

ORIGINAL ARTICLE

The effect and mechanism of action of carbocysteine on airway bacterial load in rats chronically exposed to cigarette smoke

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ABSTRACT

Background and objective: Carbocysteine (S-carboxymethylcysteine) is a mucoactive drug with *in vitro* free radical scavenging and anti-inflammatory properties. Several clinical trials have indicated that carbocysteine reduces exacerbation rates in COPD. In the present study, the effect of carbocysteine on the airway load of *Haemophilus influenzae* was assessed in rats chronically exposed to cigarette smoke (CS). In addition, the effects of carbocysteine on airway mucus hypersecretion and mucociliary clearance (MCC) associated with the adherence and clearance of *H. influenzae* were investigated.

Methods: Wistar rats were randomly divided into control, carbocysteine vehicle, CS exposure and carbocysteine treatment groups. After 12 weeks, rats were selected for quantitative inoculation of *H. influenzae*. BAL fluid and lungs were collected aseptically after 3 h for quantitative culture of *H. influenzae*. MCC was measured by quantifying the clearance of ^{99m}Tc-Sc. Goblet cell metaplasia and the presence of mucoid matter were evaluated by Alcian blue/periodic acid-Schiff staining. Mucin 5AC (Muc5AC) expression was detected by western blotting and real-time reverse transcription-PCR.

Results: Exposure to CS increased airway *H. influenzae* load, aggravated mucus hypersecretion and delayed MCC. Treatment with carbocysteine decreased airway *H. influenzae* load, and attenuated airway mucus hypersecretion, with improved MCC associated with adherence and clearance of *H. influenzae*.

Conclusions: These results suggest that carbocysteine may be beneficial in patients with COPD by increasing the clearance of bacteria and decreasing bacterial load.

SUMMARY AT A GLANCE

This study assessed the effect of carbocysteine on airway bacterial load in rats chronically exposed to cigarette smoke. Carbocysteine treatment decreased airway *Haemophilus influenzae* load and attenuated airway mucus hypersecretion. This resulted in improved mucociliary clearance with reduced adherence and more effective clearance of *H. influenzae*.

Key words: bacterial load, carbocysteine, *Haemophilus influenzae*, mucociliary clearance, mucus hypersecretion.

INTRODUCTION

COPD is often aggravated by exacerbations, which can accelerate the decrease in lung function and increase the economic burden of disease, as well as mortality rates.¹ The PEACE study, conducted in China, demonstrated a clinically and statistically significant reduction in exacerbation rates in patients with moderate-to-severe COPD who received carbocysteine over a 12-month period, as compared with those who received placebo.² However, the precise mechanism of action of carbocysteine remains uncertain.

Acute exacerbations of COPD have many causes, but as many as 70% may be due to bacterial infections.³ The local inflammatory response of the host parallels the increase in airway bacterial load,^{4,5} and there is a threshold above which the inflammatory reaction is severe enough to elicit an acute exacerbation.⁶ Mucociliary clearance (MCC) is a non-specific airway defence mechanism. Pathogens can be cleared by MCC, resulting in attenuation of the airway bacterial load. Mucin 5AC (Muc5AC) is a major component of mucus in the respiratory tract and is a vehicle for MCC.⁷ Muc5AC is also closely linked to goblet cell metaplasia and mucus hypersecretion. *Haemophilus influenzae* is the most common pathogen in the lower respiratory tract of COPD patients and adheres to Muc5AC through

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outer membrane proteins P2 and P5.⁸ There is currently no evidence linking airway bacterial load with Muc5AC expression or mucus hypersecretion and MCC *in vivo*.

Carbocysteine is a mucolytic agent that was recently shown to scavenge reactive oxygen species⁹ and reduce neutrophil elastase-induced mucin production *in vitro*.¹⁰ Carbocysteine also reduces bacterial adherence to mammalian cells¹¹ and inhibits rhinovirus infection.¹² Cigarette smoke (CS) is the main aetiological factor for COPD and induces emphysematous changes with mucus hypersecretion.^{13–15} Based on these observations, we hypothesized that reduction of bacterial load by treatment with carbocysteine may be of clinical benefit in COPD. This *in vivo* study was performed to examine the effect of carbocysteine on the airway load of *H. influenzae* in rats exposed to CS. In addition, the effect of carbocysteine on airway mucus hypersecretion and MCC associated with the adherence and clearance of *H. influenzae* was investigated. The purpose of the study was to provide an experimental basis for the clinical application of carbocysteine.

