ERJ Express. Published on January 7, 2009 as doi: 10.1183/09031936.00055908

Effect of sildenafil on acrolein-induced airway inflammation and mucus production in rats

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Short running title: Effect of sildenafil on airway inflammation

This study was supported by grants #30425007, 30370627, 30670921 from National Natural Science Foundation of China and 00-722, 06-834 from China Medical Board of New York to Dr. F.Q. Wen.

ABSTRACT

Airway inflammation with mucus overproduction is a distinguishing pathophysiological feature of many chronic respiratory diseases. Phosphodiesterase (PDE) inhibitors have shown anti-inflammatory properties. In this study, we examined the effect of sildenafil, a potent inhibitor of PDE5 that selectively degrades cGMP, on acrolein-induced inflammation and mucus production in rat airways.

Rats were exposed to acrolein for 14 and 28 days. Sildenafil or distilled saline was administered intragastrically prior to acrolein exposure. Bronchoalveolar lavage fluid (BALF) was acquired for cell count and detection of proinflammatory cytokine levels. Lung tissue was examined for cGMP content, nitric oxide (NO)-metabolite levels, histopathologic lesion scores, goblet cell metaplasia and mucin production.

The results suggested that sildenafil pretreatment reversed the significant decline of cGMP content in rat lungs induced by acrolein exposure and suppressed the increase of lung NO metabolites, the BALF leukocyte influx and proinflammatory cytokine release. Moreover, sildenafil pretreatment reduced the acrolein-induced Muc5ac mucin synthesis at both mRNA and protein levels, and attenuated airway inflammation, as well as epithelial hyperplasia and metaplasia.

In conclusion, sildenafil could attenuate airway inflammation and mucus production in the rat model, possibly through the NO/cGMP pathway, and, thus, might have therapeutic potential for chronic airway diseases.

Key words: airway inflammation, goblet cell metaplasia, guanosine 3',5'-cyclic monophosphate, mucin synthesis, phosphodiesterase-5 inhibitor

INTRODUCTION

The lung airway is a primary interface with the outside world and a key target of the environmental stimuli which may trigger inflammatory immune responses. Mucus composed of water, ions, proteins, lipids and mucin glycoproteins coats on the luminal surface of airway as an important part of the innate immune defense system against toxins and pathogens (1). However, excessive mucus accumulation with epithelial hyperplasia could contribute to airway obstruction in patients with chronic obstructive pulmonary diseases (COPD), asthma, or cystic fibrosis (CF), and it is an important factor associated with the morbidity and mortality of these diseases (2). Enhanced airway inflammation with mucus hypersecretion in response to inhaled noxious particles or gases from tobacco smoke or other environmental agents is considered to be a fundamental characteristic in COPD patients (3).

Acrolein, a reactive α,β-unsaturated aldehyde, is a toxin to which humans are exposed through a variety of environmental situations, especially as a component of cigarette smoke and automobile exhaust. It has also been identified as both a product and initiator of lipid peroxidation (4). The toxicity of acrolein in mammal cells is mainly due to its downstream signaling via cellular oxidative stress, such as glutathione (GSH) depletion and subsequently reactive oxygen species (ROS) simulation (5). Recently, it has been evidenced that acrolein is one of the major mediators of cigarette smoke-induced macrophage activation, since it releases both tumor necrosis factor (TNF)-α and interleukin (IL)-8 from human alveolar macrophages (6). Acrolein-induced proinflammatory responses are also mediated through the induction

of cyclooxygenase-2 and prostaglandin in both endothelial cells and human bronchial epithelial cells (7, 8). From in vivo studies, it is well established that exposure to acrolein can induce bronchial hyperresponsiveness, mucus overproduction and lung inflammation in animal models (9, 10, 11). Moreover, acrolein has been recently reported to markedly inhibit the generation of guanosine 3',5'-cyclic monophosphate (cGMP), an important second messenger, in rats (12). However, the underlying mechanisms remain unclear.

cGMP serves as a second messenger participating in various signaling pathways and regulating many aspects of cell function. Besides the classical regulatory role of cGMP in smooth muscle relaxation and vascular tone regulation, numerous other physiological roles have recently been discovered. For example, it has been reported that cGMP signaling is involved in the down-regulation of P-selectin expression and leukocyte recruitment in mice (13). Pharmacologically increasing cGMP levels in CF respiratory epithelial cells can correct several aspects of the downstream pathology in CF (14). cGMP analogues also have shown some anti-inflammatory activity through inhibiting LPS-induced TNF-α secretion in both human monocytes and murine macrophages (15, 16). Furthermore, elevated intracellular cGMP levels have been found to inhibit the TNF-α-induced increase of nitric oxide (NO)-metabolite levels and inducible NO synthase (iNOS) expression in rat tracheal smooth muscle cells (17). The concentration of intracellular cGMP depends on a balance between synthesis by soluble guanylate cyclases (sGCs) and degradation by cyclic nucleotide phosphodiesterases (PDEs). The activity or expression of sGCs and PDEs could be modified in various

situations. For instance, ROS, TNF-α and excessive NO are all indicated to reduce sGC expression (18, 19, 20), while PDE activities are largely increased in oxidative stress and inflammatory processes (21, 22). Therefore, sGC activators or PDE inhibitors may have therapeutic potential in the treatment of inflammatory diseases. Among these pharmacological agents, PDE5 inhibitors appear to be particularly applicable in treating pulmonary diseases, since PDE5 is expressed in high levels in lung tissue and is highly specific for hydrolysis of cGMP (23).

Sildenafil is a potent and selective PDE5 inhibitor that promotes the accumulation of intracellular cGMP. It has been reported to be 240 times more potent than the earlier generation PDE5 inhibitor, zaprinast, against PDE5 (24). Sildenafil is now commonly used in clinical treatment for erectile dysfunction. Recently, it has been intensively studied in the treatment for pulmonary arterial hypertension (25, 26), hyperoxia or hypoxia-induced lung injury (27, 28), and airway diseases (14, 29). Thus, the aim of this study is to examine the effect of sildenafil on airway inflammation, goblet cell metaplasia and mucin synthesis in a rat model induced by acrolein inhalation. The results imply for the first time that the PDE5 inhibitor could attenuate acrolein-induced airway inflammation and mucus production.

