Perspective

Carbocysteine restores steroid sensitivity by targeting histone deacetylase 2 in a thiol/GSH-dependent manner

Yun Song 1, Hao-Zhong Lu 1, Jian-Rong Xu, Xiao-Lin Wang, Wei Zhou, Li-Na Hou, Liang Zhu, Zhi-Hua Yu, Hong-Zhuan Chen 2*, Yong-Yao Cui 1

Department of Pharmacology, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

ABSTRACT

Steroid insensitivity is commonly observed in patients with chronic obstructive pulmonary disease. Here, we report the effects and mechanisms of carbocysteine (S-CMC), a mucolytic agent, in cellular and animal models of oxidative stress-mediated steroid insensitivity. The following results were obtained: oxidative stress induced higher levels of interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α), which are insensitive to dexamethasone (DEX). The failure of DEX was improved by the addition of S-CMC by increasing histone deacetylase 2 (HDAC2) expression/activity. S-CMC also counteracted the oxidative stress-induced increase in reactive oxygen species (ROS) levels and decreases in glutathione (GSH) levels and superoxide dismutase (SOD) activity. Moreover, oxidative stress-induced events were decreased by the thiol-reducing agent diithiothreitol (DTT), enhanced by the thiol-oxidizing agent diamide, and the ability of DEX was strengthened by DTT. In addition, the oxidative stress-induced decrease in HDAC2 activity was counteracted by S-CMC by increasing thiol/GSH levels, which exhibited a direct interaction with HDAC2. S-CMC treatment increased HDAC2 recruitment and suppressed H4 acetylation of the IL-8 promoter, and this effect was further ablated by addition of buthionine sulfoximine, a specific inhibitor of GSH synthesis. Our results indicate that S-CMC restored steroid sensitivity by increasing HDAC2 expression/activity in a thiol/GSH-dependent manner and suggest that S-CMC may be useful in a combination therapy with glucocorticoids for treatment of steroid-insensitive pulmonary diseases.

© 2014 Elsevier Ltd. All rights reserved.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major public health concern in both developed and developing countries. Cigarette smoke, which is a primary cause of COPD, contains a complex mixture of damaging oxygen-derived free radicals and oxidants (reactive oxygen species, ROS) as well as a variety of other chemical compounds, including reactive aldehydes and semiquinones, which are known to cause oxidative stress in lung tissue [1,2]. Glucocorticoid (GC) therapy is somewhat beneficial for COPD, although treatment is limited by uncertain and variable clinical responses in some patients. Even high doses of GCs have a minimal effect on the rapid decline in lung function in COPD patients and have only a minimal effect on reducing COPD exacerbation [3]. An extensive literature search of articles concerning steroid-insensitive COPD indicated that oxidative stress can reduce histone deacetylase 2 (HDAC2) expression and increase inflammatory cytokine production in alveolar macrophages of the airway and peripheral lung tissues in COPD patients [4,5]. Such a reduction in HDAC2 activity may be correlated with GC sensitivity. Moreover, a progressive reduction in total HDAC activity, which reflects disease severity, was observed in lung tissue specimens obtained from COPD patients with varying degrees of airflow obstruction [4].

GCs activate several anti-inflammatory pathways and suppress the induction of many inflammatory responses, but can also recruit HDAC2 to the promoter of pro-inflammatory genes, thereby suppressing pro-inflammatory gene transcription [2]. It has recently been reported that the oxidative stress associated with cigarette smoke can inhibit HDAC2 activity, thereby blocking the anti-inflammatory effects of GCs [6]. In patients with COPD, the reduction in HDAC2 expression may thus account for GC insensitivity observed in this disease. GCs provide limited clinical benefits to COPD patients and fail to reduce disease progression [7]. Thus, restoring corticosteroid sensitivity in patients with COPD

1 Corresponding author at: Department of Pharmacology, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, China.
Tel.: +86 21 6384 6590x776451; fax: +86 21 64674721.
E-mail addresses: hongzhuan_chen@hotmail.com (H.-Z. Chen), yongyaocui@hotmail.com (Y.-Y. Cui).

* These authors are contributed equally to this article.

http://dx.doi.org/10.1016/j.phrs.2014.12.002
1043-6618/© 2014 Elsevier Ltd. All rights reserved.
by targeting HDAC2 influenced by oxidative stress is expected, as corticosteroid sensitivity was achieved by administration of low concentrations of theophylline [8] and curcumin [9], which may act as HDAC activators.

