acid was significantly increased (Figure 6). The administration of L-Arg (3 mM, 10 breaths every 5 min for 30 min), but not the administration of D-Arg (3 mM, 10 breaths every 5 min for 30 min), following the aerosolization of L-NMMA, reversed the potentiation induced by L-NMMA on citric acid–induced bronchoconstriction (Figure 6).

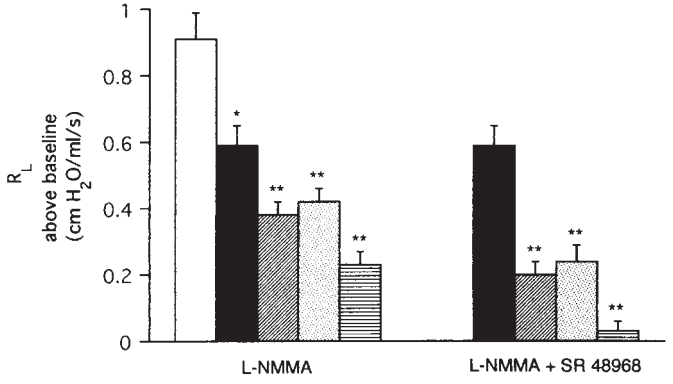

Figure 7. Effect of the bradykinin B_2 receptor antagonist HOE 140 (0.1 μmol · kg^{-1}, given intravenously, 15 min before the stimulus) (*hatched columns*), the tachykinin NK_1 receptor antagonist CP-99,994 (8 μmol · kg^{-1}, given intravenously, 15 min before the stimulus) (*dotted columns*), or the combination of the bradykinin B_2 (HOE 140) and the tachykinin NK_1 (CP-99,994) receptor antagonists (*horizontal-lined columns*) on the maximum increase in total pulmonary resistance (R_L) above baseline value induced by five breaths of aerosolized citric acid (0.4 M; pH: 1.7) in animals pretreated with L-NMMA (10 breaths every 5 min for 30 min) in the absence (*left group*) or in the presence (*right group*) of the tachykinin NK_2 receptor antagonist SR 48968 (0.3 μmol · kg^{-1}, given intravenously, 15 min before the stimulus). *Open and filled columns* indicate animals pretreated with the vehicle of SR 48968 (control of the left group: 10% dimethyl sulphoxide in 0.9% saline, 15 min before the stimulus) and with the tachykinin NK_2 receptor antagonist SR 48968 (control of the right group), respectively. Each column is the mean ± SEM of at least five experiments. *$p < 0.05$ versus control; **$p < 0.01$ versus their respective control.

Effect of SR 48968, HOE 140, and CP-99,994 on Citric Acid–induced Bronchoconstriction Increased by L-NMMA

Bradykinin B_2 receptor antagonist (HOE 140, 0.1 μmol · kg^{-1}, intravenous, 15 min before the stimulus), and NK_1 (CP-99,994, 8 μmol · kg^{-1}, intravenous, 15 min before the stimulus) and NK_2 (SR 48968, 0.3 μmol · kg^{-1}, intravenous, 15 min before the stimulus) tachykinin receptor antagonists partially reduced the L-NMMA–potentiated bronchoconstriction induced by five breaths of aerosolized citric acid (55%, 50%, and 35%, respectively) (Figure 7). The combination of HOE 140 (0.1 μmol · kg^{-1}, intravenous, 15 min before the stimulus) and CP-99,994 (8 μmol · kg^{-1}, intravenous, 15 min before the stimulus) further decreased, in a statistically significant manner compared with the group of guinea pigs treated with HOE 140 alone ($p < 0.05$), the bronchial response to five breaths of citric acid after treatment with L-NMMA (1 mM, 10 breaths every 5 min for 30 min) (Figure 7).

