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S-CMC-Lys Protective Effects on Human Respiratory Cells During Oxidative Stress

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Key Words

CFTR • Glutathione • Oxidative stress • S-carbocysteine lysine salt monohydrate

Abstract

The mucoactive drug S-carbocysteine lysine salt monohydrate (S-CMC-Lys) stimulates glutathione (GSH) efflux from respiratory cells. Since GSH is one of the most important redox regulatory mechanisms, the aim of this study was to evaluate the S-CMC-Lys effects on GSH efflux and intracellular concentration during an oxidative stress induced by the hydroxyl radical (·OH). Experiments were performed on cultured human respiratory WI-26VA4 cells by means of patch-clamp experiments in whole-cell configuration and of fluorimetric analyses at confocal microscope. ·OH exposure induced an irreversible inhibition of the GSH and chloride currents that was prevented if the cells were incubated with S-CMC-Lys. In this instance, the currents were inhibited by the specific blocker $CFTR_{inh}$-172. CFT1-C2 cells, which lack a functional CFTR channel, were not responsive to S-CMC-Lys, but the stimulatory effect of the drug was restored in LCFSN-infected CFT1 cells, functionally corrected to express CFTR. Fluorimetric measurements performed on the S-CMC-Lys-incubated cells revealed a significant increase of the GSH concentration that was completely hindered after oxidative stress and abolished by $CFTR_{inh}$-172. The cellular content of reactive oxygen species was significantly lower in the S-CMC-Lys-treated cells either before or after ·OH exposure. As a conclusion, S-CMC-Lys could exert a protective function during oxidative stress, therefore preventing or reducing the ROS-mediated inflammatory response.

Introduction

Carbocysteine lysine salt monohydrate (S-CMC-Lys) is widely used for its mucolitic and decongestant activities in acute and chronic inflammatory lung pathologies [1, 2]. As years passed, many more potential therapeutic effects have became evident for this drug, most of them being barely explained considering its effects on mucous rheology. Attention have been paid to the potential therapeutic anti-inflammatory efficacy of the drug, i.e. decrease in neutrophil infiltration into the airway lumen [3], decrease in IL-8 and IL-6 cytokine lev-

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els, and in the prostaglandin-like compound 8-isoprostane exhaled during chronic obstructive pulmonary disease [4]. Even if these effects were firstly ascribed to a direct scavenger effect of the drug thioether group to reactive oxygen species (ROS) [5, 6], recent evidences [7] suggested that the treatment of respiratory cells with S-CMC-Lys stimulates a CFTR-like channel, leading to a significant increase of Cl^- and glutathione (GSH) fluxes through the cell membrane. GSH is one of the most important protective antioxidant in cells and it takes a prominent role in the control of the inflammatory response in the epithelial lining fluid of the airways. The intracellular GSH/GSSG ratio was proven to have a crucial effect for the activation of the redox signal transduction pathway and of transcriptional factors such as the nuclear factor-kB and the activator protein-1, that subsequently regulate the genes of proinflammatory cytokines, and activate protective mechanisms, such as antioxidant gene expression [8].

The aim of this research was to evaluate the effects of S-CMC-Lys on respiratory cells after exposure to the hydroxyl radical (·OH), which is one of the strongest radical generated by atmospheric particulate matter and pollutants. By means of the patch-clamp technique, the inward Cl^- and the outward GSH fluxes were measured, before and after ·OH exposure, both in control and in S-CMC-Lys-treated cells. GSH intracellular concentration and ROS cell content were assessed by fluorimetric approaches at confocal microscope.

Materials and Methods

Cell line and solutions for cell maintenance

WI-26VA4 cells (purchased from ATCC-LGC Promochem) were cultured in Eagle's minimum essential medium, 2% L-glutamine, 10% fetal bovine serum (5% CO_2, 37°C). WI-26VA4 cells, which were already used to test S-CMC-lys effects [7, 9], are an SV40 virus transformed derivative of WI-26, a human cell line from embryonic lung tissue that underwent to a change in morphology towards "epithelial-like" cells, after SV40 transformation. The expression of CFTR in these cells had been verified by Western Blot experiments [7] and by PCR amplification after reverse transcription followed by Southern hybridization [10]. The presence of a functional CFTR channel in the plasma membrane of WI26-VA4 cells had been evidenced by patch-clamp experiments both in cell-attached and in whole-cell configuration [7, 9].

Human tracheobronchial epithelial CF (CFT1-C2, homozygous for F508 deletion of the CFTR gene [11]), and functionally corrected for CFTR (LCFSN-infected CFT1 cells[12]) cells were grown in F12 Ham's nutrient mixture medium supplemented with hormones, as reported[7]. All media components were purchased from Sigma (St. Louis, MO, USA).

100 μM S-CMC-Lys (Dompé, Milan, Italy) was added and it had been kept into the cell medium for 5 hours before the beginning of the experiments. During patch-clamp (before seal formation) and fluorimetric experiments cells were maintained in a S-CMC-Lys-free physiological salt solution (pH 7.4) buffered with HEPES (HBSS).

Electrophysiological measurements

The whole-cell patch-clamp technique was used to measure the macroscopic currents at room temperature. Bath and pipette solutions were chosen to enable selective Cl^- and GSH current recordings. The extracellular solution contained (mM): 143 N'-methyl-D-glucamine chloride, 5 glucose, 10 HEPES/NMG (pH 7.4), 1 $MgCl_2$, and 1.8 $CaCl_2$. The pipette solution contained (mM): 143 NMDG-glutathione, 3 theophylline, 5 glucose, 10 HEPES/NMG (ph 7.2), 1.9 $CaCl_2$, and 3 EGTA (free $[Ca^{2+}]$: 2×10^{-7} M). All salts were purchased from Sigma (St. Louis, MO, USA). These experimental conditions were already used [7] to evaluate the GSH flux. A Mg^{2+}-free, theophylline-containing intracellular solution was used to slow down CFTR channel rundown [13-15]. The existence, in S-CMC-Lys-incubated cells, of a functionally active CFTR channel had been verified both in excised and in whole-cell experiments [7, 9].