MATERIALS AND METHODS

Animals, acrolein exposure and sildenafil treatment

Specific pathogen-free, male Sprague-Dawley rats, weighing 200-250 g, were used in this study. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Sichuan University. A solution of acrolein (Sigma, St. Louis, MO, USA) at a concentration of 3 ppm (6.87 μg/l in sterile water) was nebulized into a 0.12 m^3 (40 x 50 x 60 cm) exposure chamber with an air flow rate of 30 l/min. The concentration of acrolein in the chamber was monitored using the method previously described (30). Briefly, chamber air was passed through a series of two glass-fritted impingers, each of which contained a solution of ethanol (49.4%), trichloroacetic acid (29.7 M), 4-hexylresorcinol (50 mM) and mercuric chloride (2.1 mM). The absorbance was determined at 605 nm by spectrophotometry 1 h after collection. The actual acrolein vapor concentration in this study was found to be 2.46 ± 0.39 ppm. The exposure time was 6 h/day, 7 days/week, for either 14 or 28 days. Control rats were exposed to filtered air under similar conditions. Viagra (sildenafil citrate) tablets were crushed into a fine powder, and 25 mg/kg sildenafil for each rat was suspended in 1 ml saline and then administered intragastrically 0.5 h before acrolein exposure.

Tissue preparation

On day 14 and day 28, rats were anesthetized intraperitoneally with 3 ml/kg chloral hydrate and then sacrificed by exsanguination from the abdominal aorta. The trachea was cannulated, and the chest cavity was opened by a midline incision. The left lung

was used for bronchoalveolar lavage (BAL). The right middle lobe was fixed and embedded in paraffin for use in morphometric and histochemical studies. The right upper and lower pulmonary lobes were snap frozen in liquid nitrogen and stored at -80ºC for homogenization in the following multiple assays.

cGMP and nitrite/nitrate measurement

cGMP generation in rat lungs was measured by iodine $(I)^{125}$ radioimmunoassay, using a cGMP assay kit (Nuclear Medical Laboratory, Shanghai University of Traditional Chinese Medicine, Shanghai, China) with the reagents provided according to the manufacturer's instructions. Briefly, 50 mg of the snap-frozen lung tissue was homogenated in 2 ml ice-cold 50 mM sodium acetate buffer (pH 4.75) and then precipitated by addition of 2 ml 100% ethanol. After centrifugation at 3,500 rpm for 15 min at 4 °C, the supernatant was collected and kept on ice. The remaining precipitate was washed again with 75% ethanol and centrifuged. The second supernatant was mixed with the first one and dried at 60°C, and it was then re-dissolved in 1-ml acetate buffer. Then 100 μl solution of each sample was incubated with an acetylating reagent (triethylamine/acetic anhydride = 2:1), ^{125}I-cGMP and rabbit antiserum against cGMP overnight at 4 °C. Rabbit serum and sheep anti-rabbit IgG were added to the mixture, which was then incubated for 3 hours at room temperature. After centrifugation at 3,000 rpm for 5 min, the pellets were counted for radioactivity by a γ counter. cGMP contents were expressed as picomoles per milligram fresh weight (pmol/mg FW).

The nitrite and nitrate concentration, an indicator of NO synthesis, was measured in lung homogenate as previously described (31). Briefly, the frozen lungs were homogenized in 10 mM Tris buffer (pH 7.4) containing 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The nitrate in the supernatant was first reduced to nitrite by incubation with nitrate reductase and nicotinamide adenine dinucleotide phosphate (NADPH). Then total nitrite concentration in tissue sample was measured spectrophotometrically at 550 nm through the Griess reaction. The protein concentration in the lung homogenate was determined by the BCA method (Pierce, Rockford, IL, USA). Data were expressed as millimicromoles per milligram protein (nmol/mg protein).

Bronchoalveolar lavage fluid (BALF) and cell count

After rats were exsanguinated via the abdominal aorta, the trachea was cannulated, and the chest cavity was opened by a midline incision. The right main-stem bronchus was ligated, and the left lung was lavaged with 2 ml followed by two times of 1 ml PBS containing 5 mM EDTA, 5 mM DTT, and 5 mM PMSF. Fluid recovery was always above 85%. Total cell counts were obtained in 0.5 ml BALF aliquots using a hemocytometer. The remaining BALF samples were centrifuged at 15,000 g for 10 min, and the supernatants were removed and stored at -80°C for the cytokine enzyme-linked immunosorbent assay (ELISA). The deposited cells were re-suspended in 0.2 ml PBS, and the differential cell counts were determined in cytospin preparations stained with Wright-Giemsa, counting 200 cells from each animal. An experienced

investigator who was blinded to the experimental conditions did all the enumerations based on standard morphological criteria.

Cytokine detection in BALF and lung homogenate

Lung homogenate was prepared as described in the nitrite/nitrate measurement. TNF-α and CINC-1 (rat homologue for human IL-8) levels in the BALF and lung homogenate were determined using commercially available ELISA kits for rat cytokines (R&D Systems, Minneapolis, MN, USA). The detection limit for the TNF-α assay was less than 5 pg/ml, and the minimum detectable dose of rat CINC-1 was 1.1 pg/ml.

Lung histopathology and immunohistochemistry

Lung tissue was fixed in 4% formaldehyde (pH 7.4), embedded in paraffin, cut into 4 μm-thick sections and then stained with hematoxylin and eosin (H&E) and Alcian blue and periodic acid-Schiff (AB/PAS). The degree of lung inflammation was evaluated by the previously described method (32). Briefly, lung lesions, including alveolar septal infiltrates, perivascular infiltrates, combined bronchus-associated lymphoid tissue hyperplasia, and peribronchiolar infiltrates, were subjectively graded on a numeric scale of 1–4, corresponding to minimal, mild, moderate and marked severity by an experienced pathologist who was blinded to the treatments. Overall histopathologic scores were generated from the average of the individual lesion scores for each rat. For the immunohistochemical detection of Muc5ac, the sections were stained with mouse monoclonal antibody to Muc5ac (clone 45 M1, 1:200; Neomarkers, Fremont, CA,

USA), using the VECTASTAIN® ABC kit (Vector laboratories, Burlingame, CA, USA). Images of the small airways that were smaller than 800 μm in diameter (10) were recorded by a semiautomatic imaging system. At least five complete airways per rat were examined. Percentages of positive stained area, by either AB/PAS or Muc5ac antibody, to the total airway epithelial area were measured by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Western blot analysis