In another series of experiments in the presence of the NK_2 tachykinin receptor antagonist (SR 48968, 0.3 μmol · kg^{-1}, intravenous, 15 min before the stimulus), we studied the effect of adding HOE 140, CP-99,994, or the combination of both bradykinin B_2 and tachykinin NK_1 receptor antagonists on the L-NMMA–potentiated bronchoconstriction induced by five breaths of citric acid: HOE 140 and CP-99,994 alone reduced the bronchoconstriction by 60% and 55% in comparison with the control (Figure 7). In animals pretreated with HOE 140, CP-99,994, and SR 48968, the bronchoconstriction induced by five breaths of citric acid after L-NMMA (1 mM, 10 breaths every 5 min for 30 min) was blocked (Figure 7).

DISCUSSION

In this study we have demonstrated that aerosol administration of citric acid induced a dose-related bronchoconstriction in guinea pigs, an effect that seems to be due completely to the activation of sensory nerves and the release of tachykinins from their peripheral terminals. This conclusion is based on the observation that the increase in RL caused by a half-submaximum dose of citric acid was abolished by a tachykinin NK_2 receptor antagonist and that the combination of antagonists for NK_2 and NK_1 receptors blocked the bronchoconstriction caused by a submaximum and maximum dose of citric acid. Furthermore, our study rules out the potential involvement of another neural component, such as the cholinergic pathway, in citric acid–induced bronchoconstriction in guinea pigs. In fact, the bronchial response to different doses of aerosolized citric acid (5, 10, and 20 breaths) was not affected by atropine.

These findings are consistent with previous data showing that pretreatment with a dose of capsaicin, which desensitized primary sensory neurons, abolished (10, 11), and that SR 48968 produced a partial inhibition of the bronchoconstriction (14) and of the airway hyperresponsiveness (11) induced by citric acid inhalation in guinea pigs. The partial inhibitory effect of the NK_2 receptor antagonist SR 48968, in comparison with the complete blockade obtained by capsaicin pretreatment, is not surprising. In fact, it has been shown that bronchoconstriction in response to a high dose of capsaicin or NKA in guinea pigs cannot be abolished completely by NK_2 receptor blockade (9), because in this animal species NK_1 receptors also contribute to tachykinin-mediated bronchoconstriction. In agreement with this notion, the present study shows that the moderate bronchoconstriction evoked by a low dose of citric acid was fully inhibited by SR 48968, whereas addition of CP-99,994 was necessary to produce complete inhibition of bronchoconstriction caused by a high dose of citric acid.

A major finding of the present study is that bradykinin plays a role in the bronchoconstriction induced by citric acid inhalation in guinea pigs. The bradykinin B_2 receptor antagonist HOE 140 reduced by about 50% the increase in RL caused by inhalation of half-submaximum, submaximum, and maximum doses of citric acid. The combination of the tachykinin NK_1 or NK_2 antagonist with the bradykinin B_2 receptor antagonist did not cause any further reduction in citric acid–induced bronchoconstriction. Thus, the hypothesis may be proposed that part of the bronchial response evoked by citric acid inhalation is due to bradykinin formation in the airways, which leads to tachykinin release and NK_2 and NK_1 receptor activation. There is strong evidence that exogenous bradykinin administration, especially if applied locally into the airways, exerts its proinflammatory action by releasing tachykinins from terminals of capsaicin-sensitive primary sensory neurons (25, 26). It has also been demonstrated that endogenously released bradykinin may cause bronchial responses by activating this neurogenic inflammatory pathway (27). Particularly relevant for the present study are the findings that the plasma extravasation induced by hydrochloric acid instillation to the guinea pig conjunctiva (15) and the increase in blood flow in the rat stomach caused by acid back diffusion (16) were due to stimulation of sensory neuropeptides release by bradykinin.

