ABSTRACT

Background and objective: Reactive oxygen species (ROS) play an important role in the pathogenesis of various respiratory diseases. Carbocisteine, a mucoregulatory drug, is used in the treatment of several disease states but little information is available about its scavenger effects on ROS. The present study was designed to examine the scavenger effects of carbocisteine on ROS.

Methods: The oxidation-reduction potential of carbocisteine was measured, and its scavenger effects on hypochlorous acid (HOCl), hydrogen peroxide (H₂O₂), hydroxyl radical (OH⁻) and peroxynitrite (ONOO⁻) were examined in cell-free conditions. The effects of carbocisteine on ROS generated from rat neutrophils, intracellular oxidative stress and release of inflammatory cytokines (IL-8 and IL-6) from IL-1β-induced airway epithelial cells, NCI-H292 cells, were investigated.

Results: Carbocisteine provided a reducing stage and showed scavenger effects on H₂O₂, HOCl, OH⁻ and ONOO⁻ in cell-free conditions. Carbocisteine inhibited ROS generation from rat neutrophils, intracellular oxidative stress and release of IL-8 and IL-6 from NCI-H292 cells. N-acetyl-L-cysteine, a radical scavenger, also inhibited these events related to ROS as well as carbocisteine.

Conclusions: These results suggest that carbocisteine could exert anti-inflammatory and anti-oxidant effects through directly scavenging ROS in addition to its previously known mucoregulatory effect.

Key words: biochemistry, chronic bronchitis, COPD, emphysema.
and then examining the scavenger effects of S-CMC on hypochlorous acid (HOCl), hydrogen peroxide (H₂O₂), hydroxyl radical (OH•) and peroxynitrite (ONOO⁻) in a cell-free system. The anti-oxidant effect of S-CMC on the generation of ROS from neutrophils, intracellular oxidative stress and the release of IL-6 and IL-8 from NCI-H292 cells, human airway epithelial cells was also explored.

METHODS

Oxidation-reduction potential at pH 7

S-CMC and carbocisteine sulfoxide (S-CMC=O) were produced at Kyorin Pharmaceutical Co., Ltd (Tokyo, Japan). S-CMC, S-CMC=O, NAC (Sigma, St. Louis, MO, USA) and l-glutathione reduced (GSH; Sigma) were dissolved in McIlvaine buffer (100 mmol/L citric acid, and 200 mmol/L Na₂HPO₄ at pH 7.0), and the solution made up to 10 mmol/L. The oxidation-reduction potential and pH were measured by using a pH/oxidation-reduction potential meter (HORIBA, Kyoto, Japan).

Detection of S-CMC=O

S-CMC (10 mmol/L) was added to phosphate buffer (140 mmol/L NaCl, 5 mmol/L KCl and 10 mmol/L Na₂HPO₄/KH₂PO₄ at pH 7.4) with and without 10 mmol/L H₂O₂ (Wako Pure Chemical, Osaka, Japan), and to PBS (Nissui Pharmaceutical, Tokyo, Japan) with and without 10 mmol/L sodium hypochlorite (NaOCl; ACROS, Geel, Belgium). After incubation at 37°C for 5 h (S-CMC=O was detected immediately after the addition of H₂O₂ or HOCl, but its contents increased slowly until 5 h), the amounts of S-CMC and S-CMC=O in the H₂O₂ and HOCl scavenging assays were measured using HPLC. In brief, Develosil C30-UG-3 column (150 x 4.6 mm inner diameter, 3 µm particle size) was used at 30°C in the HPLC system (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA). Mobile phase was a mixture of 0.1% phosphoric acid with 5 mmol/L sodium 1-octanesulfonate/acetonitrile (95 : 5, v/v), and flowed at 1.0 mL/min. The detection wavelength was set at 240 nm.

