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Induction of airway remodeling and persistent cough by repeated citric acid exposure in a guinea pig cough model

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ABSTRACT

Background: A previous study involving guinea pigs showed that repeated cough could increase peripheral airway smooth muscle area, which can also aggravate cough. The airway pathologic changes produced by prolonged cough are still unknown.
Objective: To study the airway pathologic changes in prolonged cough models of guinea pigs.
Methods: Guinea pigs were assigned to three treatment groups: citric acid inhalation (CA) alone, citric acid inhalation with codeine pretreatment (COD), or saline solution inhalation (SA). Animals were challenged with citric acid or saline solution three times weekly. The intervention period was 22 or 43 days. Animals were challenged with citric acid on the first and last days of exposure. Lung specimens were obtained for pathologic analysis 72 h after the last exposure.
Results: Compared with the other two groups, the CA group had increased frequency of cough on both 22 and 43 days of exposure. Tracheal basement membrane (BM) thickness was increased after 43 days of exposure, correlating with the frequency of cough. The area of airway smooth muscles (ASM index) in small airways increased in the CA group after both 22 and 43 days of exposure, compared with the SA group. Compared with the COD group, the ASM index in small airways increased in the CA group after 22 days of exposure instead of 43 days of exposure.
Conclusions: An increase in peripheral smooth muscle area by repeated cough was confirmed. Moreover, this is the first study to show that tracheal BM thickness increased after prolonged exposure (43 days). Repeated cough may lead to airway remodeling, which was also associated with an increased frequency of cough.

1. Introduction

Cough is a common manifestation of respiratory diseases and is known to be the most frequent complaint for which patients seek medical care. This may be associated with asthma or asthma-related conditions, such as cough variant asthma (CVA) and eosinophilic bronchitis. Other recognized common causes of chronic cough are gastroesophageal reflux disease (GERD), upper airway cough syndrome, and unexplained cough. (Irwin et al., 2006)

Airway inflammation and remodeling are established features of chronic cough due to asthmatic or non-asthmatic origins (Niimi and Chung, 2004; Niimi et al., 2005). With regards to etiology, the literature has shown the following: (1) eosinophilic inflammation has been primarily found in cough due to classical asthma, cough variant asthma, and non-asthmatic eosinophilic bronchitis (Niimi et al., 2005, 1998; Gibson et al., 2002); and (2) cellular inflammation characterized by an increase in mast cells or lymphocytes may be involved in non-asthmatic chronic cough, such as upper airway cough syndrome, GERD, and unexplained cough (Boulet et al., 1994; Lee et al., 2001; Birring et al., 2003; Mund et al., 2005).

Abbreviations: CA, citric acid inhalation; COD, citric acid inhalation with codeine pretreatment; SA, saline solution inhalation; BM, basement membrane; ASM, airway smooth muscles; ASM index, area of airway smooth muscles; SBM, sub-basement membrane; IP, intraperitoneal injection; SC, subcutaneous injection

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Airway remodeling is another prominent feature in patients suffering from chronic cough. The pathologic features of airway remodeling in asthmatic patients include sub-basement membrane (SBM) thickening, goblet cell hyperplasia, airway smooth muscle (ASM) hypertrophy and hyperplasia, submucosal gland hyperplasia, vascular proliferation, and airway wall thickening (Niimi and Chung, 2004; Niimi et al., 2005). Although with varying airway inflammation, the features of airway remodeling, such as SBM thickness and goblet cell hyperplasia, were also observed in non-asthmatic cough patients (Niimi et al., 2005; Xie et al., 2009). In addition, increased ASM area was found in patients with non-asthmatic cough, even if there was no airflow obstruction or bronchial hyperresponsiveness (Niimi et al., 2005). These findings imply that there may be a common mechanism for structural changes in both asthmatic and non-asthmatic-related chronic cough.

Generally, airway inflammation has been considered an important mechanism involved in airway remodeling (Minshall et al., 1997; Molet et al., 2001), but some studies and observations also indicated that mechanical stimuli in the airways evoked by chronic cough were other causes of airway structural changes (Niimi et al., 2005; Matsumoto et al., 2007). The mechanism may be due to direct (Ito et al., 2008) and indirect (Ressler et al., 2000; Tschumperlin, 2003; Mohamed and Boriek, 2010) effects of mechanical stimuli. In our previous study, chronic cough was induced in guinea pig models to observe the changes in airway structure. After 22 days of challenge, small ASM area was increased significantly, and this was correlated with cough frequency; no structural changes were observed in the large airways (Nakaji et al., 2016). Therefore, the main aim of the present study was to explore whether repeated cough under prolonged intervention could elicit airway remodeling, especially in the large airways of animal models.

2. Methods

2.1. Animals

Male Dunkin–Hartley guinea pigs, weighing 350–400 g, were obtained from Nihon SLC, Inc. (Hamamatsu, Japan) and kept in quarantine at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. Animal experiments were approved by the Department of Respiratory Medicine, and all procedures conformed to Kyoto University's regulations on animal experimentation.