Recording pipettes were prepared from borosilicate capillaries (outer diameter 1.5 mm; Garner Glass, Claremont, CA) with resistance of 2-7 MΩ. Patch-clamp recordings were performed as described before [7].

Cell capacitance, which was 29.5 ± 2.33 pF (n=19) and 32.1 ± 2.66 pF (n=15) in untreated and treated WI26-VA4 cells, 45.6 ± 4.5 pF (n=11) and 44.8 ± 3,0 pF (n=21) in CFT1-C2 and LCFSN-infected CFT1 cells, was checked throughout the experiments, and it was constant.

To test the effects of hydroxyl radical (OH) on the ionic currents, the cells were bathed simultaneously and for four minutes with two 145 mM NMDGCL bath solutions, one containing 10^{-5} M $Fe(SO_4)_2(NH_4)_2$ and the other containing 10^{-6} or 10^{-5} M H_2O_2. As verified by electron spin resonance, all H_2O_2 is converted to ·OH very rapidly (10^{-9} s) during the reaction, producing ·OH according to the Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + \cdot OH + HO^-$ [16-18]. A continuous superfusion with the two solutions was therefore necessary.

Fluorimetric measurements

Changes of intracellular GSH concentrations, compared to control conditions, have been evaluated using the dye monochlorobimane (MCB, Invitrogen S. R. L, Italy) [19]. Cells, rinsed from the S-CMC-lys containing medium with HBSS, were incubated for 20 min with 40 μm MCB (dissolved in HBSS) at 37°C, washed gently with warm HBSS, and placed in HBSS without MCB; therefore, S-CMC-Lys was not present when cells were incubated with MCB. CFTR inhibition was achieved by the addition of 20 μM $CFTR_{inh}$-172 to the culture medium for three days before the beginning of an experiment, as reported [20]. To test the effects of ·OH on the fluorescence intensity, cells were bathed simultaneously and for four minutes with two HBSS solutions: one containing 10^{-5} M $Fe(SO_4)_2(NH_4)_2$ and the other containing 10^{-2} or 10^{-3} M H_2O_2, as indicated. The

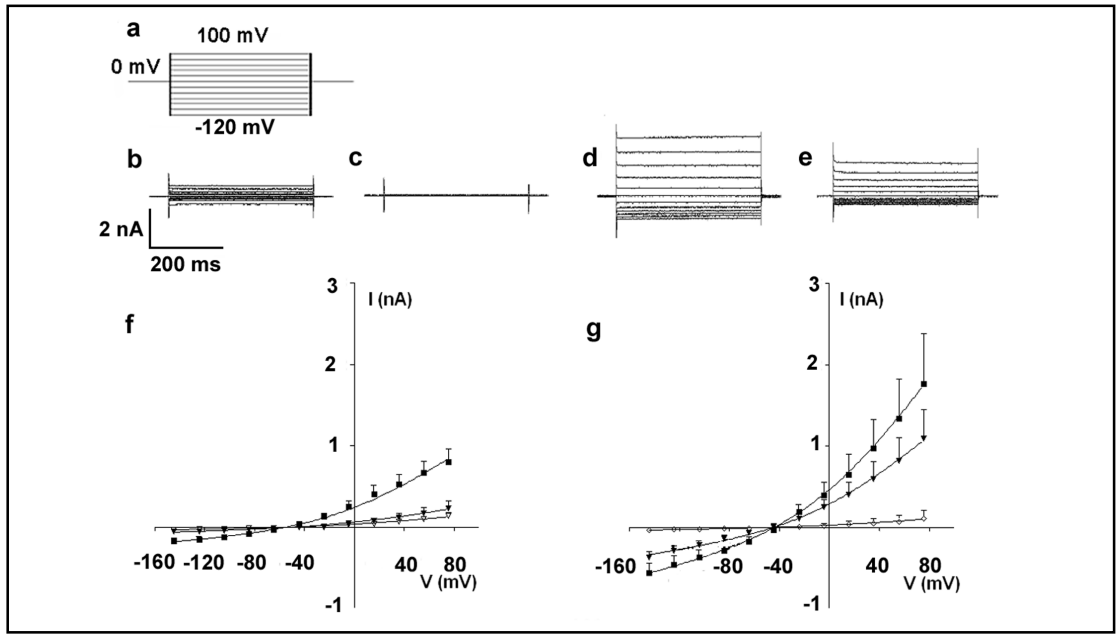


Fig. 1. WI26-VA4 cells: chloride (outward) and GSH (inward) currents recorded during patch-clamp experiments in whole-cell configuration. a) Impulse protocol. b) Currents in asymmetrical solutions ($[GSH]_{in}$=143 mM, $[Cl^-]_{in}$=3,8 mM, $[GSH]_{out}$=0 mM, $[Cl^-]_{out}$=148.6 mM); c) after 4 minutes exposure to ·OH (10^{-5} M Fe^{2+} and 1μM H_2O_2); d) currents recorded from S-CMC-Lys treated cells in asymmetrical solution; e) after 4 minutes exposure to ·OH. f) Current/Voltage (I/V) relationship in S-CMC-Lys untreated cells (■: asymmetrical solution, n= 19; ▼: asymmetrical solution after 4 minutes exposure to ·OH, n=16; ▽: 4 minutes wash out in asymmetrical solution without ·OH, n=9. Mean ± S.E.M.). G) Current/Voltage (I/V) relationship in S-CMC-Lys treated cells (■: asymmetrical solution, n= 15; ▼: asymmetrical solution after 4 minutes exposure to ·OH, n=12; ▽: NPPB 100 μM , n=3. Mean ± S.E.M.). **: $p<0.01$, n.s.: not significant between the IV curves recorded before and after exposure to ·OH (two way Anova).