The dose of HOE 140 used in the present study has been found to block diverse effects produced by bradykinin administration completely (18, 25), and this dose of the antagonist caused only a partial inhibition of the tachykinin-mediated increase in RL caused by citric acid. Therefore, it is possible that additional mechanism(s) are involved in citric acid–induced activation of sensory nerves. Protons have been proposed to affect directly the capsaicin-operated channel/receptor (28). The recently cloned vanilloid cation channel appears to be stimulated by heat and sensitized by exposure to low pH media (29). The observation that the capsaicin antagonist, capsazepine, was able to inhibit citric acid–induced bronchoconstriction in guinea pigs (14), supports the hypothesis that at least part of the ability of citric acid to stimulate sensory nerves might be due to a direct activity and/or sensitization of the vanilloid channel by protons liberated by citric acid.

NO seems to be the main mediator of the NANC relaxation within the airways of various mammals, including humans (30, 31). Recent findings showed that the bronchoconstrictor action of inhaled bradykinin both in guinea pigs (19) and humans (32, 33) is potentiated by the inhibition of the L-Arg-NO synthase pathway. There is also evidence that bronchoconstriction caused by cold air inhalation in guinea pigs, an effect mediated by bradykinin-induced release of tachykinins, is increased following NO synthase inhibition (34). Pharmacologic manipulations of this effect of cold air by the tachykinin NK_2 and the bradykinin B_2 receptor antagonists suggested that bradykinin was responsible for the simultaneous release of bronchoconstrictor tachykinins and bronchodilator NO (34). Because we observed that bradykinin appears to be involved in citric acid–induced bronchoconstriction, we also examined whether NO release could occur following exposure to citric acid. The observation that stereoselective inhibition of NO synthase caused a potentiation of the bronchoconstriction induced by citric acid inhalation, an effect that was reversed by L-Arg, but not by D-Arg, indicates that endogenous NO is involved prominently in the modulation of citric acid–induced bronchoconstriction. Furthermore, the present results showed the role of tachykinins and kinins in the increased bronchial response to citric acid after NO synthase inhibition. They also suggest that citric acid inhalation causes the release of bronchoconstrictor mediators, the tachykinins, and simultaneously

the release of bronchodilator NO. Both tachykinins and NO may be released following B_2 receptor activation (34, 35). However, there is evidence that tachykinin NK_1 receptors also may have a role in NO release from guinea pig airway epithelium (36).

Inhalation of acidic substances, including citric acid, acetic acid, and sulfuric acid aerosol, have been shown to induce cough, bronchoconstriction, and increased bronchial responsiveness in patients with asthma (2, 37). The mechanisms of these effects of acidic solutions in humans is unknown, although activation of neural pathways is likely. The present investigation in guinea pigs shows that different mechanisms and mediators (with opposing functional effects) are involved in acid-induced bronchoconstriction and suggests that if a similar complexity is also present in patients with asthma and gastroesophageal acid reflux into the respiratory tract, the usage of tachykinin and bradykinin antagonists will be a relevant future treatment.