2.2. Experimental procedure and method for inhalation

The experimental protocol is shown in Fig. 1. Five to six animals were assigned to each treatment group: saline solution (SA), citric acid (CA), and citric acid with codeine pretreatment (COD). The guinea pigs were challenged by their assigned treatment according to either the 3-week or 6-week protocol (Fig. 1b and c). Guinea pigs were placed in a chamber equipped with a nebulizer. All animals were challenged with 0.5 M CA (Chung et al., 2003) (Sigma–Aldrich Chemical Co, St. Louis, MO) on the first and last days of exposure. Animals were repeatedly challenged with CA or saline solution three times weekly. Delivery of 0.5 M CA or 0.9% saline was by nebulizer, with an output of approximately 500 ml/min for 10 min. Terbutaline (Sigma Chemical Co. St. Louis, MO) 0.05 mg/kg IP (intraperitoneal injection) was administered 5 min prior to CA exposure to prevent bronchoconstriction (Leung et al., 2007). Terbutaline was also administered to animals in the SA control group prior to saline solution exposure. Codeine phosphate (Takeda Pharmaceutical Co., Osaka, Japan) 4.5 mg/kg SC (subcutaneous injection) was delivered 40 min prior to CA exposure to inhibit cough in the COD group. The frequency of cough was measured during each challenge, with the method described in Section 2.3. Then, 72 h after the last exposure, the animals were sacrificed, and lung specimens were obtained for histologic analysis (Fig. 1a).

2.3. System for cough measurement

Guinea pigs were allowed free movement in the chamber that was equipped with an internal microphone and a pressure transducer. The chamber was provided with airflow at 1500 ml/min. Changes in airflow induced by respiration and cough were detected and recorded by a pneumotachograph. Sounds of cough were amplified and recorded with the microphone. Guinea pig behavior was captured with an external camera. Data acquisition was performed with eDacq software (EMMS, Hampshire, UK). The frequency of cough during each 10-minute exposure was counted; cough was distinguished from sneeze based on the posture of the subject, as well as the sound and pressure changes detected (Chung et al., 2003).

2.4. Sacrifice and tissue processing

At 72 h after the last exposure, all animals were anesthetized with 60 mg/kg of sodium pentobarbital IP. The trachea and lungs were excised together. A cannula was inserted into the proximal portion of the trachea, and the lungs were inflated with around 6 ml of 4% paraformaldehyde (Kistemaker et al., 2016), which made the lungs the same size. After fixation with 4% paraformaldehyde, the specimens were divided into two parts: trachea and left lobe of the lung. Specimens were sectioned into 3-μm-thick axial slices from the distal end of the trachea and at 1 cm distal to the incisional edge of the left lung. Sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS), according to standard procedures.

2.5. Inflammatory cells in the airways

For each animal, eight 200-μm-square photomicrographs of one section of the trachea, at a magnification of ×400, were examined; neutrophils and eosinophils in the epithelial layer and submucosal area were counted. The number of inflammatory cells was not counted because these were rarely observed in the small airways. Data are expressed as the mean number of cells observed per high power field (HPF); for example, neutrophils/HPF or eosinophils/HPF.

2.6. Histologic measurements and quantification

Sections were examined and photographed using a microscope (Olympus IX81; Tokyo, Japan). The images were analyzed using quantification software (ImageJ; U.S. National Institutes of Health, Bethesda, MD). The portion of the trachea that was surrounded by cartilage was defined as large airways, whereas the portion without cartilage and with an internal diameter of 100–200 μm was defined as small airways. For every section, three pictures were photographed, and data are presented as means of the parameters.

2.6.1. Large airways

Parameters that were measured in the large airways included ASM area, submucosal area, epithelium thickness, and basement membrane thickness on HE staining. Goblet cell area was measured with PAS staining. The ASM index was defined as the proportion of ASM area in the submucosal area. Goblet cell area% was determined as the proportion of the PAS-positive area within the epithelial layer.

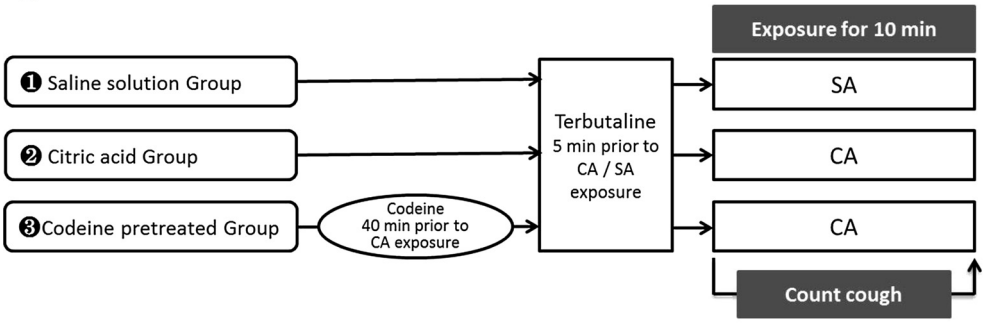
2.6.2. Small airways

In the small airways, ASM area, total wall area, and epithelium thickness were measured. The small ASM index was defined as the proportion of ASM area in the total wall area. Because PAS staining was negative in the small airways, goblet cell area was not measured.

