

Airway Hyperresponsiveness Induced by Repeated Esophageal Infusion of HCl in Guinea Pigs

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Abstract

Gastroesophageal reflux is a common disorder closely related to chronic airway diseases, such as chronic cough, asthma, chronic bronchitis, and chronic obstructive disease. Indeed, gastroesophageal acid reflux into the respiratory tract causes bronchoconstriction, but the underlying mechanisms have still not been clarified. This study aimed to elucidate functional changes of bronchial smooth muscles (BSMs) isolated from guinea pigs in an animal model of gastroesophageal reflux. The marked airway inflammation, hyperresponsiveness and remodeling were observed after guinea pigs were exposed to intraesophageal HCl infusion for 14 days. In addition, contractile responses to acetylcholine (ACh), KCl, electrical field stimulation, and extracellular Ca^{2+} were greater in guinea pigs infused with HCl compared with control groups. The L-type voltage-dependent Ca^{2+} channels (L-VDCC) blocker, nifedipine, significantly inhibited ACh- and Ca^{2+} -enhanced BSM contractions in guinea pigs infused with HCl. The Rho-kinase inhibitor, Y27632, attenuated ACh-enhanced BSM contractions in guinea pigs infused with HCl. Moreover, mRNA and protein expressions for muscarinic M_2 and M_3 receptors, RhoA, and L-VDCC in BSM were detected by real-time PCR and Western blot. Expressions of mRNA and protein for muscarinic M_3 receptors, RhoA, and L-VDCC were greater than in BSM of HCl-infused guinea pigs, whereas levels of muscarinic M_2 receptors were unchanged. We demonstrate that acid infusion to the lower esophagus and, subsequently, microaspiration into the respiratory tract in guinea

pigs leads to airway hyperresponsiveness and overactive BSM. Functional and molecular results indicate that overactive BSM is the reason for enhancement of extracellular Ca^{2+} influx via L-VDCC and Ca^{2+} sensitization through Rho-kinase signaling.

Keywords: gastroesophageal reflux; airway hyperresponsiveness; muscarinic receptors; L-type voltage-operated Ca^{2+} channels; Rho-kinase

Clinical Relevance

Marked airway inflammation, hyperresponsiveness, and remodeling were observed in guinea pigs by intraesophageal HCl infusion. Greater bronchial smooth muscle (BSM) contraction responses to acetylcholine (ACh), extracellular Ca^{2+} , KCl, and electrical field stimulation were found in guinea pigs infused with HCl. Nifedipine, an L-type voltage-dependent Ca^{2+} channel (L-VDCC) blocker, inhibited ACh and Ca^{2+} -enhanced BSM contractions, and Y27632, a Rho-kinase inhibitor, attenuated ACh-enhanced BSM contractions in guinea pigs infused with HCl. Expression of mRNA and protein for muscarinic M_3 receptors, RhoA and L-VDCC, was greater in BSM of guinea pigs infused with HCl. These findings suggest a role for Rho A or L-VDCC in esophageal acid reflux disease.

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Gastroesophageal reflux disease (GERD) is characterized by specific symptoms, including heartburn and regurgitation (1, 2). GERD has been associated with numerous pulmonary diseases, chronic bronchitis, asthma, cystic fibrosis, and chronic obstructive disease (3–5). In general, gastroesophageal reflux-induced bronchoconstriction and/or cough, as well as associated pulmonary pathology, are mainly mediated by vagal neural reflex (reflex mechanism) or repetitive acid microaspiration into the respiratory tract (reflux mechanism) (6, 7). Recently, the finding of pepsin in bronchial lavage fluid has been supposed to be strong evidence for direct acid reflux into airways, leading researches to reappraise the reflux mechanism (8).

Neural reflex mechanisms occur when reflux events are limited to the lower esophagus (distal gastroesophageal reflux), and several studies have demonstrated that distal gastroesophageal reflux may increase airway resistance and enhance airway inflammation by releasing of proinflammatory mediators, such as T helper (Th) 2 cytokines (3, 8, 9). Increase of airway bronchoconstriction and microvascular leakage by single HCl intraesophageal instillation in guinea pig showed involvement of activation of muscarinic M3 receptors (10), and the release of acetylcholine (ACh) by activation of muscarinic receptors contributes to the airway responses (11).

Activation of muscarinic M₃ receptors stimulates airway smooth muscle contraction via interaction with GTP-binding proteins (G proteins), such as G_q, to hydrolyze phosphoinositide and generate inositol triphosphate (IP₃), which activates the IP₃ receptor to deliver Ca²⁺ from internal stores (12, 13). Under healthy conditions, the muscarinic-mediated

smooth muscle contractions in airway are also associated with external Ca²⁺ influx secondary to L-type voltage-dependent Ca²⁺ channel (L-VDCC) opening (14, 15). It is possible that changes in the contractile properties of smooth muscle in airway hyperresponsiveness (AHR) by repetitive acid aspiration are subordinate to disturbances in Ca²⁺ homeostasis, because of its fundamental effect of regulating contractions. Thus, the present research aimed to study the functional alterations to receptor-dependent (response to muscarinic agonists) and -independent (response to external Ca²⁺ and KCl) contractility of bronchial smooth muscle (BSM) in guinea pig with repeated esophageal infusion of HCl (16, 17). Another goal of this research was to determine the action of Ca²⁺ sensitization by activation of Rho-kinase and of Ca²⁺ influx through L-VDCC on agonist-induced BSM contraction in guinea pig by repeated esophageal infusion of HCl.

