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Oral N-acetylcysteine or S-carboxymethylcysteine inhibit cigarette smoke-induced hypersecretion of mucus in rat larynx and trachea *in situ*

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Oral N-acetylcysteine or S-carboxymethylcysteine inhibit cigarette smoke-induced hypersecretion of mucus in rat larynx and trachea in situ. *D.F. Rogers, N.C. Turner, C. Marriott, P.K. Jeffery.*

ABSTRACT: Two weeks exposure of rats to cigarette smoke (CS) significantly ($p<0.05$) increased the secretion of fucose-containing glycoconjugates above normal in an *in situ* preparation of larynx and trachea. After equilibration mean basal secretion in CS-exposed rats was 24 μg (per 30 min collection) which was 8 times higher than that of unexposed animals ($p<0.01$). N-acetylcysteine (NAC) or S-carboxymethylcysteine (SCMC) given as 1% of the drinking water, before and after daily exposure to CS, significantly inhibited the development of the CS-induced increase in fucose secretion reducing the mean for basal secretion in each group to 7 and 5 μg, respectively ($p<0.05$). Neither NAC nor SCMC had significant effects on baseline glycoconjugate secretion in control animals. Albumin was inconsistently present in the secretions of both control and CS-exposed animals, whereas in those exposed to CS and also given one of the two cysteine derivatives there was a consistent increase in albumin transudation.

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Mucus hypersecretion and the associated hypertrophy and hyperplasia of mucus-secreting tissues are features of chronic bronchitis, cystic fibrosis and often asthma [1–8]. In humans, the increased amount of mucus-secreting tissue is associated with increased output of radioactively-labelled mucous glycoproteins as measured *in vitro* [9, 10].

Sub-acute exposure of specific pathogen free rats to an atmosphere of cigarette smoke (CS) induces not only epithelial secretory cell hyperplasia [11, 12] but also an associated hypersecretion of mucus, determined either by the measurement of radio-labelled glycoconjugates *in vitro* [13] or of fucose *in situ* [14]. The experimentally induced histological changes can be inhibited by a number of steroidal and non-steroidal anti-inflammatory agents [11, 15–18] and also the two cysteine derivatives which we investigate here [12, 19]. In this study we have investigated the capacity of NAC or SCMC to inhibit the mucus hypersecretion associated with CS-induced secretory cell hyperplasia. In addition, we have attempted to determine the effects of CS alone or in combination with cysteine derivatives on the contribution of serum transudate to the airway secretions.

Materials and methods

Thirteen week old male, albino, specific pathogen-free Wistar COBS (Charles River UK Ltd), with a mean body weight on the first day of the experiment of 350 g (SEM 3 g), were used. They were housed in laminar flow cabinets (Forth Tech Services Ltd, Scotland) to preserve lung "cleanliness" [20]. Water and dry pellet food (Heygate and Sons Ltd, Northampton, England) were freely available except during the period of exposure to CS when they were withdrawn from both experimental and control animals. Six groups of rats were used: 1) normal, untreated control rats; 2) normal rats given NAC only; 3) normal rats given SCMC only; 4) rats made "bronchitic" by sub-acute exposure (14 days) to CS; and two groups of rats exposed to CS but which also received either 5) NAC; or 6) SCMC in their drinking water. At the end of the 2 week exposure period luminal tracheal secretions were measured and the response to an acute administration of CS was investigated *in situ* in all rats.

Cigarette smoke exposure

The cigarette smoke exposure system and measurement of cabinet carbon monoxide (CO) has been described in detail previously [12]. Smoke, from 25 "middle to high tar" cigarettes yielding 25 mg tar, 2.4 mg nicotine and 14 mg carbon monoxide per cigarette [21] was generated over a 4 h period each day for 14 consecutive days by automatic smoking machines and blown into cabinets in which the rats were housed. A similar regimen has been found to give airways secretory cell hyperplasia and epithelial thickening after two weeks [12–13].

