Carbocysteine lysine salt monohydrate (SCMC-LYS) is a selective scavenger of reactive oxygen intermediates (ROIs)

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ABSTRACT. Carbocysteine lysine salt monohydrate (SCMC-Lys) is a well-known mucoactive drug whose therapeutic efficacy is commonly related to the ability of SCMC-Lys to replace fucomucins by sialomucins. The aim of this study was to determine if SCMC-Lys could exert an anti-oxidant action by scavenging reactive oxygen intermediates (ROIs). Our results show that SCMC-Lys proved effective as a selective scavenger of hypochlorous acid (HOCl) and hydroxyl radical (OH•), this effect being related to the reactivity of the SCMC tioether group. The scavenger activity of SCMC-Lys was observed in free cellular system as well as in activated human polymorphonuclear neutrophils (PMNs). SCMC-Lys scavenger activity on HOCl was paralleled by a powerful protection from HOCl-mediated inactivation of α1-antitripsin (α1-AT), the main serum protease inhibitor. Production of interleukin-8 (IL-8), a major mediator of PMN recruitment in inflammatory diseases, is known to be mediated by intracellular OH•. SCMC-Lys significantly reduced IL-8 production on stimulated human peripheral blood mononuclear cells (PBMCs) in the same range of concentrations affecting OH• activity. It is concluded that SCMC-Lys could exert, in addition to its mucoactive capacity, an anti-oxidant action, thus contributing to the therapeutic efficacy of SCMC-Lys.

Keywords: SCMC-Lys; reactive oxygen intermediates; scavenger activity; polymorphonuclear neutrophils; interleukin-8.

INTRODUCTION

Although the generation of reactive oxygen intermediates (ROIs) is involved in host defence mechanisms, high concentrations of ROIs produced in inflammatory processes can damage cells by peroxidizing lipids, disrupting proteins and deoxyribonucleic acid (DNA) [1]. Among the inflammatory cells, polymorphonuclear neutrophils (PMNs) play a key role in the pathogenesis of inflammatory diseases including acute and chronic inflammatory pathologies of the respiratory tract [2]. PMNs respond to a variety of inflammatory mediators by migrating from the blood stream to the site of inflammation [3]. Activation of PMNs is known to generate high amount of ROIs such as superoxide anion (O2•−), hydroxyl radical (OH•) and hypochlorous acid (HOCl), this effect being implicated in the extensive tissue injury observed in inflammatory lung diseases [4] such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, idiopathic pulmonary fibrosis and adult respiratory distress syndrome (ARDS) [5-7]. Among the ROIs, OH• has a rapid and non-specific reactivity, which makes this radical particularly dangerous [8], and HOCl is known to be the most powerful, cytotoxic PMN oxidant [9], degrading structural proteins such as α1-antitripsin inhibitor (α1-AT), the major protease inhibitor [10]. At moderate levels, ROIs may also exert signalling functions and regulate the transcription of inflammatory mediators [11]. Indeed, ROIs may directly induce cytokine synthesis [12] or act as a transduction signal in cells activated by exogenous stimuli such as IL-1β or lipopolysaccharide (LPS) [13]. Sodium 2-mercaptoethane sulphonate (mesna) and N-acetylcysteine (NAC) are thiol-containing mucolytic drugs. The therapeutic effect of these compounds is associated not only with their ability to reduce mucus viscosity but also with their capacity to interact directly with ROIs [14]. Carbocysteine lysine salt monohydrate (SCMC-Lys) is a well-known mucoactive drug effective in acute and chronic inflammatory lung pathologies [15]. The therapeutic efficacy of SCMC-Lys is commonly related to its ability to replace fucomucins by sialomucins, but its capacity as a scavenger of ROIs has been overlooked until now.

The aim of this study was to see if SCMC-Lys could exert an anti-oxidant action by scavenging ROIs. For this purpose, the scavenger activity of SCMC-Lys was evalua-
Scavenger capacity of SCMC-Lys

Methods

Reagents and chemicals

SCMC-Lys was from Dompé S.p.A. (L’Aquila, Italy). Potassium iodide (KI), glucose, sodium-ethylenediaminetetraacetic acid (Na₂EDTA), ferric chloride (FeCl₃), ascorbate, sodium hydroxide (NaOH), trichloroacetic acid (TCA), thiobarbituric acid (TBA), taurine, 2-deoxy-D-ribose, Paraquat (PQ), catalase (from human erythrocytes), α₁-antitripsin (α₁-AT, from human plasma), phorbol 12-myristate 13-acetate (PMA), glutathion (GSH) and trypan blue were from Sigma Chemical Co. (St. Louis, MO, USA). Sodium hypochlorite (NaOCl) and hydrogen peroxide (H₂O₂) were from Aldrich Chemical Co. (Milwaukee, WI, USA). Elastase (HSPE; from human spume) was from Elastin Product Co. (Owensville, MO, USA). MeO-succinyl-Ala-Ala-Pro-Val-p-nitroanilide was from Calbiochem (San Diego, CA, USA).