The use of carbocysteine (S-CMC), a mucoactive drug, remains common in Europe and Asia for the treatment of COPD. However, a new understanding of the importance of exacerbarion frequency in the progression of COPD and the role of systemic inflammation in the disease process, together with publication of several trials demonstrating positive effects of mucoactive drugs on exacerbarion rates and quality of life [10–12], have stimulated interest in the potential of this drug. In fact, the clinical efficacy of S-CMC seems to be more related to its antioxidant and anti-inflammatory effects than its mucolytic activity [13–15]. Recent clinical experiments have shown that S-CMC exhibited preventative effects when used for concomitant use of inhaled corticosteroids in the exacerbation of COPD [12]. However, relatively few studies have addressed the potential efficacy or mechanism of action of S-CMC contributing to steroid-insensitive COPD.

The aim of the present study was to identify factors associated with steroid insensitivity in an oxidative stress-induced cellular model and to explore the hypothesis that steroid sensitivity can be improved by S-CMC administration, thereby identifying S-CMC as a potential therapeutic tool for treatment of steroid-insensitive COPD.

Materials and methods

Cell culture and treatments

The human type II alveolar epithelial cell line A549 was maintained in F-12k medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 U/mL). Unless otherwise stated, cell culture reagents were purchased from Gibco (USA).

Cigarette smoke extract (CSE) was prepared as previously reported with minor modifications [16]. CSE was prepared by the combustion of one cigarette (12 mg tar/cigarette; Double Happiness, China), using a pump and passing the smoke through 10 mL of non-FBS culture medium at a rate of 5 min/cigarette. The resulting solution was adjusted to pH 7.4 with 1.0 M NaOH and strained through 0.22-μm gauge filters. The obtained solution represented 100% strength and was diluted to the desired concentration with culture medium. The fresh CSE was used within 30 min.

Cells were pre-incubated with or without dexamethasone (DEX; Tocris Bioscience, UK), S-CMC (Sigma–Aldrich, USA), dithiothreitol (DTT)/diadime (Sigma–Aldrich, USA) or buthionine sulfoximine (BSO; Sigma–Aldrich, USA) for 1 h and then exposed to 3% CSE for 6 h or H2O2 (100 μM; Sigma–Aldrich, USA) for 4 h. Each of these treatments showed no significant cytotoxic effects as measured with a cck-8 counting kit (Dojindo, Japan) and trypan blue exclusion method.

Animals and treatment

Animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals set by Shanghai JiaoTong University (Shanghai, China). In each experiment, Sprague-Dawley (SD) rats (age, twelve-week-old; 220 ± 20 g; Shanghai SLAC Laboratory Animal Company, China) were exposed to cigarette smoke from four cigarettes (Double Happiness, China) burning simultaneously and each exposure lasted 75 min consuming 48 cigarettes totally. The cigarette smoke (CS) exposure was performed twice per day and 5 days per week for 12 weeks by using a custom-designed and purpose-built nose-only, directed

flow inhalation and smoke-exposure system (handmade) housed in a fume and laminar flow hood. DEX (5 mg/kg) was administered intraperitoneally 3 times per week and S-CMC (300 mg/kg) was administered intragastrically every day both from week 6 to week 12 of smoke exposure. Control group was sham–treated with normal saline. The rats were anesthetized with 40 mg/kg intraperitoneal sodium pentobarbital for sample collection.

Measurement of proinflammatory cytokines in bronchoalveolar lavage fluids and cell culture supernatant

To prepare the bronchoalveolar lavage fluids (BALF), SD rats were anesthetized with sodium pentobarbital, and the trachea of each rat was surgically exposed and cannulated for BALF collection. The right lung was lavaged three times with a single volume of 1 mL of PBS, and the supernatants of BALF were collected by 1000 rpm centrifugation (4 °C, 5 min).

An enzyme-linked immunosorbent assay (ELISA; R&D Systems, USA) was used to evaluate the levels of interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) in the collection from BALF and cell culture supernatants as described by the manufacturer’s instructions.

Western blot

For the analysis of proteins expression, the A549 cells lysate was prepared by RIPA Lysis Buffer (+FMSF, 100:1; Beyotime Institute of Biotechnology, China). The total protein was extracted by centrifugation (14,000 × g, 4 °C, 10 min) in the supernatant for gel electrophoresis. After western transfer, PVDF membranes were probed with mouse anti-HDAC2 antibody, followed by horseradish peroxidase-conjugated anti-mouse (1:5000) antiserum (Santa Cruz Biotech, USA). Protein bands were visualized with SuperSignal (Pierce). The membranes were lastly re-probed with an anti-Histone H2A.X antibody (Signalway Antibody, USA) as loading controls.

