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Effects of carbocisteine on altered activities of glycosidase and glycosyltransferase and expression of Muc5ac in SO_2-exposed rats

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Abstract

Carbocisteine is a mucoregulatory drug regulating fucose and sialic acid contents in mucus glycoprotein. To investigate the mechanism of carbocisteine action, we evaluated the effects of carbocisteine on the activity of fucosidase, sialidase, fucosyltransferase and sialyltransferase, and on the expression of Muc5ac mRNA in the airway epithelium of SO_2-exposed rats. Wistar rats were repeatedly exposed to a 300-ppm SO_2 gas for 44 days. Carbocisteine (125 and 250 mg/kg × 2/day) was administered for 25 days after 20 days of SO_2 gas exposure. These enzyme activities were measured by fluorogenic substrate or glycoproteinic exogenous acceptor method. The expression levels of Muc5ac mRNA and protein were determined with real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Carbocisteine (250 mg/kg × 2/day) inhibited all the changes in these enzyme activities and the expressions of Muc5ac mRNA and protein in the lung after repeated SO_2 exposure. These findings suggest that carbocisteine may normalize fucose and sialic acid contents in mucin glycoprotein through regulation of these enzyme activities, and inhibition of both Muc5ac mRNA and protein expressions in SO_2-exposed rats.

Keywords: Carbocisteine; Fucosidase; Sialidase; Fucosyltransferase; Sialyltransferase; Muc5ac

1. Introduction

Goblet cell hyperplasia and metaplasia on the airway epithelium are associated with some changes in glycoprotein components in the mucus. Hyperplasia or metaplasia is often found in respiratory diseases such as chronic bronchitics (Miyata et al., 1998), asthma (Ordonez et al., 2001), COPD (Lamblin et al., 2001) and cystic fibrosis (Scalin and Glick, 1999). Alterations of fucose and sialic acid contents in mucus glycoproteins are frequently noted with goblet cell hyperplasia (Kobayashi et al., 1996). These alterations have been reported to co-exist with impaired mucociliary transport, vulnerability to bacterial infection, inflammatory cell infiltration, and increased airway resistance, and hyperreactivity (Rogers and Lethem, 1997). It is known that fucose and sialic acid are present as components of carbohydrate chains at C- and N-terminal positions of mucus glycoproteins (Van Halbeek et al., 1994), and these contents in mucus glycoproteins are regulated by fucosidase, sialidase, fucosyltransferase and sialyltransferase, which belong to glycosidase or glycosyltransferase. Berry et al. (1992), for example, reported that sialyltransferase activity was increased in pulmonary fractions of a bronchitis model induced by SO_2 gas exposure in rats. Baker and Sawyer (1975) showed that fucosyltransferase activity was increased both in lungs of patients with bronchitis and of dog models of SO_2-induced bronchitis but little information is available concerning pulmonary fucosidase and sialidase in respiratory disease and animal models. In this connection, mucin peptides, which constitute the protein backbones of mucus glycoproteins, are products of mucin genes. MUC5AC gene, which is one of the mucin genes, is known to be a major gene in airway. Zuhdi Alimam et al. (2000) showed that the increased expression of Muc5ac gene correlates with goblet cell metaplasia in mice, and Jany et

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al. (1991) also reported that mucin mRNA expression and goblet cell increase are induced in SO_2-exposed rats.

Carbocisteine, chemically called *S*-carboxymethylcysteine (*S*-CMC) or (2R)-2-amino-3-carboxy-methylsulfanyl-propanoic acid, is a mucoregulatory drug, which regulates fucose and sialic acid contents in mucus glycoprotein (Kobayashi et al., 1996), and is used for acute and chronic respiratory diseases to improve the expectoration of accumulated secretions in the respiratory tract (Brown, 1988). In addition, many studies have shown that *S*-CMC improves or reduces the changes in: mucus rheology (Barga et al., 1990), bacterial attachment to airways (Zheng et al., 1999; Ndour et al., 2001), cough sensitivity (Katayama et al., 2001), inflammatory cell infiltration (Hirata and Ohashi, 1995; Asti et al., 1995), ciliary cell damage (Ishibashi et al., 2001) and goblet cell hyperplasia (Miskovits et al., 1982). We also reported that *S*-CMC inhibits the increased levels of fucose and sialic acid in bronchoalveolar lavage fluid from SO_2-exposed rats (Ishibashi et al., 2001), and Yasuoka et al. (1986) suggested that *S*-CMC may correct the sialic acid/fucose ratio in mucus glycoproteins from patients with bronchitis. Baker and Sawyer (1975) showed that *S*-CMC improved an SO_2-induced change in rat pulmonary sialyltransferase. Taken together, these findings suggest that *S*-CMC may normalize the sialic acid contents of mucus glycoprotein by modulating regulatory sialyltransferase activity in airways. However, little information is still available about whether *S*-CMC would modulate the enzymes, fucosidase, sialidase and fucosyltransferase as well as MUC5AC, though *S*-CMC improved sialic acid/fucose ratio. In addition to sialyltransferase activity, we hypothesized that the primary action of *S*-CMC may be to inhibit the changes of fucosidase, sialidase and fucosyltransferase activities, and expression of Muc5ac mRNA, which correlates with goblet cell hyperplasia and metaplasia.

In this study, we investigated the effects of *S*-CMC on the activities of these enzymes regulating the fucose and sialic acid contents in mucus glycoproteins, as well as on the Muc5ac mRNA and protein expressions associated with goblet cell hyperplasia in the lung fraction of SO_2-exposed rats.