2.7. Statistical analysis

Data are expressed as means ± SEM. Statistical comparisons

(a)



(b)

3 weeks	D1	D4	D6	D8	D11	D13	D15	D18	D20	D22	D25
❶ Saline solution Group	CA	SA	SA	SA	SA	SA	SA	SA	SA	CA	Sacrifice
❷ Citric acid Group		CA	CA	CA	CA	CA	CA	CA	CA		
❸Codeine pretreated Group		COD	COD	COD	COD	COD	COD	COD	COD		

(c)

6 weeks	D1-20	D22	D25	D27	D29	D32	D34	D36	D39	D41	D43	D46
❶ Saline solution Group	Same as 3 weeks of intervention	SA	SA	SA	SA	SA	SA	SA	SA	SA	CA	Sacrifice
❷ Citric acid Group		CA	CA	CA	CA	CA	CA	CA	CA	CA		
❸Codeine pretreated Group		COD	COD	COD	COD	COD	COD	COD	COD	COD		

Fig. 1. Experimental protocols (a) Animals were assigned to three treatment groups and challenged according to the drug delivery procedure. They were exposed to SA or CA for 10 min. The frequency of cough for every 10-minute exposure was counted. (b)(c) Animals were repeatedly exposed three times weekly according to the protocol for 3 weeks and 6 weeks. At 72 h after the last exposure, the animals were sacrificed, and lung specimens were obtained for analysis. SA, saline solution; CA, citric acid; COD, citric acid with codeine pretreatment; DX, day of exposure, e.g. "D1" = "Day 1".

between groups were performed using Student's *t*-test. Multiple groups were compared using one-way ANOVA followed by Fisher's Least Significant Difference (LSD) *post hoc* test to show individual differences. Correlation analysis was by the two-tailed Pearson's test. A p-value of < 0.05 was considered significant.

3. Results

3.1. Frequency of cough

Frequency of cough was calculated as the average number of coughs on day 1 to day 22 (3-week protocol) or to day 43 (6-week protocol). Compared with the SA and COD groups, the CA group had a significantly increased frequency of cough in both the 3-week (12.12 ± 2.68 in the CA group vs. 0.74 ± 0.13 in the SA group, $p < 0.01$; vs. 1.70 ± 0.39 in the COD group $p < 0.01$) and 6-week protocols (8.04 ± 1.55 in the CA group vs. 0.48 ± 0.14 in the SA group, $p < 0.01$; vs. 3.18 ± 0.64 in the COD group $p < 0.05$) (Fig. 2a). In the 6-week protocol, the average cough frequency in the first 3 weeks (days 1–22) was compared with that in the latter 3 weeks (days 25–43). There was no difference in the SA group (0.63 ± 0.28 vs. 0.31 ± 0.11, respectively, $p > 0.05$), but the cough frequency increased significantly in the latter 3 weeks, compared with the first 3 weeks, in both the CA group (5.91 ± 1.10 vs. 10.41 ± 2.27, $p < 0.05$) and the COD group (1.45 ± 0.35 vs. 5.11 ± 1.24, $p < 0.05$) (Fig. 2b).

3.2. Inflammatory cells in the airways

In the tracheal epithelium, there was no difference in the number of neutrophils among the three groups after 3 weeks and 6 weeks of exposure (Fig. 3a). Eosinophils were not observed in the tracheal epithelium. In the submucosal area of the trachea, the number of neutrophils was not different among the three groups after 3 weeks and 6 weeks of exposure (Fig. 3b). Eosinophils were rarely seen in the submucosal area in all three groups.

3.3. Histology

3.3.1. Large airways

There was no remarkable difference in tracheal basement membrane (BM) thickness among the three groups after 3 weeks of exposure (Figs. 4 and 5a). After 6 weeks of exposure, tracheal BM thickness increased significantly in the CA group compared with that in the SA group ($5.66 \pm 0.48\,\mu m$ vs. $3.68 \pm 0.43\,\mu m$, $p = 0.003$) and the COD group ($5.66 \pm 0.48\,\mu m$ vs. $3.54 \pm 0.23\,\mu m$, $p = 0.002$) (Figs. 4 and 5a). When analyzing all animals of the three groups combined, tracheal BM thickness was correlated with the frequency of cough ($r = 0.610$, $p = 0.012$) (Fig. 5b). There was no significant difference in tracheal BM thickness between number of coughs > 4 and ≤ 4 (4.90 ± 0.60 vs. $3.74 \pm 0.24\,\mu m$, $p > 0.05$) (Fig. 6a). The difference in goblet cell area % among the three groups was insignificant after 3 or 6 weeks of exposure (3 weeks: CA 32.81% ± 1.78% vs. SA 26.99% ± 2.85% vs. COD 29.05% ± 3.92%, $p > 0.05$) (6 weeks: CA 34.41% ± 3.32% vs. SA 25.70% ± 2.64% vs. COD 30.44% ± 1.42%, $p > 0.05$) (Fig. 7). Other parameters, including the ASM index and epithelial thickness, showed no significant differences among the three groups after 3 weeks and 6 weeks of exposure (data not shown).