Measurement of cellular thiol levels, and activities of superoxide dismutase (SOD) and HDAC2

After the above indicated treatment, the cell lysate of A549 cells and the left lungs homogenate were used to measure intracellular active thiol levels (Nanjing Jiancheng Chemical Industrial Co., China), SOD activity (Dojindo, Japan), and HDAC2 activity (Genned Sciences, USA) as described by the manufacturer’s instructions.

High content screening (HCS) assay

HDAC2 expression in A549 cells were measured by HCS technology based on immunocytofluorescence. Anti-HDAC2 primary antibody (mouse anti-human; Cell Signaling Technology, USA) was incubated overnight, followed by incubation with secondary antibodies (DyLight 555 nm, goat anti-mouse; Cell Signaling Technology, USA) for 60 min in the dark and stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich, USA). Fluorescence was detected using a HCS Reader (Thermo Fisher Scientific, USA) at 10× magnifications to quantify HDAC2 expression.

Immunohistochemistry

After bronchoalveolar lavage and tissue isolation, the right lungs were immersed in 4% paraformaldehyde. After tissue fixation and paraffin embedding, 5 μm sections were prepared and stained with antibodies against the marker of steroid resistance HDAC2. The quantitative analysis of HDAC2 in the sections was performed by Image Pro Plus 6.0.
Flow cytometry

ROS production was measured by flow cytometry. Briefly, A549 cells were washed in cold PBS, digested with trypsin, and incubated with the H2DCFDA fluorescence probe (10 μM; Sigma–Aldrich, USA) at 37 °C for 1 h. Then, ROS levels were measured using an Accuri C6 flow cytometer (BD Biosciences, USA) at an excitation wavelength of 488 nm.

Short hairpin RNA (shRNA) interference

HDAC2 knockdown was performed by transfecting A549 cells with HDAC2 shRNA (Shanghai Gima Co., Ltd., China) according to the manufacturer’s instructions. A549 cells grown to 30–50% confluency were transfected by lentiviral-delivered shRNA (multiplicity of infection = 20), strengthened by polybrene (5 μg/ml) for 24 h. Then, the medium was replaced with fresh medium without shRNA and polybrene, and the culture was incubated for another 48 h. The infection efficiency was >50% according to green fluorescent protein analysis. Cells then were screened with puromycin (2.5 μg/ml) for 3–5 days and monoclonal cells were maintained for further experimentation.

High performance liquid chromatography–tandem mass spectrometry (LC–MS)

The level of GSH was measured by HPLC–MS/MS. HPLC grade acetonitrile and water were purchased from Merck (KGaA, Germany). HPLC–MS grade formic acid, ammonium formate and Analytical grade GSH were purchased from Sigma–Aldrich (United States). After indicated treatment, A549 cells lysis was collected and dissolved in acetonitrile to measure the intracellular GSH concentration. The LC–MS/MS system consisted of a Shimadzu HPLC auto sampler, and an API-4000 mass spectrometer with a turbo-ion spray source (Applied Biosystem, Foster City, CA). Chromatography separation was performed on ACQUITY BEH HILIC, 2.1 mm × 100 mm, 1.7 μm UPLC column (Waters Corporation, USA) maintained at 40 °C. The ionization source parameters were as follows: temperature 650 °C, nebulizer gas (GAS1) 60 psi, turbo heater gas (GAS2) 60 psi, curtain gas 20 psi, ionspray voltage 5500 V, and collision gas (CAD) 8 psi. The collision energy (CE) was 28 V, declustering potential (DP) was 78 V, and entrance potential (EP) was 10 V. Detection of the ions was conducted in the multiple reaction monitoring (MRM) mode, monitoring the transition of the m/z 308.4/76.0.

Surface plasmon resonance (SPR)

SPR was measured using an optical biosensor (T200; Biacore International AB, Sweden). The purified HDAC2 (>95%, active; Sigma–Aldrich, USA) was immobilized on a CM5 sensor chip (GE Health Sciences, USA). All association–dissociation measurements were made at 25 °C in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid, 0.005% Tween-20). Parameter evaluations were obtained using Biacore evaluation software (GE Healthcare, USA).

Chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction (PCR) assays

The ChIP assay was performed using the SimpleChip Enzymatic Chromatin IP kit (Cell Signaling Technology, USA) following the manufacturer’s instructions. The eluted DNA was checked for 100–300-bp fragment enrichment by 8% polyacrylamide gel electrophoresis. The immunoprecipitated DNA was used amplified using specific gene (IL-8 for humans) promoter qPCR assays. The following PCR primer sequences were used for the IL-8 promoter: forward, 5′-TTCCTCCGCTGTTCTTC-3′ and reverse, 5′-GGGCCATCATGCGAAATC-3′.