fluorescence was imaged at room temperature by means of a confocal microscope Leica TCS SP2 AOBS (Leica Microsystem, Heidelberg, Germany) with a 40.0x1.25 OIL UV HCX PL APO CS objective and an UV laser. Bimane fluorescence was observed between 400 and 550 nm, after excitation at 351 nm. All fluorescence values were calculated allotting arbitrarily a value of 100 to the fluorescence intensity measured in S-CMC-Lys-untreated cells incubated with an HBSS solution (control condition). Images were analyzed with the program imagej, considering the mean grey levels of region of interest (ROI) comprising the whole volume of several cells.

Intracellular ROS accumulation was monitored with the fluoroprobe 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein-diacetate, acetil ester (CM-H_2DCF-DA, Invitrogen S. R. L, San Giuliano Milanese, Italy) [21]. After 20 min. of incubation with 7 μM CM-H_2DCF-DA dissolved in HBSS, the cells were rinsed with warm HBSS, and placed in an HBSS solution without CM-H_2DCF-DA for 10 minutes before the beginning of the experiment. Fluorescence was monitored by means of a confocal microscope Leica TCS SP2 AOBS using excitation wavelengths of 496 nm, and probing emission between 510 and 560 nm. A value of 100 was arbitrarily assigned to the fluorescence measured in control conditions (HBSS bathed cells not treated with S-CMC-Lys).

Statistical analysis

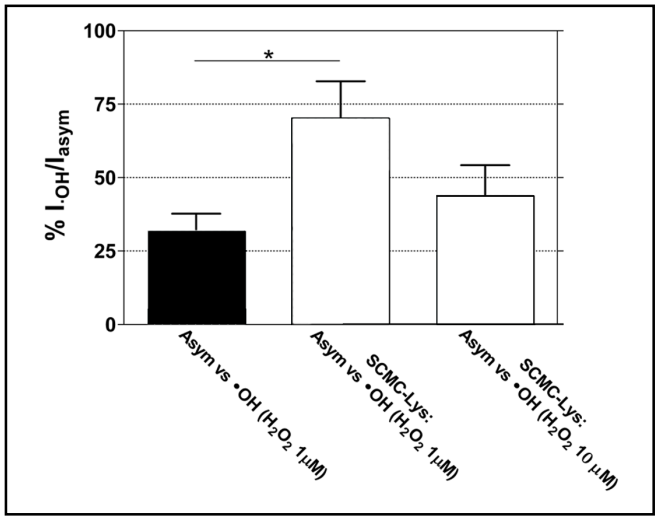
All the data are presented as mean ± SEM. Statistical analyses were performed using an unpaired Student *t* test or one-way ANOVA (to analyze multiple data), and a two way ANOVA (applied to data from experiments with more than one variable). Statistical assessments were done using the statistical package of Prism version 4 (GraphPad, San Diego, Calif.). The criterion for statistical significance was a p-value of ≤ 0.05.

Results

·OH effects on anionic currents: whole cell configuration

The whole-cell patch recordings were performed on both S-CMC-Lys-untreated or treated WI26-VA4 cells expressing CFTR (Fig. 1). In asymmetrical solutions a small GSH outward electrogenic flux and a greater inward chloride flux were observed in the untreated cells

Fig. 2. Percentage of residual current after ·OH exposure ($\%I_{OH}/I_{asym}$). This ratio was calculated by dividing the inward GSH current recorded after exposure to solution containing 1 or 10 μM H_2O_2 and 10^{-5}M Mohr salt to the same current obtained at the beginning of the experiments. (Untreated cells: H_2O_2 1μM, n=16; SCMC-Lys treated cells: H_2O_2 1 μM, n=12; SCMC-Lys treated cells: $H_2O_2$10 μM, n=12. Mean ± S.E.M.). * : $p<0.05$ comparing the value obtained S-CMC-lys treated to untreated cells (one way Anova, Dunnet post test).



(Fig. 1 b, f), as detected before [7]. The current reversal potential (V_{rev}) was -56.2 ± 2.8 mV (n=19) that was equivalent to a P_{GSH}/p_{Cl}, calculated by the Goldman Hodgkin and Katz equation, of 0.10 ± 0.02 (n=19).

Both the outward and the inward currents were significantly ($p<0.01$) inhibited after ·OH exposure (Fig. 1c, f), whereas Mohr's salt alone was ineffective (data not shown). The V_{rev} (-50.3 ± 6.1 mV, n=16) did not change significantly, thus implying that the small residual current was still attributable to channel activity and suggesting that the extent of the inhibitory effect was nearly the same on both the outward and the inward current. The wash-out with an asymmetrical ·OH-free solution did not induce a significant change in current entity or V_{rev} if compared to the same parameters observed during ·OH exposure (Fig. 1f), indicating an irreversible or a long term effect of the hydroxyl radical on the GSH and chloride currents.

An increase in the inward GSH current, coupled to a shift in the V_{rev} (-40.9 ± 4.6 mV, n=15), and a significant increase in the GSH permeability (P_{GSH}/p_{Cl} = 0.22 ± 0.04, n=15) were observed for S-CMC-Lys-incubated cells (Fig. 1 d, g). Both the inward and the outward currents were not significantly reduced after ·OH exposure but they were nearly totally blocked after exposure to the anion channel inhibitor NPPB (100 μM) (Fig 1g).

