 doi: 10.1111/j.1440-1681.2007.04857.x

S-CARBOXYMETHYLCYSTEINE INHIBITS CARBACHOL-INDUCED CONSTRICTION OF EPITHELIUM-DENUDED RAT AND HUMAN AIRWAY PREPARATIONS

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SUMMARY

1. The effects of S-carboxymethyl-L-cysteine (S-CMC), either administered orally to rats or incubated with tissue preparations from rats and humans, on isometric contractions of tracheal smooth muscle were investigated in the present study using an improved *in vitro* model of tracheal tube or ring preparations. The involvement of the tracheal epithelium in the observed effects was also investigated.

2. The experimental model permitted selective perfusion of the airway tube, luminal-IN or serosal–OUT, and measurement of airway smooth muscle contraction or relaxation in preparations with (+) or without (–) epithelium (Ep), excluding direct effects of airway mucus.

3. We found that oral pretreatment of rats with S-CMC (mixed with water; 200 mg/kg per day for 2 weeks), but not short pre-incubation of preparations *in vitro* (10^{-3} mol/L S-CMC for 1 h), diminished the sensitivity of –Ep preparations to carbachol compared with controls (EC_{50} ($-\log_{10}$ mol/L) values: 5.5 ± 0.1 *vs* 5.8 ± 0.1, respectively, for IN perfusion ($P < 0.005$); 5.6 ± 0.1 *vs* 5.9 ± 0.1, respectively, for OUT perfusion ($P < 0.005$)), whereas the sensitivity of preparations to aminophylline was not affected. Normal sensitivity to carbachol stimulation was re-established if preparations were pre-incubated with capsaicin.

4. It was also found that longer pre-incubation (4 h) of ring-preparations of human bronchus with S-CMC (10^{-5} mol/L) *in vitro* resulted in a diminished response to carbachol stimulation.

5. In conclusion, S-CMC had small inhibitory effects on the sensitivity of rat and human airway smooth muscle to carbachol, particularly in endothelium-denuded preparations. Whether the epithelium was responding to S-CMC by producing some contracting factor(s) requires further investigation.

Key words: epithelium, rat trachea, S-carboxymethylcysteine, tracheal ring, tracheal tube.

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Received 16 January 2007; revision 20 November 2007; accepted 21 November 2007.

INTRODUCTION

The airway epithelium, which separates inhaled gas from the underlying airway tissue, forms an important barrier between living structures and the environment. Preservation of the normal function of the bronchial epithelium is a precondition for maintenance of an effective air–tissue barrier. That barrier may be disturbed in airway inflammation and asthma. One objective of our earlier and the present study has been to test the hypothesis that the bronchial epithelium, either as a diffusion barrier or by actively secreting yet not well defined mediators (prostaglandins, leukotrienes or other mediators), can modulate bronchial smooth muscle sensitivity to various stimulating agents.[1–3] Experimentally, we use a system of perfused tracheal tubes because a tube airway preparation allows selective pharmacological stimulation of the epithelial or serosal side of the airway *in vitro*. In the present study, the model described earlier[2,4,5] was improved so that it allowed investigation of contractions of the entire airway tube first and then as a ring preparation.

We centred our study on the mucolytic agent S-carboxymethylcysteine (S-carboxymethyl-L-cysteine; S-CMC; carbocysteine), used across Europe. Improving mucociliary transport is one of the therapeutic approaches in asthma therapy, in which, in addition to anti-inflammatory agents,[6,7] mucolytic agents are occasionally used.[8,9] Indeed, beneficial effects of S-CMC for chronic obstructive bronchitis have been demonstrated in a large multicentre controlled trail.[10] Nevertheless, the mechanism of action of S-CMC remains unclear. It was found that S-CMC could enhance the production of sialomucins at the expense of fucomucins,[11,12] which may result in an improvement in mucociliary transport. Several other findings have implicated either reduced or increased sputum viscosity[13–16] or even an anti-inflammatory property[17–19] that may be related to increased sialoglycoprotein secretion as a mechanism of action of S-CMC.

Nevertheless, asthmatic subjects often have airway hyperresponsiveness that is accompanied by secretion of thick mucus and various degrees of inflammation. Recent evidence indicating that S-CMC may indirectly favour smooth muscle relaxation[18,20,21] further justified the aim of the present study, which was to test the effects of S-CMC on airway smooth muscle (ASM) responsiveness. More precisely, we wanted to examine the effect of S-CMC on bronchial epithelium function and/or tracheal smooth muscle in healthy animals and its effect on the isometric contraction of bronchial smooth muscle in cannulated preparations or ring preparations. The second objective of the study was to construct and test a slightly