Drug administration during sub-acute CS exposure

NAC and SCMC were given orally as 1% (w/v in tap water) of the drinking water. The pH of the solution was adjusted to 6.5, equivalent to that of the tap water used. Administration of NAC and SCMC began two days before exposure to CS to acclimatize the animals. A fresh solution of the drugs was given at least every second day. Rats were caged in pairs and drank the solutions from single calibrated water bottles. The water consumption of rats given the drugs was initially low but recovered to normal levels within 2–3 days: the average daily dose ($mg \cdot kg^{-1}$ body weight) for NAC or SCMC was 1,419 (SEM 42, n=57 determinations) or 831 (SEM 40, n=72 determinations), respectively. The dose is the same as that used previously for NAC [12] and is considerably higher that that currently used in man albeit consumed in a different way.

Laryngotracheal secretion

Between two and four days after the end of the sub-acute exposure period laryngotracheal secretion in normal and "bronchitic" rats, with and without drug treatment, (mean body weight now 423 g; SEM 10) was studied *in situ* (fig. 1) using a method described in detail previously [14]. Rats were anaesthetized with pentobarbitone sodium BP (60–80 $mg \cdot kg^{-1}$, *i.p.*). The larynx and trachea were cannulated and perfused with warmed, oxygenated, physiological saline for 30 min periods up to three and a half hours. At the end of each 30 min period the perfusion fluid (containing secretions) was collected and the system refilled. During the first 5 min of the 5th collection animals of all groups were exposed directly to an acute exposure with cigarette smoke (diluted 1:3 with air) generated from a single "middle to high tar" cigarette and delivered *via* the emptied larynx and tracheal segment: the system was refilled and perfused for the remaining 25 min of the collection. We have previously shown that acute administration of air has no consistent effect on secretion [14]. At the end of each collection the samples were frozen at -20°C before further analysis.

Sample preparation and assays

Each of the collected samples was prepared and assayed as described in detail previously [14]. Briefly,

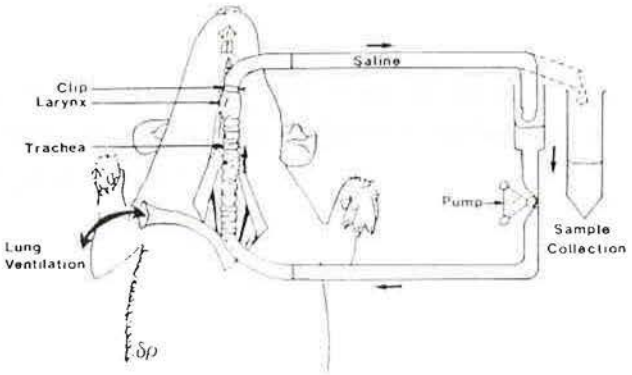


Fig. 1. – *In situ* preparation for the collection of laryngotracheal secretions in the anaesthetized rat. Physiological saline perfuses the isolated airway. Evey half hour the saline, containing secretions, is collected [14].

the frozen samples were freeze-dried and the proteins, including glycoproteins, precipitated with ethanol. After centrifugation, the precipitate of each sample was re-suspended in 0.1 M sodium hydroxide, sonicated, and aliquots assayed for fucose [22], hexose [23], protein [24], and rat serum albumin [25]. The minimum detectable concentration of albumin was 2 μg per collection. The mean coefficient of variation of the assay was 10%, and the mean error of repeat measurement 3% [14].

Statistical analysis

Data obtained for the concentration of secretions were not normally distributed and a two-tailed Mann-Whitney U-test was used to compare groups. Responses to acute administrations of CS diluted with air were analysed using a two-tailed Wilcoxon signed-rank test for paired observations [26]. P-values equal to or less than 0.05 were taken as significant. For ease of presentation, data have been summarized and given as means and their standard errors (SEM). Inhibition of secretion by either drug was considered to be "complete" when the value for CS+NAC was significantly less than that for CS alone and not significantly different to the corresponding control value.