We estimated the percentage of residual current after ·OH exposure ($\%I_{OH}/I_{asym}$) by calculating the ratio between the currents recorded at -144.4 mV after and before exposure to solutions that contained 1 or 10 μM H_2O_2 and 10^{-5}M Mohr salt (Fig. 2). The percentage of residual current after ·OH exposure ($\%I_{OH}/I_{asym}$) was significantly larger in S-CMC-Lys-incubated cells treated with 1 μM H_2O_2 and 10^{-5}M Fe^{2+} compared to untreated cells. In S-CMC-Lys-incubated cells, 10 μM H_2O_2 did not induced any significant variation of $\%I_{OH}/I_{asym}$ compared to the same parameters observed after 1 μM H_2O_2 exposure.

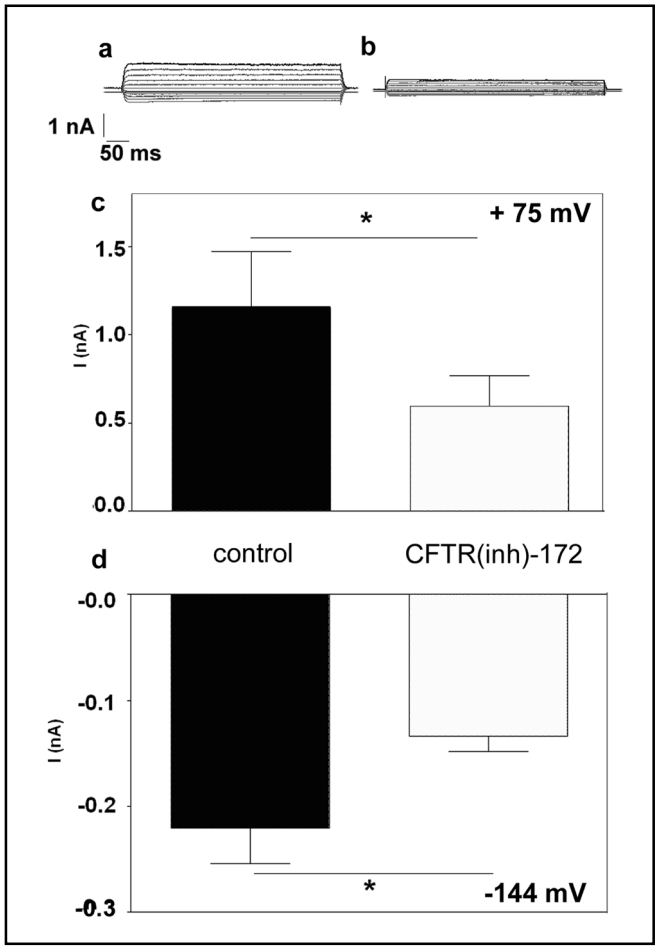


Fig. 3. S-CMC-Lys stimulated anionic current is sensitive to $cftr_{inh}$-172. Currents in S-CMC-Lys treated cells a) before (control) or b) after 2 μM thiazolidinone $CFTR_{inh}$-172 exposure ($CFTR_{(inh)}$-172). c) Outward chloride current recorded at +75.6 mv (before or after current inhibition with $CFTR_{inh}$-172, n=4. Mean ± S.E.M.). d) Inward GSH currents taken at -144.4 mv (before or after current inhibition with the thiazolidinone, n=4. Mean ± S.E.M.). *: $p<0.05$ with Student's t-test.