METHODS

Materials and reagents

Mouse Muc5AC mAb (clone 45M1) was from Sigma-Aldrich, St Louis, MO, USA. Trizol reagent was from Invitrogen, Carlsbad, CA, USA, real-time reverse transcription (RT)-PCR kits were from TaKaRa Co., Dalian, China, and the ABI 7500 real-time fluorescence quantitative PCR instrument was from ABI, Foster City, CA, USA. *H. influenzae* was kindly provided by Peking Union Hospital, Beijing, China, and the ^{99m}Tc-Sc kit was from Peking Shihong Medicine Research Center, Beijing, China. Cigarettes were Da Qianmen brand (carbon monoxide content 14 mg, tar oil content 15 mg), and carbocysteine was from Guangzhou Baiyunshan Co., Guangzhou, China.

Experimental protocol

Specific pathogen-free, male Wistar rats, 6–8 weeks old and weighing 180 ± 30 g were purchased from China Medical University, Shenyang, China. The animal experiments were approved by the institutional animal care and use committee. Ninety-six rats were randomly divided into four groups of 24 using random number tables. Group A, the normal control group breathed fresh air; group B the carbocysteine vehicle group breathed fresh air, and carbocysteine (500 mg/kg) was administered intragastrically from the sixth week, as in group D; group C, the CS exposure group was exposed to CS in a glass box ($0.8 \times 0.6 \times 0.6$ m) with a 2×2 cm vent hole on top. The rats were exposed to CS twice a day, for 2 h/day, 5 days/week for 12 weeks, to a total of 30 cigarettes/day, at a smoke concentration of 5% (v/v);^{13–15} group D, the carbocysteine treatment group, was exposed to CS as

for group C, and carbocysteine was administered intragastrically 30 min before exposure to CS from the first day of the sixth week.

Tissue preparation and morphometric analyses

After 12 weeks, six rats in each group were selected randomly. After induction of anaesthesia by intraperitoneal administration of chloral hydrate (3 mL/kg, 10%) and sacrifice by exsanguination, the trachea was exposed and cannulated, the right main-stem bronchus was ligated and the left lung was subjected to BAL. The middle lobe of the right lung was formalin-fixed, paraffin-embedded and sectioned for histology. The remainder of the right lung was stored at -80°C for subsequent quantitative (q)PCR and western blot analysis. Morphological alterations in the lungs were observed under an optical microscope at a magnification of $\times 200$, using a cross-line. Mean linear intercept (MLI) (or mean alveolar intercept) and mean alveolar number (MAN) were measured using a HPIAS-100 automated image analyser. For each rat, data were obtained from at least eight fields and the mean values were calculated.

Measurement of pulmonary function

Six rats in each group were randomly selected for pulmonary function measurements according to the methods described by Xu *et al.*¹⁴ Rats were anaesthetized by intraperitoneal administration of chloral hydrate, and a 'Y'-type endotracheal cannula was connected to a flow transducer (type HX200) for measurement of forced expiratory volume in 0.3 s (FEV_{0.3})/FVC.

Immunohistochemistry

Lung sections were deparaffinized and stained with Alcian blue/periodic acid-Schiff (AB-PAS) for detection of mucin glycoproteins and goblet cell metaplasia. Images of the bronchiolar epithelium were obtained at a magnification of $\times 200$. AB-PAS-positive staining in each airway image was semi-quantitatively assessed by an investigator who was blinded to the treatment conditions. The AB-PAS-stained areas were quantified using Image-Pro plus 4.5 software.

Real-time reverse transcription-PCR analysis

Total RNA was prepared from lung tissue of each rat in the four groups, using Trizol (Invitrogen) according to the manufacturer's instructions. Forward and reverse primer sequences for rat Muc5AC were 5'-CTGCCAACCCTTACCG-3' and 5'-GCACAGGCGTCATTCA-3', respectively.¹⁰ GAPDH expression was measured as a housekeeping gene control using rat forward and reverse primer sequences of 5'-GCAAGTTCAACGGCACA-3' and 5'-CATTTGATGTTAGCGGGAT-3', respectively. Total RNA concentrations in each sample were determined by

measurement of absorbance at 260 nm. All samples were assayed in duplicate with each primer set.