Lung and stomach homogenates were prepared in lysis buffer, containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM NaF, 2 mM EDTA, 0.1% SDS, and a protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN, USA). Western blot analysis for Muc5ac was performed as previously described (33) with a few modifications. Briefly, 100 μg total protein for each sample except the stomach sample, which contained 20μg total protein, was separated by SDS-polyacrylamide gel electrophoresis in 6% acrylamide-bisacrylamide (60:1) gel for 4 h and then transferred electronically to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated with a 1:1000 dilution of mouse monoclonal antibody against Muc5ac (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The signals were developed using Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) from the frozen tissue. First-strand cDNA was synthesized from 5 μg of total RNA for each sample using MMLV reverse transcriptase (MBI Fermentas Inc, Ontario, Canada) and random hexamer primer, according to the manufacturer's instructions. The PCR program for both Muc5ac and β-actin was initiated by a 2 min denaturation step at 94°C, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 5 min. Primers for Muc5ac PCR (500 bp) were (forward) 5'-GCT CAT CCT AAG CGA CGT CT-3' and (reverse) 5'-GGG GGC ATA ACT TCT CTT GG-3'. Primers for β-actin PCR (200 bp) were (forward) 5'-CCT CAT GAA GAT CCT GAC CG-3' and (reverse) 5'-ACC GCT CAT TGC CGA TAG TG-3'. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. Densitometry was carried out using a Bio-Rad ChemiDoc image acquisition system and Quantity One (v4.6) quantitation software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All values were expressed as mean ± SD. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's HSD test (equal variances) or Dunnett's T3 test (unequal variances) for *post hoc* multiple comparisons (SPSS for Windows version 13.0, Chicago, IL, USA). A significant difference was accepted at $P < 0.05$.

RESULTS

Effect of acrolein exposure and sildenafil treatment on levels of cGMP and NO-metabolites in rat lungs

Previously, it was shown that acrolein inhibited cGMP generation in rat plasma (12). To test whether cGMP levels are altered in the lung by acrolein and sildenafil, rats pretreated with sildenafil or not were exposed to 3.0 ppm acrolein for either 14 or 28 days, and the cGMP levels in lung homogenate were examined using radioimmunoassay. As shown in figure 1A, acrolein significantly decreased (nearly 50%) the cGMP levels in the lungs of the acrolein-exposed rats compared with the control rats ($P < 0.05$; n = 4-6). No significant difference was found among the control groups, the groups treated with sildenafil alone and the groups treated with acrolein plus sildenafil at both day 14 and day 28. Meanwhile, there was a significant difference between the groups exposed to acrolein only and the groups treated with acrolein plus sildenafil, which suggested that the decline of cGMP levels induced by acrolein exposure could be restored by sildenafil administration.

To determine whether NO synthesis in the lung was also changed by acrolein exposure and sildenafil intervention, the levels of stable end-products of NO metabolism (nitrite/nitrate) were examined. The results demonstrated that nitrite and nitrate levels in rat lungs were significantly elevated after subchronic acrolein inhalation, and a trend toward even higher NO metabolite levels was observed in rats exposed to acrolein for 28 days compared to those for 14 days. Sildenafil administration markedly reduced the elevated NO-metabolite levels induced after

acrolein exposure at both day 14 and day 28, indicating that the acrolein inhalation-induced excessive NO production could be attenuated by sildenafil treatment (fig. 1B).

Effect of sildenafil on acrolein-induced leukocyte influx in BALF

To investigate the effect of sildenafil on acrolein-induced leukocyte influx in rat airways, total and differential cell counts were performed in the BALF. Total BALF leukocyte counts were significantly higher in acrolein-exposed groups than those in control groups at both day 14 and day 28 but decreased over time (fig. 2A). BALF cell differential counts revealed that the numbers of lymphocytes did not differ among all treated and control groups (fig. 2D). In contrast, the numbers of neutrophils and macrophages in the BALF were significantly increased after acrolein inhalation for 14 and 28 days, as compared with the controls (fig. 2B and fig. 2C). In the acrolein-exposed groups, macrophage accumulation remained elevated for the duration of the subchronic exposure period, whereas the numbers of neutrophils tended to decrease over time. Sildenafil administration remarkably reduced the acrolein-induced recruitment of neutrophils and macrophages in the BALF, and there were significant differences of neutrophil and total cell counts in sildenafil treatment between 14 and 28 days of acrolein exposure (fig. 2). The results suggested that sildenafil treatment could partially suppress the increase of inflammatory cell influx in the BALF induced by acrolein exposure.

Effect of sildenafil on acrolein-induced proinflammatory cytokine release in BALF and lungs

Since acrolein was previously reported to release both TNF-α and IL-8 from human macrophages (6), ELISA for TNF-α and CINC-1 (rat analogue of human IL-8) in the BALF and lung tissue was used to determine the effect of sildenafil on acrolein-induced proinflammatory response in rats. After acrolein exposure for 14 and 28 days, TNF-α and CINC-1 levels were both significantly elevated in the BALF (fig. 3A, C) and the lung homogenate compared to the controls (fig. 3B, D). Intragastrical administration of sildenafil before acrolein challenge significantly reduced the increase of TNF-α and CINC-1 release induced after acrolein inhalation in the BALF, as well as in the lung tissue (fig. 3). No statistically significant differences in TNF-α and CINC-1 levels were found in sildenafil treatment between 14 and 28 days of acrolein exposure.

Effect of sildenafil on histopathological changes in rat airways induced by acrolein exposure

Histopathological changes of rat airways were examined by H&E and AB/PAS. After the repeated inhalation of acrolein, thickening of the airway epithelium, peribronchial inflammatory cell infiltration and lumen obstruction by mucus and cell debris could be detected by H&E staining (fig. 4A). Inflammatory lesion scores were markedly increased in the acrolein-exposed rats, which were significantly inhibited by sildenafil pre-administration (fig. 4B). Moreover, a prominent increase in the numbers of goblet cells along the airway surface epithelium, as determined by AB/PAS, was observed

after acrolein exposure (fig. 5A). Since goblet cell metaplasia extending to the small airways is a distinguishing feature of the lungs in acrolein-exposed rats (10), the percentages of the AB/PAS-positively stained area to the total epithelial area in small airways were measured. The results suggested that the increase of AB/PAS staining in airway epithelium, induced by acrolein exposure, was significantly inhibited by sildenafil treatment on both day 14 and day 28. However, rats treated with acrolein plus sildenafil for 28 days tended to have a slight increase in pathological lesions as compared with the rats under the same treatment for 14 days, but the difference was not statistically significant (fig. 4B and fig. 5B). Taken together, these results implied that sildenafil could effectively attenuate, though failed to fully blocked, acrolein-induced airway inflammation and goblet cell metaplasia in rats.