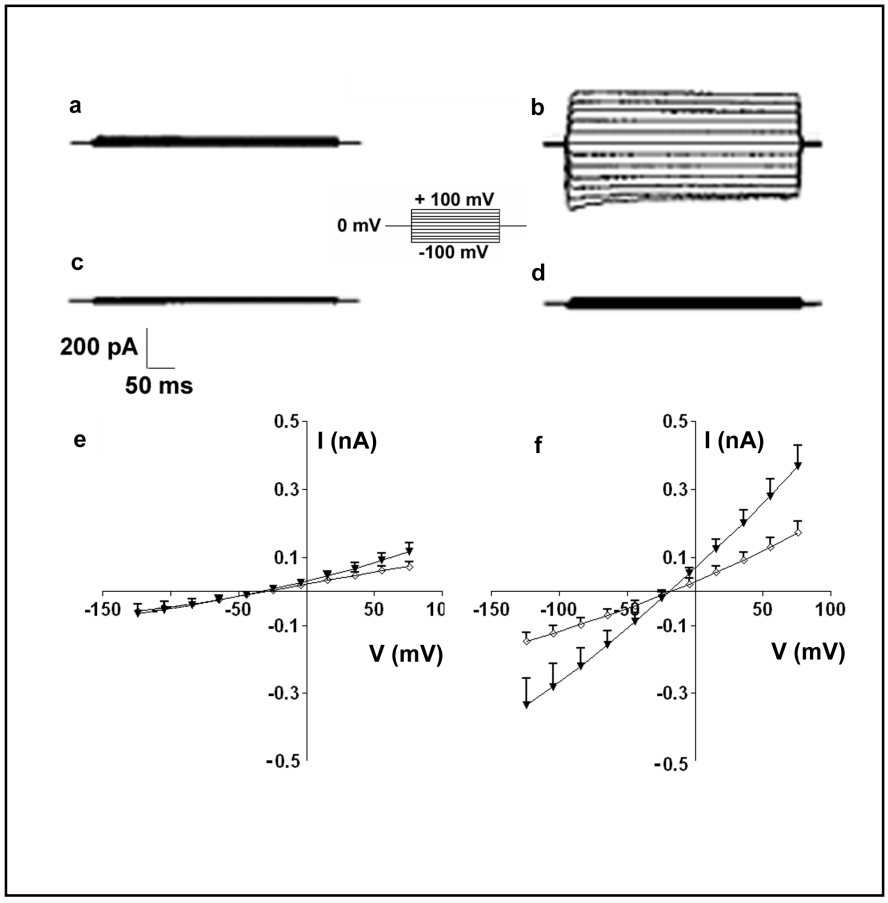


Fig. 4. Whole-cell experiments performed on CFT1-C2 and LCFSN-infected CFT1 cells incubated with S-CMC-Lys. a and b) Currents in CFT1-C2 and in LCFSN-infected CFT1 cells in that order (asymmetrical solutions: $[GSH]_{in}$=143 mM, $[Cl^-]_{in}$=3.8 mM, $[GSH]_{out}$=0 mM, $[Cl^-]_{out}$=148.6 mM). C and d) Currents in CFT1-C2 and LCFSN-infected CFT1cells respectively after 100 μM NPPB exposure. E) Current/Voltage (I/V) relationship in CFT1-C2 cells (▼: asymmetrical solutions, n=11; ◊: after 100 μM NPPB addition, n=9. Mean ± S.E.M.). F) Current/Voltage (I/V) relationship in LCFSN-infected CFT1 cells (▼: asymmetrical solutions, n=21; ◊: after 100 μM NPPB addition, n=17. Mean ± S.E.M.). **: $p<0.01$ between the IV curves recorded before and after NPPB treatment (two way Anova).

S-CMC-Lys activated anionic currents are related to CFTR channel activity

In order to gain further clues to better characterize the anionic currents activated after S-CMC-Lys incubation, we performed a new set of whole-cell experiments aimed to evaluate the current sensitivity to the CFTR channel inhibitor $CFTR_{inh}$-172 thiazolidinone[22]. Furthermore we carried out additional experiments to assess the effect of S-CMC-Lys on CFT1-C2 cells lacking a functional CFTR expression at the plasma membrane. All the experiments were performed with the same experimental conditions (asymmetrical GSH/Cl^- solution, pre-incubation of the cells to 100 μM S-CMC-Lys for 5 hours before whole-cell experiment).

After exposure of S-CMC-Lys-treated cells to 2 μM $CFTR_{inh}$-172, both the outward chloride (Fig. 3) and the inward GSH currents were significantly ($p<0.05$) reduced. In CFT1-C2 cells pre-treated with S-CMC-Lys (Fig. 4a) we recorded small inward and outward currents that were not significantly reduced in the inward direction after 100 μM NPPB exposure (Fig. 4c, e). Whereas the effect of S-CMC-Lys was lacking in CFT1 cells, we observed currents significantly greater in LCFSN-infected CFT1 cells, which are transfected to express a functional CFTR channel (Fig. 4b, f). In these cells both the outward and the inward current were significantly inhibited by NPPB (Fig. 4d, f).

Fluorimetric determination of GSH cellular concentrations

In S-CMC-Lys-untreated cells, intracellular fluorescence intensity of MCB varied as a function of the oxidative-stress strength. It decreased significantly from the value observed in control conditions (Fig. 5a, g) to the value recorded when cell were exposed to an HBSS solution containing 1 μM H_2O_2 and 10^{-5}M Mohr salt (Fig. 5 b, g) and it was further reduced when H_2O_2 was increased to 10 μM (fig. 5 c, g). In S-CMC-Lys-incubated cells, a significant increase in fluorescence intensity was observed (Fig. 5d, g). This parameter was again significantly reduced after exposure to an HBSS solution containing 1 (Fig. 5e, g) and 10 μM H_2O_2 (Fig. 5f, g). The increase of fluorescence intensity observed in S-CMC-Lys-incubated cells was abolished in cells treated with the specific CFTR blocker $CFTR_{inh}$-172, which was instead ineffective on untreated cells (Fig. 6).