Materials and Methods

Animals

Adult male (300–350 g) guinea pigs were used. The experimental protocol was approved by the ethics committee of Shanghai University of Traditional Chinese Medicine.

Animal Model of Esophageal Acid Reflux

The animal model was conducted as previously reported (16).

In Vivo Airway Responsiveness

Guinea pigs were anesthetized and lung function was investigated in spontaneously breathing guinea pigs as previously described (18).

Collection of Bronchoalveolar Lavage Fluid and Leukocyte Analysis

Bronchoalveolar lavage fluid (BALF) was collected by lavage of lungs three times with 1.0 ml PBS via a tracheal cannula. A differential cell count was obtained by microscopic counting of a minimum of 200 cells/slide using standard morphological and staining criteria.

Quantitative Analysis of Cytokines in BALF

Cytokine levels, including IL-13, IL-5, IL-4, and IFN- γ , in BALF (50 μ l) were measured using Bio-Plex Pro rat cytokine assays (Bio-Rad Laboratories, Hercules, CA). TNF- α , IL-6, IL-2 (eBioscience, Vienna, Austria) and granulocyte/macrophage colony-stimulating factor (Abcam, Cambridge, UK) were measured according to the product manuals.

Determination of *In Vitro* BSM Function

Methods were carried out as previously described (19). Briefly, the left main bronchus was isolated, and epithelium was scraped by gently rubbing with a thin stainless rod. For all rings, one end was fixed to the bottom of the organ bath and other was connected to a highly sensitive isometric force transducer (MLT0202; ADInstruments, Castlehill, New South Wales, Australia) for the measurement of isometric force.

Analysis of Lung Histopathology

Lung tissue sections were stained with periodic acid–Schiff (PAS) to identify mucus secretion, and Masson's trichrome to determine collagen deposition (20).

To identify α -smooth muscle actin (α -SMA), the paraffin-embedded sections were immunostained with the primary antibody against rat SMA (sc-53015; Santa Cruz Biotechnology, Inc., Santa Cruz, CA),

Table 1. Primers Used for Quantitative Real-Time PCR

	Forward Primer	Reverse Primer
M2	5'-CCGTCAAGTCCAATGTCTTCTA-3'	5'-TTGTAATGTTTCGGAGGCAATAG-3'
M3	5'-GAAATGCCAGTAAGCCTCAGAC-3'	5'-TGGATGGGAAGATTGGAGAAG-3'
Cacna1c	5'-CGGGAGAAAAAGTGTGAGACAG-3'	5'-GGGCAGGTAGAAGTTGATGATG-3'
RhoA	5'-TAGTTGGGTCTTCGGGTGAC-3'	5'-GCAGGTTGCTTAGTCATCTTCA-3'
GAPDH	5'-GCTGGAAACATACTCGTCATCA-3'	5'-GCAAGGGACATCAGGAAATAGT-3'

Definition of abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

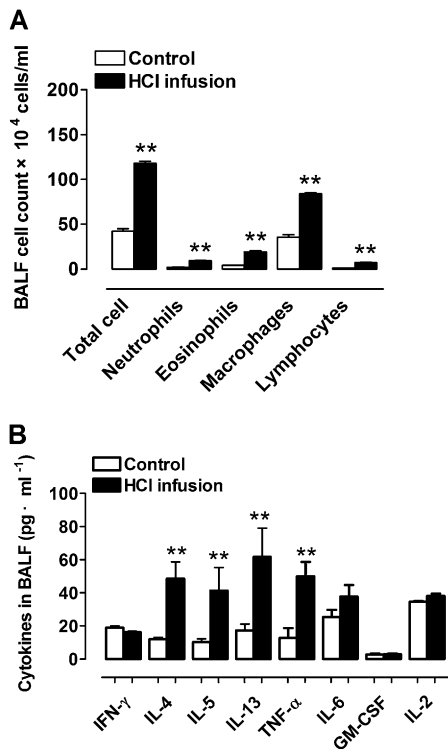


Figure 1. Determination of inflammatory cells and cytokines in bronchoalveolar lavage fluid (BALF) in a model of esophageal acid reflux. (A) Number of inflammatory cells and differential count of inflammatory cells in the BALF ($n = 10$). (B) Cytokine levels in the BALF ($n = 7-10$). Results are expressed as means ($\pm$ SEM). ** $P < 0.01$ compared with control animals. GM-CSF, granulocyte/macrophage colony-stimulating factor.

and the secondary antibody goat anti-rabbit IgG (H+L) conjugated to AffiniPure (no. 115-035-003; Jackson ImmunoResearch Labs, Inc., West Grove, PA).

Real-Time PCR Analysis

Real-time PCR analysis was conducted according to the manufacturers' procedures (Tissue RNA mini kit [Biomiga, San Diego, CA]; RevertAid first strand cDNA synthesis kit and Maxima SYBR Green/ROX qPCR Master Mix [Thermo, Fisher Scientific, Waltham, MA]). The primer sets used for the quantitative PCR analysis are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. PCR was performed with ViiA7 Real-Time PCR Detection System (Applied Biosystems, Grand Island, NY). The $2^{-\Delta\Delta}$ threshold cycle value was calculated after GAPDH normalization.