2. Materials and methods

2.1. Animals

Male Wistar-strain rats weighing 150 to 160 g were obtained from Japan Laboratory Animals (Tokyo, Japan). They were cared in a well-ventilated room under controlled environmental conditions in the animal care unit of Research Center, Kyorin Pharmaceutical (Tochigi, Japan). The rooms were lit between 7 a.m. and 7 p.m. and maintained at a temperature of 22 ± 2 °C and humidity of 50–60%. All animals tested were freely given solid food (Funabashi Farm, Funabashi, Japan) and tap water.

2.2. SO_2-treated model and drug treatment

Rats were exposed to 300 ppm of SO_2 gas, 4 h a day for 44 days by using a gas exposure apparatus (CIS-1000G, Japan Clear, Tokyo, Japan). The SO_2 gas concentration in the chamber was monitored with a detector tube (Gastec, Ayase, Japan). Rats were randomly divided into four groups: (1) normal group (non-SO_2-exposed); (2) control group (SO_2-exposed and treated with 0.3% carboxymethylcellulose sodium solution as the vehicle); (3) *S*-CMC 125 mg/kg, p.o. group [SO_2-exposed and treated with *S*-CMC (Kyorin Pharmaceutical, Tokyo, Japan) at 125 mg/kg, p.o.]; and (4) *S*-CMC 250 mg/kg, p.o. group (SO_2-exposed and treated with *S*-CMC at 250 mg/kg, p.o.). After exposure of rats to SO_2 gas for 20 days, the treatments were administered twice a day for 24 days while continuing daily exposure of SO_2 gas. On the day after the final exposure (thus, without gas exposure), these rats were drug-treated once more. Then, the animals were sacrificed by decapitation under anesthesia using ether 1 h after the last treatment, and lungs were removed for subsequent steps.

2.3. Determination of glycosidase and gycosyltransferase activities

2.3.1. Fucosidase activity

Fucosidase activity was assayed by using 4-methylumbelliferyl-α-L-fucosidase as the substrate (Turner et al., 1975). About 0.5 g of lung tissues each from SO_2-treated and normal rats was homogenized in 50 mM Tris–HCl buffer (pH 7.0, 250 mM sucrose; 7 ml/g tissue) at 4 °C. Fucosidase fractions were prepared by centrifuging the homogenate at $1000 \times g$ for 10 min at 4 °C. Then, the supernatant was centrifuged at $10{,}000 \times g$ for 60 min at 4 °C. The resultant pellet was suspended in 5 ml of 0.1 M citrate buffer (pH 5.5). An aliquot (100 μl) of the suspension was diluted to 2.4 ml with 0.1 M citrate buffer (pH 5.5). The diluted sample (100 μl) was mixed with 100 μl of 1.2 mM 4-methylumbelliferyl-α-L-fucosidase (Sigma, Missouri) and incubated for 3 h at 37 °C. The reaction was terminated by addition of 1.8 ml of 0.1 M glycine–NaOH buffer (pH 10.4). Fluorescence intensity was measured with a fluorometer (excitation at 366 nm, and emission at 440 nm; Hitachi F-2000, Tokyo, Japan). α-L-Fucosidase (Sigma) was used as a standard. Proteins were determined according to the method by Lowry et al. (1951).

2.3.2. Sialidase activity

Sialidase activity was assayed by using 2-(4-methylumbelliferyl)-α-D-scethyl-neuraminic acid as the substrate (Taniguchi et al., 1982). About 0.5 g of lung tissues each from SO_2-treated and normal rats was homogenized in 50 mM Tris–HCl buffer (pH 7.0, 250 mM sucrose; 7 ml/g tissue) at 4 °C. Sialidase fractions were prepared by centrifuging the homogenate at $1000 \times g$ for 10 min at 4 °C. The supernatant (2.1 ml) was centrifuged at $10{,}000 \times g$

for 60 min at 4 °C. The resultant pellet was suspended in 0.5 ml of 0.025 M sodium acetate buffer (pH 4.5). An aliquot (100 μl) of the suspension was mixed with 100 μl of 0.125 mM 2-(4-methylumbelliferyl)-α-D-scethyl-neuraminic acid (Sigma) and incubated for 3 h at 37 °C. The reaction was terminated by addition of 1.8 ml of 0.1 M glycine–NaOH buffer (pH 10.4). Fluorescence intensity was measured with a fluorometer (excitation at 366 nm, and emission at 440 nm; Hitachi F-2000). Sialidase (Neuraminidase, Sigma) was used as a standard. Proteins were determined according to the method by Lowry et al. (1951).