For RT, samples were incubated at 37°C for 15 min and heated at 85°C for 5 s in order to inactivate the RT enzyme. SYBR Green I-based real-time PCR was performed on an ABI PRISM 7500 instrument (ABI, Foster City, CA, USA), and each reaction contained 2 µL of cDNA, 800 nmol/L of each primer, 1×ROX reference dye,

1×SYBR Premix Ex Taq (TaKaRa CO, Dalian, China), and nuclease-free water to a final volume of 25 µL. The PCR parameters were initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s and 72°C for 60 s. The efficiency of each primer pair was assessed using serial dilutions of pooled RT products. The expression of Muc5AC mRNA was standardized to that of GAPDH mRNA and data were expressed as the ratio relative expression of Muc5AC mRNA: relative expression of GAPDH mRNA. The threshold cycle (Ct) for each sample was taken as a quantitative measure of the amount of PCR product. The Muc5AC signal was normalized to that of GAPDH and expressed as $\Delta Ct = (Ct_{\text{Muc5AC}} - Ct_{\text{GAPDH}})$. Changes in the Muc5AC signal relative to the total amount of genomic DNA were expressed as $\Delta Ct = Ct_{\text{treatment}} - Ct_{\text{control}}$ and further analysis was performed using the $2^{-\Delta\Delta Ct}$ method.¹⁶

Western blot analysis

Western blot analysis was performed using lung tissue samples from each rat in the four groups. Samples were homogenized in lysis buffer containing 10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, 5 mmol/L ethylenediamine tetra-acetic acid and protease inhibitor cocktail.¹⁴ Protein content was determined by the Coomassie Brilliant Blue method and equal amounts of protein (50 µg) were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membrane by electroblotting for 1 h and membranes were then incubated in 50 mmol/L Tris-HCl, 0.1% Tween-20, 154 mmol/L NaCl, pH 7.5 (TTBS), containing 5% non-fat dry milk and 1% BSA for 1 h. Samples were then incubated overnight in TTBS, 3% BSA, 0.1% sodium azide, containing the anti-Muc5AC mAb (1:500 dilution). After several washes in TTBS, the membranes were incubated for 2 h in TTBS containing goat anti-mouse antibody coupled to horseradish peroxidase. Immunoreactive bands were visualized using an enhanced chemiluminescence kit. Levels of Muc5AC protein were evaluated by measuring the optical densities of the protein bands using Scion Image for Windows image analysis software. As a reference, expression of β-actin was measured in the same protein extracts and did not change.

Measurement of mucociliary function in rats by scintigraphy

Labelled colloid solution was freshly prepared, according to the manufacturer's instructions, and

the amount of labelling was quantified to verify the procedure. Six rats were randomly selected from each group for measurement of mucociliary function. As described by Foster *et al.*,¹⁷ rats were anaesthetized by intraperitoneal injection of ketamine (0.15 g/kg) and suspended by their upper incisors from a rubber band on a 60-degree board. The tongue was gently extended and a 0.1 mL volume of liquid ^{99m}Tc-Sc (200 µCi = 7,400,000 Bq) was delivered into the distal region of the oropharynx. Animals were immediately imaged for 5 min to demonstrate the initial distribution of ^{99m}Tc-Sc from the dorsal aspect, using a gamma-camera. The rats were then permitted to recover and imaged periodically to demonstrate subsequent clearance, by means of positioning and administration of ketamine after 1, 2 and 3 h. To quantify the clearance of ^{99m}Tc-Sc, lung images were stored on a computer and manipulated to identify regions of interest. Activity time plots were constructed for the regions of interest after correction for background and isotopic decay.

Assay of airway bacterial load in the rat model of *Haemophilus influenzae* inoculation

Six rats in each group were randomly selected for *H. influenzae* inoculation. The rats were anaesthetized and intratracheally inoculated with 1.5×10^7 colony-forming units of *H. influenzae* using a blunt-end feeding needle.^{18,19} After 3 h the rats were sacrificed, the trachea was intubated and BAL fluid was collected aseptically for cell counts and bacterial cultures. The lungs were then removed aseptically, homogenized, plated at appropriate dilutions on chocolate agar and incubated at 37°C for 24 h in a candle extinction jar for quantitative bacterial culture.^{18,19}

Statistical analysis

Data are presented as mean ± SEM, and was analysed using SPSS 13.0 for Windows software (SPSS Inc., Chicago, IL, USA). The *t*-test was used for comparisons between two groups and analysis of variance followed by the post-hoc Fisher's least significant difference (LSD) test was used for comparisons among multiple groups. A *P*-value < 0.05 was regarded as statistically significant.