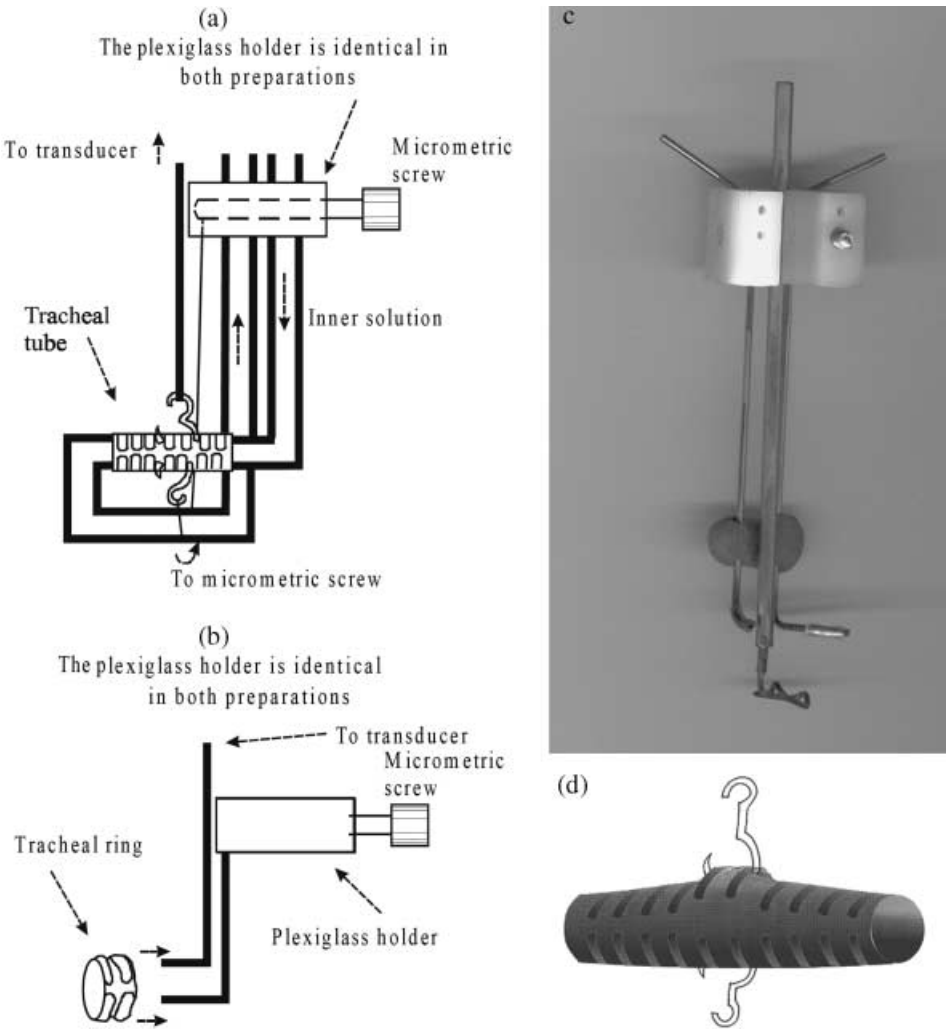

Fig. 1 (a) Schematic representation of the experimental apparatus. (a) The new tracheal tube preparation with the 'carrier' block. Inner luminally (IN) and outer (OUT) perfusion solutions are maintained at 37°C and bubbled with 95% O_2–5% CO_2 and a constant flow rate of 2 mL/min is maintained. Under microscopic control, two stainless-steel hooks were passed through the tracheal wall around two adjacent cartilagineous rings as close as possible to the tracheal muscle insertion. The lower hook, which served as a 'fixed point', was attached via silk thread to the micrometric screw serving to adjust hook tension on the tracheal wall. This allowed precise adjustment of resting tension. The upper hook was connected to a force transducer. (b) Tracheal ring preparation. The 'carrier block' for cannulated trachea could be easily removed and replaced by an identical one which the lower part has been adapted for the mounting of tracheal rings. (c) A photograph of the new tracheal tube preparation. (d) Tracheal tube with the hooks that are placed as close as possible to the end of the tracheal rings and close to the tracheal muscle.

modified *in vitro* apparatus that can be used with an airway tube or, alternatively, an airway ring preparation.

METHODS

Animal preparation

Experiments were performed on tracheas taken from male Sprague-Dawley rats, weighing 390–420 g. Animal treatment and experimental procedures were in accordance with the recommendations of INSERM and with the local Instructions for Animal Care of Greifswald University. All animals were housed in individual cages and received water and food *ad libitum*. The pretreated group of animals (S-CMCpr) received S-CMC mixed with water (200 mg/kg per day) for 2 weeks. During the 3rd week, rats were killed for the experiments by stunning and quick exsanguination. The tracheas were immersed in modified Krebs'–Henseleit solution (KH; composition (in mmol/L): NaCl 113; KCl 4.8; $MgCl_2 \cdot 6H_2O$ 1.3; KH_2PO_4 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; glucose 5.7) and cleaned from surrounding tissue.

Perfusion studies

Proximal tracheal ends (10 tracheal rings long) were used for the experiments. In half the preparations the epithelium was removed (–Ep) by gently rubbing the luminal side with a cotton-wrapped metal stick; in the other half, the epithelium was left intact (+Ep).

Under magnification (×2–4), two stainless-steel hooks were passed through the tracheal wall around two adjacent cartilaginous rings as close as possible to the tracheal muscle insertion. The tracheal segment was then connected longitudinally to steel tubes built in the 'carrier block' of the apparatus (in–out system) and firmly tightened with silk thread. The apparatus used (Fig. 1a–c; EMKA Technologies, Paris, France) was an improved version of the cannulated tracheal system described previously.[2] The improvement[4] consisted of the fact that the lower hook, which served as a fixed point, was attached via the silk thread to the micrometric screw serving to adjust hook tension on the tracheal wall. This allowed precise adjustment of the resting tension. The upper hook was connected to a force transducer (IT1-25; EMKA Technologies), the latter being attached to a micromanipulator that permitted displacement of the upper hook along a strict vertical axis. Any change in tension at the level of the tracheal muscle was registered by the recorder (AT 550; Gould Instrument Systems, Valley View, OH, USA), to which the amplified signal (EMKA Technologies four-way amplifier) from the transducer was connected. The 'carrier block' for cannulated trachea could be removed easily and replaced by one suitable for mounting tracheal rings (Fig. 1b; ring system and human airway, see below).

The KH solution (37°C, pH 7.4, gassed with 95% O_2/5% CO_2) was perfused at a constant flow rate (2 mL/min) through the organ bath (outer perfusion–OUT) and through the lumen of the tracheal segment (inner perfusion–IN) using peristaltic pumps (Watson Marlow 5025, Falmouth, Cornwall, UK).