Results

During the pre-treatment period all rats appeared well and maintained a constant mean body weight. Table 1 shows the mean (SEM) amounts of fucose (μg) during equilibration (collections 1–3) to baseline at collection 4 for normals and rats exposed to CS for 2 weeks, with or without concurrent NAC or SCMC treatment. The CS-exposed animals tolerated SCMC less well than NAC, in that they failed to gain weight as rapidly, showing reduced water consumption and weight loss towards the end of the experiment. For each group mean baseline values were not significantly different to those of collections 2 and 3. The baseline values allow for comparison of the effects of sub-acute CS and the extent

Table 1. – The effect of N-acetylcysteine (NAC) and S-carboxymethylcysteine (SCMC) on the secretion of fucose-containing glycoconjugates into the trachea and larynx of rats sub-acutely exposed to tobacco smoke: mean values in µg per collection (±SEM)

Period		Control n=6–10	CS exposed n=7	+NAC n=6–8	+SCMC n=6–7
A	1	24.3 (7.3)	85.6 (37.6)*	21.2 (3.1)†	23.1 (5.3)†
	2	4.5 (1.2)	47.4 (30.2)*	10.1 (3.9)†	7.5 (1.0)†
	3	3.1 (1.3)	41.2 (25.7)*	7.1 (2.1)†	5.0 (0.7)†
B	4	2.9 (1.1)	23.9 (6.2)*	7.4 (2.6)†	4.9 (1.0)†
C	5	5.5 (1.6)!	33.9 (9.4)*	8.4 (1.9)†	7.2 (1.1)
D	6	1.8 (1.2)	25.0 (6.1)*	5.7 (1.7)†	5.3 (0.6)†
	7	2.9 (1.6)	17.6 (4.2)*	5.3 (1.7)†	6.5 (2.4)†

CS exposed rats were given smoke from 25 cigarettes, generated over 4 h, for 14 days. Two groups of rats (+NAC and +SCMC) received NAC or SCMC as 1% solutions in their drinking water during the two weeks of smoke exposure period. Control animals were treated similarly but were not exposed to cigarette smoke. CS: cigarette smoke; A: periods of equilibration after end of dissection and cannulation of the air-way segment; B: baseline values; C: effect of acute-exposure to CS given directly into the segment; D: recovery periods after acute exposure; *: $p<0.05$ compared to controls; †: $p<0.05$ compared to CS exposed; !: $p<0.05$ compared to period 4; n: number of animals.

Table 2. – The effect of NAC or SCMC on the secretion of hexose into the tracheal lumen of rats sub-acutely exposed to cigarette smoke (CS). Mean µg per collection (±SEM)

Period		Control n=5–9	CS exposed n= 8	+NAC n= 9–10	+SCMC n= 7
A	1	126.2 (28.4)	276.7 (42.4)*	196.2 (25.1)*	176.3 (25.4)
	2	53.6 (10.1)	140.2 (34.2)**	118.8 (32.1)	56.1 (9.9)†
	3	54.5 (16.7)	124.9 (38.0)	68.2 (16.9)	30.0 (7.5)
B	4	41.2 (8.1)	101.4 (26.4)	69.4 (25.1)	36.4 (14.5)†
C	5	100.8 (12.2)!!	147.8 (33.1)	77.8 (21.0)	83.3 (18.1)
D	6	54.2 (13.4)	116.7 (27.4)	54.9 (17.6)	52.5 (13.5)
	7	49.4 (10.8)	81.8 (24.3)	52.5 (15.5)	47.4 (22.5)

NAC: N-acetylcysteine; SCMC: S-carboxymethylcysteine; *: $p<0.05$; **: $p<0.01$ compared to controls; †: $p<0.05$ compared to CS exposed; !!: $p<0.01$ compared to period 4.