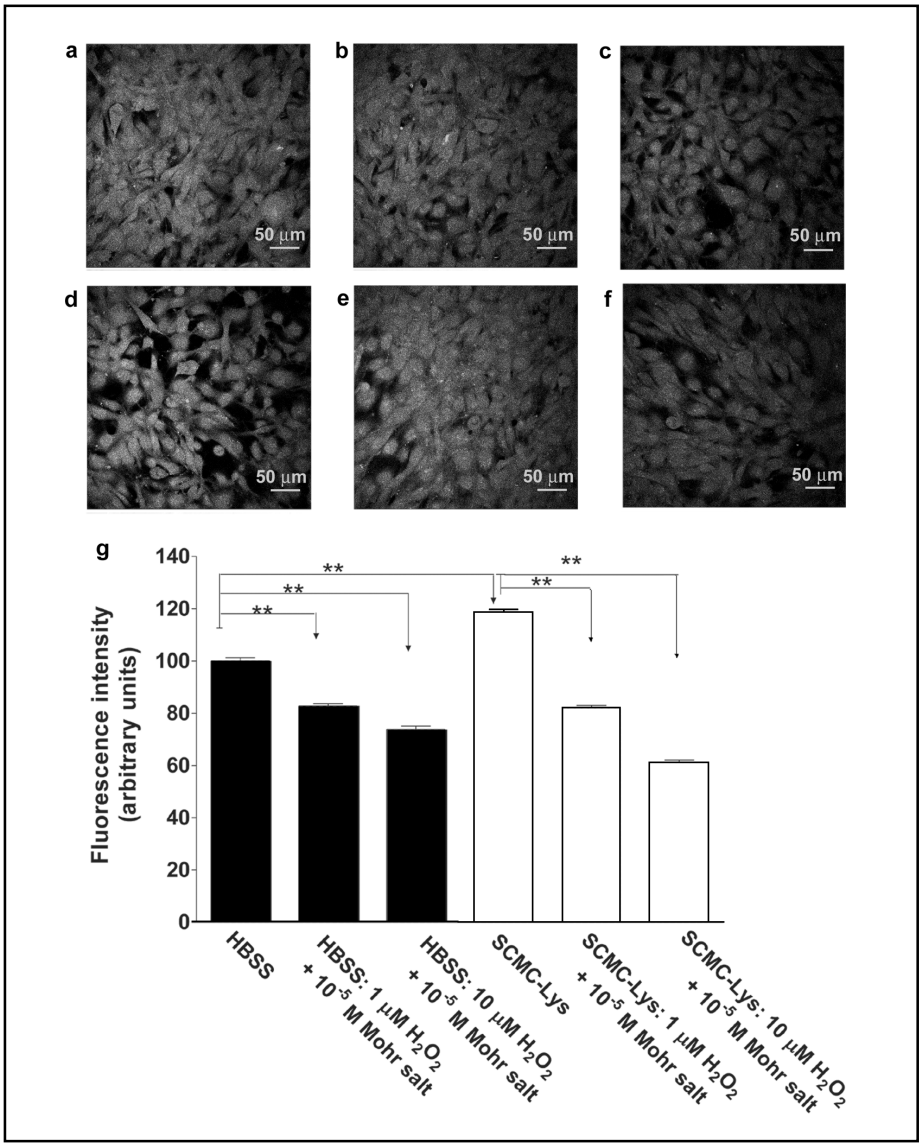


Fig. 5. GSH intracellular concentration measured in WI26-VA4 cells by means of the GSH sensitive dye monochlorobimane (MCB). Confocal microscope original images. Cells bathed with a) HBSS solution, b and c) HBSS plus 1 or 10 μM H_2O_2 and 10^{-5}M Mohr salt respectively. S-CMC-Lys-incubated cells bathed with d) HBSS solution, e and f) HBSS plus 1 or 10 μM H_2O_2 and 10^{-5}M Mohr salt respectively. g) Mean fluorescence intensities recorded in untreated cells (bathed with: HBSS, n=225; HBSS plus 1 mM H_2O_2 and 10^{-5}M Mohr salt, n=250; HBSS plus 10 μM H_2O_2 and 10^{-5}M Mohr salt, n=240.) and in S-CMC-Lys treated cells (exposed to: HBSS, n= 271; HBSS plus 1 μM H_2O_2 and 10^{-5}M Mohr salt, n=270; HBSS plus 10 μM H_2O_2 and 10^{-5}M Mohr salt, n=266). Mean ± SEM. ** $p< 0.01$ with one way Anova test.

Fluorimetric determination of ROS cellular levels

In S-CMC-Lys-treated cells, the fluorescent signal was significantly smaller then the signal measured in control cells either with or without H_2O_2 and 10^{-5}M Mohr salt in the HBSS bathing solution. Fluorescence intensity in HBSS bathed cells (Fig. 7a, g) was significantly reduced compared to the same parameter measured after exposure to 1 (Fig. 7 b, g) or 10 μM H_2O_2 (Fig 7c, g) and 10^{-5}M Mohr salt. In S-CMC-Lys-incubated cells, the fluorescence signal was significantly smaller then control when the cells were bathed with HBSS (Fig. 7d, g). Furthermore the fluorescence was not significantly increased after 1μM H_2O_2 and Mohr salt exposure as well (Fig. 7e, g). A slight increase of fluorescence was observed when cells were bathed with HBSS plus 10 μM H_2O_2 and Mohr's salt (Fig. 7f, g), but even in these conditions the signal was not significantly different from the one recorded in control cells.

Discussion

Recent findings [7] showing that S-CMC-Lys could stimulate an electrogenic GSH secretion by human respiratory cells and the observation that oxidant and anti-oxidant balance in the airways could be responsible of acute and chronic airway diseases [23], prompted us to evaluate if the drug could play a valuable role on respiratory cells during oxidative stress induced by ·OH. Among the other ROS, the hydroxyl radical has a rapid and non-specific reactivity, that makes it particularly dangerous.

Inhibitory or stimulatory effects of ROS on anion channels have been evidenced [17, 24-31]. Here we shown

that in WI-26VA4 cells both the inward and the outward glibencamide-insensitive [7] anionic fluxes were nearly totally inhibited in an irreversible way after hydroxyl radical exposure. After incubation with S-CMC-Lys an increase in the chloride and GSH anionic fluxes, paralleled to an increase in the GSH permeability, was observed. The S-CMC-Lys-stimulated currents were inhibited by the CFTR selective blocker $CFTR_{inh}$-172, they were not detectable in CFT1-C2 cells that lack a functional CFTR expression at the plasmamembrane, and they were restored in LCFSN-infected CFT1 cells that are transfected to express a functional CFTR channel at the plasma membrane. As still suggested [7, 32], this datum confirmed that the S-CMC-Lys-stimulated currents were dependent on CFTR activity. The CFTR-mediated GSH and chloride currents, which were maximally activated after S-CMC-Lys treatment [9], were not significantly inhibited after 1 μM H_2O_2 and 10^{-5}M Fe^{2+} exposure, well in agreement with the observation that CFTR activity is not reduced after oxidative stress [25, 33]. This outcome cannot be due to a direct scavenger effect of the S-CMC-Lys thiol group, as already observed by others [6], because the treatment with the drug came before the exposure to the radical ·OH during the patch-clamp experiments.