RESULTS

Morphological analysis and pulmonary function

HE staining of lung tissues revealed complete alveolar walls and no evidence of inflammatory changes in groups A or B (Fig 1a,b), whereas in group C, there was infiltration of lymphocytes accompanied by focal emphysema. Some alveolar walls were broken and merged, with significantly enlarged alveolar spaces (Fig. 1c). MLI was significantly increased, whereas

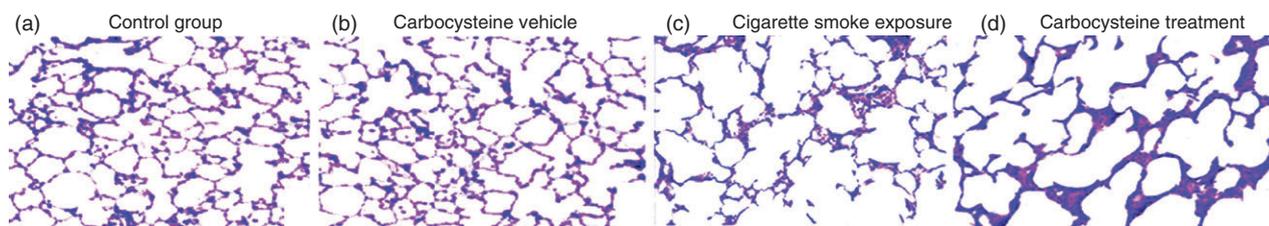


Figure 1 HE staining of lung tissues (original magnification, $\times 200$). (a) Normal control group; (b) carbocysteine vehicle group, showing complete alveolar walls and no evidence of inflammatory changes; (c) cigarette smoke exposure group, showing broken alveolar walls, resulting in merged and enlarged alveolar spaces; (d) carbocysteine treatment group, showing less disruption of alveolar walls, resulting in fewer merged and enlarged alveolar spaces.

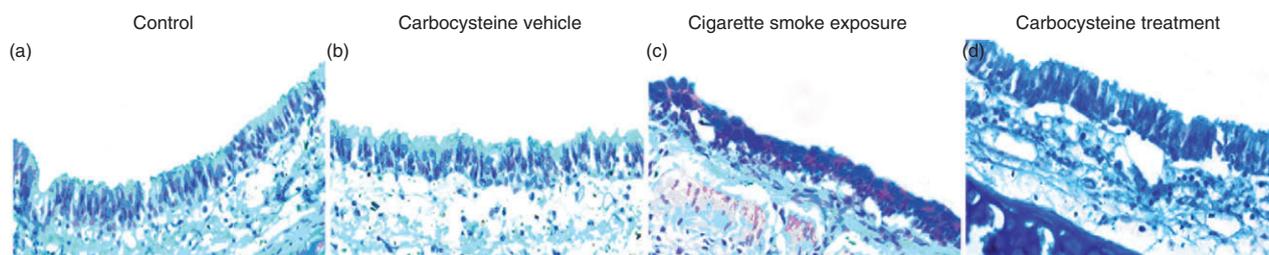


Figure 2 Changes in expression of mucin and goblet cell metaplasia in rat airways. Positive Alcian blue/periodic acid-Schiff staining is indicated by blue colour (original magnification, $\times 200$). (a) Control group and (b) carbocysteine vehicle group, both showing few positively stained blue areas; (c) cigarette smoke exposure group, showing an obvious increase in the blue stained area; (d) carbocysteine treatment group, showing a decrease in the blue stained area compared with group C.

Table 1 Assessment of lung tissue morphology and lung function in the different groups of rats

Group	<i>n</i>	MLI (μm)	MAN (per mm^2)	FEV _{0.3} /FVC
A	6	8.5 \pm 1.0	377.6 \pm 33.2	80.0 \pm 4.1 [†]
B	6	9.1 \pm 1.3	369.6 \pm 26.9	81.3 \pm 3.9 [†]
C	6	24.9 \pm 2.7*	172.2 \pm 44.6*	73.2 \pm 3.5 [†]
D	6	18.6 \pm 1.1 [†]	215.3 \pm 24.8 [†]	77.8 \pm 4.2 [†]

Data are mean \pm SEM.

* $P < 0.01$ versus control rats, MLI (or mean alveolar intercept) was increased and MAN was decreased in group C compared with groups A and B.

[†] $P = 0.015$ versus rats exposed to cigarette smoke, MLI was decreased and MAN was increased in group D compared with group C.

[‡] There were no statistically significant differences in FEV_{0.3}/FVC among the different groups ($P = 0.089$).

FEV_{0.3}, forced expiratory volume in 0.3 s; MAN, mean alveolar number; MLI, mean linear intercept.

MAN was decreased in group C compared with groups A and B (Table 1, $P = 0.015$). FEV_{0.3}/FVC was not lower than 70%, with no statistically significant differences between groups (Table 1, $P = 0.089$).

Expression of mucin and goblet cell metaplasia

Expression of mucin, as evidenced by blue staining, was mainly observed in goblet cells. A prominent

Table 2 Expression of mucin and goblet cell metaplasia, as assessed by AB-PAS staining of lung tissue in the different groups of rats

Group	<i>n</i>	Percentage AB-PAS positively stained area
A	6	27.8 \pm 7.9
B	6	31.5 \pm 9.2
C	6	76.3 \pm 7.8*
D	6	44.7 \pm 7.7 [†]

Data are mean \pm SEM.