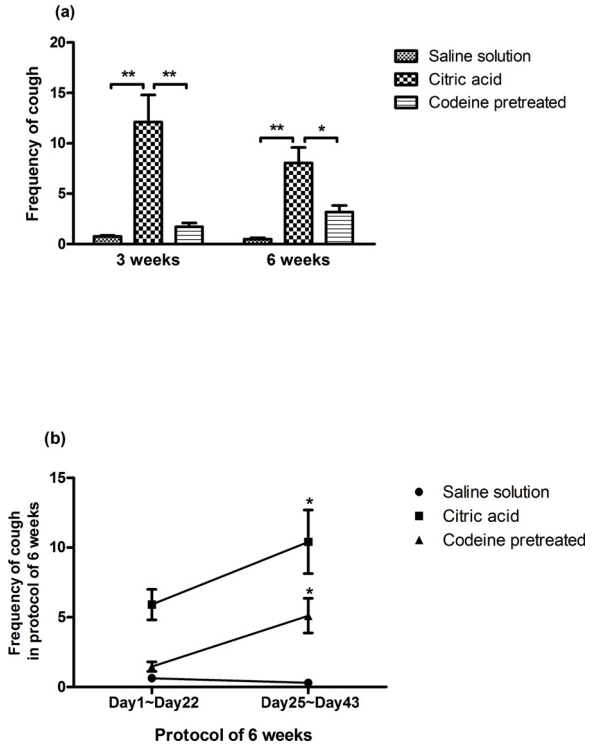


Fig. 2. Comparison of the frequency of cough among the three treatment groups after 3 weeks and 6 weeks of exposure (a) Differences in frequency of cough among the three groups are compared between the 3-week and 6-week protocols. (b) In the 6-week protocol, the frequency of cough in the latter 3 weeks is compared with the first 3 weeks among the three treatment groups. Values are expressed as means ± SEM.
** $p < 0.01$ and * $p < 0.05$ by one-way ANOVA, LSD *post hoc* test in (a) and by Student's *t*-test in (b).

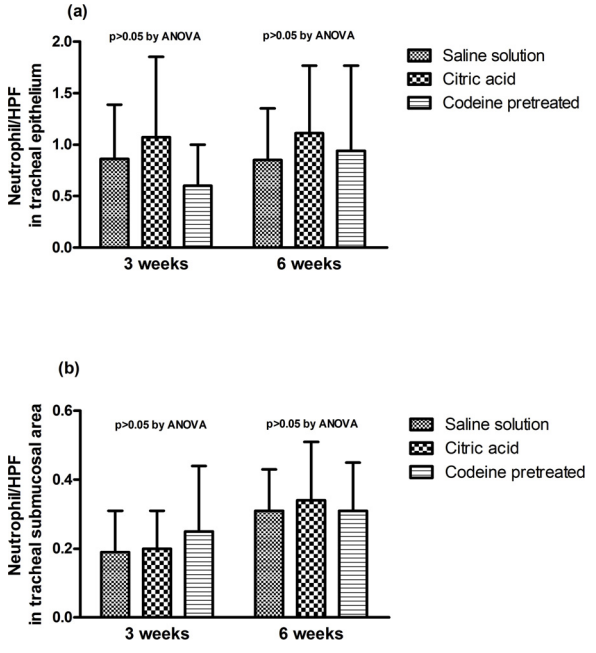


Fig. 3. Inflammation in the large airways Comparison among the three treatment groups after 3 weeks and 6 weeks of exposure in terms of mean ± SEM neutrophils/HPF (a) in the tracheal epithelium and (b) in the submucosal area. HPF, high-power field.