Effect of sildenafil on acrolein-induced Muc5ac mucin synthesis in rat lungs

Muc5ac is the predominant mucin gene expressed in goblet cells. In order to investigate the effect of sildenafil on acrolein-induced Muc5ac mucin synthesis in rat airway epithelium, immunohistochemistry for Muc5ac was performed on lung sections. Consistent with AB/PAS staining results, the area positively stained by anti-Muc5ac monoclonal antibody in airway epithelium was increased markedly after acrolein exposure for 14 and 28 days, which could be significantly attenuated by sildenafil pretreatment (fig. 6). To quantify the changes of Muc5ac protein levels in rat lungs, western-blot analysis was performed using lung homogenates. Results showed that

sildenafil treatment significantly reduced the strong signal of immunostained band for Muc5ac in the rat model (fig. 7A, B; $P < 0.05$, n = 3); meanwhile there were no significant differences in sildenafil treatment between 14 and 28 days of acrolein exposure. To further examine Muc5ac gene expression at transcriptional level, the steady-state mRNA levels for Muc5ac and β-actin in lung tissue were analyzed by RT-PCR (fig. 7C) and quantified by densitometry (fig. 7D). The acrolein-induced accumulation of Muc5ac mRNA in rat lungs was also significantly inhibited by sildenafil administration ($P < 0.05$; n = 3). Taken together, the results suggested that sildenafil could partially reduce acrolein-induced synthesis of Muc5ac at both mRNA and protein levels.

DISCUSSION

In this study, we demonstrated the effect of sildenafil, a PDE5 inhibitor, on airway inflammation and mucus overproduction induced by acrolein inhalation in rats. Repeated exposure to acrolein, a major toxin and irritant in cigarette smoke, significantly decreased cGMP levels in lung tissue and increased leukocyte cell counts, as well as proinflammatory cytokine release in the BALF. Acrolein also induced lung NO-metabolite accumulation, airway inflammation, goblet cell hyperplasia, and Muc5ac mucin synthesis in this rat model. The major finding of this study is that sildenafil treatment significantly attenuated the above pathological changes induced by acrolein exposure. The results also imply that the inhibitory effect of sildenafil on airway inflammation may act through the NO/cGMP pathway.

Cyclic nucleotide second messengers (cAMP and cGMP) play a central role in diverse signal transduction and regulation of various pathophysiological responses. Intracellular cGMP levels regulate cellular processes by activating protein kinases, directly gating specific ion channels, or altering other intracellular cyclic nucleotide (e.g., cAMP) concentrations through regulation of PDEs (34). At the same time, cGMP levels in tissue are controlled by a balance between the activities of sGCs, which catalyze the generation of cGMP, and the cyclic nucleotide PDEs, which catalyze the degradation of cGMP. These biochemical reactions are often affected by endogenous or exogenous compounds, including hormones, neurotransmitters, and toxins, etc. (35). Acrolein is one of the most reactive α,β-unsaturated aldehydes present in tobacco smoke and is also formed during lipid peroxidation (4). The acute toxicological effects

of acrolein have been extensively studied, but the effects of subacute long-time exposure of acrolein on the NO/cGMP pathway are yet unknown. A recent research reported that acrolein administration (4mg/kg) for 3 or 7 days produced an increase in blood pressure associated with a decrease of cGMP levels in rat plasma (12). Our study discovered that 3.0 ppm acrolein inhalation for 14 and 28 days remarkably increased lung levels of NO-metabolites by 3 to 5 times while significantly inhibiting cGMP generation by nearly 50% in rats (fig. 1). Despite the presence of ample amounts of NO that could activate the sGC in rat lungs, it is interesting that cGMP levels were markedly reduced. One of reasons may be that excessive NO synthesis from iNOS could contribute to sGC down-regulation in the lung (20, 36). Since the acrolein-induced decline of cGMP levels could be reversed by sildenafil treatment in this study, PDE5 activity enhancement could also cause the cGMP reduction. Further study is warranted to more thoroughly address whether sGCs or PDEs or both were affected by acrolein exposure.

There is growing evidence that cGMP reduction is associated with lung injury and inflammation. It has recently been reported that sGC expression is reduced in lungs of mice sensitized and challenged with ovalbumin (OVA) (36). In a murine model of LPS-induced lung injury, sGC inhibition worsens lung inflammation, which can be reversed by a cell-permeable cGMP analogue (37). In CF respiratory epithelial cells, increasing cGMP levels correct multiple aspects of the CF pathological cascade, including defective protein glycosylation, bacterial adherence and proinflammatory responses (14). In the present study, acrolein exposure caused a significant cGMP

reduction in rat lungs, along with inflammatory responses characterized by leukocyte influx, TNF-α and CINC-1 release and increased lung histopathologic lesions. Therefore, it is worthwhile to test whether cGMP enhancers can exert some inhibitory effects on acrolein-induced airway inflammation. Since PDE5 is the predominant PDE enzyme responsible for degradation of cGMP in the lung, PDE5 inhibitors appear to hold potential for treating pulmonary disease. In primary cultures of rat alveolar epithelial cells, blockading PDE5 with zaprinast can reduce LPS-mediated IL-6 and TNF-α biosynthesis (38). In tracheal smooth muscle cells, zaprinast also inhibits the TNF-α induced increase of iNOS expression and NO generation and reverses the TNF-α induced reduction of sGCs (17). Compared with zaprinast, the earlier generation of PDE5 inhibitor, sildenafil is much more potent at inhibiting PDE5. Recently, sildenafil has shown promise in clinical studies for treatment of lung diseases, such as primary or COPD-associated pulmonary hypertension (25, 26). It also shows anti-inflammatory effects in certain animal models. For instance, in guinea pig models of airway disease, sildenafil inhibits airway hyperreactivity, leukocyte infiltration, and NO generation after allergen exposure and exposure to endotoxin (29). In mice challenged with bacterial aerosol, sildenafil reduces lung neutrophil infiltration (14). Our data showed that sildenafil pretreatment significantly reduced acrolein-induced leukocyte influx and NO generation, as well as TNF-α and CINC-1 release, in both the BALF and lung tissue, which could be attributed to the consequent reversed intracellular cGMP levels in the lung.