Procedure

Perfusion studies

In this experimental model, studies were conducted using preparations with and/or without epithelium taken from animals pretreated with S-CMC (S-CMCpr) or from control animals preparations.

After a period of stabilization (45–60 min), the tracheal muscle was stretched to its optimal length, corresponding to approximately 1.5 g passive pretension. Preliminary assays were performed to determine the optimal stretch of the muscle, as described previously.[4] The length–tension relationship did not differ significantly between S-CMCpr and control preparations.

In the initial set of experiments, cumulative concentrations of carbachol (10^{-7} to 10^{-3} mol/L) were perfused IN (+Ep or –Ep) or OUT (+Ep or –Ep) in S-CMCpr or controls preparations ($n = 8$ for each). We concluded from these experiments that responses to IN and OUT stimulation in –Ep preparations were identical (on the basis of EC_{50} values and maximal responses) and, to further examine the effects of S-CMC in –Ep preparations on IN stimulation with carbachol, we designed another four sets of experiments.

First, to examine the effect of pre-incubation, preparations taken from untreated animals were incubated with 10^{-3} mol/L S-CMC (S-CMCinc; –Ep; $n = 8$) for 60 min and cumulative concentrations of carbachol were perfused luminally (IN).

In the second set of experiments, S-CMCpr (–Ep) preparations were pre-incubated with 10^{-6} miol/L indomethacin (INDinc; both sides; $n = 8$) or 10^{-5} mol/L capsaicin (CAPSinc; both sides; $n = 6$) for 60 min and prior to perfusion of cumulative concentrations of carbachol IN. The effects of S-CMC on the relaxant effects of aminophylline were examined in S-CMCpr (–Ep) preparations that were first precontracted with a medium concentration (10^{-6} mol/L) of carbachol OUT and then perfused IN with 10^{-8} to 10^{-3} mol/L aminophylline ($n = 10$) and compared with control responses to aminophylline ($n = 6$). In the third set of experiments, to examine direct relaxant effects of S-CMC, preparations taken from control animals were precontracted OUT with an EC_{50} concentration of carbachol (10^{-6} mol/L) and then perfused IN with cumulative concentrations of S-CMC, ranging from 10^{-7} to 10^{-3} mol/L (+Ep, $n = 7$; –Ep, $n = 7$).

Human airway

Preparations of human bronchi (3–4 mm in diameter, $n = 4$ in each group) were obtained during thorax surgery (partial lung resection due to lung cancer), stored for approximately 1 h in cold (+4°C) physiological salt solution (PSS; NaCl 0.9%) and then dissected into 2–3 mm ring preparations and incubated with 10^{-5} mol/L S-CMC for 4 h. Paired controls were left in cold PSS during that time. In either one of the paired preparations, the epithelium was removed before the preparations were mounted for *in vitro* experiments. Owing to scarcity of the specimens, cannulation was not possible and therefore the experiments were performed only on bronchial rings. In these preparations only dose–response curves to carbachol were obtained.

Substances

The following substances were used: carbachol (carbamylcholine chloride, Sigma Chimie, St Quentin Fallavier, France), indomethacin (Sigma Chimie), aminophylline (theophylline–ethylenediamine; Pharmacie Centrale des Hopitaux, Paris, France), capsaicin (Sigma Chimie) and S-CMC (Park-Davis, Orleans, France). The S-CMC was diluted in 10% NaOH, whereas indomethacin was diluted in methanol; final dilutions of both stock solutions were made in KH solution. Final solutions contained less then 0.01% methanol and NaOH; all controls were exposed to the same concentrations of solvent as the treatment groups.

Statistical analyses

Data are expressed as a percentage of the maximal response and in absolute values (g or s) and given as the mean±SEM. Half-maximal concentration (EC_{50}) values were calculated by means of non-linear regression using the Hill–Langmuir equation implemented in GraphPad Prism (GraphPad Software, San Diego, CA, USA) and the results given as the mean of $-\log_{10}$ EC_{50} values obtained. Statistical analysis was perfomed using analysis of variance and Student's *t*-test for paired or unpaired data adjusted for multiple comparisons (Bonferroni), as appropriate. $P < 0.05$ was regarded as being statistically significant. The False Discovery Rate (FDR) procedure was also used for multiple comparisons.[22,23] All statistical analyses were performed using the software package Graph Pad Prism 4 for Windows (Graph Pad Software), except for the FDR, for which a short routine was written in Microsoft Excel (Microsoft Office 2000; Microsoft, Redmond, VA, USA).

RESULTS

Animals pretreated with oral S-CMC did not show any clinical signs of disease or metabolic disturbances. They showed normal cage activity, no disturbances in the sleep–wake pattern, normal spontaneous food and water intake, weight gain ordinary stool consistency and normal eyes and no piloerection.

Perfusion studies

Effects of oral pretreatment and pre-incubation with S-CMC and capsaicin and indomethacin

In preparations taken from S-CMCpr amimals, we found diminished sensitivity to carbachol in –Ep, but not +Ep, preparations (Tables 1,2; Fig. 2). Interestingly, the diminished sensitivity following epithelial application (inside, IN) of carbachol in –Ep preparations from S-CMCpr was absent in preparations pre-incubated with 10^{-5} mol/L capsaicin (CAPSinc; Table 3; Fig. 3), but was maintained in preparations pre-incubated with 10^{-6} mol/L indomethacin

Table 1 EC_{50} values for carbachol (IN)

	+Ep	–Ep	*P*
Control	5.40 ± 0.11	5.85 ± 0.06	< 0.005
S-CMCpr	5.19 ± 0.08	5.52 ± 0.09	< 0.005
P	NS	< 0.005	

Data are the mean±SEM of EC_{50} values ($-\log_{10}$; mol/L), obtained in preparations with (+Ep) and without (–Ep) epithelium, taken from controls or animals pretreated for 2 weeks with with s-carboxymethyl-L-cysteine (S-CMC; 200 mg/kg per day, p.o.; S-CMCpr), following stimulation from the epithelial side (IN) with cumulative concentrations of carbachol ($F = 10.45$).