Isolation of mononuclear leukocytes and PMNs

Human peripheral blood mononuclear cells (PBMCs) and PMNs were obtained from buffy coats of heparinized blood from normal volunteers by courtesy of Centro Trasfusionale, Ospedale Regionale S. Salvatore, L’Aquila, Italy. PBMCs were obtained by centrifugation on Ficoll/Hipaque [16]. PMNs were prepared to 95% purity by dextran sedimentation followed by hypotonic lysis of contaminating red blood cells [17]. Cellular viability was >95% in all experiments, as measured by Trypan blue dye exclusion.

Scavenging of HOCl

Free cellular experiments

SCMC-Lys solution was freshly prepared in phosphate-buffered 10 mM, pH 7. NaClO concentration was determined before use by iodometric titration. SCMC-Lys 1 mM and NaClO 1 mM were mixed together as a 1 ml final volume, and incubated at 37°C in a thermostated autosampler. Determination of SCMC concentration changes and oxidised product (carbocysteine sulphinic acid; SCMC = O) formation was performed at different time intervals during incubation, using HPLC analysis (25 mM phosphate-buffered saline pH 3/CH₃CN 95:5, 1.2 ml/min; Merck supersphere Select B 250×4 mm column).

In a separate set of experiments, SCMC-Lys (3 mM-1 mM) or GSH (3 mM-1 mM), NaClO 140 μM and taurine 15mM were mixed in PBS buffer at pH 7.4 in a final volume of 1 ml, and incubated at 25°C for 5 min. After the addition of 20 mM KI, the absorbance was measured against a reference cuvette at 350 nm with a Perkin-Elmer Lambda 2 UV/Vis Spectrometer. The amount of reactive HOCl was determined according to the method of Weiss et al. [18].

Cellular experiment

PMNs (1.5×10⁶/ml) were resuspended in PBS buffer, pH 7.4 containing 7.5 mM glucose. Cell aliquots of 1 ml supplemented with 15 mM taurine, were stimulated with PMA (160 nM) at 37°C for 60 min, in the presence or absence of various concentrations of SCMC-Lys (3 µM-1 mM). Assays were stopped by the addition of catalase (50 µg), and the cells removed by centrifugation. After the addition of 20 mM KI to the cell-free supernatants, absorbance was measured spectrometrically against a reference cuvette at 350 nm. The differential spectrum was directly proportional to the amount of reactive HOCl calculated using the extinction coefficient E₅₅₀ = 22900M⁻¹cm⁻¹.

Scavenging of OH⁻

Since OH⁻ interacts with the deoxyribose to form a malondialdehyde (MDA)-like product, a reaction mixture containing 2-deoxy-D-ribose 2.8 mM, H₂O₂ 15 mmol/ml, FeCl₃ 3 mM, Na₂EDTA 3 mM and ascorbate 3 mM in PBS buffer at pH 7.4 was prepared in a final volume of 1 ml. Solutions of FeCl₃ and ascorbate were prepared immediately before use, in denaturated water. This OH⁻ generating system was incubated at 37°C for 30 min in the presence of different concentrations of SCMC-Lys (0.1-3 mM) or GSH (0.1-3 mM). At the end of incubation, samples were mixed with 1ml/60g TCA and 0.5 ml TBA solution (1g TBA in 100 ml 0.05N NaOH), and boiled for 20 min. When heated, samples developed a pink chromogen and their absorbance was measured spectrometrically at 532 nm (Argus 400, Packard). The percentage of inhibited deoxyribose oxidation by SCMC-Lys was determined according to the method of Halliwell [19].