A link between airway inflammation and mucus production has been well suggested by both experimental and clinical observations over the past 15 years (1). In healthy humans and animals, peripheral airways contain few or no identifiable goblet cells, but the number of AB/PAS positive goblet cells and the amount of mucin produced within the surface of the epithelium are greatly increased after inflammatory stimulation (2). Inflammatory mediators released during airway inflammation can interact with airway epithelial cells and activate intracellular signaling pathways, resulting in overproduction of mucins. TNF-α can activate nuclear factor (NF)-κB in airway epithelium, leading to goblet cell metaplasia and mucus production in mice (39). IL-1β is capable of inducing MUC5AC expression in human airway epithelium mediated by cyclooxygenase-2 and prostaglandin E2 (40). In addition, NO has recently been reported to trigger protein kinase C (PKC) activation, resulting in MUC5AC over-expression in respiratory epithelial cells (41). In the present study, sildenafil significantly attenuated acrolein-induced goblet cell metaplasia in rat airway epithelium, as suggested by histological analysis; and decreased Muc5ac synthesis at both mRNA and protein levels in the lung, as evidenced by RT-PCR and western blotting. The inhibitory effects of sildenafil on acrolein-augmented Muc5ac mucin synthesis in rat airways may be ascribed to the combined effects of the down-regulation of proinflammatory mediators, such as TNF-α, CINC-1 and NO. However, one limitation of this study is that the secretion of mucin was not measured. Intracellular mucin is synthesized and stored in cytoplasmic membrane-bound secretory granules. Upon external stimulation, the granules translocate to the cell periphery, where the

granule membranes fuse with the plasma membrane and the mucin content is released into the airway lumen in a process of exocytosis regulated by a variety of interactive proteins or chemicals (42). Mucus hypersecretion ultimately leads to airway plugging and bronchial obstruction. Further study is worthwhile to test whether mucin exocytosis is also altered by acrolein and sildenafil treatment.

In summary, subchronic inhalation of acrolein vapor could cause pronounced airway inflammation in rats characterized by leukocyte influx, proinflammatory cytokine release, NO metabolite accumulation and goblet cell metaplasia with augmented mucin synthesis, which was associated with a significant reduction in lung cGMP levels. We demonstrated for the first time the benefits of sildenafil, a PDE5 inhibitor, in attenuating acrolein-induced airway inflammation and mucus production, possibly through restoring the cGMP pathway. Since cigarette smoking is the most important risk factor initiating the development of COPD, and among those chemical components of cigarette smoke, acrolein appears to play a critical role in mediating inflammatory processes, the results suggested that sildenafil might have therapeutic potential for treating chronic airway diseases. The relative tissue specificity of sildenafil, its tolerable side effects, and its proven safety makes it an attractive therapeutic option and warrants further study.

ACKNOWLEDGEMENTS

This study was supported by grants #30425007, 30370627, 30670921 from National Natural Science Foundation of China and 00-722, 06-834 from China Medical Board of New York to Dr. F.Q. Wen.

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FIGURE LEGENDS

Fig. 1 Levels of cGMP and nitrite/nitrate in rat lungs. Rats were exposed to 3.0 ppm acrolein as described in the METERIALS AND METHODS section. cGMP levels were determined by I^{125} radioimmunoassay (A). Nitrite/nitrate levels in the lung homogenate were measured with Griess reagent after the conversion from nitrate to nitrite (B). Values are expressed as means ± SD (n = 4-6). *$P < 0.05$, significant difference from the control group. $^{\#}P < 0.05$, significant difference from the acrolein-exposed group. $^{\&}P < 0.05$, significant difference between 14 and 28 days of acrolein exposure with or without sildenafil treatment.

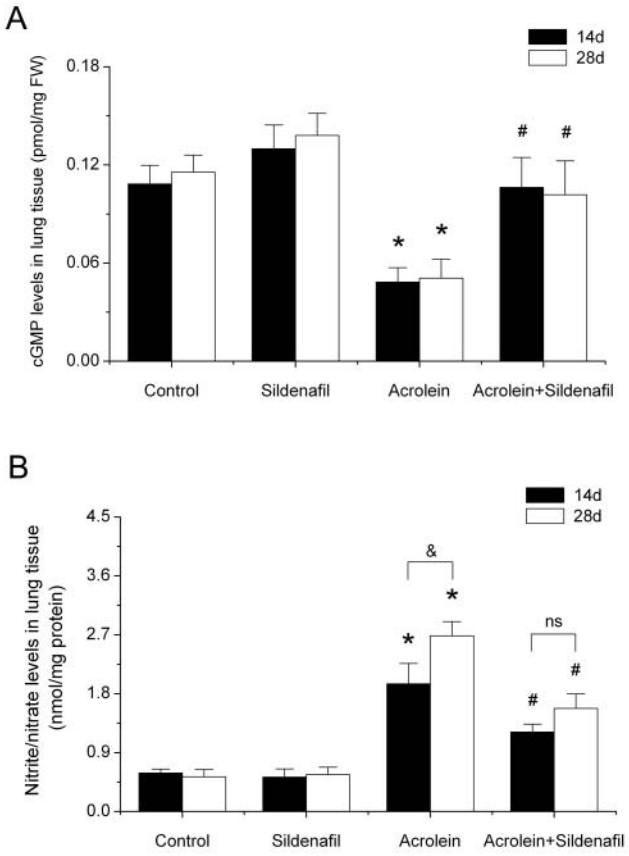


Fig. 2 Total and differential cell counts in the BALF. The numbers of total cells (A), neutrophils (B), macrophages (C) and lymphocytes (D) in the rat BALF were measured at day 14 and day 28 after acrolein challenge. Values are expressed as means ± SD (n = 4-6). *$P < 0.05$, significant difference from the control group. $^{\#}P < 0.05$, significant difference from the acrolein-exposed group. $^{\&}P < 0.05$, significant difference between 14 and 28 days of acrolein exposure with or without sildenafil treatment.