Table 2 EC_{50} values for carbachol (OUT)

	+Ep	–Ep	*P*
Control	6.01 ± 0.09	5.92 ± 0.06	NS
S-CMCpr	6.17 ± 0.08	5.61 ± 0.05	< 0.005
P	NS	< 0.005	

Data are the mean±SEM of EC_{50} values ($-\log_{10}$; mol/L), obtained in preparations with (+Ep) and without (–Ep) epithelium, taken from controls or animals pretreated for 2 weeks with with s-carboxymethyl-L-cysteine (S-CMC; 200 mg/kg per day, p.o.; S-CMCpr), following stimulation from the serosal side (OUT) with cumulative concentrations of carbachol ($F = 11.14$).

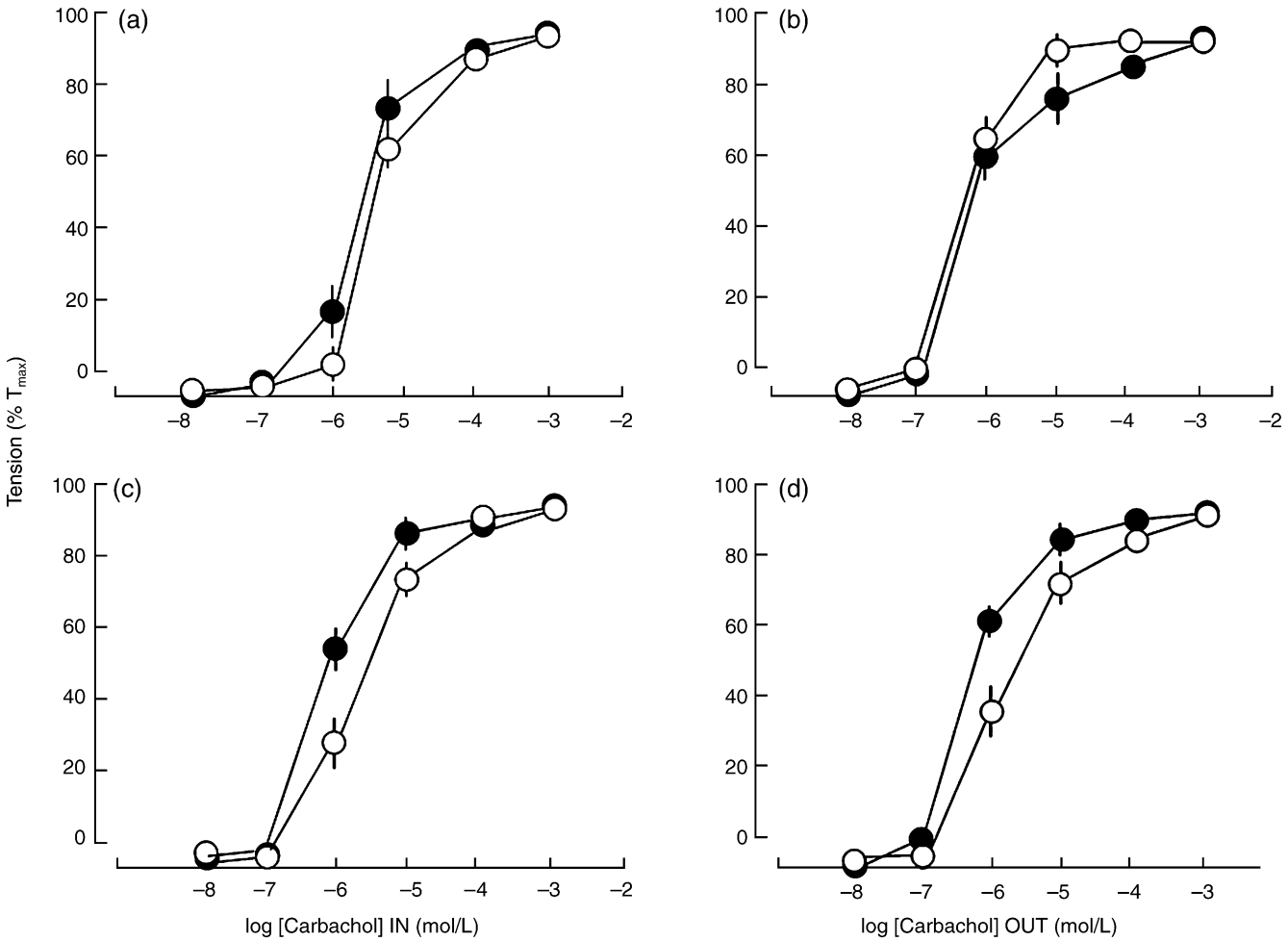

Fig. 2 Cumulative concentration–responses curves constructed after administration of carbachol (a,c) epithelialy (IN) or (b,d) serosaly (OUT) in rat isolated trachea with (a,b) and without (c,d) epithelium. Preparations were taken from control animals or animals pretreated for 2 weeks with S-carboxymethyl-L-cysteine (S-CMC; 200 mg/kg per day, p.o.). (●), control; (○), S-CMC pretreated. Tension is expressed as a percentage of the maximal tension (T_{max}) obtained and presented as the mean±SEM.