Statistical analysis

Quantitative data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance followed by the Dunnett’s test for multiple comparisons. All analyses were performed using Prism GraphPad 5.0 statistical software. A probability (p) value <0.05 was considered statistically significant.

Results

Effects of S-CMC on oxidative stress-induced steroid insensitivity by suppression of pro-inflammatory cytokine release

ELISA analysis demonstrated significantly higher levels of IL-8 and TNF-α in A549 cells exposed to CSE or H2O2, which were resistant to DEX pre-treatment (10−3 M) (p < 0.01). We also found that S-CMC (10−4 M) alone did not inhibit production of IL-8 or TNF-α. Interestingly, the failure of DEX to suppress IL-8 and TNF-α production in response to CSE or H2O2 was reversed by S-CMC. Enhanced action of DEX by S-CMC was concentration-dependent, ranging from 10−7 to 10−4 M (Fig. 1A–D).

Next, we assessed the efficacy and potency of DEX in the presence or absence of S-CMC. The mean steroid sensitivity was determined by the concentration of DEX required to achieve 50% of maximal inhibition (IC50), which reflects steroid potency, and the maximum anti-inflammatory effect (I(max)), which represents steroid efficacy. From a potency point of view, the IC50 value of DEX alone (10−14–10−6 M) on IL-8 and TNF-α release was 1.67 × 10−9 M and 2.16 × 10−9 M, respectively. DEX concentration–response curves for inhibition of CSE-induced IL-8 and TNF-α release, however, showed a left shift in the presence of S-CMC by increasing the concentration from 10−7 to 10−4 M. The IC50 values of DEX in the presence of S-CMC at concentrations of 10−7 M, 10−6 M, 10−5 M, and 10−4 M were 1.02 × 10−9, 1.13 × 10−10, 1.19 × 10−11, and 8.36 × 10−12 M, respectively, for IL-8 (Fig. 1E), and 1.22 × 10−9, 1.48 × 10−10, 3.14 × 10−11, and 4.26 × 10−12 M, respectively, for TNF-α (Fig. 1F). From an efficacy point of view, the I(max) value of DEX alone on IL-8 and TNF-α release was only 32.4% and 16.3%, respectively. Interestingly, S-CMC increased the I(max) value of DEX in a concentration-dependent manner. The J(max) value of DEX in the presence of S-CMC (10−4 M) reached 59.3% for IL-8, an increase of 83% (Fig. 1E), and 27.2% for TNF-α, an increase of 66.9%, compared to DEX alone (Fig. 1F). These results demonstrated that oxidative stress-induced steroid insensitivity can be improved by S-CMC administration in terms of DEX potency and efficacy.

Consistent with the results of in cellular models, ELISA analysis also demonstrated significantly higher levels of IL-8 and TNF-α in rat models of COPD which were resistant to DEX pre-treatment. We found that DEX or S-CMC alone did not inhibit production of IL-8 or TNF-α. However, the failure of DEX to suppress IL-8 and TNF-α production in rat exposed to cigarette smoke can be improved in the presence of S-CMC (Fig. 1E and F).

Effects of S-CMC on oxidative stress-induced steroid insensitivity by modulating HDAC2 expression/activity

Furthermore, we investigated the impact of S-CMC on down-regulation of HDAC2 expression/activity, which involves steroid sensitivity in response to a CSE-induced inflammatory response. Western blot (Fig. 2A and B) and HCS analyses (Fig. 2C) showed that
S-CMC reverses oxidative stress-induced steroid insensitivity by suppression of pro-inflammatory cytokine release. A549 cells were pre-incubated with DEX (10^{-8} M) or S-CMC (10^{-4} M) alone, or DEX (10^{-8} M) combined with S-CMC (10^{-6}–10^{-8} M) for 1 h, followed by exposure to 3% CSE for 6 h or H_{2}O_{2} (100 \mu M) for 4 h (A–D). A549 cells were pre-treated with increasing concentrations of DEX (10^{-14}–10^{-6} M) in the presence or absence of S-CMC (10^{-4}–10^{-6} M) for 1 h, followed by exposure to 3% CSE for 6 h (E and F). Concentration-dependent curves were generated using GraphPad Prism 5.0 software in a nonlinear regression with dose–response inhibition (variable slope) equation. The maximum inhibition of inflammatory cytokines release (I_{max}) and the corresponding DEX concentration were calculated as the index of steroid sensitivity. The levels of IL-8 (A, C, and E) and TNF-\alpha (B, D, and F) in the indicated groups were measured by ELISA. The levels of IL-8 (G) and TNF-\alpha (H), in the BALF of rats from indicated groups were measured. Values are presented as means ± SEM (n = 6–10). **p < 0.01 compared to controls; ††p < 0.01, and †p < 0.05 compared to DEX treatment alone.
Fig. 2. S-CMC improves oxidative stress-induced decrease of HDAC2 expression/activity. A549 cells were pre-treated with DEX in the presence or absence of S-CMC at the indicated concentrations for 1 h prior to exposure to 3% CSE for 6 h. HDAC2 expression in A549 cells was measured by western blot analysis (A and B) and high content screening analysis (C). HDAC2 expression in the lung tissues was detected by immunohistochemistry quantified by analysis of integral optical density (E and F, ×200). The activity of HDAC2 in A549 cells (D) or the lung tissues (G) was detected using a HDAC2 activity assay kit according to the manufacturer’s instructions. Values are presented as means ± SEM (n = 6–8). *p < 0.01 compared to controls. †† p < 0.01 compared to the CSE/CS exposure group. ‡‡ p < 0.01 and ‡ p < 0.05 compared to DEX treatment alone.