* $P < 0.01$ versus control rats, percentage AB-PAS positively stained area was increased in group C compared with groups A and B.

[†] $P = 0.013$ versus rats exposed to cigarette smoke, percentage AB-PAS positively stained area was decreased in group D compared with group C.

AB-PAS, Alcian blue/periodic acid-Schiff.

increase in the numbers of goblet cells along the airway surface epithelium, as assessed by AB-PAS staining, was observed after CS exposure (Fig. 2c), as compared with groups A and B (Fig. 2a,b). Moreover, a decrease in the positively stained area was detected in group D (Fig. 2d). Analysis of the percentage positive staining with AB-PAS revealed increased staining in group C compared with groups A and B and decreased staining in group D compared with group C (Table 2, $P = 0.013$).

Table 3 Expression of mRNA for Muc5AC relative to GAPDH in the different groups of rats

Group	Muc5AC Ct average	GAPDH Ct average	Δ Ct average (Muc5AC Ct – GAPDH Ct)	$\Delta\Delta$ Ct average (Δ Ct Muc5AC Ct – Δ Ct GAPDH Ct)	$2^{-\Delta\Delta$ Ct relative to GAPDH
A	26.56 ± 0.15	18.25 ± 0.62	8.47 ± 0.29	0	1.0 ± 0.29
B	27.24 ± 0.41	18.77 ± 0.58	8.31 ± 0.42	-0.17	0.90 ± 0.42
C	31.35 ± 1.20	20.25 ± 0.40	11.0 ± 0.87	2.53	5.78 ± 0.87*
D	30.53 ± 1.46	20.53 ± 0.16	10.0 ± 0.54	1.53	2.89 ± 0.54†

Data are mean ± SEM ($n = 6$).

* $P = 0.013$ versus control rats, relative Muc5AC mRNA expression was increased in group C compared with groups A and B.

† $P = 0.021$ versus rats exposed to cigarette smoke, relative Muc5AC mRNA expression was decreased in group D compared with group C.

Ct, threshold cycle; Muc5AC, mucin 5AC.

Muc5AC mRNA expression

To further examine Muc5AC gene expression at the transcriptional level, the steady state mRNA levels for Muc5AC and GAPDH in lung tissue were analysed by real-time RT-PCR. GAPDH was considered to be an appropriate gene for normalization and remained unchanged in all samples. Real-time RT-PCR demonstrated similar expression of Muc5AC mRNA in group B (0.9-fold) compared with group A, and increased expression of Muc5AC in group C (5.78-fold) and group D (2.89-fold) (Table 3). There was no significant difference between groups A and B, whereas the expression of Muc5AC relative to GAPDH was significantly increased in group C compared with groups A and B (Fig 3, $P = 0.009$). Furthermore, there was decreased expression of Muc5AC relative to GAPDH in group D compared with group C (Fig 3, $P = 0.021$).

Muc5AC protein expression

Western blotting revealed that Muc5AC protein expression was higher in group C than in groups A or B (Fig 4, $P = 0.008$), whereas it was lower in group D than in group C (Fig 4, $P = 0.026$).

Mucociliary function in rats

Mucociliary function in rats was quantified *in vivo* by the clearance of ^{99m}Tc -Sc. The clearance of ^{99m}Tc -Sc was delayed in group C compared with groups A and B, whereas it was increased in group D compared with group C (Fig. 5). The absolute values for retention of ^{99m}Tc -Sc particles in the lungs are shown in Table 4. There was no significant difference between groups A and B, whereas the clearance rate decreased significantly in group C compared with groups A and B ($P = 0.037$). Furthermore, the clearance rate was increased in group D compared with group C ($P = 0.030$).

Airway bacterial load in the rat model of *Haemophilus influenzae* inoculation

Rapid influx of large numbers of neutrophils and alveolar macrophages is crucial for the effective early