3.3.2. Small airways

Compared with the SA group, the CA group had a significantly increased ASM index after exposure for 3 weeks (25.23% ± 0.95% vs. 13.51% ± 2.70%, $p = 0.001$) and 6 weeks (41.35% ± 6.63% vs. 20.04% ± 2.36%, $p = 0.011$) (Figs. 4b and 8a). Although the ASM index increased significantly in the CA group compared with the COD group after 3 weeks of exposure (25.23% ± 0.95% vs. 15.37% ± 1.81%, $p = 0.004$), the increase was not significant after 6 weeks of exposure (41.35% ± 6.63% vs. 30.51% ± 6.20%, $p = 0.17$) (Figs. 4b and 8a). The increases in the ASM index in the CA and COD groups were significant after 6 weeks of exposure compared with 3 weeks of exposure (CA group: 41.35% ± 6.63% after 6 weeks vs. 25.23% ± 0.95% after 3 weeks, $p = 0.043$; COD group: 30.51% ± 6.20% after 6 weeks vs. 15.37% ± 1.81% after 3 weeks, $p = 0.047$) (Figs. 4b and 8a). The difference was not significant in the SA group (20.04% ± 2.36% after 6 weeks vs. 13.51% ± 2.70% after 3 weeks, $p = 0.10$) (Figs. 4b and 8a). When analyzing all animals of the three groups combined, the ASM index correlated with the frequency of cough after exposure for 3 weeks ($r = 0.685$, $p = 0.005$) (Fig. 8b) and 6 weeks ($r = 0.633$, $p = 0.009$) (Fig. 8c). The ASM index increased in animals with cough frequency > 4 when compared with animals with cough ≤4 (39.90% ± 5.48% vs. 22.25% ± 2.80%, $p < 0.01$) (Fig. 6b). Because PAS staining was negative in the small airways, goblet cell area was not measured. There was no significant difference in small airway epithelial thickness after 3 weeks and 6 weeks of exposure (data not shown).

4. Discussion

In this study, whether repeated cough will induce airway remodeling in animal models was investigated. In a previous study, we found that small ASM thickness (ASM index) increased after 3 weeks of cough induction by CA and was correlated with cough frequency (Nakaji et al., 2016). To clarify the differences in airway structural changes after a prolonged period of cough, a 6-week protocol of cough induction was performed, and there was a more significant increase in small ASM thickness compared with that after 3 weeks of exposure. Moreover, tracheal BM thickness was found to be increased in the 6-week protocol and was also correlated with the frequency of cough. The results confirmed our hypothesis that mechanical stress due to cough can elicit airway remodeling, and that the severity of cough may influence the level of structural changes in the airways.

In our study, we used an experimental guinea pig model to simulate chronic cough. The unique feature of this study, compared with other methods of induction (Hara et al., 2008; Takahama et al., 1997), was the repetitive CA stimulation over 3 weeks, which can more satisfactorily simulate the disease process of chronic cough in humans; in addition, other methods require anesthesia, surgical procedures, or mechanical ventilation (Hara et al., 2008; Takahama et al., 1997) and may not be reproducible as a chronic cough model. According to published papers on chronic cough and asthma induction in animal models, the duration of the challenge protocol varied from 18 days to 8 weeks and was performed by either cigarette smoke or ovalbumin (Mutoh et al., 2000; Luo et al., 2013; Xu et al., 2013; Park et al., 2012). We designed a 6-week protocol of cough induction to analyze the pathologic features of airway remodeling, because structural changes in small airways were observed in our previous study after 3 weeks of cough induction by CA. Therefore, we doubled the duration of induction to measure the extent of airway remodeling.

The present findings are, in part, consistent with some clinical studies. When airway wall thickness in patients with chronic cough was examined by computed tomography, the walls of central airways were thickened in patients with CVA and non-asthmatic cough (NAC) (Matsumoto et al., 2007); this finding of airway wall thickening in NAC may be associated with cough hypersensitivity. Bronchoscopic biopsy showed that basement membrane thickening and increased ASM area were prominent features in the airways of both asthmatic and non-asthmatic cough patients (Niimi et al., 2005). Cough consists of forced