Western Blot

Dissected lungs from guinea pigs were homogenized in RIPA lysis buffer and centrifuged. Lung lysates (40 μ g) from each group were subjected to Western blot analysis. RhoA (2117S; Cell Signaling Technology, Boston, MA), M2 (LS-C124189; LifeSpan BioScience, Inc., Seattle, WA), M3 (H00001131-A01; Abnova Corporation, Taipei, Taiwan), Cacna1c (ab84814; Abcam), and GAPDH (LS-C110488; Life Span) were used following the manufacturer's instructions.

Statistical Analysis

Data are expressed as the mean ($\pm$ SEM), and n is the number of animals. The difference between two groups was analyzed with the unpaired Student's t test, and the differences among multiple groups were analyzed by one-way ANOVA. A P value less than 0.05 was considered to indicate statistical significance. All statistical analysis was performed using Prism version 4.03 (GraphPad Software, San Diego, CA).

Results

Airway Inflammation, Remodeling, and Responsiveness

We used an animal model of esophageal acid reflux to induce the Th-directed response, comparing leukocyte levels in BALF of both control and HCl-infused animals. Macrophages represented the majority of leukocytes in both control and guinea pigs infused with HCl. We observed a remarkable enhancement in BALF leukocyte numbers (> 2.8 -fold), with neutrophils increased by 4.65-fold, eosinophils by 4.63-fold, macrophages by 2.49-fold, and lymphocytes by 7.0-fold in HCl-infused animals compared with those of controls (Figure 1A). Cytokines in BALF arrays revealed significantly increased contents of the Th2 cytokines in HCl-infused animals: IL-4 ($416.9 \pm 76.2\%$; $n = 10$), IL-5 ($517.6 \pm 155.5\%$; $n = 10$), IL-13 ($335.3 \pm 32.8\%$; $n = 10$), and TNF- α ($303.3 \pm 46.9\%$; $n = 7$) compared with those of controls. Furthermore, there was

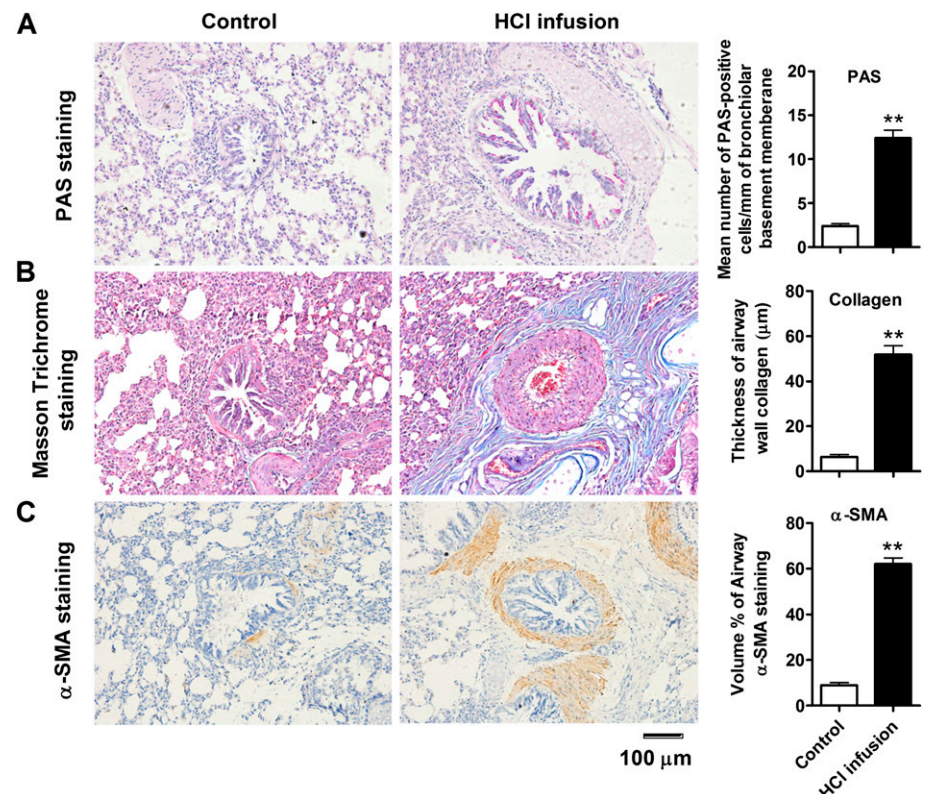


Figure 2. Photomicrographs illustrating periodic acid-Schiff (PAS) staining (A), α -smooth muscle actin (α -SMA) staining (B), and Masson's trichrome staining (C) from control and HCl-infused guinea pigs ($n = 10$). (A) The average number of PAS-positive goblet, (B) α -SMA expression, and (C) collagen thickness in airway wall. Results are shown as means ($\pm$ SEM). ** $P < 0.01$ compared with control animals. Scale bar, 100 μ m.