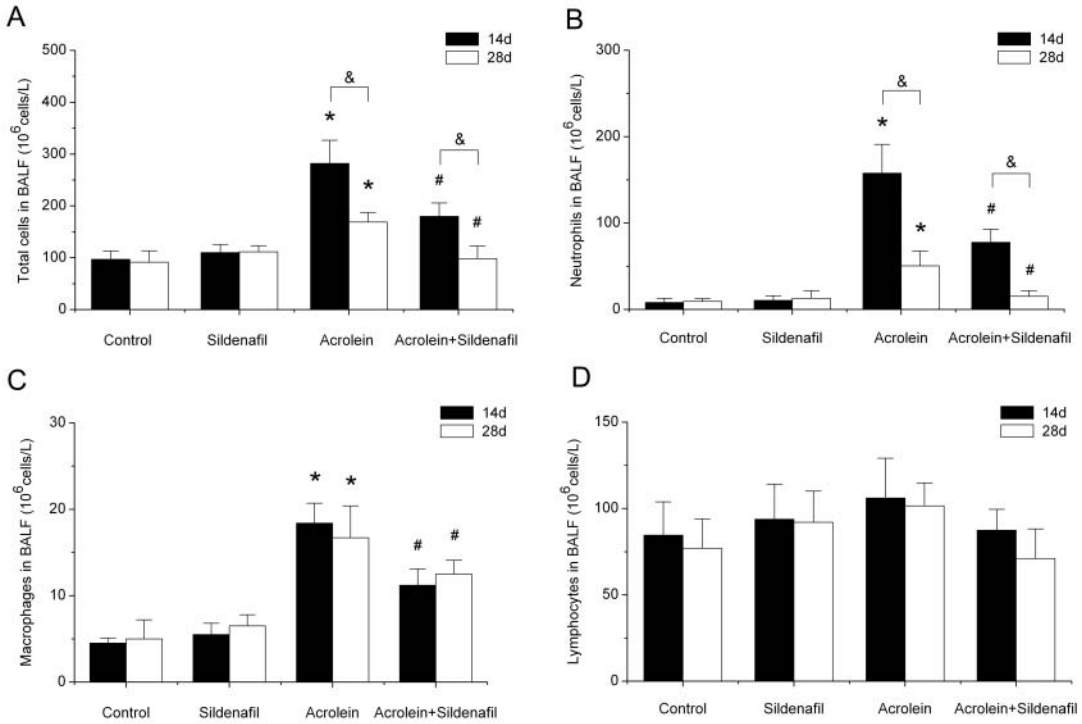


Fig. 3 Levels of TNF-α and CINC-1 in the BALF and lungs. Cytokines were determined by ELISA in both the BALF (A, C) and lung homogenate (B, D). Values are expressed as means ± SD (n = 4-6). *$P < 0.05$, significant difference from the control group. $^{\#}P < 0.05$, significant difference from the acrolein-exposed group.

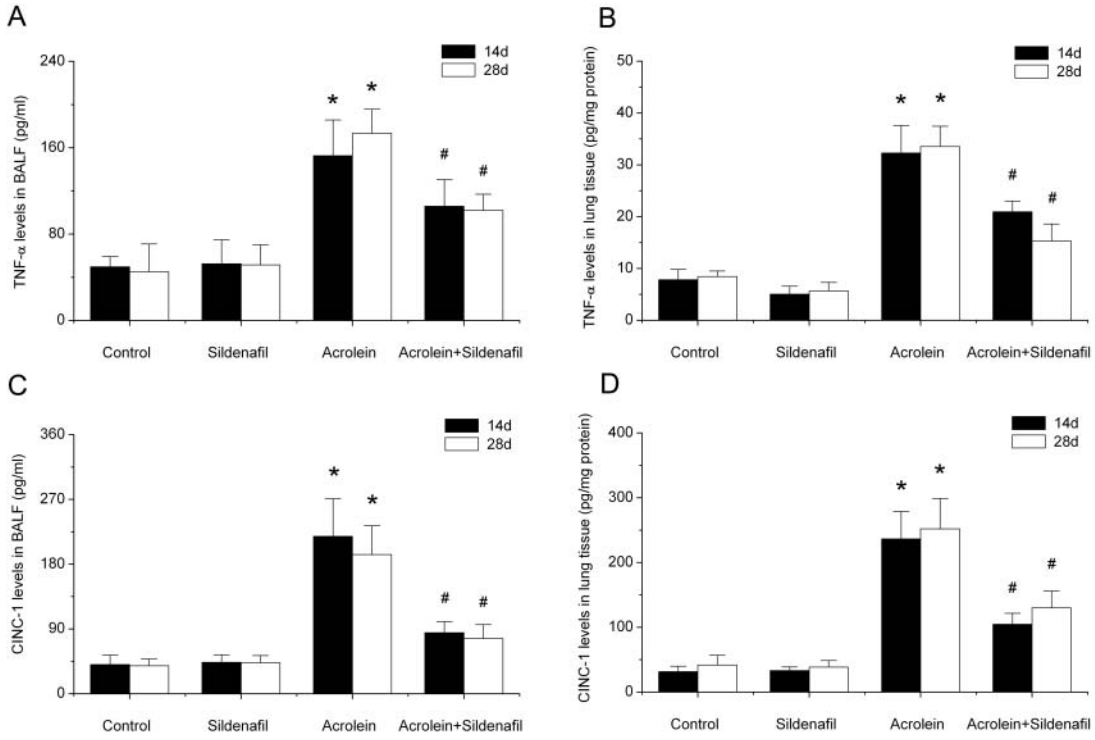


Fig. 4 Histological changes in rat airways. A: lung tissues from control rats at day 14 (a) and day 28 (c), rats treated with sildenafil alone at day 14 (b) and day 28 (d), rats exposed to acrolein-only at day 14 (e) and day 28 (g), and rats treated with acrolein plus sildenafil at day 14 (f) and day 28 (h) were all analyzed by H&E staining. Bar = 100 μm. B: lung inflammatory lesions for each animal were scored as described in the MATERIALS AND METHODS section. Values are expressed as means ± SD (n = 4-6). *$P < 0.05$, significant difference from the control group. $^{\#}P < 0.05$, significant difference from the acrolein-exposed group.