H₂O₂ scavenging activity

The H₂O₂ scavenger effect was measured by using luminol-enhanced chemiluminescence. With or without S-CMC, NAC and GSH, 2 mmol/L H₂O₂ and 15 mmol/L taurine were mixed with or without S-CMC, NAC and GSH in PBS at pH 7.4, and then incubated at 25°C for 5 min. After adding 20 µmol/L potassium iodide (Sigma), absorbance was measured at 350 nm with a spectrophotometer.

OH• scavenging activity

The percentage of inhibited OH• was determined as previously described. In brief, 2.8 mmol/L 2-deoxy-d-ribose (Sigma), 3 mmol/L H₂O₂, 3 mmol/L FeCl₃, 3 mmol/L Na₂EDTA and 3 mmol/L ascorbate were mixed with or without S-CMC, NAC and GSH in 500 µL PBS at pH 7.4. After incubation at 37°C for 30 min, the mixture was added to 500 µL of %-6 % trichloroacetic acid and 250 µL of 1% 2-thiobarbituric acid in 50 mmol/L NaOH, and then boiled for 20 min. The absorbance was measured at 532 nm with a spectrophotometer.

ONOO⁻ scavenging activity

The ONOO⁻ scavenging activity was measured by monitoring the oxidation of dihydrorhodamine 123. In brief, 1 µmol/L sodium peroxynitrite (Cayman Chemical, Ann Arbor, MI, USA) and 5 µmol/L dihydrodoramidine 123 (Invitrogen, Carlsbad, CA, USA) were mixed with or without S-CMC, NAC and GSH in rhodamine buffer (90 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L Na₂HPO₄/NaH₂PO₄ at pH 7.4). Argon gas was introduced to the rhodamine buffer until saturation. The background and final fluorescent intensities were measured immediately before and 5 min after adding peroxynitrite with a microplate fluorescence spectrophotometer (ARVO-MX) at the excitation and emission wavelengths of 485 and 535 nm, respectively.

ROS generation from rat neutrophils

Peritoneal neutrophils were collected from Wistar-strain rats (Charles River Laboratories Japan, Figure 1 Molecular structures of N-acetyl-L-cysteine (NAC; a) and carbocisteine (S-carboxymethylcysteine, S-CMC; b).
Kanagawa, Japan), which were kept in a well-ventilated room under controlled environmental conditions, 19.5 h after an intraperitoneal injection of 1% sodium casein solution (120 mL/kg). The neutrophils were suspended in Hanks’ buffer (Nissui Pharmaceutical) at a concentration of 2.6 × 10⁶ cells/mL. To 155 μL of the cell suspension, 20 μL of S-CMC, NAC or Hanks’ buffer (control) and 20 μL of the 1 mmol/L luminol solution were added, and pre-incubated at 37°C for 1 h. After adding 5 μL of 200 μmol/L N-formyl-methionyl-leucyl-phenylalanine (fMLP; Wako Pure Chemical), chemiluminescence was measured with a microplate luminometer (ARVO-MX) for 1 s. All animals were cared for throughout the study in accordance with the institutional animal care/use committee.

**Cell culture and drug treatment**

NCI-H292 cells (ATCC, Manassas, VA, USA), a human pulmonary mucoepidermoid carcinoma cell line, were cultured in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum (ICN Biomedicals, Irvine, CA, USA). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ until sub-confluent, and then the medium was replaced with fresh medium. The cells were stimulated with human recombinant IL-1β (R&D Systems, Minneapolis, MN, USA) for 1.5 h (intracellular oxidative stress assay) or 24 h (IL-8 and IL-6 assays). To examine the effect of S-CMC, the cells were pre-incubated for 24 h with 0.1, 1, 10 mmol/L S-CMC and 10 mmol/L NAC.

**Intracellular oxidative stress in NCI-H292 cells**

2′, 7′-Dichlorodihydrofluorescein diacetate (DCF, Invitrogen) is an intracellular oxidation indicator. In the presence of intracellular ROS, DCF is oxidized to the fluorescent compound dichlorofluorescein. After stimulation with IL-1β for 1.5 h, the cells were washed with fresh medium, and placed immediately in the medium containing 10 μmol/L DCF, then incubated for 20 min. After DCF staining, the cells were washed and placed in PBS buffer. The fluorescent intensities were determined with a microplate fluorescence spectrophotometer (ARVO-MX) at the excitation and emission wavelengths of 485 and 535 nm, respectively.