2.3.3. Fucosyltransferase activity

Fucosyltransferase assay was performed by the method described previously (Lenoir et al., 1995). About 0.25 g of lung tissues each from SO_2-treaed and normal rats was homogenized in 10 mM Tris–HCl buffer (pH 7.0, 250 mM sucrose, 10 mM KCl, and 10 mM $MgCl_2$; 9 ml/g tissue) at 4 °C. Fucosyltransferase fractions were prepared by centrifuging the homogenate at 30,000 × *g* for 30 min at 4 °C. The supernatant was centrifuged at 200,000 × *g* for 60 min at 4 °C. The resultant pellet was suspended in 0.4 ml of 10 mM Tris–HCl buffer (pH 7.4, 10 mM KCl and 10 mM $MgCl_2$). An aliquot (50 μl) of the suspension was mixed with 50 μl of 10 mM Tris–HCl buffer (pH 7.4, 0.625% Triton X-100, 10 mM KCl, and 10 mM $MgCl_2$), 50 μl of a 60-fold dilution of commercially available GDP-fucose-[^{14}C]-10 mM Tris–HCl solution (NEN Life Science Products, Boston, MA), and 100 μl of 25 mM AMP (2 mg/ml asialofetuin; Sigma), and then incubated for 30 min at 23 °C. The reaction was terminated by addition of 500 μl of 20% trichloroacetic acid, and then the reaction mixture was centrifuged at 300 rpm for 10 min. The pellet was washed twice with 3 ml each of distilled water and transferred in a vial. Radioactivity was measured with a liquid scintillation counter (Packard Tri-Carb 1900TR, USA), using the corresponding, non-incubated sample as a blank control. Proteins were determined according to the method by Lowry et al. (1951).

2.3.4. Sialyltransferase activity

Sialyltransferase assay was performed by the method described previously (Berry et al., 1992). About 0.5 g of lung tissues each from SO_2-treaed rats and normal rats was homogenized in 50 mM Tris–HCl buffer (pH 7.0, 250 mM sucrose; 7 ml/g tissue) at 4 °C. Sialyltransferase fractions were prepared by centrifuging the homogenate at 1000 × *g* for 10 min at 4 °C. The supernatant was centrifuged at 10,000 × *g* for 60 min at 4 °C. The resultant pellet was suspended in 0.6 ml of 50 mM Tris–HCl buffer (pH 7.4). An aliquot (100 μl) of the suspension was mixed with 400 μl of 50 mM Tris–HCl buffer (pH 7.4). An aliquot (100 μl) of this mixture as a sample was mixed with 200 μl of 100 mM morpholinoethanol sulfate, 40 μl of 1 mM $MgCl_2$, 40 μl of 1 mM $MnCl_2$, 20 μl of 33 mg/ml asialofetuin, and 100 μl of a 100-fold dilution of commercially available CMP-asialic acid-[^{14}C]-50 mM Tris–HCl solution (NEN Life Science Products), and then incubated for 120 min at 37 °C. The reaction was terminated by addition of 500 μl of 20% trichloroacetic acid to precipitate and collect the glycoproteins on Glass Microfiber Filter G/B filter (Whatman, UK). The glycoproteins collected were washed twice with 5 ml each of distilled water and once with 1 ml methanol and transferred in a vial. Radioactivity was measured with a liquid scintillation counter (Packard Tri-Carb 1900TR), using the corresponding, non-incubated sample as a blank control. Proteins were determined according to the method by Lowry et al. (1951).

2.3.5. RNA isolation and real-time RT-PCR for Muc5ac mRNA analysis

Total RNA was extracted from lung tissue samples, using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction based on the guanidium–phenol–chloroform procedure described by Chomczynski (1993). The purity of RNA was estimated by measuring the 260- to 280-nm absorption ratio with a spectrophotometer (Hitachi U-3210, Tokyo, Japan). Muc5ac mRNA was measured according to the method by Borchers et al. (1998). For real-time RT-PCR, primer sequences for Muc5ac were selected to minimize primer dimerization (Gen-Bank). These primers were confirmed to recognize specifically Muc5ac mRNA by performing BLASTN searches against dbEST and nr from a library (a nonredundant set of GenBank, EMBL and DDBJ database sequences). Primers for β-actin were selected from published sequences. The primer sequences (and amplicon sizes in parentheses) were as follows: for Muc5ac lower primer, 5′-TTC TAT GAA GAA GGC TGC TTA TTT GAC-3′, and for Muc5ac upper primer, 5′-TTT GAA TGG CCA AGC TTA GG-3′ (243 bp); and for β-actin lower primer, 5′-CAG GAT GGC GTG AGG GAG AGC-3′, and for β-actin upper primer, 5′-AAG GTG TAG TGG TGG GAA TGG-3′ (409 bp). Reverse transcription of Muc5ac and β-actin mRNA was carried out in a fluorometric thermal cycler (PCR express, Tokyo, Japan) and in a 10 μl mixture of 2450 ng of total RNA with 25 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a buffer containing 10 mM dithiothreitol, 1 mM deoxynucleotide triphosphates (dNTP), 10 units of RNasin, and 0.2 μM 3′-oligonucleotide. The first-strand synthesis consisted of primer annealing (25 °C, 10 min) and template extension (50 °C, 50 min). The synthesized cDNA was amplified by PCR in a 25 μl mixture of 0.75 units of *Taq* polymerase (Invitrogen), 1.5 mM $MgCl_2$, 0.2 μM each lower primer, 0.2 μM each upper primer, and a 4000-fold dilution of SYBR Green I (TAKARA SHUZO, Otsu, Japan). PCR amplification and fluorescence detection were accomplished using a Smart Cycler System (SC100, Cepheid, Sunnyvale, CA) (Belgrader et al., 2001). Fluorometric thermal cycler settings for PCR were as follows: for Muc5ac, an initial hold at 94 °C for 10 min, and then 40 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s and

84 °C for 6 s; and for β-actin, an initial hold at 94 °C for 90 s, and then 35 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 45 s and 86 °C for 6 s. Each intended PCR product was cloned into the TA cloning vector pCR4 (Invitrogen) and sequenced for confirmation of the correct insert sequence. The PCR products were also checked by electrophoresis on a 2% agarose gel and stained with ethidium bromide. All Muc5ac mRNA expression data were normalized to β-actin expression level from the same individual samples.