CSE-induced down-regulation of HDAC2 expression was markedly reversed by DEX (10⁻⁸ M) in the presence of S-CMC (10⁻⁶–10⁻⁴ M), while DEX (10⁻⁸ M) and S-CMC (10⁻⁴ M) treatment alone had no significant impact. Although HDAC2 activity was restored by S-CMC alone (10⁻⁴ M), the efficacy of S-CMC did not reach the ability of DEX + S-CMC at concentrations of 10⁻⁴ or 10⁻⁶ M (Fig. 2D).

In rat model of COPD, immunohistochemistry (Fig. 2E and F) showed that DEX treatment alone had no significant impact on HDAC2 expression, while combined treatment of DEX and S-CMC markedly increased HDAC2 expression in the lung sections and in the lung homogenates, which is more obvious than S-CMC treatment alone. Moreover, similar results of HDAC2 activity were found in the lung homogenates (Fig. 2G). These results suggested HDAC2 played a crucial role in the treatment effects of S-CMC and DEX.

To further confirm whether HDAC2 mediated the ability of S-CMC to improve steroid sensitivity, lentiviral-delivered shRNA was constructed and transfected to A549 cells to silence HDAC2 gene expression. HDAC2 gene silencing typically was achieved by
HDAC2-siRNA761 and down-regulation of HDAC2 was estimated at about 80% (Fig. 3A). We found that DEX (10^{-8} M) combined with S-CMC (10^{-4} M) did not affect CSE-induced cytokine release in HDAC2dim cells compared with control cells (Fig. 3B and C). Although a slight decrease was observed in cells treated by either DEX or S-CMC alone or in association with DEX, the decrease in cytokine production did not reach statistical significance. These results provide compelling evidence that restoring CSE-induced steroid insensitivity can be achieved using S-CMC by modulating HDAC2 expression/activity.

Next, we investigated the role of S-CMC on the CSE-induced oxidative stress response and found that CSE significantly decreased GSH levels (Fig. 4A), impaired SOD activity (Fig. 4B), and increased ROS production (Fig. 4C). The CSE-induced oxidative stress response was significantly improved by addition of 10^{-4} M S-CMC. DEX at a concentration of 10^{-8} M also improved the CSE-induced oxidative stress response, but not to a statistically
significant level. Interestingly, the effect of DEX was significantly enhanced by the addition of S-CMC in a concentration-dependent manner (Fig. 4A–C). These results demonstrated that S-CMC was beneficial in improving CSE-induced cellular oxidative stress to enhance the treatment efficacy of DEX.

Effects of S-CMC on HDAC2 expression/activity and pro-inflammatory cytokine release via thiol molecules

Given that S-CMC is a thiol compound/donor, we investigated whether the effect of S-CMC on restoration of HDAC2 activity and subsequent increased steroid sensitivity was influenced by the addition of the thiol-reducing agent DTT and thiol-oxidizing agent diamide (as a probe for thiols).