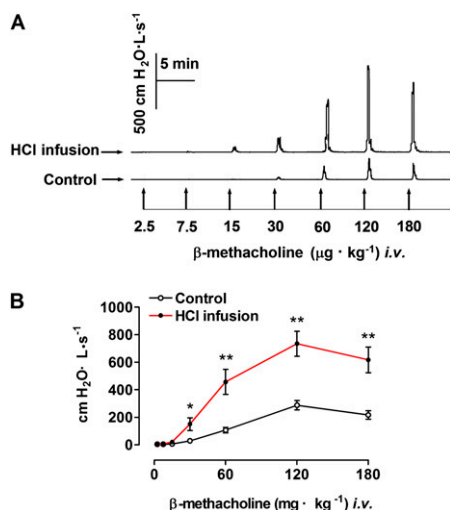


Figure 3. Airway hyperresponsiveness (AHR) was determined from control and HCl-infused guinea pigs. Air flow and tidal volume were determined by cannulating and connecting the trachea via a flow head to a spirometer (ML140; ADInstruments). A physiological pressure transducer (SP844; ADInstruments) measured the transpulmonary pressure by the difference between the tracheal cannula and an esophageal cannula. (A) Airway resistance, as a measurement of bronchoconstriction, in anesthetized guinea pigs in response to cumulative intravenous administration of β -methacholine and is expressed as total airway resistance ($\text{cm H}_2\text{O} \cdot \text{L} \cdot \text{s}^{-1}$; $n = 10$). (B) Summary of concentration-contraction response curve data collected from experiments as shown in A. Results are shown as means ($\pm$ SEM) ($n = 10$). Significance of differences was analyzed using the unpaired Student's *t* test. * $P < 0.05$ and ** $P < 0.01$ compared with control animals. i.v., intravenous.

no difference in the content of IFN- γ , IL-6, granulocyte/macrophage colony-stimulating factor, or IL-2 between control and HCl-infused animals (Figure 1B).

The degrees of mucus hyperproduction and goblet cell hyperplasia were analyzed by PAS staining. The average numbers of PAS-positive cells were significantly enhanced in HCl-infused animals compared with control animals (Figure 2A). Masson's trichrome and morphometrically semiquantitated intensity of collagen deposition showed that HCl infusion gave rise to increased subepithelial collagen deposition, as illustrated in Figure 2B. Moreover, α -SMA expression was significantly increased in HCl-infused animals compared with that of controls (Figure 2C).

To investigate the relationship with airway remodeling, AHR to β -methacholine was examined in control and HCl-infused animals. Figure 3A shows typical traces of airway resistance in the lung as airway responsiveness in control and HCl-infused animals via intravenous administration of increasing concentrations of β -methacholine. In HCl-infused animals, airway responsiveness to β -methacholine was increased distinctively compared with control animals at concentrations of β -methacholine from 60 to 180 $\mu\text{g} \cdot \text{kg}^{-1}$ (Figure 3B).

Measurement of *In Vitro* Function

Figure 4A shows that adding the muscarinic agonist, ACh, (0.01–1,000 μM) to the bath induced contractions in BSM in a concentration-dependent pattern. The

maximal contractions induced by ACh were markedly higher in BSM from the HCl-infused animals ($P < 0.01$) compared with the control animals (maximal response: 0.16 ± 0.01 and $0.34 \pm 0.01 \text{ g} \cdot \text{mg tissue}^{-1}$, respectively; $n = 6-8$), and with negative logarithm of half-maximal effective molar concentration of ACh values of $5.31 (\pm 0.07)$ and $5.62 (\pm 0.11)$. To determine the BSM contractions independently of receptor stimulation, a concentration-response curve to KCl was generated ($n = 6-8$; Figures 4C and 4D). KCl (5–100 mM) induced a significant contraction in BSM from HCl-infused animals ($P < 0.01$) compared with that in control animals (0.11 ± 0.01 and $0.25 \pm 0.01 \text{ g} \cdot \text{mg tissue}^{-1}$ for control and HCl infusion, respectively).