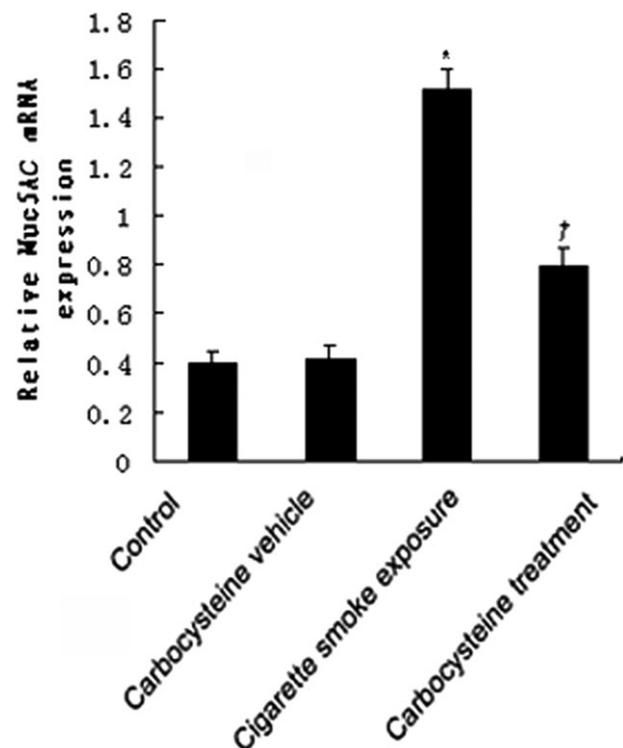


Figure 3 Expression of mRNA for mucin 5AC (Muc5AC) and GAPDH in lung tissue of the different groups of rats, as assessed by real-time PCR. Data are mean ± SEM ($n = 6$). * $P = 0.009$ versus control rats, mRNA expression for Muc5AC was higher in group C (1.51 ± 0.09) than in group A (0.4 ± 0.05); † $P = 0.026$ versus rats exposed to cigarette smoke, mRNA expression for Muc5AC was significantly lower in group D (0.8 ± 0.1) than in group C (1.51 ± 0.09).

clearance of bacteria from the lungs. Therefore, the total number of BAL fluid cells, as well as neutrophil and macrophage counts, was assessed 3 h after inoculation with *H. influenzae*. There were no significant differences in the total number of cells, or the numbers of neutrophils or alveolar macrophages

Figure 4 Representative images showing expression of (a) mucin 5AC (Muc5AC) and (b) β -actin proteins in lung tissue of the different groups of rats, as assessed by western blot analysis. (c) Quantitation of Muc5AC protein relative to β -actin protein in lung tissue of the different groups; * $P=0.008$ versus control rats, Muc5AC protein was increased in group C compared with group A; † $P=0.026$ versus rats exposed to cigarette smoke, Muc5AC protein was decreased in group D compared with group C. Data are mean \pm SEM ($n=6$).

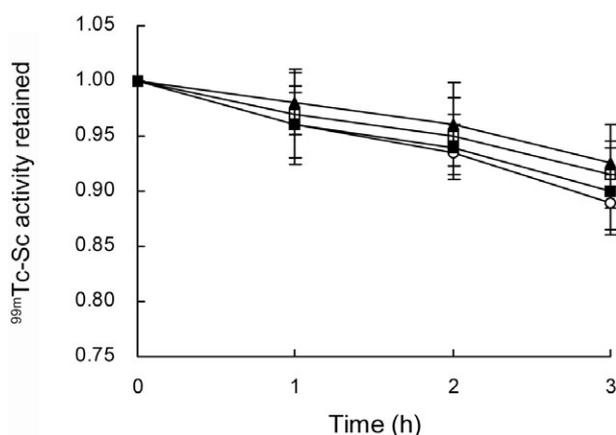
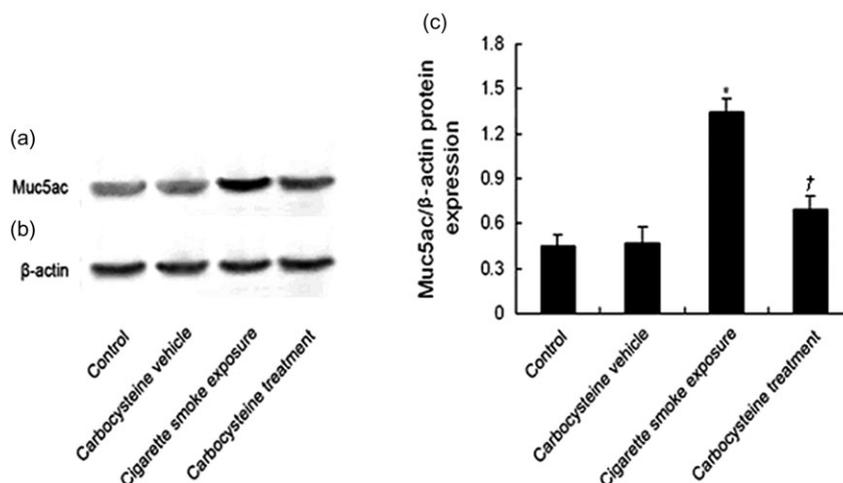