As shown in Fig. 5A and B, stimulation of cells with CSE resulted in an increase in IL-8 and TNF-α levels when compared with unstimulated control cells. CSE-induced inflammatory cytokine production was significantly inhibited by DTT and enhanced by diamide (p < 0.05) when compared to untreated cells. Moreover, the ability of DTT was enhanced by the thiol compound/donor S-CMC when compared to the CSE-stimulated group. Interestingly, the failure of DEX to inhibit cytokine release was improved by the addition of DTT (p < 0.01), similar to the effect of S-CMC. HDAC2 expression/activity on CSE-stimulated cells was significantly decreased when compared to the unstimulated control cells (Fig. 5C and D). The CSE-induced decrease in HDAC2 expression/activity was significantly counteracted by DTT (p < 0.01) and enhanced by diamide when compared to untreated cells (Fig. 5C and D). In addition, the ability of DTT or diamide to promote HDAC2 expression/activity was significantly enhanced by S-CMC when compared to the CSE-stimulated group (Fig. 5C and D).

To further confirm whether the ability of S-CMC to promote CSE-induced events is mediated through thiol molecules, we monitored thiol levels. Fig. 5E illustrated that thiol levels, either basal or DTT or diamide-treated levels on CSE-stimulated cells, were significantly decreased when compared to the untreated control group. However, thiol levels were significantly increased in DTT-treated cells and decreased in diamide-treated cells when compared to treatment in the presence of CSE. Interestingly, S-CMC was more effective in enhancing thiol levels in cells with or without treatment with DTT or diamide when compared to the CSE-stimulated group, and S-CMC treatment increased thiol levels, which were decreased in response to CSE (Fig. 5E). Similarly, S-CMC was more effective in enhancing GSH levels in cells with or without treatment with DTT or diamide when compared to the CSE-stimulated group, and S-CMC increased GSH levels, which are decreased in response to CSE (Fig. 5F). We also measured GSH/thiol levels in the lung in COPD rats, we found that, similar to the results from in vitro models, cigarette smoke-induced decreased GSH/thiol levels were significantly improved by treatment with S-CMC, that effect was enhanced by combined treatment with DEX, although DEX alone did not have a statistically significant effect on GSH/thiol levels impacted by cigarette smoke (Fig. 5G and H). These results demonstrated that thiol/GSH is critical for CSE-induced events and that S-CMC, by increasing thiol/GSH cellular concentrations, restored HDAC2 expression/activity.

S-CMC increases HDAC2 expression/activity in a thiol/GSH-dependent manner

Given that thiol/GSH molecules are critical to HDAC2 expression/activity, we assessed whether an interaction existed between thiol/GSH and HDAC2. For this purpose, we used an enzyme activity assay technique and found that S-CMC did not improve HDAC2 activity, while GSH was effective in countering the CSE-induced decrease in HDAC2 activity in a concentration-dependent manner under cell-free conditions. In addition, GSH also increased HDAC2 activity without CSE stimulation compared to the control group (Fig. 6A). The SPR technique, which is used to assess the specificity and affinity of small molecule–protein interactions (thiol/GSH molecules and HDAC2) under cell-free in vitro conditions [17,18], also demonstrated that GSH (10⁻⁶–10⁻² M) increased RU value in a concentration-dependent manner with a KD value of 1.63 × 10⁻⁸ M for GSH–HDAC2 interactions, whereas the KD values for S-CMC and DTT to HDAC2 were above 10⁻⁴ M and 10⁻³ M, respectively, which were much higher than that of GSH (Fig. 6B), demonstrating that a high specificity interaction exists between GSH and HDAC2.

We next assessed whether S-CMC increased HDAC2 expression by increasing GSH biosynthesis and found that the ability S-CMC to counteract the CSE-induced decrease in HDAC2 expression was suppressed in the presence of buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis (Fig. 6C). S-CMC also failed to decrease H4 acetylation in the IL-8 promoter in CSE-exposed cells in the presence of BSO (Fig. 6C and D). Taken together, these results indicate that S-CMC restored GC sensitivity by increasing HDAC2 expression/activity in a thiol/GSH-dependent manner in A549 cells.

Discussion

The results of the present study provide the first evidence that the failure of steroid therapy to suppress oxidative stress–induced pro-inflammatory cytokine production can be restored by increasing HDAC2 expression/activity through the action of S-CMC. We also found that S-CMC can increase thiol/GSH levels and S-CMC treatment increased the recruitment of HDAC2 and suppressed H4 acetylation of the inflammatory cytokine promoter, and this effect was further ablated by inhibition of GSH synthesis. These findings suggest that S-CMC can restore steroid sensitivity by increasing HDAC2 expression/activity in a thiol/GSH-dependent manner, thus S-CMC presents a therapeutic tool for enhancement of the efficacy of DEX treatment.

Lung epithelial cells are not only simple structural cells of the lung, but also serve as a barrier to invading allergens, produce enzymes and mediators that maintain normal airway homeostasis, and play an important role in prevention of pulmonary diseases. Lung epithelial cells also play a critical role in the lung inflammatory response by releasing inflammatory mediators and may act as important inflammatory cells, as with neutrophils, macrophages, and T lymphocytes in COPD [19,20]. In fact, A549 cells are a widely used model cell line for such biological evaluations.