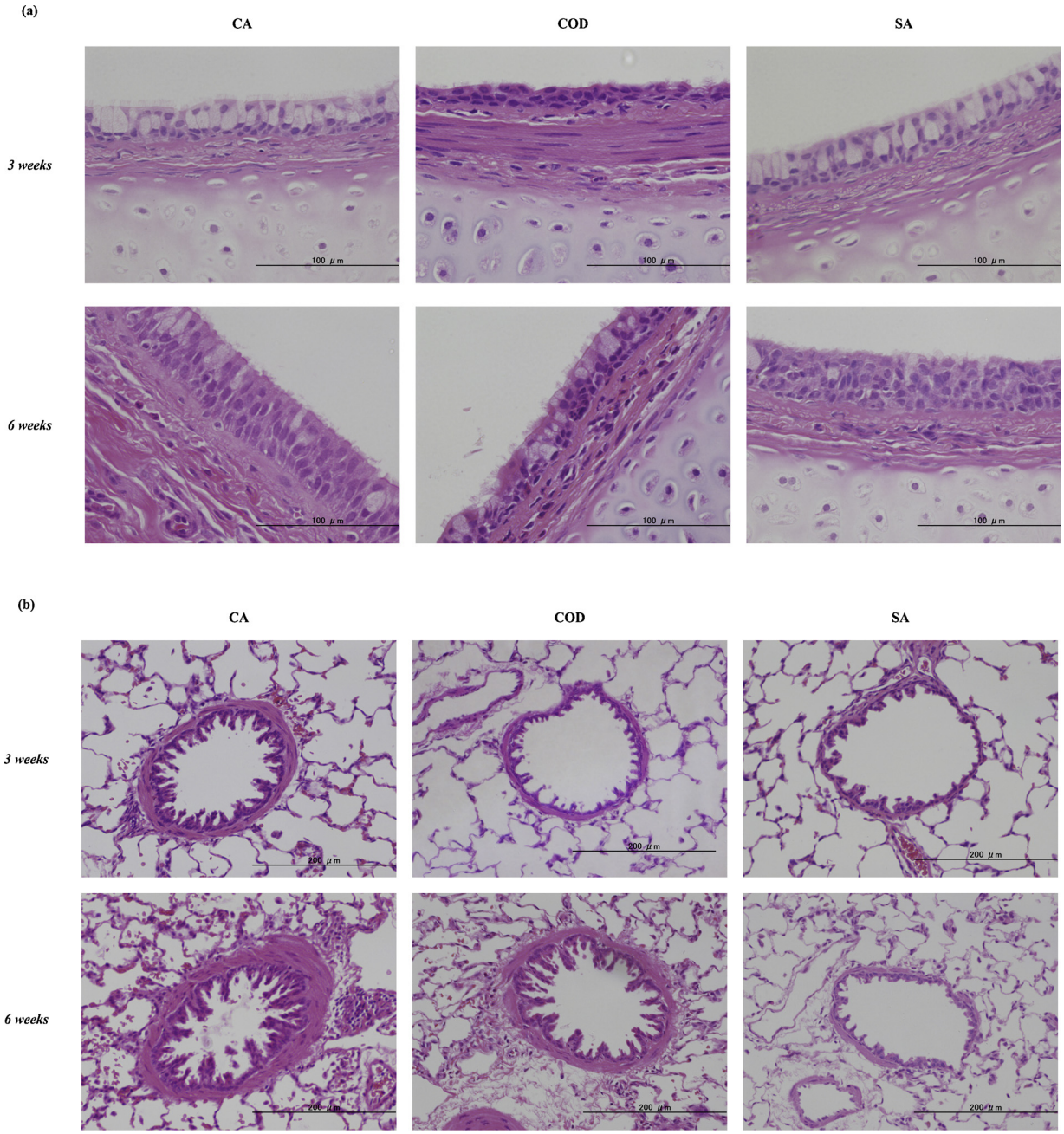


Fig. 4. Photomicrographs of airway sections in the three treatment groups after 3 weeks and 6 weeks of exposure.
(a) In the large airways, basement membrane thickness is increased after 6 weeks of treatment in the CA group compared with the other two groups. (b) In the small airways, airway smooth muscle is increased after 3 weeks in the CA group compared with the other two groups. After 6 weeks, airway smooth muscle is thicker than that after 3 weeks in both the CA and COD groups. (hematoxylin and eosin, ×400).
CA, citric acid; SA, saline solution; COD, citric acid with codeine pretreatment.

rapid expiration after deep inspiration. The bronchi and intrathoracic trachea are compressed and narrowed during forced rapid expiration of coughing. This narrowing results from a transmural pressure gradient between extraluminal and intraluminal pressures (Langlands, 1967). The lung and airway cells, including ASM cells, are exposed to the mechanically dynamic environment. Cellular functions and development of the respiratory system are influenced by mechanical stimuli (Hasaneen et al., 2005; Edwards, 2001; Yang et al., 2000). Some studies showed that mechanical stress may act directly to stimulate lung cell growth. After exposure to intermittent stretch, the number of lung epithelial and ASM cells increased and [^{3}H]thymidine incorporation, which was used to determine cell proliferation, was higher; this effect was not mediated by prostaglandins or leukotrienes (Liu et al., 1992; Smith et al., 1994). Moreover, a study found that mechanical stretch can increase calcium ion (Ca^{2+}) influx through stretch-activated channels in human bronchial smooth muscle cells (Ito et al., 2008). It has been considered that abnormal Ca^{2+} mobilization affected ASM cell functions (Amrani and Panettieri, 2002). Therefore, mechanical stretch from chronic cough may upregulate proliferation of ASM cells, which are among the main structural cells in the pathogenesis of airway remodeling.

Aside from the direct effects of mechanical stress that may affect airway structural changes, mechanical stimuli can also regulate mediators related to the process of airway remodeling through various cell