References

1. Simpson, W. G. 1995. Gastroesophageal reflux disease and asthma: diagnosis and management. *Arch. Intern. Med.* 155:798–804.
2. Boyle, J. T., D. N. Tuchman, S. M. Altschuler, T. E. Nixon, A. I. Pack, and S. Cohen. 1985. Mechanisms for the association of gastroesophageal reflux and bronchospasm. *Am. Rev. Respir. Dis.* 131(Suppl.): S16–S20.
3. Sontag, S. J. 1997. Gastroesophageal reflux and asthma. *Am. J. Med.* 103:84S–90S.
4. Mendelson, C. L. 1946. Aspiration of stomach contents into lungs during obstetric anesthesia. *Am. J. Obstet. Gynecol.* 52:191–205.
5. Geppetti, P., E. Del Bianco, R. Patacchini, P. Santicioli, C. A. Maggi, and M. Tramontana. 1991. Low pH-induced release of calcitonin gene-related peptide from capsaicin-sensitive sensory nerves: mechanism of action and biological response. *Neuroscience* 41:295–301.
6. Martling, C.-R., and J. M. Lundberg. 1988. Capsaicin sensitive afferents contribute to acute airway edema following tracheal instillation of hydrochloric acid or gastric juice in the rat. *Anesthesiology* 68:350–356.
7. Holzer, P. 1991. Capsaicin: cellular targets, mechanisms of actions, and selectivity for thin sensory neurons. *Pharmacol. Rev.* 43:143–201.
8. Solway, J., and A. R. Leff. 1991. Sensory neuropeptides and airway function. *J. Appl. Physiol.* 71:2077–2087.
9. Bertrand, C., J. A. Nadel, P. D. Graf, and P. Geppetti. 1993. Capsaicin increases airflow resistance in guinea pigs *in vivo* by activating both NK_2 and NK_1 tachykinin receptors. *Am. Rev. Respir. Dis.* 148:909–914.
10. Forsberg, K., J.-A. Karlsson, E. Theodorsson, J. M. Lundberg, and C. G. A. Persson. 1988. Cough and bronchoconstriction mediated by capsaicin-sensitive sensory neurons in the guinea-pig. *Pulm. Pharmacol.* 1:33–39.
11. Girard, V., J. C. Yavo, X. Emonds-Alt, and C. Advenier. 1996. The tachykinin NK_2 receptor antagonist SR 48968 inhibits citric acid-induced airway hyperresponsiveness in guinea pigs. *Am. J. Respir. Crit. Care Med.* 153:1496–1502.
12. Emonds-Alt, X., P. Vilain, P. Goulaouic, V. Proietto, D. V. Broeck, C. Advenier, E. Naline, G. Neliat, J. C. Breliere, and G. Le Fur. 1992. A potent and selective non-peptide antagonist of the neurokinin A (NK_2) receptor. *Life Sci.* 50:PL101–PL106.
13. Bevan, S., S. Hothi, G. Hughes, I. F. James, H. P. Rang, K. Shah, C. S. J. Walpole, and J. C. Yeats. 1992. Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br. J. Pharmacol.* 107:544–552.
14. Satoh, H., Y.-P. Lou, and J. M. Lundberg. 1993. Inhibitory effects of capsazepine and SR 48968 on citric acid-induced bronchoconstriction in guinea-pigs. *Eur. J. Pharmacol.* 236:367–372.
15. Figini, M., P. Javdan, F. Cioncolini, and P. Geppetti. 1995. Involvement of tachykinins in plasma extravasation induced by bradykinin and low pH medium in the guinea-pig conjunctiva. *Br. J. Pharmacol.* 115:128–132.
16. Pethö, G., M. Jocic, and P. Holzer. 1994. Role of bradykinin in the hyperaemia following acid challenge of the rat gastric mucosa. *Br. J. Pharmacol.* 113:1036–1042.
17. Bhoola, K. D., C. D. Figueroa, and K. Worthy. 1992. Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol. Rev.* 44:1–80.
18. Ricciardolo, F. L. M., J. A. Nadel, P. D. Graf, C. Bertrand, S. Yoshihara, and P. Geppetti. 1994. Role of kinins in anaphylactic-induced bronchoconstriction mediated by tachykinins in guinea-pigs. *Br. J. Pharmacol.* 113:508–512.
19. Ricciardolo, F. L. M., J. A. Nadel, S. Yoshihara, and P. Geppetti. 1994. Evidence for reduction of bradykinin-induced bronchoconstriction in guinea-pigs by release of nitric oxide. *Br. J. Pharmacol.* 113:1147–1152.