2.3.6. Relative expression levels of Muc5ac protein

Relative expression levels of Muc5ac protein were measured according to the method by Takeyama et al. (1999). Lung lysates were prepared using lysis solution and diluted with phosphate buffered saline (PBS), and 50 μl of the lysate solution was incubated with 50 μl of bicarbonate–carbonate buffer at 40 °C in a 96-well plate until dryness. The wells were washed three times with PBS and blocked with 2% BSA (fraction V, Sigma) for 15 min at room temperature. The wells were again washed three times with PBS and then incubated for 15 min after adding 50 μl of a dilution of mouse monoclonal MUC5AC antibody (1:100; NeoMarkers, Fremont, CA) in PBS containing 0.05% Tween 20 (Sigma) into each well. Then, the wells were washed three times with PBS, and 100 μl of horseradish peroxidase-goat anti-mouse IgG conjugate (1:10,000; Pierce, Rockford, IL) was dispensed into each well. After 10 min, the wells were washed five times with PBS. Color reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase solution (KPL, Gaithersburg, MD) and terminated with 1 M HCl. Absorbance was read at 450 nm with a microplate reader (Corona Electric MTP-32, Tokyo, Japan). Muc5ac protein was expressed as O.D. (450 nm) per g dry weight.

2.4. Statistical analysis

Data were expressed as mean ± standard error, and first tested by Bartlett's test for homogeneity variance. If Bartlett's test confirmed homogeneity of variance, then one-way analysis of variance and Dunnett's multiple comparison tests were applied to compare each dose group with the control group. If, however, Bartlett's test indicated heterogeneity of variance, Kruskal–Wallis rank test and Dunnett's-type multiple comparison test were applied. Probability of less than 0.05 was regarded as a significant level.

3. Results

3.1. Regulation of glycosidase and glycosyltransferase activities in SO_2-exposed rats

3.1.1. Fucosidase and sialidase

In order to investigate the mechanism of *S*-CMC action on glycosidase activity, we evaluated first the effects of *S*-CMC on the pulmonary fucosidase and sialidase activities in SO_2-exposed rats. Fucosidase and sialidase activities were measured by using a fraction of the 10,000 × *g* lung pellets rich in Golgi apparatuses. The effect of *S*-CMC on fucosidase activity in SO_2-exposed rats is shown in Fig. 1A. The enzyme activity was significantly decreased in repeatedly SO_2-exposed rats compared with normal rats ($P<0.01$). *S*-CMC tended to inhibit an SO_2-induced decrease in fucosidase activity at a dose of 125 mg/kg × 2/day, and significantly inhibited this decrease at a dose of 250 mg/kg × 2/day compared with control ($P<0.01$). The effect of *S*-CMC on sialidase activity in SO_2-exposed rats is shown in Fig. 1B. The activity was decreased in repeatedly SO_2-exposed rats, though not significantly compared with normal rats. *S*-CMC inhibited this SO_2-induced, apparent trend of decrease in sialidase activity at doses of 125 and 250 mg/kg × 2/day. These results show that *S*-CMC inhibited the SO_2-induced decrease in pulmonary fucosidase and sialidase activities in rats.

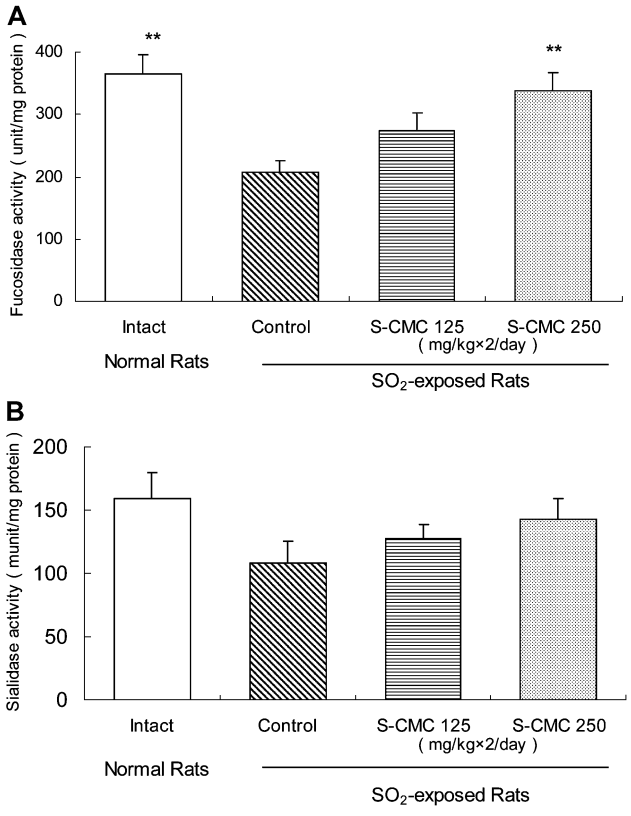

Fig. 1. Effect of S-CMC on fucosidase and sialidase activities in subcellular lung fractions prepared from SO_2-exposed rats. Fucosidase activity in 1.000–10.000 × *g* fraction of lung was assayed using 4-methylumbelliferyl-α-L-fucosidase as the substrate (A). Sialidase activity in 1.000–10.000 × *g* fraction in lung was assayed using 2-(4-methylumbelliferyl)-α-D-scethyl-neuraminic acid as the substract (B). Enzyme activity is expressed as unit per mg protein. Each column represents the mean ± S.E.M. (N = 10). $^{**}P<0.01$ vs Control (Dunnett's multiple comparison test).