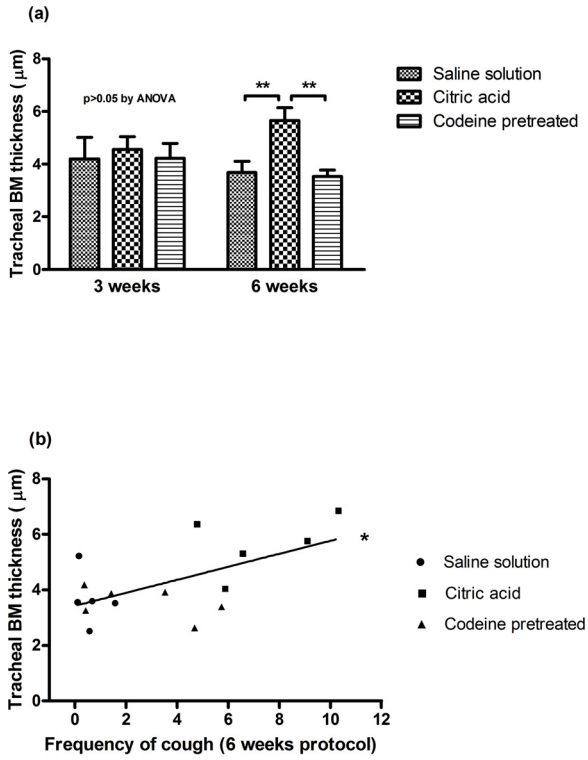


Fig. 5. Histologic changes in tracheal BM thickness in the three treatment groups after 3 weeks and 6 weeks.
(a) Comparison of mean ± SEM tracheal BM thickness among the three treatment groups after 3 weeks and 6 weeks of exposure. (b) Correlation between the frequency of cough and tracheal BM thickness after 6 weeks of treatment exposure.
** $p < 0.01$ by one-way ANOVA, LSD *post hoc* test.
* $p < 0.05$ by two-tailed Pearson's correlation test.
BM, basement membrane.

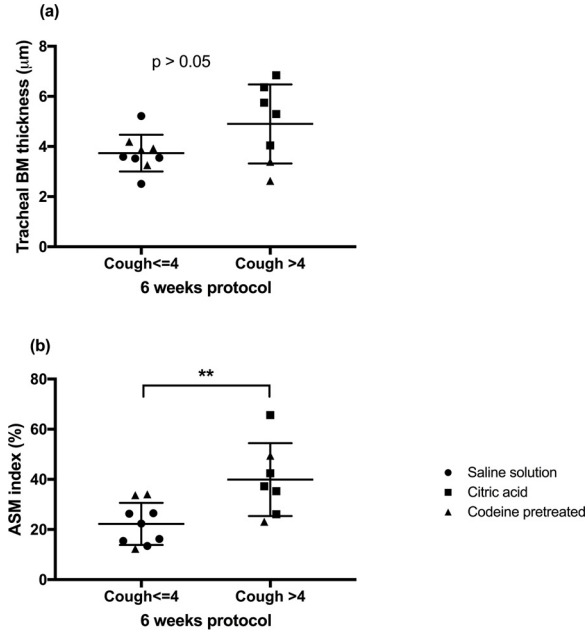


Fig. 6. Comparisons of tracheal BM thickness and of the ASM index between different frequencies of cough after 6 weeks of exposure (a) Tracheal BM thickness is compared between animals who coughed > 4 and ≤4 times. (b) The ASM index is compared between animals who coughed > 4 and ≤4 times. Values are expressed as means ± SEM.
** $p < 0.01$ by Student's *t*-test.

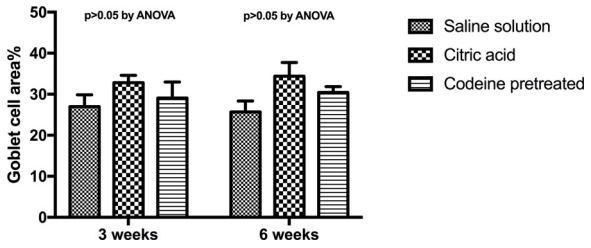


Fig. 7. Histologic changes in goblet cell area in the three treatment groups after 3 weeks and 6 weeks Comparison of goblet cell area among the three treatment groups after 3 weeks and 6 weeks of exposure. Values are expressed as means ± SEM.