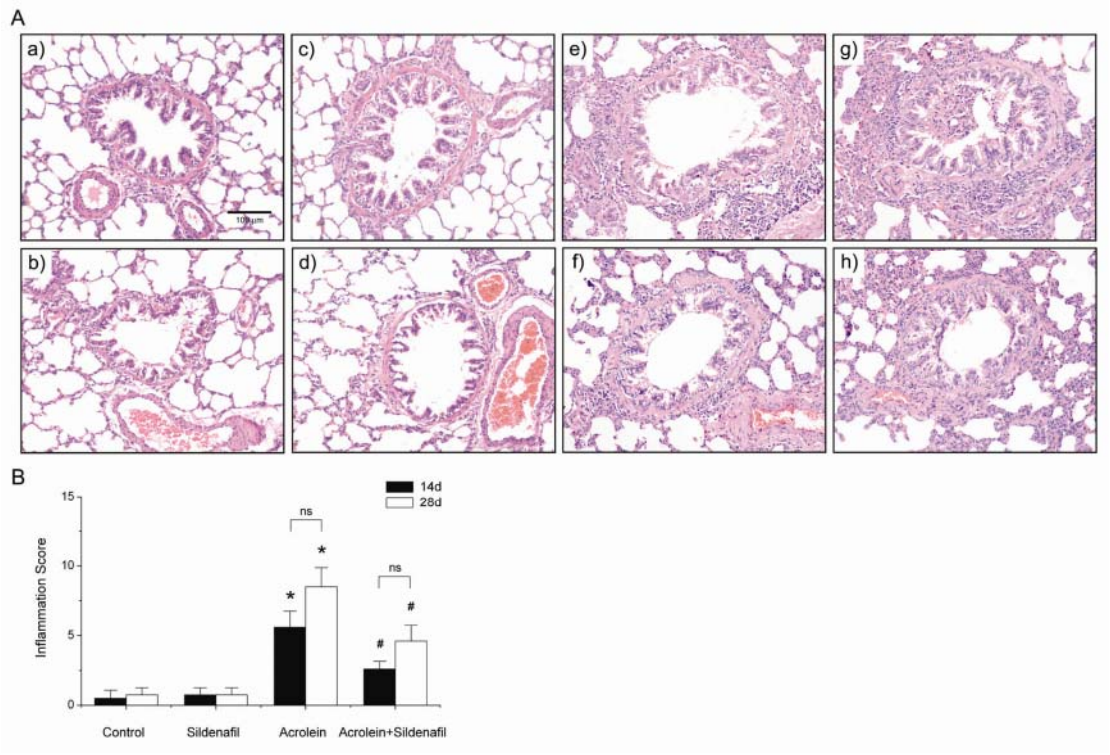


Fig. 5 Changes of AB/PAS staining in rat airways. A: lung tissues from control rats at day 14 (a) and day 28 (c), rats treated with sildenafil alone at day 14 (b) and day 28 (d), rats exposed to acrolein at day 14 (e) and day 28 (g), and rats treated with acrolein plus sildenafil at day 14 (f) and day 28 (h) were analyzed by AB/PAS staining. Bar = 100 μm. B: the percentages of AB/PAS positive staining area to total epithelial area in rat airways. Values are expressed as means ± SD (n = 4-6). *$P < 0.05$, significant difference from the control group. $^{\#}P < 0.05$, significant difference from the acrolein-exposed group. $^{\&}P < 0.05$, significant difference between 14 and 28 days of acrolein exposure with or without sildenafil treatment.

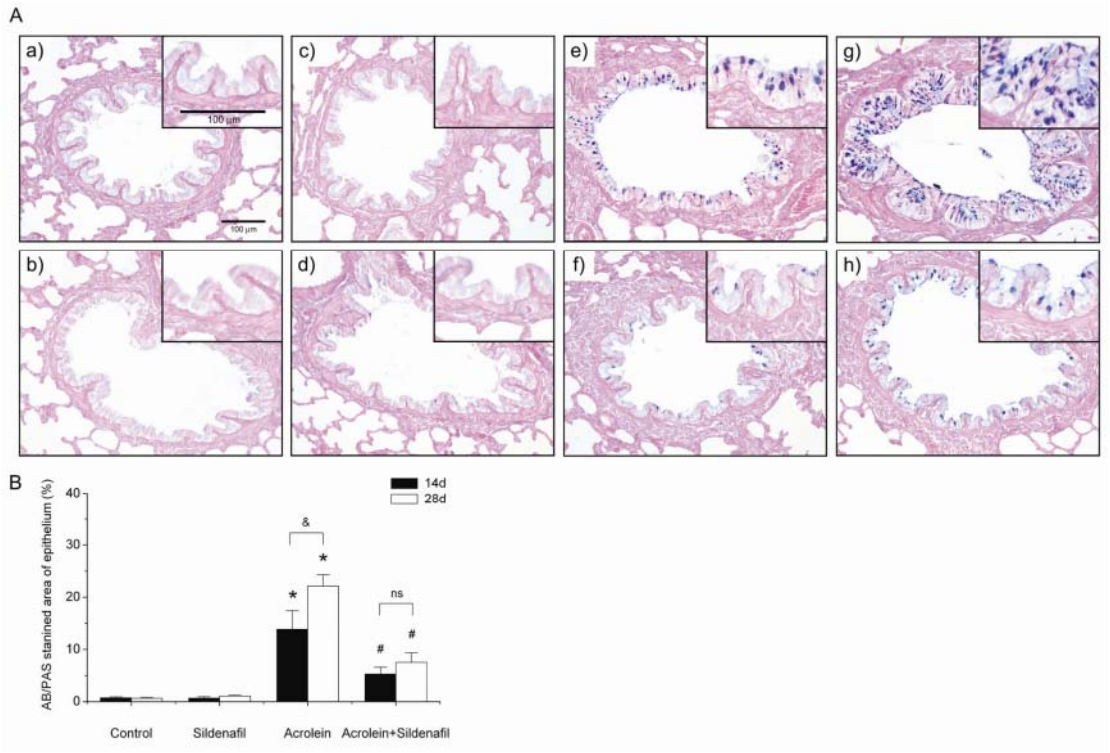


Fig. 6 Changes in Muc5ac immunohistochemical staining in rat airways. A: lung tissues from control rats at day 14 (a) and day 28 (c), rats treated with sildenafil alone at day 14 (b) and day 28 (d), rats exposed to acrolein at day 14 (e) and day 28 (g), and rats treated with acrolein plus sildenafil at day 14 (f) and day 28 (h) were analyzed by Muc5ac immunohistochemistry. Bar = 100 μm. B: the percentages of Muc5ac positive staining area to total epithelial area in rat airways. Values are expressed as means ± SD (n = 4-6). $^{*}P < 0.05$, significant difference from the control group. $^{\#}P < 0.05$, significant difference from the acrolein-exposed group. $^{\&}P < 0.05$, significant difference between 14 and 28 days of acrolein exposure with or without sildenafil treatment.