Table 3 EC_{50} values for carbachol following pre-incubation with indomethacin or capsaicin

	–Ep (IN)
Control	5.85 ± 0.06*
S-CMCinc	5.71 ± 0.07
S-CMCpr	5.52 ± 0.09
S-CMCpr (INDinc)	5.59 ± 0.09†‡
S-CMCpr (CAPSinc)	5.99 ± 0.15*†

Data are the mean±SEM of EC_{50} values ($-\log_{10}$; mol/L), obtained in preparations without epithelium (–Ep) following stimulation from the epithelial side (IN) with cumulative concentrations of carbachol. Preparations were taken from controls and pre-incubated in S-carboxymethyl-L-cysteine (S-CMC; 10^{-3} mol/L; S-CMCinc) or from animals pretreated for 2 weeks with oral S-CMC (200 mg/kg per day, p.o.; S-CMCpr) and pre-incubated with 10^{-6} mol/L indomethacin (INDinc) or 10^{-5} mol/L capsaicin (CAPSinc). *$P < 0.03$ compared with S-CMCpr ($F = 5.444$; see also Table 2); †$P < 0.05$ compared with S-CMCinc (FDR procedure); ‡$P < 0.05$ compared with S-CMCpr (FDR procedure).

(INDinc; Table 3). In control experiments, we observed that pre-incubation of ring preparations with capsaicin did not affect their sensitivity (EC_{50}) or the maximal force developed (T_{max}) following carbachol stimulation.

In contrast with the effects of oral pretreatment, pre-incubation of tissues with S-CMC (S-CMCinc) for 60 min did not affect the sensitivity of –Ep preparations to carbachol IN stimulation (Table 3). However, all +Ep preparations were more sensitive to carbachol OUT stimulation then to carbachol IN stimulation. Removal of the epithelium increased sensitivity to carbachol in all preparations except for control preparations stimulated with carbachol (OUT; Tables 1,2).

Direct effect of S-CMC, T_{max} and aminophylline

In +Ep or –Ep preparations precontracted with carbachol (10^{-6} mol/L) OUT, S-CMC alone in the concentration range 10^{-7} to 10^{-3} mol/L IN had neither contracting nor relaxant effects. In addition, the T_{max} developed following stimulation with carbachol did not depend on the side of stimulation (OUT or IN) and was not affected by removal of the epithelium, pretreatment with S-CMC or pre-incubation

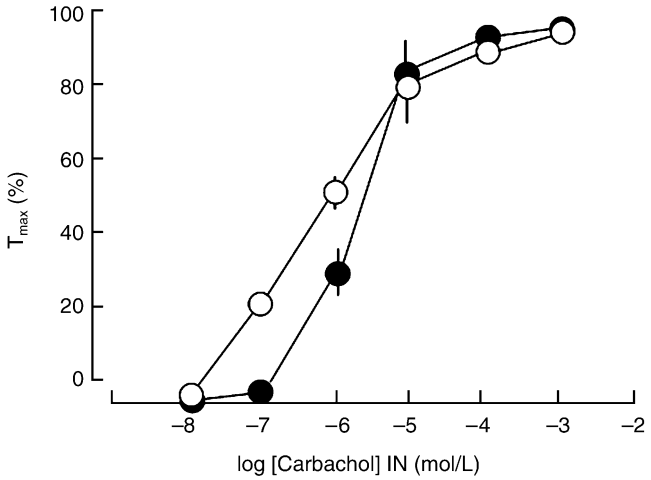

Fig. 3 Cumulative concentration–responses curves constructed after administration of carbachol epithelialy (IN) in rat isolated trachea without epithelium. Preparations were taken from animals pretreated for 2 weeks with s-carboxymethyl-L-cysteine (S-CMC; 200 mg/kg per day, p.o.) and either not incubated (●) or incubated (○) for 60 min with 10^{-6} mol/L capsaicin. Tension is expressed as a percentage of the maximal tension (T_{max}) obtained and presented as the mean±SEM.

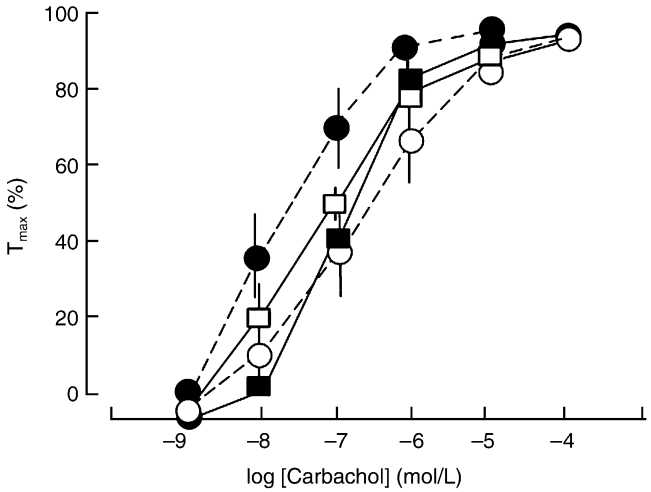

Fig. 4 Cumulative concentration–responses curves constructed after the administration of carbachol in human isolated bronchial rings. Half the bronchial rings were stored at 4°C, whereas the other half were incubated for 4 h in s-carboxymethyl-L-cysteine (S-CMC; 10^{-5} mol/L). Then, in paired preparations, the epithelium was either removed or left intact (–Ep and +Ep, respectively). Tension is expressed as a percentage of the maximal tension (T_{max}) obtained and presented as the mean±SEM. (●), no S-CMC, –Ep; (○), no S-CMC, +Ep; (■), S-CMC, –Ep; (□), S-CMC, +Ep.

with 10^{-3} mol/L S-CMC, 10^{-6} mol/L indomethacin or 10^{-5} mol/L capsaicin (data not shown). Sensitivity to aminophylline IN in –Ep preparations precontracted with carbachol 10^{-6} mol/L OUT was not affected by pretreatment with S-CMC ($-\log_{10}$ EC_{50}: 3.2 ± 0.1 *vs* 3.66 ± 0.11 for S-CMCpr *vs* controls, respectively; NS).