3.1.2. Fucosyltransferase and sialyltransferase

Next, we evaluated the effect of *S*-CMC on the activities of pulmonary fucosyltransferase and sialyltransferase, both of which are gylcosyltransferase, in SO_2-exposed rats. Fucosyltransferase activity in a lung fraction of 200,000 × *g* pellets containing mitochondria was assayed. The effect of *S*-CMC on fucosyltransferase in SO_2-exposed rats is shown in Fig. 2A. The activity was significantly increased in repeatedly SO_2-exposed rats compared with normal rats ($P<0.01$). *S*-CMC was not effective at a dose of 125 mg/kg × 2/day in inhibiting an SO_2-induced increase in fucosyltransferase activity, but significantly effective at a dose of 250 mg/kg × 2/day compared with control ($P<0.05$). Pulmonary sialyltransferase activity in a fraction of 10,000 × *g* pellets containing Golgi apparatus was assayed. The effect of *S*-CMC on sialyltransferase activity in SO_2-exposed rats is shown in Fig. 2B. The activity tended to be increased in repeatedly SO_2-exposed rats, though not significantly compared with normal rats. *S*-CMC tended to inhibit this SO_2-induced trend of increase in sialyltransferase activity at doses of 125 and 250 mg/kg × 2/day. These results show that *S*-CMC inhibited the SO_2-induced increase in pulmonary fucosyltransferase and sialyltransferase activities in rats. The improving effect of *S*-CMC (250 mg/kg × 2/day) on this sialyltransferase activity in the present study is supported by a previous report (Berry et al., 1992).

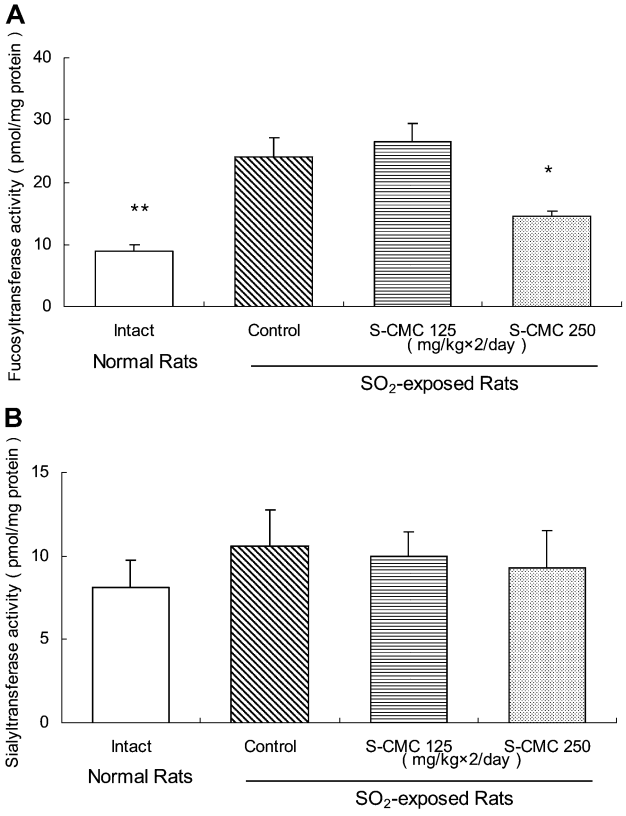

Fig. 2. Effect of S-CMC on fucosyltransferase and sialyltransferase activities in sub-cellular lung fractions prepared from SO_2-exposed rats. Fucosyltransferase activity in 30.000–200.000 × *g* fraction of lung was measured with GDP-fucose-[^{14}C] substrate (A). Sialyltransferase activity in 1.000–10.000 × *g* fraction of lung was measured with CMP-asialic acid-[^{14}C] substrate (B). Enzyme activity is expressed as pmol per mg protein. Each column represents the mean ± S.E.M. (N = 10). *$P<0.05$ and **$P<0.01$ vs Control (Dunnett's multiple comparison test).