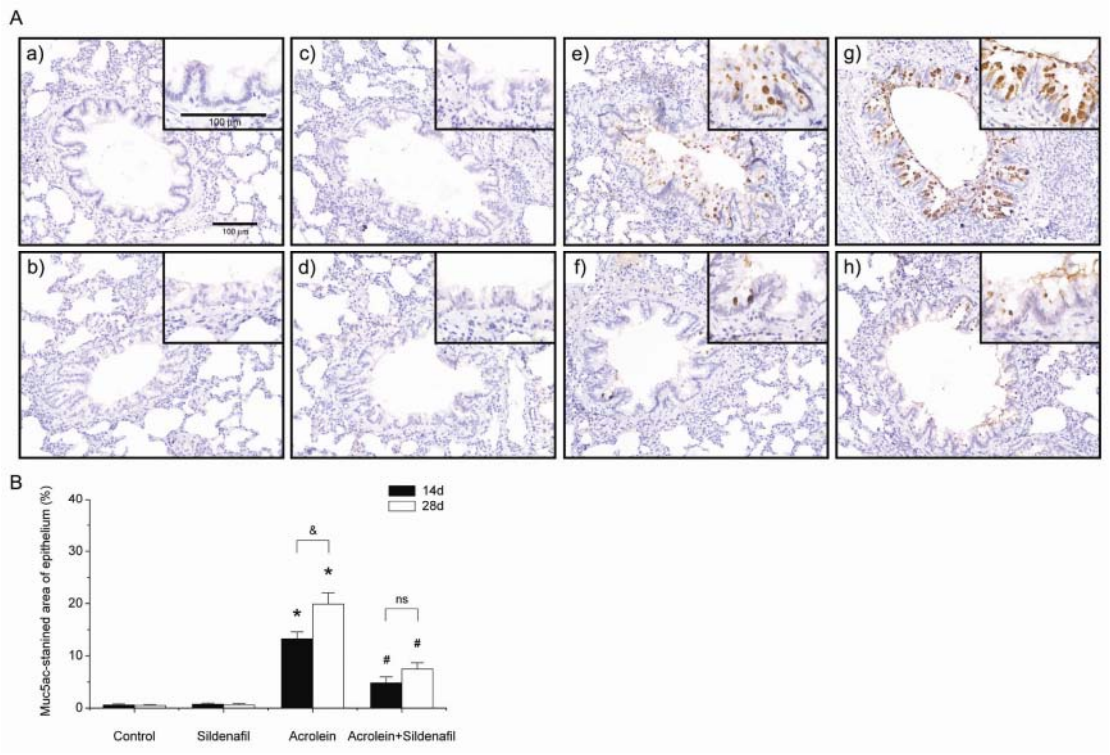
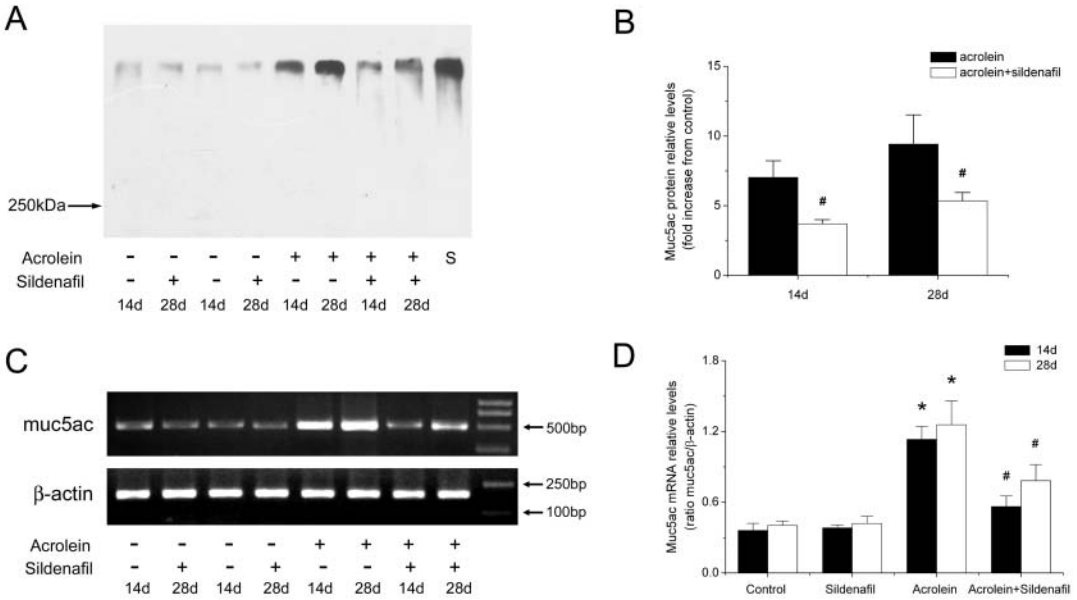


Fig. 7 Changes of Muc5ac mRNA and protein levels in rat lungs. Muc5ac protein levels were measured by western blotting (A) and quantified densitometrically (B). The stomach Muc5ac glycoprotein was used as a positive control (S). The steady-state of mRNA levels for Muc5ac was analyzed by RT-PCR (C) and quantified by densitometry (D). Results shown in A and C are representatives of three independent experiments. Values are expressed as means ± SD (n=3). *$P < 0.05$, significant difference from the control group. $^{\#}P < 0.05$, significant difference from the acrolein-exposed group.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28d
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β-actin
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Muc5ac mRNA relative levels
(ratio muc5ac/β-actin)
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0.0
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Control
Sildenafil
Acrolein
Acrolein+Sildenafil