The activation of the S-CMC-Lys-stimulated currents could exert a protective role during oxidative stress. GSH concentration is high in pulmonary epithelial lining fluid (ELF) in which it represents a strong antioxidative defence against free radicals and other oxidants, and it is implicated in immune modulation and inflammatory response [23]. A reduction in ELF glutathione concentration has been shown in several inflammatory condition such as, for example, idiopathic pulmonary fibrosis, acute respiratory distress syndrome, lung allograft, HIV infection. During all these pathological conditions, the efflux of GSH stimulated by S-CMC-Lys could therefore put forth a positive role.

The lack of effect of oxidative stress elicited by 1 μM H_2O_2 and 10^{-5}M Fe^{2+} exposure on CFTR-mediated chloride current after S-CMC-Lys treatment could provide a further beneficial effect. Considering that anion secretion is coupled in the respiratory tract to fluid secretion [34], and ROS are reported to stimulate mucus secretion from respiratory epithelial cells [35], the S-CMC-Lys-activated chloride current could lead to the production of a more hydrated mucous therefore improving mucociliary clearance during oxidative-stress conditions.

Modulation of the GSH concentration and of the redox status of respiratory epithelial cells is a crucial factor in affecting cell vulnerability or tolerance to oxidative insults, and it has been implicated in immune modulation and inflammatory responses [36]. Besides evaluating S-CMC-Lys effect on GSH secretion, we studied the effect of the drug on GSH and ROS intracellular levels. The anti-inflammatory S-CMC-Lys actions [3-6] could be attributed, in fact, not only to a direct scavenger effect due to the drug thioether group, as already proposed [5], but also to the modulation of the intracellular GSH levels, as suggested for the analogue cysteine containing drug N-acetylcyteine (NAC) [37].

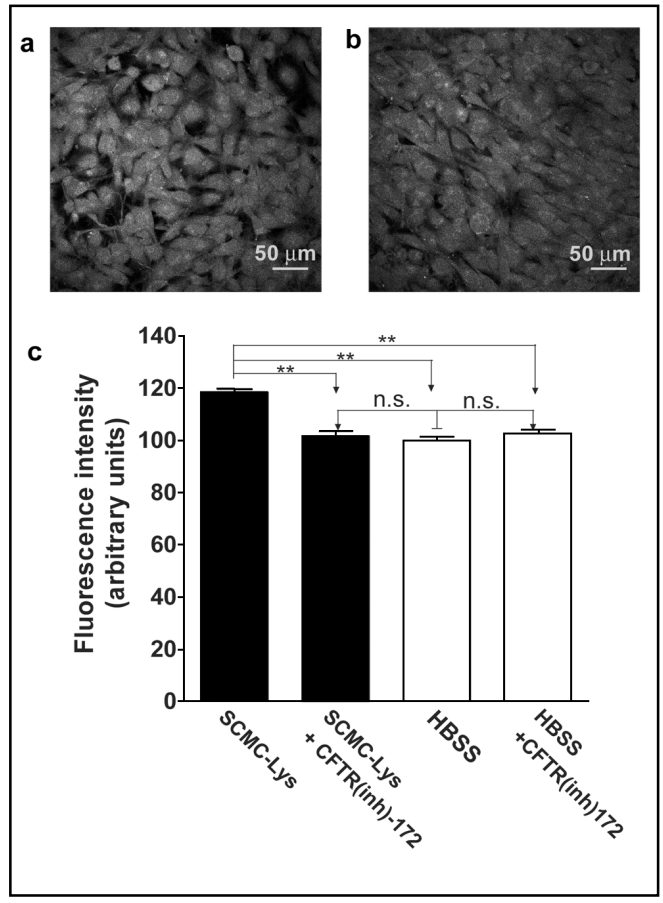


Fig. 6. Effect of 20 μM $CFTR_{inh}$-172 on GSH intracellular level measured in WI26-VA4 cells by means of the GSH sensitive dye monochlorobimane (MCB). Confocal microscope original images. a) S-CMC-Lys-incubated cells bathed with a physiological HBSS solution containing the $CFTR_{inh}$-172, b) untreated cells bathed with a $CFTR_{inh}$-172 containing HBSS solution, c) Mean fluorescence intensity (arbitrary units) in S-CMC-Lys treated cells (bathed with: HBSS, n= 271; HBSS plus 20 μM $CFTR_{inh}$-172, n= 250), and in untreated cells (bathed with: HBSS, n= 225; HBSS plus 20 μM $CFTR_{inh}$-172, n= 250). Mean ± SEM.