In a separate set of experiments, SCMC-Lys (1mM) was added to the reaction mixture in the absence of 2-deoxy-D-ribose. After 1, 15 or 30 min of incubation at 37°C, determination of any SCMC concentration decrease and SCMC = O formation was achieved using HPLC analysis (25 mM phosphate-buffer saline at pH 3/CH₃CN 95:5, 1.2 ml/min; Merck supersphere Select B 250×4 mm column).

Scavenging of H₂O₂

H₂O₂ (2mM) and SCMC-Lys (2 mM) solutions were freshly prepared in PBS buffer, pH 7.4. The two substances were mixed in a ratio of 1:1 to obtain a final volume of 1 ml, and then incubated in a thermostated autosampler at 37°C. Determination of any SCMC concentration decrease and SCMC = O formation was achieved using HPLC analysis (25 mM phosphate-buffer saline at pH 3/CH₃CN 95:5, 1.2 ml/min; Merck supersphere Select B 250×4 mm column).

Scavenging of peroxynitrite (ONOO⁻)

Since H₂O₂ interacts with isoaamyl nitrite to form ONOO⁻, a reaction mixture containing H₂O₂ (5 mM)
and isoamyl nitrite (5 mM) in PBS buffer, pH 7.4 was prepared in a final volume of 1 ml. SCMC-Lys (1mM) was added to the reaction mixture. After 1, 15, 30 or 60 min of incubation at 37 °C, determination of any SCMC concentration decrease and SCMC = O formation was achieved using HPLC analysis (25 mM phosphate-buffered saline, pH 3 /CH3CN 95:5, 1.2 ml/min; Merck supersphere Select B 250 × 4 mm column).

**α1-AT activity**

Different concentrations of SCMC-Lys (0.01-3 mM) were mixed with α1-AT (5 ng/ml) and NaOCl (75 µM) in PBS buffer, pH 7.4 (100 µl total volume) and incubated for at 25 °C for 30 min. After incubation, HSPE (50 µg/ml) was added. After 20 min, elastase enzymatic activity was measured spectrometrically at 410 nm, as previously described [20].

**Determination of IL-8 production**

PBMCs were resuspended (10^7/ml) in RPMI 1640 medium supplemented with 1% FCS, and plated at a density of 5 × 10^5/well in 96-well plates. SCMC-Lys (1mM or 3 mM) or vehicle were then added to the cells and 30 min later PQ (1 mM) or IL-1β (0.3 ng/ml) were added. IL-8 production was determined in cell-free supernatants, 24 hours after PQ or IL-1β addition (ELISA kit; Amer sham, UK; sensitivity 2 pg/ml).

**Statistical analysis**

Analysis of variance (ANOVA) for a random model test followed by Dunnett’s t test was applied in order to evaluate the treatment and the concentration effects. Homogeneity of variance was tested by the Levene test. Significance threshold was set at p < 0.05. Calculation of EC_{50} was carried out by ALLFIT 2.0 program.

**RESULTS**

**Scavenger effect of SCMC-Lys on ROIs**

The capacity of SCMC-Lys to react with HOCl was firstly investigated in cell-free assays. As shown in Figure 1A, the reaction between SCMC-Lys (1 mM) and NaOCl (mixed in a ratio of 1:1) was immediate, and about 60% of the compound was converted into SCMC = O in the first minute of reaction. The relative amount of SCMC and SCMC = O remained constant up to 30 min during the reaction:

The scavenger capacity of SCMC-Lys on HOCl was further established by spectrometrical analysis of reactive HOCl inhibition. As reported in Figure 1B, SCMC-Lys strongly reduced the amount of reactive HOCl. The scavenger effect of SCMC-Lys was concentration-dependent, and the IC_{50} value of reactive HOCl inhibition was 53.4 ± 7.6 µM.

In addition, the scavenger capacity of SCMC-Lys against HOCl was comparable to the scavenger capacity of GSH (IC_{50} 22.5 ± 1.2 µM; data not shown), the main, endogenous antioxidant. Finally, scavenging of HOCl by SCMC-Lys was investigated on PMA-stimulated PMNs. PMA (160 nM) strongly induced HOCl production by PMNs (48 nmol/ml and 2 nmol/ml in the presence or absence of PMA, respectively). As reported in Figure 2, HOCl reactive amount was blocked by SCMC-Lys. The effect was concentration-dependent, being almost complete at 300 µM (93% of inhibition; IC_{50} 82 ± 12 µM).