Table 3. – Effect of NAC or SCMC on the secretion of protein into the tracheal lumen of rats sub-acutely exposed to cigarette smoke (CS). Mean µg per collection (±SEM)

Period		Control n=6–8	CS exposed n=8	+NAC n=8–10	+SCMC n=7
A	1	3381 (682)	4204 (696)	3734 (562)	3771 (405)
	2	1431 (406)	2315 (521)	2567 (613)	1968 (140)
	3	1361 (550)	2324 (712)	1920 (484)	1643 (156)
B	4	1082 (385)	2000 (520)	2055 (599)	1414 (170)
C	5	2114 (518)!	2483 (386)	2722 (540)	2571 (434)!
D	6	1271 (599)	2152 (446)	2022 (382)	1929 (256)
	7	1245 (411)	1744 (362)	1363 (292)	1757 (539)

NAC: N-acetylcysteine; SCMC: S-carboxymethylcysteine; !: $p<0.05$ compared to period 4.

Table 4. – The effect of NAC and SCMC on albumin transudation into the tracheal lumen of rats sub-acutely exposed to cigarette smoke. Mean μg per collection (±SEM)

Period		Control (n=6–8)	CS exposed* (n=7)	+NAC* (n=8–10)	+SCMC* (n=5–7)
A	1	<2**	78.4	158.4 (59.8)*	294.6 (111.4)
	2	<2	17.1	103.2 (53.5)	198.8 (74.4)
	3	<2	15.5	101.3 (44.2)	152.8 (55.3)†
B	4	29.9+	16.1	97.8 (44.3)	160.4 (47.4)†
C	5	<2	68.1	128.2 (47.3)	212.7 (68.1)
D	6	<2	67.8	62.1	150.8 (62.3)
	7	24.7++	17.7	75.0 (32.0)	170.2 (59.1)†

Abbreviations as in table 1. SEM values only given where half or more of the animals in the group had detectable albumin; *: albumin inconsistently present in 2 of 7 animals in the CS-exposed group, in 5 of 9 treated with NAC and 6 of 7 with SCMC; **: detectable limit was 2 μg or more; +: only detectable in one animal when the value was 239.5; ++: only detected in one animal when the value was 148.0; †: $p<0.05$ compared to CS-exposed group.

of inhibition by NAC or SCMC of the CS-induced hypersecretion. Collection 5 represents the response to an acute administration of CS given directly into the tracheal segment, and collections 6 and 7 the recovery after the acute exposure.

Effects of sub-acute exposure to CS

Sub-acute exposure raised mean fucose concentrations significantly ($p<0.05$) at each of the 6 collection periods when compared with the respective collections for normal controls (table 1). The mean basal secretion of fucose in "bronchitic" rats was approximately 8 times greater than that seen in normal rats ($p<0.01$). Treatment with either NAC or SCMC completely inhibited the increased fucose concentrations seen in the "bronchitic" rats at baseline and at every subsequent collection but for collection 5 in the SCMC group. At collection 2 and at baseline SCMC also completely inhibited the CS-induced increase in hexose. NAC had no significant effect on the hexose response to CS (table 2). There were no statistically significant changes in total protein secretion between groups for any collection (table 3). The albumin content varied (table 4). In the normal control group albumin was only detected (*i.e.* ≥2 μg) in the perfusate of two animals, one at collection 4 and another at 7. In the CS-exposed group it was present in 2 out of 7 at all collections. In contrast, albumin was detected in 5 of 9 and 6 of 7 CS-exposed animals treated with NAC or SCMC, respectively. Thus, the highest mean and consistent values for albumin were found in "bronchitic" animals given the drugs.