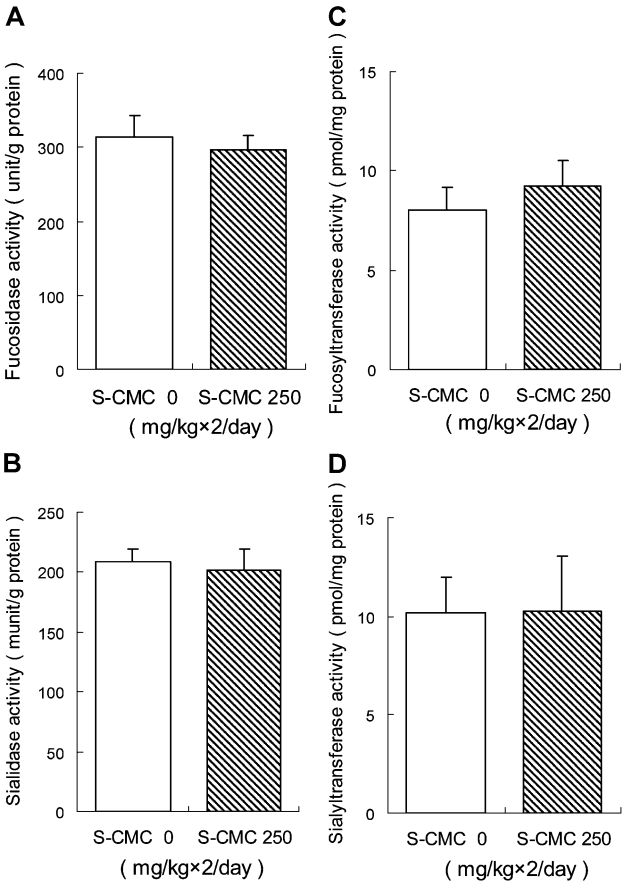

Fig. 3. Effect of S-CMC on fucosidase (A), sialidase (B), fucosyltransferase (C) and sialyltransferase (D) activities in sub-cellular lung fractions prepared from normal rats. Each enzyme activity was measured by the same method as for SO_2-exposed rats. Each column represents the mean ± S.E.M. of each group (N = 10).

3.2. Effects of glycosidase and glycosyltransferase activities in normal rats

Then, we investigated the effect of *S*-CMC on normal pulmonary fucosidase, sialidase, fucosyltransferase and sialyltransferase activities at the same fractions from non-SO_2-exposed rats. *S*-CMC had no effect on any of fucosidase, sialidase, fucosyltransferase and sialyltransferase activities in the corresponding fractions from normal rats orally treated with *S*-CMC (250 mg/kg × 2/day) for 25 days, the same period as for SO_2-exposed rats (Fig. 3). These results show that *S*-CMC have no direct activity on any of these pulmonary enzymes in a normal state. The result in the present study is in agreement with a previous study that *S*-CMC dose not affect sialyltransferase activity in non-SO_2-exposed rats (Berry et al., 1992).

3.3. Analysis of Muc5ac mRNA expression

We evaluated the state of Muc5ac mRNA expression and the effect of *S*-CMC on that state in SO_2-exposed rats, and compared with the results from normal rats. Muc5ac mRNA expression was determined with real-time RT-PCR method. The effect of *S*-CMC on Muc5ac mRNA expression in SO_2-exposed rats is shown in Fig. 4A. The molecular weight of PCR products was checked and confirmed by electrophoresis. PCR products of Muc5ac and β-actin mRNA from the rat lung yielded single fragments of about 243 and 409 bp (data not shown), respectively. These were consistent with the predicted sizes for rat Muc5ac and β-actin. Real-time RT-PCR evaluation showed that a low level of Muc5ac mRNA was expressed in the lung from normal rats. In contrast, the Muc5ac mRNA was expressed significantly more in the lung from repeatedly SO_2-exposed rats compared with normal ($P<0.01$, Fig. 4A). *S*-CMC tended to inhibit an SO_2-induced increase in Muc5ac mRNA expression at a dose of 125 mg/kg ×2/day, while it significantly inhibited the increased expression at a dose of 250 mg/kg ×2/day compared with control ($P<0.05$, Fig. 4A). This result indicates that *S*-CMC inhibits the SO_2-induced increase of pulmonary Muc5ac mRNA expression in rats.

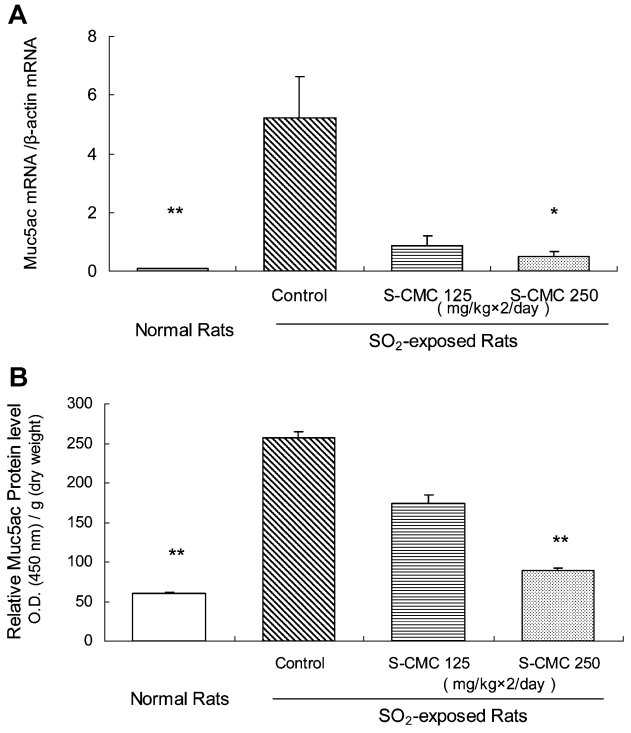

Fig. 4. Effect of S-CMC on Muc5ac mRNA expression and protein level in the lung sample from SO_2-exposed rats. RNA was extracted from rat lung. Muc5ac mRNA was measured by real-time RT-PCR and these date were normalized to β-actin level (A). Lung lysates were prepared and the relative Muc5ac protein was assayed by ELISA. Muc5ac protein levels were expressed as OD (450 nm) per g dry weight (B). Each column represents the mean ± S.E.M. (N=6-8). $*P<0.05$ and $**P<0.01$ vs Control (Dunnett's multiple comparison test).