Human bronchial rings

The carbachol concentration–response curves for human bronchial rings pre-incubated for 4 h with S-CMC, without epithelium, were less sensitive to carbachol stimulation (Fig. 4, Table 4), thus indicating that removal of the epithelium increased sensitivity to carbachol stimulation only in preparations not pre-incubated with S-CMC, whereas pre-incubation with S-CMC seemed to diminish sensitivity to carbachol.

Table 4 EC_{50} values for human bronchus

	+Ep	–Ep	*P*
Control	7.2	7.7	NS
S-CMC	6.9	6.8	NS
P	NS	< 0.05	

Data are the mean±SEM of EC_{50} values ($-\log_{10}$; mol/L), obtained in human preparations with (+Ep) and without (–Ep) epithelium and either incubated for 4 h in the presence of s-carboxymethyl-L-cysteine (S-CMC; 10^{-5} mol/L; $F = 8.54$) or not (control).

DISCUSSION

In the present study, we found that removal of the epithelium rendered preparations more sensitive to carbachol stimulation and, alternatively, that pretreatment with S-CMC rendered preparations without epithelium less sensitive to carbachol stimulation compared with controls (–Ep). These changes, although small, were significant and require an explanation. In addition, we were able to demonstrate similar small effects in human bronchus denuded of epithelium (i.e. preparations incubated with S-CMC were less sensitive to carbachol).

The results of various experimental studies, including our own, clearly demonstrate that tracheal epithelium can modulate tracheal smooth muscle contraction.[1–3,24,29] In most of these studies, the inhibitory effects of the bronchial epithelium were found to be small (except in dogs). However, it has been notoriously difficult to identify a single underlying pathophysiological mechanism. In addition, it seems that not only the contracting, but also the relaxing effects of some pharmacological agents are dependent on the presence of an intact epithelium.[25] This may be relevant for a better understanding of different airway pathologies. It has been shown that the bronchial epithelium is damaged in patients with severe asthma, indicating that bronchial epithelial damage and airway hyperresponsiveness could be linked.[26] Several studies have demonstrated that the bronchial epithelium constitutes a powerful diffusion barrier[2,27] and can attenuate the effects of pharmacological agents applied luminally. The findings of the present study, although demonstrating small effects, support this hypothesis. It appears that the epithelium in rats mediates less inhibition compared with guinea-pigs. In guinea-pigs, we have found bigger differences following serosal compared with epithelial perfusion (D Pavlovic *et al.*, unpubl. obs., 1999). However, it has been quite difficult to demonstrate airway hyperresponsiveness *in vitro* in airways already hyperresponsive *in vivo*. The significance of small *in vitro* changes that we observed may (but do not have to) correspond to more important *in vivo* changes, which we would like to verify in further experiments.

Incubation with S-CMC appears to influence human ring preparations but not rat preparations. However, in the present study, rat preparations were incubated for 60 min whereas human ring

preparations were incubated for 4 h and this may explain the differences observed.

In a recent animal study, it was shown that in SO_2-exposed rats carbocysteine diminished fucose, sialic acid and protein content, as well as the number of inflammatory cells, and reduced free radicals and elastase activity in bronchoalveolar lavage fluid.[20] In addition, an increase in cAMP in tracheal tissue was observed[20] and patch-clamp techniques revealed increased activity and density of cAMP-dependent Cl^- channel.[21] These findings could, in principle, explain our observations. However, numerous other mechanisms may also be involved.

There is evidence that blocking enkephalinase (which degrades kinins) by phosphoramidon increases bronchoconstriction similar to removal of the epithelium.[28] Indeed, mechanical removal of the epithelium could promote the release of different mediators from mast cells, which could be responsible, at least in part, for the bronchial hyperresponsiveness observed experimentally.[29] It has also been shown that S-CMC increases the production of sialomucins[11,12] and suggested that sialomucins could have an antikinin action.[17] The anti-inflammatory activity of S-CMC was confirmed in an animal study where it reduced neutrophil infiltration provoked by intratracheal injection of interleukin (IL)-1.[18] The same study demonstrated that S-CMC diminished smoke-induced bronchial hyperresponsiveness in guinea-pigs. Very recently, it was demonstrated that treatment with S-CMC effectively reduces airway hyperreactivity and airway inflammation at different phases of the response to secondary allergen challenge in mice, implicating the possible importance of the timing of S-CMC administration.[19]

Indeed, capsaicin-sensitive nerve terminals secrete various, although only partially identified, mediators.[30–33] It is conceivable that some as yet unidentified mediator/s originating from capsaicin-sensitive nerve terminals could have inhibitory effects on tracheal smooth muscle contraction. In addition, mechanical removal of the epithelium could have contributed to the release of such mediator/s.[29] Nerve terminal destruction by capsaicin and the disappearance of the putative inhibitory agent could, in turn, have increased the sensitivity of tracheal smooth muscle to carbachol compared (in the present experiments) with S-CMCpr preparations not pre-incubated with capsaicin. It is tempting to hypothesize that S-CMS binds nitric oxide (NO) released from sensory nerves in epithelium-denuded tissues and thereby suppresses the responsiveness to carbachol.[34] As a result of treatment with capsaicin, the NO stores may be depleted and S-CMS will not inhibit carbachol-induced contractions any further.