Figure 5 Activity time plot showing retention of $^{99m}\text{Tc-Sc}$ in the lungs of different groups of rats following delivery into the distal region of the oropharynx. The clearance of $^{99m}\text{Tc-Sc}$ was delayed in group C compared with group A ($P=0.037$), whereas it was faster in group D compared with group C ($P=0.030$). Data are mean \pm SEM ($n=6$). (—○—) Control; (—■—) carbocysteine vehicle; (—▲—) cigarette smoke exposure; (—□—) carbocysteine treatment.

among the different groups (Table 5, $P=0.069$). In this study, neutrophils and macrophages did not appear to play important roles in the early clearance of bacteria 3 h after inoculation with *H. influenzae*. The airway *H. influenzae* load in BAL fluid and homogenized lung was increased in group C compared with groups A and B (Table 6, $P=0.035$), whereas it was decreased in group D compared with group C (Table 6, $P=0.041$).

DISCUSSION

Carbocysteine (S-carboxymethylcysteine) is a mucocactive drug with *in vitro* free radical scavenging and anti-inflammatory properties.²⁰ Several trials demonstrating the positive effects of carbocysteine on exacerbation rates and quality of life have rekindled

interest in this family of drugs and may offer new insights into the care of patients with COPD.^{21–23}

Smoking is a major cause of COPD. Emphysematous changes induced in rat models by CS and mucus hypersecretion have been shown to occur in direct proportion to the duration and amount of smoke inhaled.^{13–15} In this study, emphysematous changes in Wistar rats exposed to CS for 12 weeks were demonstrated by measurements of MLI and MAN. Mucus hypersecretion, that is, goblet cell metaplasia and upregulation of Muc5AC expression, was also observed in the rat lungs. Moreover, airflow limitation was measured using a lung function instrument for small animals and $\text{FEV}_{0.3}/\text{FVC}$ was not less than 70%. All this evidence indicates that this was a successful rat model of CS-induced emphysema and mucus hypersecretion.

Colonization or infection of the airways by bacteria may cause persistent inflammation and mucus hypersecretion, thereby facilitating a vicious cycle that accelerates the progression of COPD.²⁴ Simple antibacterial treatment is insufficient to disrupt this vicious cycle.^{25,26} *H. influenzae* is a common pathogen of the lower respiratory tract in COPD patients, both during the stable state and in acute exacerbations. Therefore, *H. influenzae* was selected for this study and inoculated into the airways of the different groups of rats. Airway loads of *H. influenzae* in the different groups were assessed after 3 h, revealing significant differences among the groups. There was an increased airway load of *H. influenzae* in group C compared with groups A and B, and an obvious decrease in group D compared with group C. Airway loads of *H. influenzae* after 6 h also showed significant differences among the groups; there was an increased *H. influenzae* load in group C compared with groups A and B, and a decreased *H. influenzae* load in group D compared with group C.

The mechanism of bacterial clearance involves infiltration of neutrophils, phagocytosis by macrophages and the MCC system. Impaired MCC increases the airway bacterial load in COPD.⁶ At present, no studies have investigated the relevance of host airway mucus hypersecretion, MCC and bacterial load in

Table 4 Mucociliary function in the different groups of rats, as assessed by the absolute values for retention of ^{99m}Tc -Sc particles in the lungs

Group	Lung retention of ^{99m}Tc -Sc particles (kilo counts)			
	0 h	1 h	2 h	3 h
A	3092 ± 21	2968 ± 27	2891 ± 23	2783 ± 25
B	3083 ± 31	2960 ± 26	2899 ± 19	2790 ± 23
C	3097 ± 23*	3045 ± 18*	2963 ± 17*	2849 ± 29*
D	3089 ± 25 [†]	3004 ± 21 [†]	2935 ± 24 [†]	2811 ± 27 [†]

Data are mean ± SEM ($n = 6$).

* $P = 0.035$ versus control rats, lung retention of ^{99m}Tc -Sc particles was increased in group C compared with groups A and B.

[†] $P = 0.041$ versus rats exposed to cigarette smoke, lung retention of ^{99m}Tc -Sc particles was decreased in group D compared with group C.

Table 5 Cell counts in BAL fluid from the different groups of rats, 3 h after inoculation with *Haemophilus influenzae*

Group	Total cell count ($\times 10^5$)	Neutrophils ($\times 10^5$)	Alveolar macrophages ($\times 10^5$)
A	3.8 ± 0.3	0.89 ± 0.3	2.72 ± 0.6
B	3.9 ± 0.5	0.92 ± 0.3	2.83 ± 0.4
C	4.3 ± 0.4	1.24 ± 0.3	3.25 ± 0.5
D	4.1 ± 0.4	1.07 ± 0.2	3.02 ± 0.5

Data are mean ± SEM ($n = 6$). There were no significant differences in the numbers of BAL fluid cells among the different groups, 3 h after inoculation with *H. influenzae* ($P = 0.069 > 0.05$).