**IL-8 and IL-6 release from NCI-H292 cells**

IL-8 and IL-6 proteins were measured using IL-8 ELISA kit (R&D Systems) and IL-6 ELISA kit (R&D Systems), respectively, following the manufacturer’s instructions. The supernatant (cell medium) was harvested after incubation for 24 h with 0.5 ng/mL IL-1β, and stored frozen until assayed.

**Statistical analysis**

All data are expressed as mean ± SEM. One-way ANOVA was used to determine statistically significant differences between groups. Dunnett’s multiple comparison test was used when statistical significances were identified in the ANOVA. A probability of $P < 0.05$ for the null hypothesis was accepted as indicating a statistically significant difference.

**RESULTS**

**Oxidation-reduction potential at pH 7**

The oxidation-reduction potential is a simple parameter that identifies whether an agent in solution has oxidizing or reducing power. The oxidation-reduction potentials of S-CMC and other agents were measured at pH 7, the physiological pH. The oxidation-reduction potentials of S-CMC, S-CMC=O, NAC and GSH were −12, +6, −191 and −204 mV, respectively. These results showed that S-CMC had reducing power (< 0 mV) and its oxidation-reduction potential was higher than those of NAC and GSH, indicating that the reducing potency of S-CMC was lower than those of NAC and GSH at physiological pH (reducing power: NAC > S-CMC > S-CMC=O).

**Detection of S-CMC=O**

Conversion of S-CMC into S-CMC=O was examined after the reaction of S-CMC with H₂O₂ or HOCl. After the chemical reaction between H₂O₂ and S-CMC at 37°C for 5 h, the relative amount of S-CMC=O was 22.3% (the initial amount of S-CMC was considered as 100%, Fig. 2). Similarly, the relative amount of S-CMC=O was 18.1% after the reaction between HOCl and S-CMC (Fig. 2). These results suggest that S-CMC reacted with H₂O₂ and HOCl in cell-free conditions.

**H₂O₂ scavenger effect**

The scavenger effect of S-CMC was examined along with that of NAC and GSH. The H₂O₂ scavenging...
ability of S-CMC was determined using luminol-dependent chemiluminescence in a cell-free system. S-CMC significantly reduced the luminol-dependent chemiluminescence at 10 μmol/L (Fig. 3a). NAC also significantly suppressed this reaction at 100 μmol/L, whereas GSH was significantly effective at 0.1 μmol/L. These results suggest that S-CMC had a scavenger effect on H₂O₂ as did NAC and GSH.

**HOCl scavenger effect**

The effects of S-CMC and other agents on HOCl activity were assayed by measuring changes in absorbance after the reaction between HOCl and taurine. HOCl activity was significantly reduced after treatment with S-CMC at 1 and 10 μmol/L (Fig. 3b). HOCl activity was also significantly reduced with NAC and GSH at 10 μmol/L. These results suggest that S-CMC had a scavenger effect on HOCl activity as did NAC and GSH.

**OH• scavenger effect**

The effects of S-CMC and other agents on OH• activity were evaluated. S-CMC and GSH significantly reduced iron-catalysed OH•-mediated oxidation of deoxyribose at 1000 μmol/L, whereas NAC significantly reduced OH• activity at 300 μmol/L (Fig. 3c). These results suggest that S-CMC had a scavenger effect on OH• activity as did NAC and GSH.

**ONOO− scavenger effect**

The ONOO− scavenging capability of S-CMC was measured by monitoring the oxidation of dihydrorhodamine 123 (d). In contrast, NAC and GSH significantly inhibited ONOO− activity at 100 and 10 μmol/L, respectively. These results suggest that S-CMC had a scavenger effect on ONOO− but its scavenging effect was lower than those of NAC and GSH.