Frequency-dependent BSM contractions produced by electrical field

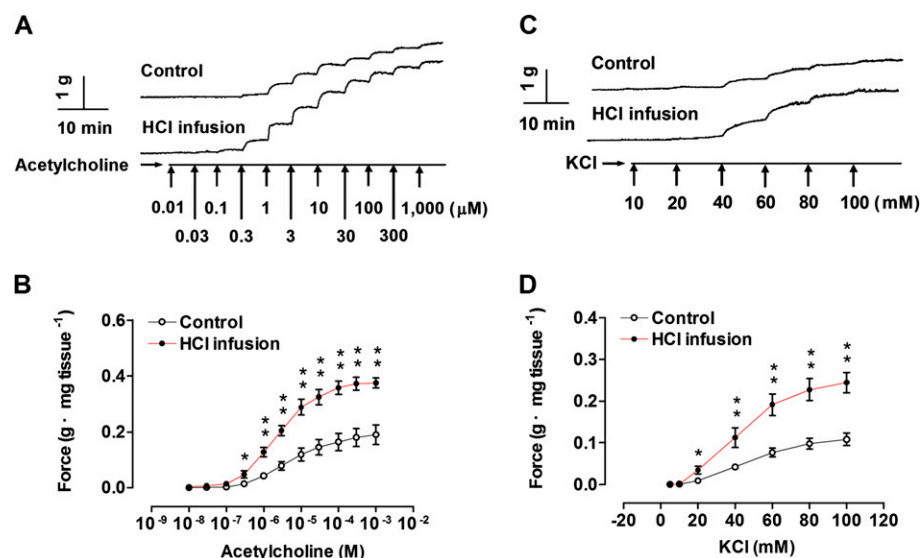


Figure 4. Bronchial smooth muscle (BSM) contraction in response to acetylcholine (ACh) and KCl from control and HCl-infused guinea pigs. For all rings, one end was fixed to the bottom of the organ bath, while the other was connected to a high-sensitivity isometric force transducer (MLT0202; ADInstruments) for the measurement of isometric force. The resting tension was adjusted to 1.0 g at the beginning of the experiment. Krebs-Henseleit buffer solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 25.0 mM NaHCO_3 , 1.2 mM KH_2PO_4 , and 10.0 mM glucose) was changed every 15 minutes, and the equilibration period was more than 60 minutes. After the equilibration period, 1.0 g of ring preparations was then allowed to return to a steady plateau as determined by contractile sensitivity of the preparation with 10 μM ACh, and allowed to equilibrate at baseline passive tension for a further 30 minutes before starting the experiment. At the end of the test, the wet weight of each ring was measured. The contractile response of rings was expressed as the ratio of force to ring weight ($\text{g} \cdot \text{mg tissue}^{-1}$). (A) Original recording trace showed that 0.01–1,000 μM ACh enhanced the contraction of BSM. ACh was applied to organ bath at points indicated by arrows. (B) Summary of concentration-contraction response curve data collected from experiments shown in (A) ($n = 6-8$). (C) Original recording trace shows that 10–100 mM KCl enhance the contraction of BSM. KCl was applied to the organ bath at points indicated by arrows. (D) Summary of concentration-contraction response curve data collected from experiments shown in (C) ($n = 6-8$). Results are shown as means ($\pm$ SEM). * $P < 0.05$ and ** $P < 0.01$ compared with control animals.

stimulation (EFS; $n = 9-10$), were higher in HCl-infused animals compared with control animals at all frequencies employed (from 0.5 to 20 Hz; Figures 5A and 5B). Preincubation of BSM preparations with

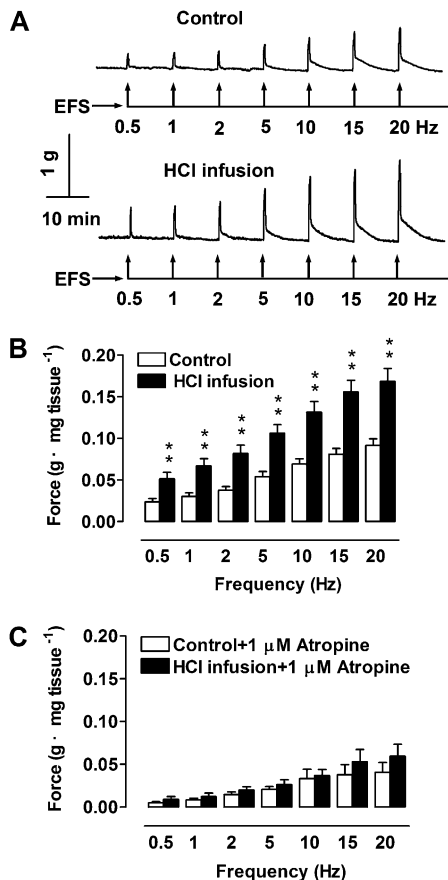


Figure 5. Cholinergic contractions in response to electrical field stimulation (EFS; 25 V for 1 ms, 0.5–20 Hz for 60 s) in control and HCl-infused guinea pigs. To determine EFS-induced BSM contractions, frequency–response curves (0.5–20 Hz) were generated by stimulating the tissues for 60 seconds with pulses of 1 millisecond duration at 25 V, with 10-minute intervals between stimulations. Subsequently, after incubation periods of 40 minutes, frequency–response curves were repeated in the presence of the muscarinic receptor antagonist, atropine (1 μ M), to conform the responses that were mediated by muscarinic (ACh) receptor activation. (A) Original traces showing EFS-induced contractions. (B) Frequency-dependent contractions induced by EFS ($n = 9-10$). (C) EFS-induced contractions were significantly inhibited by 1 μ M atropine after 30-minute preincubation ($n = 5-6$). Results were obtained from bronchial rings of control or HCl-infused guinea pigs. Data represent the means ($\pm$ SEM) in each group. ** $P < 0.01$ compared with control animals.

atropine (1 μ M), a muscarinic receptor antagonist, strongly inhibited EFS-induced BSM contractions in both control and HCl-infused animals ($n = 5-6$; Figure 5C), indicating that EFS-evoked contractions are mainly mediated by muscarinic receptor. In addition, preincubation of BSM preparations with tetrodotoxin (1 μ M), a voltage-gated sodium channel blocker, almost abolished the EFS-evoked contractions in all tested frequencies ($n = 4$).