Corticosteroid resistance is a major feature of COPD, which is characterized by neutrophilic inflammation [21–24]. Expression of IL-8, which is strong neutrophil chemoattractant, is increased in the lung tissues of COPD patients [4]. This increase in IL-8 expression is strongly associated with decreased HDAC2 expression, leading to subsequent activation of gene transcription [21,23,24]. In cultured cell experiments, H₂O₂ administration decreased HDAC2 expression [25], which is a key molecule for the resolution of inflammatory reactions by corticosteroids [24,25]. Our further results presented that failure of steroid therapy to suppress pro-inflammatory cytokine production, which were completely resistant to steroid therapy can be reproduced in cellular as well as animal models in the oxidative stress condition, and can also be restored by increasing HDAC2 expression/activity through the action of S-CMC, suggesting a potential role of S-CMC in a combination steroid therapy for treatment of steroid-insensitive pulmonary disease.

Oxidative stress caused by cigarette smoking is considered a disturbance in the oxidant–antioxidant balance, resulting in potential cell damage. Moreover, increased oxidative stress can trigger production of pro-inflammatory cytokines, which are increased
Fig. 5. Effects of S-CMC on HDAC2 expression/activity and pro-inflammatory cytokines release via thiol molecules. A549 cells were pre-incubated with DEX (10^{-8} M) or S-CMC (10^{-4} M) alone, or in combination for 1 h, followed by exposure to 3% CSE for 6 h in the presence of DTT (10^{-4} M) or diamide (10^{-4} M). The levels of IL-8 (A) and TNF-α (B) in the indicated groups were measured by ELISA. HDAC2 expression was measured by high content screening analysis (HCS) (C) and HDAC2 activity was measured using an HDAC2 activity assay kit (D). Intracellular active thiol levels in A549 cells or the lung tissue homogenate were examined using a colorimetric assay (E, G). Intracellular GSH levels in cell extracts or the lung tissue homogenate were determined by LC-MS/MS (F, H). Values are presented as means ± SEM (n=6–10). *p < 0.05 and **p < 0.01 compared to baseline values. ††p < 0.01 and †p < 0.05 compared to the CSE/CS exposure group. ‡‡p < 0.01 and ‡p < 0.05 compared to the DEX-treated group.
in the lungs of smokers and patients with COPD [26–28]. S-CMC, a mucolytic agent, is effective to reduce the severity and rate of exacerbation of disease in COPD patients [12]. The clinical efficacy of S-CMC seems to be more related to its anti-oxidant and anti-inflammatory effects than to its mucolytic activity [14,29]. The results obtained in the present study further support the opinion that S-CMC has beneficial effects by inhibiting oxidative stress-induced inflammatory responses. It is well established that S-CMC possesses antioxidant properties by scavenging reactive oxygen intermediates. The scavenger capacity of S-CMC act at relative high concentrations. It is somewhat surprising that the action of S-CMC on restoring steroid insensitivity through increasing HDAC2 activity was observed at relative lower concentrations, suggesting that S-CMC could achieve some other novel mechanisms to restore steroid sensitivity. The identification of the ability of S-CMC to restore HDAC2 activity and steroid efficacy raises interesting questions regarding the mechanisms driving these responses.

The most interesting finding in this study was that S-CMC-incubated cells revealed a significant increase in active thiol/GSH levels, which was completely hindered by oxidative stress. This finding supports another observation, in which DTT, a thiol reducing agent, strengthened the counteractive effect of S-CMC in CSE-stimulated A549 cells. In contrast, diamide, an oxidizing agent, weakened the counteractive effect of S-CMC in CSE-induced A549 cells. These findings also demonstrated a correlation between S-CMC and thiol/GSH in the regulation of oxidative stress-induced events. An association with a loss of intracellular thiols with the onset and progression of pulmonary diseases has been reported elsewhere [30,31], thus increasing thiol/GSH concentrations might be an effective approach for the treatment of COPD.