Other hypotheses, such as an anti-oxidant action for S-CMC, similar to one recently described for carbocysteine lysine salt monohydrate (SCMC-Lys),[35] should be examined. Last, but not least, one simple feed-back mechanism could be proposed that could explain our finding of diminished sensitivity in –Ep preparations from S-CMCpr: removal of the epithelium and removal of one or more excitatory agent(s) secreted by the epithelium renders tracheal smooth muscle less sensitive to carbachol. The prostaglandins, products of arachidonic acid metabolism, do not seem to be involved because pre-incubation of preparations with indomethacin did not affect the sensitivity of the preparations. The therapeutic effects of S-CMC may be variable and it has been proposed that variation in the efficacy of S-CMC and pharmacogenomics may be underlying factors in this variation.[36–38] Certainly, further insights into the mechanisms of action of S-CMC are needed to understand its effects, especially in human tissues.

In the present study, we used a slightly improved *in vitro* model that permitted independent perfusion of the epithelial (luminal) and serosal (outside) layers of the airway.[2,4,5,24,39–41] However, an alternative model is also available.[42] This system incorporates interchangeable carriers for tracheal or bronchial ring preparations that facilitate comparative experiments and is not a significant financial burden when the use of both techniques is necessary.

The principle finding of the present study is that pretreatment of animals, or longer incubation of human bronchus, with S-CMC induces relatively small decrease of reactivity of rat or human airway smooth muscle denuded of epithelium. Whether S-CMC has beneficial effects in hyperresponsive airways with injured epithelium has to be examined. Because S-CMC is widely used throughout Europe as a supplementary therapeutic in various pathologies characterized by airway obstruction and hyperresponsiveness, further explanation of the mechanisms of action underlying its beneficial effects, probably in a model of sensitized, allergic animals, would be valuable.

ACKNOWLEDGEMENTS

This study was funded by the research budget of INSERM U408, Paris, and EMA University, Greifswald.

REFERENCES

1. Hay DW, Farmer SG, Raeburn D *et al*. Airway epithelium modulates the reactivity of guinea-pig respiratory smooth muscle. *Eur. J. Pharmacol*. 1986; **129**: 11–18.
2. Pavlovic D, Fournier M, Aubier M, Pariente R. Epithelial vs. serosal stimulation of tracheal muscle: Role of epithelium. *J. Appl. Physiol*. 1989; **67**: 2522–6.
3. Stuart-Smith K, Vanhoutte PM. Airway epithelium modulates the responsiveness of porcine bronchial smooth muscle. *J. Appl. Physiol*. 1988; **65**: 721–7.
4. Pavlovic D, Viires N, Zedda C, Fournier M, Aubier M. Effects of corticosteroids on epithelial structure and smooth muscle function of rat trachea. *Eur. Respir. J*. 1998; **11**: 575–82.
5. Pavlovic D, Brione E, De Vernejoul D, Aubier M. Partial inhibition by epithelium of tracheal smooth muscle relaxation induced by the potassium channel activator, BRL 38227. *Br. J. Pharmacol*. 1993; **110**: 139–44.
6. Marom Z, Shelhamer J, Alling D, Kaliner M. The effects of corticosteroids on mucous glycoprotein secretion from human airways *in vitro*. *Am. Rev. Respir. Dis*. 1984; **129**: 62–5.
7. Wanner A, Zarzecki S, Hirsch J, Epstein S. Tracheal mucous transport in experimental canine asthma. *J. Appl. Physiol*. 1975; **39**: 950–7.
8. Brown DT. Carbocysteine. *Drug Intell. Clin. Pharm*. 1988; **22**: 603–8.
9. Molina C, Aiache JM, Pradelle J, Brun J, Cheminat JC. Interet clinique des medications fluidifiantes des secretions bronchiques. *Poumon. Coeur* 1970; **26**: 91–102.
10. Allegra L, Cordaro CI, Grassi C. Prevention of acute exacerbations of chronic obstructive bronchitis with carbocysteine lysine salt monohydrate: A multicenter, double-blind, placebo-controlled trial. *Respiration* 1996; **63**: 174–80.
11. Degand P. Etude biochemique du mode d'action de la s-carboxy-methyl-cysteine. *Bull. Physiophathol. Respir*. 1973; **9**: 462–3.
12. Havez R, Degand P, Roussel P, Randoux A. Mode d'action biochemique des derives de la cysteine sur le mucus bronchique. *Poumon. Coeur* 1970; **26**: 81–90.
13. Davis SS, Deverell LC. Rheological factors in mucociliary clearance. The assessment of mucotropic agents using an *in vitro* model. *Mod. Probl. Paediatr*. 1976; **19**: 207–17.