L-VDCC and Rho-Kinase Pathway Associated with Contraction of BSM in Guinea Pigs Infused with HCl

Preincubation of BSM with nifedipine (10 μ M; 30 min), an L-VDCC blocker, slightly affected the ACh-induced contractions in control rings ($n = 5$). In contrast, 10 μ M nifedipine significantly attenuated ACh-evoked contraction in rings of the HCl infusion group (Figure 6B, $n = 5-6$). Pretreatment of rings with Y27632 (10 μ M), a Rho-kinase inhibitor, greatly affected ACh-evoked contractions in rings of HCl-infusion compared with control guinea pigs (Figure 6A). It was found that there are no significant differences for the negative logarithm of half-maximal effective molar concentration of ACh values for ACh-evoked responses in the absence and presence of either nifedipine or Y27632 in control and HCl-infused animals (Table 2). Concentration–response curves to Ca^{2+} (0.1–30 mM; $n = 6-7$) were generated in Ca^{2+} -free Krebs solution containing KCl (80 mM) and thapsigargin (1 μ M). BSM contractions to CaCl_2 were compared with those from control rings (Figure 6C; $P < 0.01$). Preincubation of rings with 10 μ M nifedipine reduced the CaCl_2 -evoked contractions in tissues of HCl-infused animals, restoring them to the similar levels as those seen in control tissue. In control rings, treatment with nifedipine was able to reduce CaCl_2 -evoked BSM contractions at a concentration of 3 mM CaCl_2 (Figure 6C).

Expressions of mRNA and Protein for Muscarinic M₂ and M₃ Receptors, L-VDCC, and RhoA

Compared with BSM in control guinea pigs, the mRNA and protein expressions of muscarinic M₃ receptor, CacnalC (L-VDCC), and RhoA were greater than those of BSM in HCl-infused animals. In contrast, the expression of M₂ receptor in

BSM of HCl-infused animals was not significantly altered when compared with that in BSM of control animals (Figure 7).

Discussion

In this study, we examined airway inflammation, remodeling, and AHR in guinea pigs subjected to infusion of HCl for 14 days. We found that macrophages represented the majority of leukocytes in both control and guinea pigs infused with HCl. Analysis of BALF revealed a remarkable enhancement in leukocyte numbers, including neutrophils, eosinophils, macrophages, and lymphocytes, in HCl-infused animals compared with those of controls. Furthermore, infusion of HCl resulted in increased IL-4, -5, and -13, and TNF- α concentration in BALF compared with controls. Guinea pigs infused with HCl showed increases of AHR in comparison with controls. We also demonstrated that remodeling was produced in guinea pigs infused with HCl in comparison with controls.

Chronic airway inflammation induced by gastroesophageal reflux is characterized by infiltration of macrophages, neutrophils, lymphocytes, and eosinophils (8, 16). The present results were consistent with these findings, as our models of infusion of HCl for 14 days induced typical chronic inflammation, with macrophages, neutrophils, lymphocytes, and eosinophils infiltrated into the airways. It has been reported that repetitive HCl aspiration in rats can initiate an inflammatory response, such as production of macrophages and T cells, which is associated with increased release of cytokines, including IL-1 α , IL-1 β , transforming growth factor- β , TNF- α , and IL-2 (3). Recently, an *in vitro* study demonstrated that acidification of human bronchial epithelial cells (16-HBE) with gastric fluid induced epithelial damage and inflammation were involved in both IL-6 and IL-8 release (21). However, in this study, we found that the inflammatory response to infusion with HCl in the airway of guinea pigs was mainly associated with increased IL-4, -5, and -13, and TNF- α concentration in BALF.

In this study, treatment with infusion of HCl in guinea pigs could successfully reproduce many of the remodeling features of human asthma, including expression of