**ROS generation from rat neutrophils**

Reactive oxygen species generated from neutrophils cause inflammation and so induce tissue injury in respiratory diseases. To clarify the anti-oxidant effect of S-CMC on ROS generation from neutrophils, rat neutrophils were stimulated with fMLP. S-CMC significantly inhibited the increase in chemiluminescence induced with fMLP at 10 mmol/L (Fig. 4). Likewise, NAC inhibited the increases at 1 mmol/L.
These results suggest that S-CMC inhibited ROS generation from rat neutrophils but its effect was lower than that of NAC.

**Intracellular oxidative stress in NCI-H292 cells**

Several reports have suggested that IL-1β can induce intracellular oxidative stress-mediated ROS signalling in the airway epithelial cells. It was hypothesized that S-CMC could reduce IL-1β-induced intracellular oxidative stress. The effect of S-CMC on intracellular oxidative stress was investigated using a fluorescent indicator of intracellular oxidation, DCF in NCI-H292 cells. IL-1β significantly increased cellular fluorescence at 0.5 and 1.0 ng/mL in NCI-H292 cells compared with untreated cells (Fig. 5a). The IL-1β (0.5 ng/mL)-induced fluorescence was significantly reduced after pretreatment with S-CMC at 1 and 10 mmol/L and NAC at 1 mmol/L (Fig. 5b). These results suggest that S-CMC inhibited intracellular oxidative stress induced by IL-1β in NCI-H292 cells as did NAC.

**IL-8 and IL-6 release from NCI-H292 cells**

The induction of inflammatory cytokines such as IL-8 and IL-6 in airway epithelial cells is regulated by intracellular oxidative stress. To determine the anti-inflammatory properties of S-CMC, the effects of S-CMC and NAC on IL-1β-induced release of IL-8 and IL-6 were examined in NCI-H292 cells. IL-1β (0.5 ng/mL) significantly increased the release of IL-8 and IL-6 after 24 h in NCI-H292 cells compared with untreated cells (Fig. 6). The IL-1β-induced release of IL-8 and IL-6 was significantly inhibited by pretreatment with S-CMC at 1 and 10 mmol/L (Fig. 6). NAC also inhibited the release at 1 mmol/L (Fig. 6). These results suggest that S-CMC inhibited IL-1β-induced release of IL-8 and IL-6 in NCI-H292 cells as did NAC.

**DISCUSSION**

The present study has shown that S-CMC has scavenger effects on HOCl and OH⁻, which is consistent with preceding information on carbocysteine lysine salt monohydrate (SCMC-Lys). SCMC-Lys, consisting of S-CMC and lysine, has been reported to have antioxidant activity by scavenging reactive oxygen intermediates, such as HOCl and OH⁻. The present study showed that S-CMC also had these effects on H₂O₂ and ONOO⁻ as did NAC. In addition, S-CMC was shown to prevent the release of ROS, including HOCl, OH⁻, H₂O₂ and ONOO⁻, from activated neutrophils. These results suggest that S-CMC has scavenger effects on ROS as does NAC, a radical scavenger; however, the magnitude of S-CMC’s scavenger effects on ROS is lower than that of NAC.
The mechanism of these radical scavenging effects was investigated, commencing with the chemical structure of S-CMC. S-CMC has no free thiol group but has a thioether structure, which is structurally distinct from NAC (Fig. 1). A compound containing a thioether group can reduce ROS by reacting with it and oxidizing to the sulfoxide or sulfone derivative. In the present study, S-CMC was a reducing agent with an oxidation-reduction potential; NAC was also a reducing agent but its oxidation-reduction potential was distinct from NAC (Fig. 1). A compound containing a thioether structure, which is structurally known mucoregulatory effect.

These results suggest that S-CMC exerts anti-inflammatory and anti-oxidant effects through directly scavenging of ROS in addition to its previously known mucoregulatory effect.

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