3.4. Analysis of relative Muc5ac protein expression

We determined the effects of *S*-CMC on relative Muc5ac protein levels in SO_2-exposed rats (Fig. 4B). Relative levels of rat Muc5ac protein were measured by ELISA, using a mouse monoclonal anti-Muc5ac antibody. A low expression level of Muc5ac protein was present in the lung of normal rats. In contrast, the level of Muc5ac protein was significantly increased after repeated SO_2-exposure ($P<0.01$, Fig. 4B). *S*-CMC tended to inhibit an SO_2-induced increase in the Muc5ac protein expression at a dose of 125 mg/kg ×2/day, while it significantly inhibited the increased expression at a dose of 250 mg/kg ×2/day compared with control ($P<0.01$, Fig. 4B). This result indicates that *S*-CMC inhibits the SO_2-induced increase on pulmonary Muc5ac protein expression as well as Muc5ac mRNA expression in rats.

4. Discussion

Miyata et al. (1998) indicated that airway secretions from rabbits exposed to SO_2 gas are composed of long chains of sugars in mucins, thus leading to increased viscosity and obstruction, similar to the characteristics of sputa from bronchitic patients. The SO_2 gas exposure also induced increases in fucose and sialic acid contents in bronchoalveolar lavage fluid (Ishibashi et al., 2001), and changes of sialyltransferase activity in pulmonary subcellular fractions in rats (Berry et al., 1992). Further, it was reported that SO_2 exposure causes mucin mRNA expression and goblet cell increase, which indicates goblet cell hyperplasia in rats (Jany et al., 1991). Thus, the SO_2-exposed model is considered suitable to estimate the effects of drugs or drug candidates on the Muc5ac mRNA expression and mucin's sugar-structure changes in airways of bronchitic patients. In this study, we used the SO_2-exposed rat model to investigate the effects of *S*-CMC on the activities of fucosidase, sialidase, fucosyltransferase and sialyltransferase, and on the expression of Muc5ac mRNA and protein.

S-CMC has little direct mucolytic activity, and is a mucoregulatory drug regulating fucose and sialic acid contents in mucus glycoprotein (Brown, 1988). Based on that study and other previous studies (Rose et al., 2000; Miskovits et al., 1982; Zuhdi Alimam et al., 2000; Temann et al., 1997; Dabbagh et al., 1999; Louahed et al., 2000; Nadel, 2001), we hypothesized that *S*-CMC may inhibit the activity changes of fucosidase, sialidase, fucosyltransferase and sialyltransferase activities to normalize fucose and sialic acid contents in the lung fraction of SO_2-exposed rats, a model of bronchitis. The effects of *S*-CMC on pulmonary fucosidase, sialidase, fucosyltransferase and sialyltransferase activities in rats were explored by using previous methods (Berry et al., 1992; Turner et al., 1975; Taniguchi et al., 1982: Lenoir et al., 1995). *S*-CMC (125 and 250 mg/kg ×2/day) corrected all of the changes in the activities of fucosidase, sialidase, fucosyl-

transferase and sialyltransferase in lungs of SO_2-exposed rats. This supports our previous finding that *S*-CMC inhibited the changes in fucose and sialic acid levels in bronchoalveolar lavage fluid from SO_2-exposed rats (Ishibashi et al., 2001). Furthermore, *S*-CMC (250 mg/kg × 2/day) caused no effects on any of these enzyme activities in normal rats. Taken together, all these findings suggest that *S*-CMC may regulate or normalize the physicochemical properties of mucus, or the balance of fucose and sialic acid in mucus glycoprotein through correcting the activity of these enzymes in a still unknown way.

Next, we evaluated the effects of *S*-CMC on pulmonary Muc5ac mRNA and protein expressions in SO_2 exposed rats, by real-time RT-PCR and ELISA, respectively. *S*-CMC inhibited the Muc5ac mRNA and protein expression induced by 300-ppm SO_2 exposure. In a separate study which will be published elsewhere, we found that *S*-CMC inhibited an increased number of goblet cells, or goblet hyperplasia, in the airway epithelium of SO_2-exposed rats (data not shown). These effects of *S*-CMC were seen at the same dose, that is, 250 mg/kg/kg × 2/day. Miskovits et al. (1982) also reported that treatment with *S*-CMC (2250 mg/day) reduced goblet cell hyperplasia in patients with chronic bronchitis.