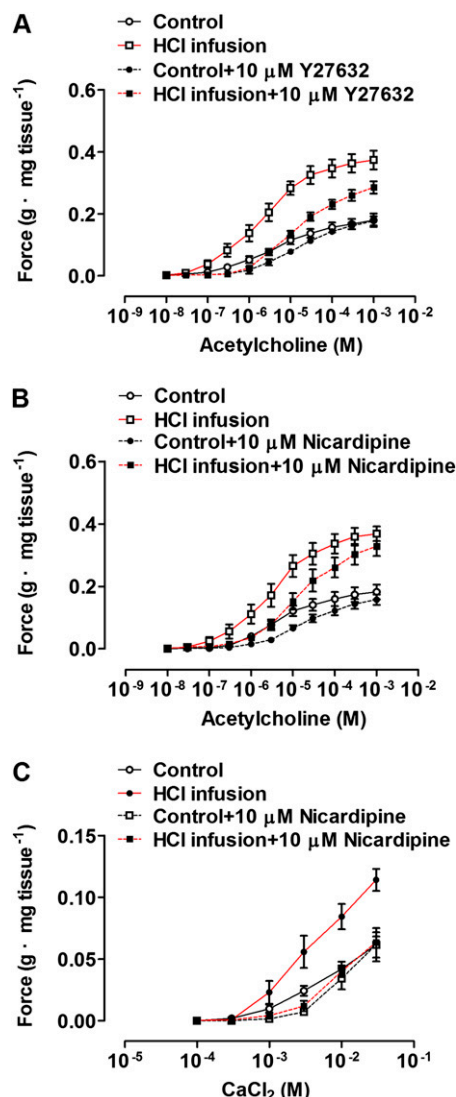


Figure 6. BSM contraction in response to ACh in the absence and presence of nicardipine, the L-type Ca²⁺ channel blocker, or Y27632, the Rho-kinase inhibitor, in control and HCl-infused guinea pigs. (A) Concentration–contractile response curves show that ACh-induced BSM contraction was significantly inhibited by Y27632 (10 μM) in HCl-infused guinea pigs, but not in control guinea pigs ($n = 5$). (B) Concentration–contractile response curves show that ACh-induced BSM contraction was inhibited by nicardipine (10 μM) in both HCl-infusion and control guinea pigs ($n = 5$). (C) To evaluate the direct effects of external Ca²⁺ influx on BSM contractions, the rings were suspended in 5-ml organ baths containing Krebs Ca²⁺-free solution containing 1 mM EGTA to sequester Ca²⁺ ions, and 1 μM thapsigargin to deplete sarcoplasmic reticulum Ca²⁺ stores. After an equilibration period of 15 minutes, the effects of external calcium from 0.1 to 30 mM on contractions of BSM were determined in the presence or absence of 10 μM

Table 2. Potency (Negative Logarithm of Half-Maximal Effective Molar Concentration of Acetylcholine) and Maximal Response Values Obtained from Concentration–Response Curves to Acetylcholine and Extracellular Ca²⁺ in Bronchial Smooth Muscle from Control and HCl-Infusion Guinea Pigs

Agonists	Groups	pEC ₅₀	E _{max} (g · mg tissue ⁻¹)
Acetylcholine	Control	5.31 ± 0.07	0.16 ± 0.01
	HCl infusion	5.62 ± 0.11	0.34 ± 0.01*
	Control + nicardipine	4.82 ± 0.12	0.14 ± 0.01
	HCl infusion + nicardipine	4.92 ± 0.10	0.29 ± 0.01*
	Control + Y27632	5.00 ± 0.21	0.16 ± 0.01
	HCl infusion + Y27632	4.97 ± 0.10	0.25 ± 0.01*
CaCl ₂	Control	—	0.06 ± 0.02
	HCl infusion	—	0.11 ± 0.01*
	Control + nicardipine	—	0.06 ± 0.01
	HCl infusion + nicardipine	—	0.06 ± 0.01

Definition of abbreviations: E_{max}, maximal response; pEC₅₀, negative logarithm of half-maximal effective molar concentration of acetylcholine. Concentration–response curves to acetylcholine and Ca²⁺ were generated in the absence or presence of either the Rho-kinase inhibitor, Y27632 (10 μM), or the L-type calcium channel blocker, nicardipine (10 μM). Data represent the mean ± SEM of six to eight animals. * $P < 0.01$ compared with respective control group.

airway mucus, peribronchial fibrosis, and thickness of airway smooth muscle (Figure 2). Increasing mass of airway smooth muscle is a feature of airway remodeling in asthma, which may result from both airway smooth muscle cell hyperplasia and hypertrophy (22). Airway remodeling has been considered to associate with AHR that was induced by long-term continuous airway inflammation. The effects of IL-13 in asthma have been highlighted, including the direct action of IL-13 on airway epithelium and smooth muscle, as well as allergic inflammation and airway remodeling after chronic allergen exposure (23).

AHR, an increased contractility of airway smooth muscle, plays a major role in the pathogenesis of bronchial asthma (24) and also in that of GERD (11). The predominance of Th2 over Th1 cytokines plays a central role in the allergic inflammation in bronchial asthma (25–27). Using receptor neutralization and IL-4 and IL-13 knockout mice, it has been showed

nicardipine from control and HCl-infused guinea pigs. Concentration–contractile response curve shows that external calcium-induced BSM contraction was inhibited by nicardipine (10 μM) in HCl-infused guinea pigs, but not in control guinea pigs ($n = 6–7$). Data represent the means (± SEM) in each group.

that these cytokines are implicated in the development of AHR (28). The recent study using primary cultured human BSM cells demonstrated that ACh-induced Ca²⁺ signaling and receptor expression were activated by cytokines IL-4 and -13, indicating that enhanced Ca²⁺ mobilization was associated with bronchial hyperresponsiveness (29). In the present experiment, we found that production of AHR in guinea pigs infused with HCl was accompanied by increases of Th2 cytokines, including IL-4, -5, -13, and Th1 cytokines, including TNF-α concentration in BALF, suggesting that production of AHR is mostly correlated with the imbalance of Th1 and Th2 expression in our model.

Functional studies of isolated bronchial rings showed that the contraction of BSM to exogenous muscarinic agonist was markedly greater in guinea pigs infused with HCl compared with control animals. BSM contractions induced by EFS in guinea pigs infused with HCl were also greater than those in the HCl infusion group. These functional results from *in vitro* study are consistent with the AHR induced by intravenous administration of methacholine seen in anesthetized animals with HCl infusion, as illustrated in Figure 4. The contractility of BSM induced by ACh and EFS could indicate alterations at the receptor level, with the related downstream