Table 6 Effect of carbocysteine on the airway load of *Haemophilus influenzae*, as measured in the BAL fluid and homogenized lung tissue of the different groups of rats

Group	BAL fluid ($\times 10^3$ cfu)	Homogenized lung tissue ($\times 10^3$ cfu)
A	13.8 ± 3.0	28.0 ± 6.2
B	14.6 ± 5.6	29.6 ± 6.2
C	30.7 ± 7.3*	54.2 ± 5.5*
D	21.5 ± 3.6 [†]	39.3 ± 5.0 [†]

Data are mean ± SEM ($n = 6$).

* $P < 0.01$ versus control rats, airway *H. influenzae* load in BAL fluid and homogenized lung tissue was increased in group C compared with groups A and B.

[†] $P = 0.041 < 0.05$ versus rats exposed to cigarette smoke, airway *H. influenzae* load was decreased in group D compared with group C.
cfu, colony-forming units.

COPD. Bacterial load and MCC were studied after 3 h because the numbers of macrophages and neutrophils did not obviously change after inoculation with *H. influenzae*, as evidenced by the BAL fluid cell counts, and consistent with the findings of Hansen

*et al.*¹⁸ Therefore, MCC appears to be the main mechanism for bacterial clearance at this time point, while infiltration of neutrophils and phagocytosis by macrophages may have marked effects at a later stage.

The clearance of ^{99m}Tc -Sc, which reflects mucociliary function in rats, was quantified by scintigraphy, according to the method described by Foster *et al.*¹⁷ Clearance of ^{99m}Tc -Sc was delayed in group C compared with groups A and B. Furthermore, increased clearance of ^{99m}Tc -Sc was observed in group D compared with group C. CS exposure decreased MCC and treatment with carbocysteine increased MCC. The changes in *H. influenzae* load were consistent with the changes in MCC in the corresponding groups.

Haemophilus influenzae adheres to Muc5AC after entering the airways.⁸ On the one hand, this adherence may initiate host defence mechanisms that eliminate the bacteria through MCC; on the other hand adherence represents the first step in bacterial colonization of the airways and can initiate a cytokine response and inflammation. Previous studies demonstrated that carbocysteine inhibits Muc5AC production in rats exposed to SO_2 ,²⁷ and reduces neutrophil elastase-induced Muc5AC expression in NCI-H292 cells *in vitro*.¹⁰ As CS is the most important risk factor for COPD, it is important to study the effect of carbocysteine on Muc5AC expression induced by CS, both *in vivo* and *in vitro*. In the present study, goblet cell metaplasia and Muc5AC expression were upregulated after CS exposure, whereas goblet cell metaplasia and Muc5AC expression were inhibited in the group treated with carbocysteine. This is the first study to report this effect *in vivo*.

Carbocysteine is a classical mucolysis agent, which splits disulphide linkages and reduces the glutinousness of the sputum.²⁸ This may partly explain the improved MCC observed in rats in the present study. Moreover, this study is the first to show that carbocysteine inhibits Muc5AC expression and goblet cell metaplasia after exposure to CS *in vivo*. *H. influenzae* adheres to Muc5AC, and therefore, downregulation of Muc5AC results in decreased adherence of *H. influenzae* in the airways. Carbocysteine decreases the adherence of *H. influenzae* by downregulating the expression of Muc5AC, and also increases the clearance of *H. influenzae* by improving MCC. This

effect may be partly related to the increased clearance of *H. influenzae* and the decreased bacterial load. N-acetylcysteine is a powerful antioxidant that has been shown to reduce exacerbation rates in COPD^{29,30} and inhibit the adherence of bacteria *in vitro*.³¹ However, compared with N-acetylcysteine, carbocysteine is safe, effective, economical and well tolerated,³² making it a more cost-effective option in developing countries. Carbocysteine may be useful as a supplemental antimicrobial agent and as a pathogen-directed therapy.³³

In summary, this study has demonstrated for the first time the benefits of the mucolytic agent, carbocysteine, in attenuating the CS-induced airway load of *H. influenzae*, as well as airway mucus hypersecretion, while improving MCC in rats. These results suggest that carbocysteine may be beneficial for COPD patients by increasing the clearance of bacteria and decreasing the bacterial load.

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