The effect of SCMC-Lys on OH^ activity was also evaluated. SCMC-Lys produced a concentration-dependent reduction of reactive OH^, being the maximal
inhibitory effect observed at 3 mM (49% of inhibition; Figure 3A). In addition, the scavenger capacity of SCMC-Lys against OH\(^\cdot\) was comparable to the scavenger capacity of GSH (Figure 3A), the main endogenous antioxidant.

Reactivity of SCMC-Lys on OH\(^\cdot\) was also evaluated by determination of SCMC = O formation. As shown in Figure 3B, exposure of SCMC-Lys (1 mM) to Fenton’s reagents converted a consistent amount of SCMC (59%) into SCMC = O in the first minute of the reaction. The relative amount of SCMC and SCMC = O remained constant up to 30 min of the reaction.

Finally, the scavenger capacity of SCMC-Lys on reactive H\(_2\)O\(_2\) and ONOO\(^\cdot\) was investigated. The reaction between SCMC-Lys and H\(_2\)O\(_2\) was weak, with only 9% of the compound converted to SCMC = O after six hours of incubation (data not shown). A similar scavenger effect was observed against ONOO\(^\cdot\), with only 10% of the SCMC-Lys converted to SCMC = O after 1 hour of incubation (data not shown).

**Effect of SCMC-Lys on HOCl-mediated inactivation of α1-AT**

Methionyl residues of α1-AT are oxidized by HOCl. The effect of SCMC-Lys on HOCl-mediated α1-AT inactivation was evaluated within the same range of concentrations found to scavenge HOCl generated by stimulated PMN. Results in Figure 4 show that SCMC-Lys dramatically reduced HOCl-mediated inhibition of α1-AT activity. The effect was concentration-dependent, being significant starting at 0.1 mM. Maximal protection of α1-AT activity was observed at 3 mM (over 80% inhibition of HSPE activity).

**Effect of SCMC-Lys on PQ and IL-1β-induced IL-8 production**

Since it has been reported that intracellular production of OH\(^\cdot\) is involved in IL-8 production [13], we investigated the effect of SCMC-Lys on PQ and IL-1β-induced IL-8 production in PBMCs. As reported in Figure 5A, preincubation of PBMCs with SCMC-Lys (1 mM and 3 mM) significantly inhibited IL-8 production induced by IL-1β (32% and 46% of inhibition at 1 mM and 3 mM, respectively). Conversely, pretreatment of PBMCs with
SCMC = O (1 mM) did not affect IL-1β-induced IL-8 production (Figure 5A).

Under the same experimental conditions, SCMC-Lys (1 mM and 3 mM) also significantly reduced PQ-induced IL-8 production (Figure 5B; about 50% inhibition at both concentrations tested).

**DISCUSSION**

In the present study we show that SCMC-Lys, a well-known mucoactive drug, possesses antioxidant properties in cell-free and cellular systems. In particular, SCMC-Lys is a selective scavenger of HOCl and OH'. The scavenger activity of SCMC-Lys was paralleled by its ability to protect α1-AT from oxydative damage and to inhibit IL-8 production.

Over recent years, several experimental studies have demonstrated the antioxidant properties of some thiolic compounds such as NAC or mesna, commonly used as mucolytic agents [14]. Conversely, few studies have been conducted on tioether-containing drugs, such as SCMC-Lys. It was recently reported that the tioether group could also react with ROIs, oxidating itself to sulphoxide and sulphon derivatives. In fact, it was demonstrated that the tioether residues of methionine present on the surface of proteins are oxidated by ROIs to the corresponding sulphoxide methionine and this reaction could play an important role in protecting the protein’s active site against ROIs cytotoxicity [21].

We have also observed that SCMC-Lys acts as a scavenger of HOCl and OH' by oxidation of its tioether group and subsequent generation of SCMC = O. The scavenger capacity of SCMC-Lys against HOCl and OH' was comparable to GSH scavenger capacity, GSH being the main endogenous antioxidant. The capacity of SCMC-Lys to interact with OH', determined by SCMC = O formation, seems to be reduced in the OH' extracellular test. The reduced scavenger capacity of SCMC-Lys and GSH in the OH' extracellular assay could be related to the high specific reactivity of the substrate 2-deoxy-D-ribose to OH', 2-deoxy-D-ribose being absent in the oxidation assay.
The chemical reaction between HOCI or OH· and SCMC is represented below:

\[
\begin{align*}
\text{SCMC} + \text{HOCI} & \rightarrow \text{SCMC-O} + \text{OH}^- \\
\text{SCMC} + \text{OH} & \rightarrow \text{SCMC-O} + \text{H}_2\text{O}
\end{align*}
\]

Conversely, SCMC-Lys did not significantly interact with \( \text{H}_2\text{O}_2 \) and \( \text{ONOO}^– \). Accordingly, the sensitivity of the thioether group to \( \text{H}_2\text{O}_2 \) and \( \text{ONOO}^– \) oxidation is strongly reduced in SCMC-Lys in the presence of the carboxyllic residue.