In the present study, the Muc5ac mRNA and protein are one of pulmonary mucin gene transcriptions, and Muc5ac protein, one of the backbone proteins of mucus glycoprotein, and were both induced in SO_2-exposed rats, as previously reported (Jany et al., 1991). It is also reported that SO_2 gas exposure induced the release of reactive oxygen radicals and elastase in bronchoalveolar lavage fluid in rats (Ishibashi et al., 2001). Many researchers have shown that relative oxygen radicals (Shim et al., 2001), interleukin (IL)-13 (Kondo et al., 2002), epidermal growth factor (EGF) (Takeyama et al., 1999, 2000, 2001; Nadel, 2001), and tumor necrosis factor-α (TNF-α) (Takeyama et al., 1999, 2000, 2001; Nadel, 2001) induced MUC5AC mRNA expression and goblet cell hyperplasia or metaplasia. Furthermore, Takeyama et al. (1999, 2000, 2001) and Nadel (2001) indicated that TNF-α causes epidermal growth factor receptor (EGFR) expression in the airway epithelium, and the EGFR ligands such as EGF and IL-13 induce mucin expression and neutrophil activation. These emerging evidences indicate that SO_2 gas exposure induces a lot of specific inflammatory mediators including reactive oxygen radicals, elastases, IL-13, TNF-α and EGF, and up-regulates the expression and activation of EGFR, which probably leads to MUC5AC mRNA expression and goblet cell hyperplasia. In this connection, we reported that *S*-CMC inhibited the release of reactive oxygen radicals and elastase in bronchoalveolar lavage fluid from SO_2-exposed rats (Ishibashi et al., 2001). Hirata and Ohashi (1995) reported that *S*-CMC inhibited murine neutrophil chemotaxis stimulated by various factors, including fMet-Leu-Phe-OH (fMLP) and leukotriene B_4. Ishii et al. (2002) reported that *S*-CMC attenuated fMLP-stimulated neutrophil activation by inhibiting phosphatidyl inositol phospholipase C-mediated signal transduction. Taken together, we suggest that inhibitory effects of *S*-CMC on the Muc5ac mRNA and protein expression induced by SO_2 gas exposure may be based on anti-inflammatory actions via the attenuation of neutrophil activation.

Recently, it is reported that the expression of fucosyltransferase and sialyltransferase activities and mRNAs were increased by TNF-α and EGF, as in the case of MUC5AC mRNA expression and goblet cell hyperplasia in human airways (Delmotte et al., 2002). Perrais et al. (2002) found that MUC5AC mRNA expression induced by TNF-α and EGF was regulated on putative binding sites for Sp1, AP2 and nuclear factor-κB transcription factors. Some of fucosyltransferase and sialyltransferase promoters also contained several putative transcriptional-factor binding sites similar to those for MUC5AC promoter, for example, Sp1 or AP2 (Taniguchi et al., 2000, 2001, 2003). These findings suggest that the inhibitory effects of *S*-CMC on all of the changes in the activities of fucosidase, sialidase, fucosyltransferase and sialyltransferase, and the expression of Muc5ac mRNA, protein and goblet cell hyperplasia induced by SO_2 gas exposure may be based on transcriptional suppression effects in addition to anti-inflammatory effects. Fig. 5 shows the two proposed effecting sites of *S*-CMC, and a typical structure of

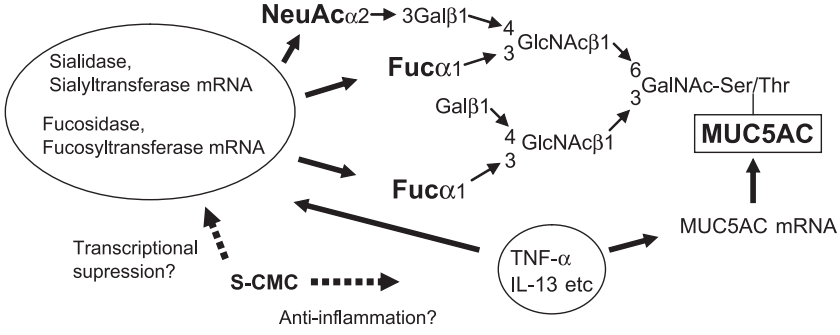

Fig. 5. Schematic representation of the two proposed effecting sites of S-CMC and a typical structure of respiratory mucin from chronic bronchitis patient. Fucose and silaic acid in mucus glycoproteins are regulated by fucosidase, sialidase, fucosyltransferase and sialyltransferase genes. MUC5AC, the protein backbone, is products of MUC5AC gene. S-CMC may have the anti-inflammatory and/or transcriptional suppression actions. GalNAc: N-acetylgalactosamine; Gal: galactose; GlcNAc: N-acetylglucosamine; Fuc: fucose; NeuAc: N-acetylneuraminic acid, sialic acid; Ser/Thr: serine or threonine.

respiratory mucin from chronic bronchitis patients (Van Halbeek et al., 1994). In the future, we consider it essential to investigate whether *S*-CMC would modify favorably the mRNA expression levels of these enzymes and Muc5ac, which can be induced by inflammatory factors, and the regions of interaction in transcriptional level.

In this study, we found that *S*-CMC regulates the activities of fucosidase, sialidase, fucosyltransferase and sialyltransferase and expression of Muc5ac using an animal model of bronchitics, or SO_2-exposed rat. In other respiratory diseases such as COPD, asthma and cystic fibrosis, alterations of mucus glycoproteins in airways are also noted (Ordonez et al., 2001; Lamblin et al., 2001; Scalin and Glick, 1999; Singer et al., 2002). We therefore suggest that *S*-CMC may correct the changes of these enzyme activities and MUC5AC expression in all respiratory diseases involving mucus alteration. Thus, it is interesting to investigate whether *S*-CMC has similar regulatory actions in other respiratory disease models such as COPD, asthma and cystic fibrosis as well as bronchitis.

In conclusion, *S*-CMC inhibited the activity changes of pulmonary fucosidase, sialidase, fucosyltransferase and sialyltransferase, and the increased expression of Muc5ac mRNA and protein in SO_2-exposed rats. We suggest that these actions of *S*-CMC may be based on anti-inflammatory and/or transcriptional suppression effects. We consider that *S*-CMC may improve or reduce the respiratory damages, for example, mucus rheology changes, bacterial attachment to airways, cough sensitivity and ciliary cell damage, induced by impaired mucociliary transport, where fucose and sialic acid contents in mucus glycoprotein have been changed.

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