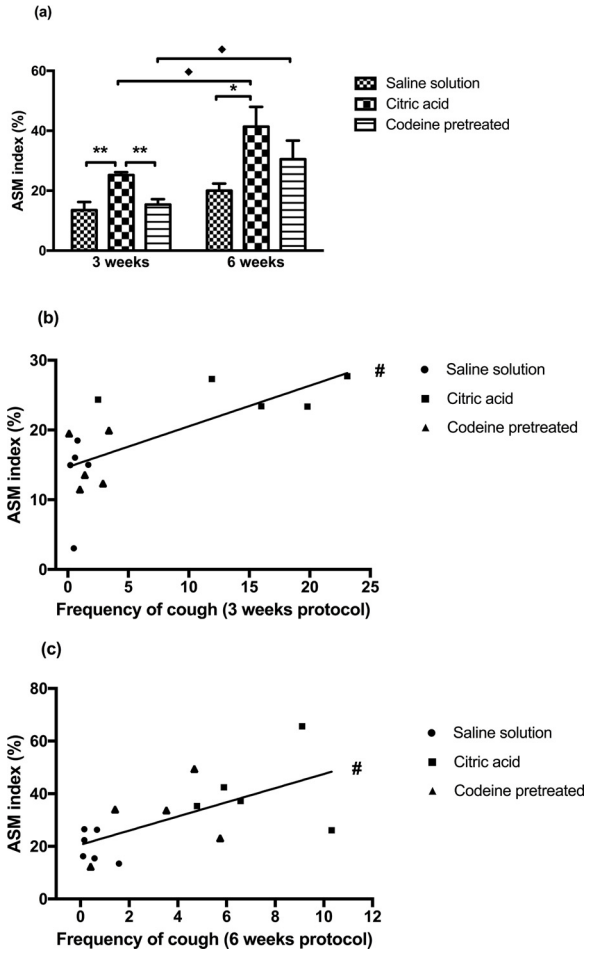


Fig. 8. Histologic changes in the ASM index in the three treatment groups after 3 weeks and 6 weeks in small airways.
(a) Comparison of the mean ± SEM ASM index among the three treatment groups after 3 weeks and 6 weeks of exposure in small airways. Correlation between the frequency of cough and the ASM index (b) after 3 weeks and (c) after 6 weeks of treatment exposure.
** $p < 0.01$, * $p < 0.05$ by one-way ANOVA, LSD *post hoc* test.
◆ $p < 0.05$ by Student's *t*-test.
# $p < 0.01$ by two-tailed Pearson's correlation test ASM, airway smooth muscle.

types, such as bronchial epithelial cells and ASM cells (Mohamed and Boriek, 2010). TGF-β is one of the important mediators that can be expressed and secreted by structural cells in the airways, such as fibroblasts, bronchial epithelial cells, and smooth muscle cells (Mohamed and Boriek, 2010; Kelley et al., 1991; Magnan et al., 1994). TGF-β can induce the expression of extracellular matrix (ECM) components released by activated ASM cells and fibroblasts; increased ECM protein

deposition results in subepithelial fibrosis and basement membrane thickening in asthma (Boxall et al., 2006; Roche et al., 1989). In patients with chronic cough, there were also significant increases in SBM thickness, the level of TGF-β in bronchoalveolar lavage fluid, and ASM cells, and the level of TGF-β was correlated with the increase of SBM thickness (Lee et al., 2001). In addition, TGF-β upregulated the expressions of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in ASM cells in the presence of platelet-derived growth factor (PDGF), thereby enhancing migration of ASM cells towards the epithelium to form new bundles (Ito et al., 2009). Hence, the effect of TGF-β in increasing ASM cell proliferation and hyperplasia eventually affected airway remodeling. Since TGF-β can be released by ASM cells and fibroblasts, the potential autocrine effects are among the possible mechanisms of airway remodeling (Xie et al., 2007). Therefore, the repetitive mechanical and physical effects of coughing bouts on airway cells could activate the release of certain mediators that can enhance chronic cough and provide a positive feedback system for cough persistence.

In the present study, the structural changes were to first occur in the smooth muscles confined in small airways, which have thinner walls that do not have cartilage. The transmural pressure would have a much greater effect on small airways during cough. This is consistent with the clinical manifestations of asthma, in terms of spirometric indices of small airway obstruction appearing earlier in the disease process (Marseglia et al., 2007).

In the CA group, although the small ASM area increased more significantly than in the SA group, there was no remarkable difference when compared with the COD group after prolonged exposure of 6 weeks. There may be two reasons for these results. In the COD group, cough frequency increased during the latter 3 weeks, compared with the first 3 weeks, of the 6-week exposure. Since the result of this study showed a correlation between the frequency of cough and the ASM index in small airways, increased cough frequency may have affected the ASM area in the COD group. Another explanation may be the effect of CA on airway bronchoconstriction in guinea pigs (Ricciardolo, 2001). In the present study, bronchoconstriction may have occurred in both the CA and COD groups during prolonged exposure of up to 6 weeks. The compressive forces on the airways could have affected the properties of smooth muscle cells and led to insignificant differences in the small ASM index between the CA and COD groups. Future studies involving this animal model should take into this into consideration in the experimental design.

The ASM index was increased while the tracheal BM thickness was not increased in the COD group with the 6-week protocol. Furthermore, during the 6-week exposure, cough was well inhibited in the first 3 weeks, but not in the latter 3 weeks. Examining the results of the 3-week protocol, the ASM index increased after 3 weeks of citric acid exposure, while BM thickness did not show significant changes. Thus, although cough was not completely inhibited during the latter 3 weeks in the 6-week protocol in the COD group, the effect of increased cough frequency on BM thickness appeared to be not significant enough to make changes in the tracheal BM in 3 weeks' time. Another possible reason is that the low frequency of cough (i.e. ≤ 4) would cause an effect on ASM that is detectable with the present method, while the effect on tracheal BM was not detectable. The phenomenon was also observed in the present study when comparing between animals with cough number > 4 and ≤ 4. Therefore, a more sensitive method could be adopted in further studies to provide more information about cough.

In summary, the present study showed that repeated cough may lead to airway remodeling, which was associated with cough frequency. The mechanical stress evoked in the airways may contribute to a vicious cycle of cough persistence by inducing airway remodeling.

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