20. Li, C. G., and M. J. Rand. 1991. Evidence that part of the NANC relaxant response of guinea-pig trachea to electrical field stimulation is mediated by nitric oxide. *Br. J. Pharmacol.* 102:91–94.
21. Lei, Y.-H., P. J. Barnes, and D. F. Rogers. 1993. Regulation of NANC neural bronchoconstriction in vivo in the guinea-pig: involvement of nitric oxide, vasoactive intestinal peptide and soluble guanylyl cyclase. *Br. J. Pharmacol.* 108:228–235.
22. Dusser, D. J., E. Umeno, P. D. Graph, T. D. Djokic, D. B. Borson, and J. A. Nadel. 1988. Airway neutral endopeptidase-like enzyme modulates tachykinin-induced bronchoconstriction in vivo. *J. Appl. Physiol.* 65:2585–2591.
23. Wirth, K., F. J. Hock, U. Albus, W. Linz, H. G. Alpermann, H. Anagnostopoulos, S. Henke, G. Breipohl, W. König, J. Knolle, and B. A. Schölkens. 1991. HOE 140, a new potent and long acting bradykinin-antagonist: in vivo studies. *Br. J. Pharmacol.* 102:774–777.
24. McLean, S., A. Ganong, P. A. Seymour, R. M. Snider, M. C. Desai, T. Rosen, D. K. Bryce, K. P. Longo, L. S. Reynolds, G. Robinson, A. W. Schmidt, C. Siok, and J. Heym. 1993. Pharmacology of CP-99,994: a nonpeptide antagonist of the tachykinin neurokinin-1 receptor. *J. Pharmacol. Exp. Ther.* 267:472–479.
25. Bertrand, C., J. A. Nadel, I. Yamawaki, and P. Geppetti. 1993. Role of kinins in the vascular extravasation evoked by antigen and mediated by tachykinins in guinea pig trachea. *J. Immunol.* 151:4902–4907.
26. Ichinose, M., M. G. Belvisi, and P. J. Barnes. 1990. Bradykinin induced bronchoconstriction in guinea-pig *in vivo*: role of neural mechanisms. *J. Pharmacol. Exp. Ther.* 253:594–599.
27. Bertrand, C., and P. Geppetti. 1996. Tachykinin and kinin receptor antagonists: therapeutic perspectives in allergic airway disease. *Trends Pharmacol. Sci.* 17:255–259.
28. Bevan, S., and P. Geppetti. 1994. Protons: small stimulants of capsaicin-sensitive sensory nerves. *Trends Neurosci.* 17:509–512.
29. Caterina, M. J., M. A. Schumacher, M. Tominaga, T. A. Rosen, J. D. Levine, and D. Julius. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816-824.
30. Barnes, P. J., and M. G. Belvisi. 1993. Nitric oxide and lung disease. *Thorax* 48:1034–1043.
31. Nijkamp, F. P., and G. Folkerts. 1994. Nitric oxide and bronchial reactivity. *Clin. Exp. Allergy* 24:905–914.
32. Ricciardolo, F. L. M., P. Geppetti, A. Mistretta, J. A. Nadel, M. A. Sapienza, S. Bellofiore, and G. U. Di Maria. 1996. Randomised double-blind placebo-controlled study of the effect of inhibition of nitric oxide synthesis in bradykinin-induced asthma. *Lancet* 348:374–377.
33. Black, P. N., and S. M. Brodie. 1998. Nitric oxide and response to inhaled bradykinin in severe asthma. *Lancet* 351:449.
34. Yoshihara, S., J. A. Nadel, M. Figini, C. Emanueli, P. Pradelles, and P. Geppetti. 1998. Endogenous nitric oxide inhibits bronchoconstriction induced by cold air inhalation in guinea pigs: role of kinins. *Am. J. Respir. Crit. Care Med.* 157:547–552.
35. Figini, M., F. L. M. Ricciardolo, P. Javdan, F. P. Nijkamp, C. Emanueli, P. Pradelles, G. Folkerts, and P. Geppetti. 1996. Evidence that epithelium-derived relaxing factor released by bradykinin in the guinea pig trachea is nitric oxide. *Am. J. Respir. Crit. Care Med.* 153:918–923.
36. Figini, M., C. Emanueli, C. Bertrand, P. Javdan, and P. Geppetti. 1996. Evidence that tachykinins relax the guinea-pig trachea via nitric oxide release and by stimulation of a septide-insensitive NK_1 receptor. *Br. J. Pharmacol.* 117:1270–1276.
37. Cockcroft, D. W., and B. A. Berscheid. 1982. Effect of pH on bronchial response to inhaled histamine. *Thorax* 37:133–136.