Response to acute CS

Tables 1 through 4 also show data for the effect of the acute administration of dilute CS on the mean values for markers. Significant changes were found in only two groups: *i.e.* normal rats and those given sub-acute CS exposure together with SCMC. In normal rats, the acute administration of CS caused significant increases of fucose ($p<0.05$), hexose ($p<0.01$) and protein ($p<0.05$), but not albumin. In rats exposed to CS for 2 weeks and given NAC or SCMC, the acute administration of CS caused only an increased content of protein in the secretions of the SCMC-treated animals ($p<0.05$). In normal animals NAC or SCMC treatment alone had no significant effect on secretion except for a raised mean secretion of fucose in collection 6 ($p<0.05$).

Discussion

Cigarette smoke (CS) exposure in man is associated with the development of mucous hypersecretion, due to hyperplasia and hypertrophy of airways mucus-secreting tissue [2, 27]. Sub-acute (2 weeks) exposure of specific pathogen free rats to an atmosphere of CS produces histological changes which mimic the salient changes to mucus-secreting tissue seen in man, *i.e.* there is an increase in the number of mucus-secreting cells, particularly in the surface epithelium [11, 12, 28] and an increase in discharge of intracellular secretion [13, 14]. We have previously demonstrated [14], using the same CS exposure regimen as that in the present study, that the secretion of fucose, a biochemical marker for epithelial-derived glycoconjugates [29], is significantly raised in bronchitic animals. The present study confirms the observation and has demonstrated further that the increased secretion of fucose-containing glycoconjugates is prevented by concurrent administration either of N-acetylcysteine or S-carboxymethylcysteine. However, inhibition of these secretory and associated cellular changes appears to be associated with increased transudation of albumin into the airway lumen.

Cigarette smoke (CS) contains a variety of potentially injurious agents including oxidants and free radicals which have been implicated in CS-induced damage to the lung. Among the intracellular anti-oxidants are the superoxide dismutases, catalase and reduced glutathione (GSH). The latter is known to be an important anti-oxidant present in large quantities in both intracellular and extracellular fluid lining the lung [30] and reduced sulphydryls are known

to be depleted during oxidant damage [31]. Furthermore, exogenous sulphydryl-containing compounds are capable of inhibiting oxidant damage to the lung [31–33]. It has been suggested that N-acetyl and S-acetyl cysteines may serve to increase and protect intracellular stores of reduced glutathione from depletion during oxidant attack [34–36] or in the case of the N-acetyl derivative may act as an oxygen radical scavenger [36]. Thus, these indirect (*via* GSH) or direct anti-oxidant (in the case of NAC) mechanisms may be responsible for the inhibitory effects of NAC and SCMC seen in the present study. Alternatively, NAC or SCMC may be inhibiting mucus hyper-secretion by preventing the development of mucous cell hyperplasia [12, 37] (which may be as a result of oxidative stress) or by direct effects on the rate of uptake of glycoprotein precursors into cells during synthesis of intracellular mucins through an, as yet, undefined mechanism of action. The latter has been suggested as a mechanism of action for selected non-steroidal, anti-inflammatory agents [38, 39].

Albumin was found inconsistently in the control animals of the present study. These *in vivo* results in the rat contrast with those obtained in the ferret trachea *in vitro* where there is evidence of active transport of albumin placed in the external buffer solution across the mucosa and into the tracheal lumen [40]. The apparent discrepancy may be related to the rate of output in rat *in situ* being less than that achieved *in vitro* with total concentrations over the 30 min collection period of the present study less than the threshold required for detection by RID. In agreement with EKLUND *et al.* [41], measurable amounts of albumin are found after exposure to CS in both bronchoalveolar lavage of healthy human smokers and in our rats exposed to CS sub-acutely. However, in contrast, NAC given sub-acutely in our study appeared to increase mucosal permeability to albumin whereas in the study by EKLUND *et al* [41] NAC (given as 200 mg *t.i.d.* for 8 weeks) reduced it, albeit not significantly.