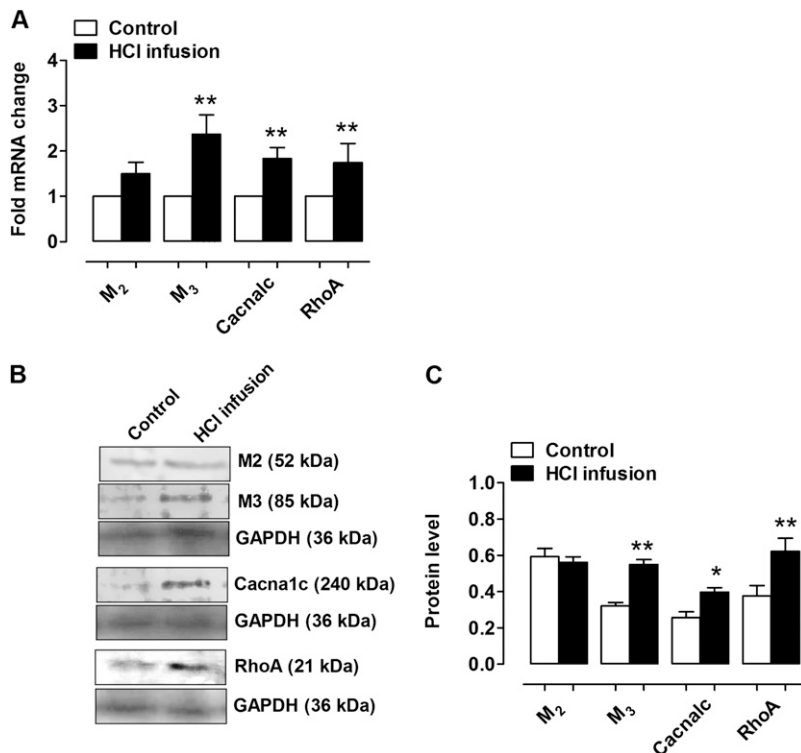


Figure 7. Relative BSM mRNA and protein expressions for muscarinic M₂ and M₃ receptors, RhoA, and L-type voltage-dependent Ca²⁺ channels in control and HCl-infused guinea pigs. (A) Real-time PCR was performed and the $2^{-\Delta\Delta}$ threshold cycle value was calculated after glyceraldehyde 3-phosphate dehydrogenase (GAPDH) normalization. Fold changes were determined relative to control group ($n = 10$). (B) Western blot was performed. (C) Relative protein level was calculated after GAPDH normalization ($n = 5$). Data are shown as the means ($\pm$ SEM) in each group. * $P < 0.05$ and ** $P < 0.01$ compared with control group.

signaling. ACh interacts with two muscarinic receptors expressed in airway smooth muscles, M₃ receptors coupled via G α_q with phosphoinositide hydrolysis and IP₃-dependent Ca²⁺ release, and M₂ receptors coupled via G α_i (30). Determination of the expression of mRNA and protein of muscarinic M₂ and M₃ receptors in the BSM from guinea pigs infused with HCl showed that the expression of muscarinic M₃ receptors was greater than that in controls, indicating that up-regulation of M₃ receptors led to overcontraction of BSM in HCl-infused animals.

It has been considered that Ca²⁺ mobilization can be altered in BSM of asthma. It is well known that the intracellular Ca²⁺-independent phosphorylation of myosin

light chain is associated with regulation of the Rho-kinase signaling pathway (31), and mediated agonist-induced Ca²⁺ sensitization in smooth muscle contraction (32, 33). It had been reported that ACh-enhanced BSM contractions in the asthma state were blocked by Rho-kinase inhibitor, such as Y27632 (31, 34). In this study using HCl-infused animals, we found that ACh-induced BSM contraction was inhibited by Y27632, combined with the higher expression of RhoA mRNA or protein in the BSM of HCl-infused animals compared with that of controls, suggesting that, in an acidified state, the increased contractions in BSM are mainly mediated by increases of RhoA expression and activation of M₃ receptors.

The elevation of cytosolic Ca²⁺ concentration via Ca²⁺ influx was activated by L-VDCC. The depolarization of the cell membrane and the activation of L-VDCC via high extracellular potassium leads to an increase in the intracellular Ca²⁺ concentration, and subsequently activated contractile response (35). In the present study, KCl induced an increase in contractile response in BSM from HCl-infused animals, suggesting an increase of extracellular Ca²⁺ influx or Ca²⁺ sensitivity. On the other hand, in physiological conditions, Ca²⁺ influx through dihydropyridine-sensitive Ca²⁺ channels is a key factor in providing the contractile response evoked by ACh in airway smooth muscles (14, 15). In the present experiment, the enhanced BSM contractions by ACh and extracellular Ca²⁺ in HCl-infused animals were inhibited by nifedipine, the L-VDCC blocker. In addition, we found that the expression of L-VDCC mRNA or protein in the BSM of HCl-infused animal was significantly increased. It is possible that, in the acidified state, activation of both the L-type Ca²⁺ channel and the M₃ receptor lead to an increase in Ca²⁺ influx, and subsequently contribute to contraction in BSM (36, 37). Acid infusion to the lower esophagus and subsequent microaspiration into the respiratory tract in guinea pigs leads to the airway smooth muscle remodeling and overactivation in the BSM, which may be associated with an alteration in L-VDCC function.

In conclusion, our data show that acid infusion to the lower esophagus and subsequent microaspiration into the respiratory tract in guinea pigs leads to the AHR and overactive BSM. Functional and molecular data suggest that overactive BSM is the results of enhancement of extracellular Ca²⁺ influx via L-VDCC and Ca²⁺ sensitization through Rho-kinase signaling. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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