Intracellular thiol/GSH plays a vital role in intracellular redox balance and regulates several important physiological reactions. If oxidant stress can directly activate any of the enzymes involved in activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), high thiol levels could prevent NF-κB activation simply by scavenging oxidants. A strong relationship between intracellular thiol/GSH and NF-κB activation has been reported in the literature. For example, low cellular thiol/GSH levels promote NF-κB activation, whereas high intracellular thiol/GSH levels reportedly degrade NF-κB [32,33]. Activation of NF-κB and other transcription factors, which promote histone acetylation activity, lead to histone acetylation and subsequent transcription of genes that encode inflammatory proteins, such as IL-8. GCs reverse this process by binding to GC receptors and recruiting HDAC2, which reverses NF-κB-induced histone acetylation and switches off activated inflammatory genes. GSH is the primary intracellular low molecular weight thiol and most intracellular active thiols are derived from GSH [34], which is the primary physiological thiol-donor within the cell. GSH serves several vital functions, including scavenging of free radicals, maintenance of the essential thiol

---

**Fig. 6.** S-CMC increases HDAC2 expression/activity in a GSH-dependent manner. Active HDAC2 protein (purified) was directly co-incubated with DEX (10^{-8} M), S-CMC (10^{-6} M), or GSH (10^{-6} M) in the presence or absence of 3% CSE under cell-free conditions at 37 °C for 2 h. HDAC2 activity was measured using a HDAC2 activity assay kit (A). Interactions of GSH with HDAC2 were performed by SPR analysis (B). HDAC2 binding and histone acetylation were analyzed by ChIP analysis in A549 cells in the presence or absence of S-CMC, BSO, or S-CMC combined with BSO for 6 h prior to exposure to CSE for 1 h (C). Basal and CSE-induced histone acetylation of the IL-8 gene promoter in A549 cells after S-CMC treatment in the presence or absence of BSO followed by exposure to CSE for 6 h (D). Values are presented as means ± SEM (n = 3). *p < 0.05 compared to controls, †p < 0.05 and ††p < 0.01 compared to the CSE group, †p < 0.01 compared to the S-CMC treatment group.
status of proteins, and providing a cysteine reservoir. In addition, GSH has been shown to modulate the activity of proteins by post-translational modification [35]. Oxidative stress is associated with accumulation of ROS and depletion of GSH that together inhibited histone deacetylases (HDACs) activity, reduced protein levels of HDAC2 [36]. Consistent with these observations, we found that the accumulation of ROS and depletion of thiol/GSH induced by oxidative stress associated with decreased HDAC2 expression/activity resulting steroid resistance both in cellular and animal models.

Several reports have shown that enhancement of GSH concentration in tissues is achieved by the addition of its sulfur-containing precursors, such as cysteine [37]. S-CMC, as a cysteine derivative, can decrease ROS production in CSE-stimulated bronchial epithelial cells [38] and increase GSH secretion by human respiratory cells in the presence of S-CMC [39]. Recent study reported that incubation with GSH restored the deacetylase activity of HDAC2 [40]. The results of other reports, combined with our current findings, strongly suggest that increased thiol/GSH levels by S-CMC are favorable toward restoring HDAC2 activity. The specific mechanism might be explained by S-CMC stimulation of GSH efflux from respiratory cells, thereby increasing intracellular active thiol levels to restore HDAC2 activity and reduce the ROS-mediated inflammatory response [15]. Enzymatic analysis and the SPR technique further confirmed that a direct interaction between GSH and HDAC2 exists under cell-free conditions. This further supports the above finding that thiol/GSH is closely related to HDAC2 activity. In contrast to prior cellular studies in which the antioxidants GSH and S-CMC act as thiol compounds to restore HDAC2 activity, only GSH showed high binding affinity to HDAC2 under cell-free conditions. A possible explanation for this might be that thiol compounds/donors, such as S-CMC, act as thiol molecules by increasing GSH concentrations to up-regulate HDAC2 activity in cells. In fact, the effect of S-CMC was ablated by BSO, as determined by the ChIP assay, confirming that S-CMC restored HDAC2 activity by increasing GSH biosynthesis. Hence, we have reason to believe that S-CMC restored HDAC2 activity by increasing thiol/GSH levels and suppressed histone acetylation of the inflammatory cytokine gene promoters [40], which could achieve further restoration of steroid-insensitivity.

Conclusion

In summary, our results indicate that S-CMC can ameliorate impaired steroid sensitivity that targets HDAC2 via thiol/GSH both in cellular and animal model of oxidative stress-induced steroid insensitivity and suggests that S-CMC can be used as a combination therapy for treatment of steroid-insensitive pulmonary diseases. Although a close interaction between thiol/GSH and HDAC2 was established in our study, the possible mechanisms remain unclear and warrant further investigations.

Role of the funding source

This work was funded by the National Natural Science Foundation of China (No. 81370144) and the Science and Technology Commission of Shanghai Municipality (No. 1214001400).

Competing interests

The authors have no competing interests (financial or otherwise) with respect to this article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jphs.2014.12.002.