Early pharmacokinetic studies in man examined the fate of ^{35}S label after a single dose (100 mg) of ^{35}S-NAC: these indicated that NAC was rapidly absorbed, passed into luminal mucus and was available in the lung in active form for at least 5 h [42]. SCMC has been reported to be concentrated in the lung resulting in high tissue to plasma ratios [43]. However, more recent studies in man by COTGREAVE *et al.* [44] failed to demonstrate free or bound NAC in bronchial lavage after oral NAC was given at 600 mg daily for 2 weeks: plasma free and total cysteine contents were unaltered but both free and total plasma glutathione increased significantly. The last mentioned authors argued that their results did not support the concept that NAC was acting through cleavage of disulphide bridges but rather that NAC, its metabolites or the consequent rise in glutathione might have "mucoregulatory" effects. Our present results support the latter concept in that they demonstrate a modulating effect of oral NAC or SCMC on the discharge of fucose-containing macromolecules in an animal model of bronchitis. The availability of the sulphydryl group to reduce S-S bonds in both molecules differs: that of NAC is free whilst that of SCMC is protected. It might be expected, therefore, that if acting by breakage of disulphide bonds, the distinct molecules would have differing potencies when given at similar doses. However, both appear to be similarly active in their "mucoregulation": it may be, therefore, that the availability of cysteine to GSH might be the more relevant and common mechanism of action.

In conclusion NAC or SCMC, when administered concurrently during a sub-acute smoking regimen, significantly inhibit the development of increased secretion of fucose-containing glycoconjugates into the rat larynx and trachea. The inhibition, however, appears to be associated with an increase in albumin transudation into the airway lumen.

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L'administration perorale de N-acétylcystéine ou de S-carboxyméthylcystéine inhibe l'hypersécrétion de mucus induite par la fumée de cigarette dans le larynx et la trachée du rat in situ. D.F. Rogers, N.C. Turner, C. Marriott, P.K. Jeffery.

RÉSUMÉ: Nous avons utilisé des rats spécifiquement libres de pathogènes, et exposés de manière subaiguë à la fumée de cigarette (CS), comme modèle pour l'hyperplasie des cellules muqueses, et nous avons déterminé les effets prophylactiques de la N-acétylcysteine (NAC) et de la S-carboxyméthylcystéine (SCMC) données par voie orale sur l'hypersécrétion associée de glycoconjugate, en utilisant une préparation *in situ* de larynx et de trachée. L'exposition de rats pendant deux semaines à la fumée de cigarette augmentait significativement ($p<0.05$) la sécrétion de glycoconjugate contenant du fucose au-dessus de la normale pendant la période de 3.5 h de l'étude *in situ*. La sécrétion basale moyenne chez les rats "bronchitiques" était de 24 µg (par 30 minutes de collection), ce qui est 8 fois supérieur à celle des animaux non exposés ($p<0.01$). NAC ou SCMC ont été données à la concentration de 1% dans l'eau à boire, respectivement avant et après l'exposition quotidienne à la fumée de cigarette. Elles ont inhibé de façon significative le développement de l'augmentation de sécrétion de fucose induite par la fumée de cigarette, en réduisant les moyennes pour la sécrétion basale de chaque groupe à 7 et 5 µg respectivement ($p<0.05$). Ni la NAC ni la SCMC n'ont eu d'effets significatifs sur la sécrétion de base des glycoconjugates chez les animaux contrôle. L'albumine était présente de façon irrégulière dans les sécrétions, à la fois chez les animaux contrôle et chez les animaux exposés à la fumée de cigarette, alors que chez ceux exposés à la fumée de cigarette et recevant aussi un de ces deux dérivés de la cystéine il y avait une augmentation régulière de la transsudation d'albumine. Nous concluons que NAC et SCMC inhibent l'hypersécrétion de glycoconjugate faisant suite à une exposition subaiguë à la fumée de cigarette, mais que l'augmentation consécutive de la transsudation d'albumine indique que l'hypersécrétion muquese des voies aériennes peut servir à limiter les lésions muqueuses et l'augmentation consécutive de la perméabilité de la muqueuse.
Eur Respir J., 1989, 2, 955–960