14. Martin R, Litt M, Marriott C. The effect of mucolytic agents on the rheologic and transport properties of canine tracheal mucus. *Am. Rev. Respir. Dis*. 1980; **121**: 495–500.
15. Edwards GF, Steel AE, Scott JK, Jordan JW. s-Carboxymethylcysteine in the fluidification of sputum and treatment of chronic airway obstruction. *Chest* 1976; **70**: 506–13.
16. Puchelle E, Aug F, Polu JM. Effect of the mucoregulator s-carboxymethyl-cysteine in patients with chronic bronchitis. *Eur. J. Clin. Pharmacol*. 1978; **14**: 177–84.
17. Muh J, Latreille J, Guerrin F. Recherche d'inhibiteurs naturels du bronchospasme a la bradykinine chez le cobaye. In: *Colloaque International de Pathologie Thoracique*. Poissot, Medic diffuser, Clichy. 1968; 193–202.
18. Asti C, Melillo G, Caselli GF *et al*. Effectiveness of carbocysteine lysine salt monohydrate on models of airway inflammation and hyperresponsiveness. *Pharmacol. Res*. 1995; **31**: 387–92.
19. Takeda K, Miyahara N, Kodama T *et al*. s-Carboxymethylcysteine normalises airway responsiveness in sensitised and challenged mice. *Eur. Respir. J*. 2005; **26**: 577–85.
20. Ishibashi Y, Okamura T, Masumoto Y, Tachiiri T, Momo K. Effects of carbocisteine on airway inflammation and related events in SO_2-exposed rats. *Nihon Kokyuki Gakkai Zasshi* 2001; **39**: 17–23.
21. Meyer G, Doppierio S, Daffonchio L, Cremaschi D. s-Carbocysteine-lysine salt monohydrate and cAMP cause non-additive activation of the cystic fibrosis transmembrane regulator channel in human respiratory epithelium. *FEBS Lett*. 1997; **404**: 11–14.
22. Hochberg Y, Benjamini Y. Controlling of the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Soc. Ser. B* 1995; **57**: 289–300.
23. Curran-Everett D. Multiple comparisons: Philosophies and illustrations. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 2000; **279**: R1–8.
24. Folkerts G, Nijkamp FP. Airway epithelium: More than just a barrier! *Trends Pharmacol. Sci*. 1998; **19**: 334–41.
25. Fedan J, Hay D, Farmer S, Raeburn D. Epithelial cells: Modulation of airway smooth muscle reactivity. In: Barnes PJ, Rodger I, Thompson N (eds). *Asthma, Basic Mechanics and Clinical Management*. Academic Press, London. 1988; 143–62.
26. Laitinen LA, Heino M, Laitinen A, Kava T, Haahtela T. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am. Rev. Respir. Dis*. 1985; **131**: 599–606.
27. Morikawa T, Webber SE, Widdicombe JG. The effect of hydrogen peroxide on smooth muscle tone, mucus secretion and epithelial albumin transport of the ferret trachea *in vitro*. *Pulmon. Pharmacol*. 1991; **4**: 106–13.
28. Frossard N, Stretton CD, Barnes PJ. Modulation of bradykinin responses in airway smooth muscle by epithelial enzymes. *Agents Actions* 1990; **31**: 204–9.
29. Franconi GM, Rubinstein I, Levine EH, Ikeda S, Nadel JA. Mechanical removal of airway epithelium disrupts mast cells and releases granules. *Am. J. Physiol*. 1990; **259**: L372–7.
30. Meini S, Evangelista S, Geppetti P *et al*. Pharmacologic and neurochemical evidence for the activation of capsaicin-sensitive sensory nerves by lipoxin A4 in guinea pig bronchus. *Am. Rev. Respir. Dis*. 1992; **146**: 930–4.
31. Kawao N, Ikeda H, Kitano T *et al*. Modulation of capsaicin-evoked visceral pain and referred hyperalgesia by protease-activated receptors 1 and 2. *J. Pharmacol. Sci*. 2004; **94**: 277–85.
32. Ono K, Saito A, Goto K, Kasuya Y, Shigenobu K. Elucidation with the protease alpha-chymotrypsin of the inhibitory modulating action of endogenous neuropeptide Y over sympathetic neurotransmission in rat vas deferens. *J. Pharmacol. Sci*. 2003; **91**: 113–24.
33. Paulino N, Dantas AP, Bankova V *et al*. Bulgarian propolis induces analgesic and anti-inflammatory effects in mice and inhibits *in vitro* contraction of airway smooth muscle. *J. Pharmacol. Sci*. 2003; **93**: 307–13.
34. Kloek J, van Ark I, Bloksma N *et al*. Glutathione and other low-molecular-weight thiols relax guinea pig trachea *ex vivo*: Interactions with nitric oxide? *Am. J. Physiol. Lung Cell Mol. Physiol*. 2002; **283**: L403–8.
35. Brandolini L, Allegretti M, Berdini V *et al*. Carbocysteine lysine salt monohydrate (SCMC-LYS) is a selective scavenger of reactive oxygen intermediates (ROIs). *Eur. Cytokine Netw*. 2003; **14**: 20–6.
36. Steventon GB. A methodological and metabolite identification study of the metabolism of s-carboxymethyl-L-cysteine in man. *Chromatographia* 1998; **48**: 561–8.
37. Steventon GB, Waring R, Sturman S, Heafield M, Williams A. The metabolism of s-carboxymethyl-L-cysteine in man: A neurodegenerative disease study. *Med. Sci. J*. 1999; **27**: 119–20.
38. Steventon GB. Diurnal variation in the metabolism of s-carboxymethyl-L-cysteine in humans. *Drug Metab. Dispos*. 1999; **27**: 1092–7.
39. Folkerts G, van der Linde HJ, Nijkamp FP. Virus-induced airway hyperresponsiveness in guinea pigs is related to a deficiency in nitric oxide. *J. Clin. Invest*. 1995; **95**: 26–30.
40. Folkerts GLH, Verheyen AK, Nijkamp FP. Endogenous nitric oxide modulation of potassium-induced changes in guinea-pig airway tone. *Br. J. Pharmacol*. 1995; **115**: 1194–8.
41. Folkerts G, Kloek J, Geppetti P, van der Linde HJ, Nijkamp FP. Factors that determine acetylcholine responsiveness of guinea pig tracheal tubes. *Eur. J. Pharmacol*. 2001; **420**: 151–7.
42. Munakata M, Huang I, Mitzner W, Menkes H. Protective role of epithelium in the guinea pig airway. *J. Appl. Physiol*. 1989; **66**: 1547–52.