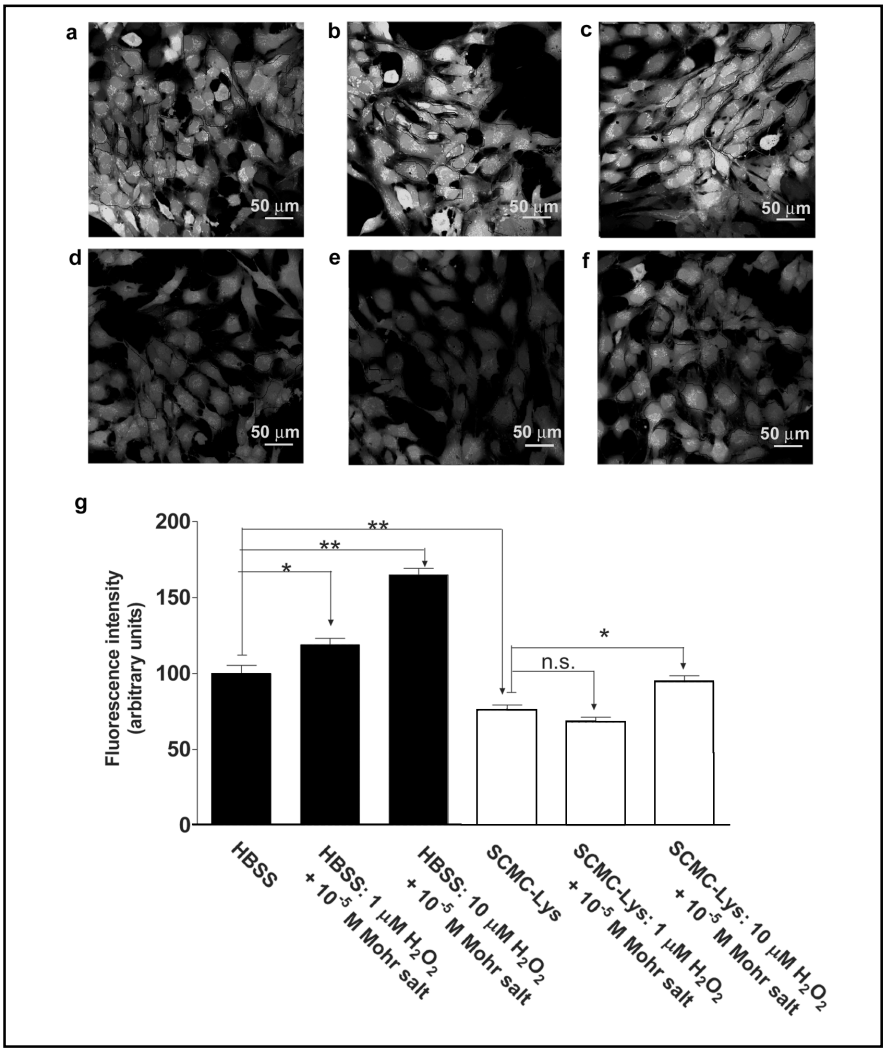


Fig. 7. Cellular ROS assay obtained loading cells with CM-H_2DCF-DA. Confocal microscope original images. Black lines surround the selected ROIs. Cells bathed with a) HBSS solution, b and c) HBSS plus 1 or 10 μM H_2O_2 and 10^{-5}M Mohr salt respectively. S-CMC-Lys treated cells bathed with d) HBSS solution, e and f) HBSS plus 1 or 10 μM H_2O_2 and 10^{-5}M Mohr salt respectively. g) Mean fluorescence intensity (arbitrary units, considering the mean intensity of control equal to 100) recorded in untreated cells (bathed with: HBSS, n=65; HBSS plus 1 μM H_2O_2 and 10^{-5}M Mohr salt, n=65; HBSS plus 10 μM H_2O_2 and 10^{-5}M Mohr salt, n=70) and in S-CMC-Lys treated cells (bathed with: HBSS, n= 70; HBSS plus 1 μM H_2O_2 and 10^{-5}M Mohr salt, n=65; HBSS plus 10 μM H_2O_2 and 10^{-5}M Mohr salt, n=65). Mean ± SEM. *, ** $p< 0.05, 0.01$ with one way Anova test.

In this study we showed that S-CMC-Lys-incubated cells had a larger amount of intracellular reduced GSH if compared to control cells. Likewise it has been proposed for NAC [37], this effect could be related to the fact that S-CMC-Lys, acting as a cysteine donor, could represent a direct precursor for the biosynthesis of GSH. Anyway, the increase in GSH levels seems to be attributable to a more complex mechanism, because this effect was completely abolished at the presence of the specific CFTR inhibitor $CFTR_{inh}$-172. The observation that the inhibitor did induce a GSH drop in S-CMC-Lys-treated cells, when CFTR channel was stimulated, but not in control cells, having no or fewer active CFTR channels, suggested that S-CMC-Lys mediated increase in GSH intracellular levels was somehow related to the presence of active CFTR channels. Further experimental trials have to be performed to better clarify this observation. Preliminary Western blot experiments suggested that this effect was not attributable to a variation of the γ-glutamilcysteine sinthetase (γ-GCS) catalytic subunit (HS) at protein expression levels. The involvement of CFTR in other pathways [38] implicated in the modulation of the GSH intracellular concentration remains possible.

After cell exposure to ·OH we observed a decrease in GSH intracellular levels both in S-CMC-Lys-treated and in untreated cells, in agreement with the observation that the exposure of respiratory epithelial cells to oxidants triggers an initial fall in GSH level due to GSSG formation [8]. Anyway, cells incubated with S-CMC-Lys showed a significant reduction of ROS intracellular levels. This decrease was unrelated to the direct scavenger effect of the drug thioether group, because cells were treated with S-CMC-Lys before exposure to ROS. CFTR activity seems related to the oxidative stress of the cell. Indeed, a debate is nowadays open concerning the possibility of an altered redox state related to cystic fibrosis

pathology [39]. Furthermore, IB-3 cells, which lack a functional CFTR channel, show greater ROS intracellular levels compared to the C38 cell line expressing active CFTR channels [40]. Therefore, the reduction of ROS intracellular levels observed in S-CMC-Lys-incubated cells could be linked to CFTR channel stimulation as well.

As a conclusion, during oxidative stress (when cells were exposed to 1 μM H_2O_2 and 10^{-5}M Fe^{2+}) S-CMC-Lys, exerting either a direct or an indirect stimulation of CFTR, stimulated both GSH and Cl^- fluxes, increased GSH concentration, and buffered ROS increase in cells expressing CFTR channel, suggesting that S-CMC-Lys could have positive effects for several lung pathologies.

S-CMC-Lys has an antioxidant action that could protect cells from ROS-mediated cell damages. This effect, which could be related to the anti-inflammatory effect of the drug, could be important in the therapeutic approaches of several respiratory pathologies, and disclose new therapeutic approaches for the drug.

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