The scavenger effect of SCMC-Lys was also observed on stimulated PMNs, suggesting that SCMC-Lys could exert an anti-inflammatory action by the scavenging of ROIs produced by activated PMNs. Indeed, among the free radicals produced by activated PMNs, OH· and HOCI are probably the most powerful species in the pathogenesis of tissue injury. The OH· radical is extremely aggressive with a short half-life and non-specific reactivity [8]. Similarly, HOCI is a powerful oxidant capable of reacting with a variety of potential biological substrates [9], including \( \alpha\)-1AT, the major serum protease inhibitor [10]. \( \alpha\)-1AT inactivation leads to an increase in proteolytic activity of PMN elastase this effect being implicated in the extensive tissue injury observed in pulmonary inflammatory diseases including ARDS [22] and chronic emphysema [23]. Our data show that SCMC-Lys scavenger capacity on the HOCI produced by activated PMNs, is paralleled by preservation of \( \alpha\)-1AT activity, further suggesting that the anti-oxidant capacity of SCMC-Lys could contribute to its therapeutic efficacy. It was recently reported that SCMC-Lys inhibits elastase-induced conversion of xanthine dehydrogenase to xanthine oxidase in human endothelial cells this effect being paralleled by a significant reduction in \( \text{O}_2^- \) production [24].

It was reported that production of IL-8 could be mediated, at least in part, by intracellular OH· [13]. The effect of OH· was found to be specific for IL-8 production, while IL-1β and tumor necrosis factor (TNF) production was not affected by OH· scavengers. Our results show that SCMC-Lys significantly inhibited IL-8 production in PBMCs stimulated by PQ or IL-1β. Reduction of IL-8 production by SCMC-Lys was observed within the same range of concentrations found to affect OH· reactivity. Our data strongly suggest that inhibition of IL-8 production by SCMC-Lys could be related to its scavenger capacity on OH·. Also, SCMC = O did not affect IL-1β-induced IL-8 production. Finally, the inhibitory effect of SCMC-Lys was specific for IL-8 since no reduction in TNF and IL-1β production was observed in LPS-stimulated PBMCs (data not shown).

This result is especially relevant in view of the physiopathological role of IL-8. Indeed, it has been suggested that the chemotactic recruitment of PMNs into the lung by IL-8 could play a crucial role in the development and maintenance of several inflammatory diseases including ARDS [25], idiopathic pulmonary fibrosis [26] and cystic fibrosis [27]. Accordingly, IL-8 production is supposed to be involved in the induction of pulmonary fibrosis by PQ as well as in its acute toxicity [28]. Finally, IL-1β is considered to be one of the main mediators in the inflammatory response of the respiratory system [29], suggesting that inhibition of IL-8 production could contribute to the therapeutic effect of SCMC-Lys.

Inhibition of OH· reactivity and IL-8 production by SCMC-Lys was observed at relative high concentrations (1 mM and 3 mM). However, NAC was reported to reduce OH· reactivity and IL-8 production in the same range of concentrations observed with SCMC-Lys [14, 30]. Since the anti-inflammatory capacity of NAC is related, at least in part, to inhibition of chemokine production and PMN recruitment [31], reduction of IL-8 production by SCMC-Lys suggests also that SCMC-Lys could exert an anti-inflammatory action. In agreement with this hypothesis, we have previously reported that SCMC-Lys significantly reduced IL-1β-induced pulmonary PMN recruitment in mice [32].

In summary, we found that SCMC-Lys is a selective scavenger of OH· and HOCI. The capacity of SCMC-Lys to scavenge OH· and HOCI is related to the reactivity of its thioether group. Scavenger capacity of SCMC-Lys is paralleled by protection of \( \alpha\)-1AT activity and inhibition of IL-8 production. These results suggest that SCMC-Lys could exert, in addition to its mucoactive capacity, an anti-oxidant action, thus contributing to its therapeutic efficacy.

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