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EVALUATION OF THIOL-BASED ANTIOXIDANT THERAPEUTICS IN CYSTIC FIBROSIS SPUTUM: FOCUS ON MYELOPEROXIDASE

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Abstract

Neutrophil-dependent reactions catalyzed by myeloperoxidase (MPO) are thought to play important roles in the pulmonary pathobiology of cystic fibrosis (CF). Aerosolized thiol antioxidants such as glutathione (GSH) and *N*-acetylcysteine (NAC) are currently being utilized as therapeutics to modify CF respiratory tract oxidative processes. We hypothesized that MPO in CF airway lining fluids may be a target of such therapeutics. MPO activity in sputum from 21 adult CF patients was found to be inversely associated with lung function (FEV_1). In contrast, systemic inflammation (assessed by plasma C-reactive protein) was not correlated with lung function. *Ex vivo* studies revealed that GSH and NAC effectively scavenged *N*-chloramines in sputum, and inhibited sputum MPO activity with potency exquisitely dependent upon MPO activity levels. Detailed kinetic analyses revealed that NAC and GSH inhibit MPO by distinct mechanisms. Activation of the key proinflammatory transcription factor NF-κB in cultured HBE1 cells was inhibited by GSH. Our findings reveal that MPO activity and its reactive products represent useful predictors of the doses of inhaled thiol antioxidants required to ameliorate airway oxidative stress and inflammation in CF patients, and provides mechanistic insight into the antioxidative/anti-inflammatory mechanisms of action of GSH and NAC when administered into the CF lung.

Keywords

myeloperoxidase; cystic fibrosis; sputum; glutathione; *N*-acetyl cysteine; *N*-chloramines; antioxidants; individualized medicine

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in a single gene encoding the transmembrane glycoprotein, CF transmembrane conductance regulator (CFTR) (1). CFTR, a cyclic adenosine monophosphate (cAMP)-activated transmembrane channel protein that primarily conducts chloride ion (2), is also involved in the regulation of other ion channels (e.g., epithelial sodium channel activity) (3,4). Loss of CFTR function in respiratory tract tissues results in thick, underhydrated mucus due to hyperabsorption of sodium chloride and reduced periciliary water and anion transport, leading to reduced mucociliary clearance, airway bacterial colonization and inflammation characterized by an intense airway neutrophil influx (4–6). The neutrophil influxes and their effector oxidative processes are believed to represent major factors in CF respiratory tract disease (7).

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Myeloperoxidase (MPO) is the most abundant protein/enzyme within neutrophils (5% of the dry weight of the cell) (8). MPO catalyzes the formation of hypochlorous acid (HOCl), a potent bactericidal agent, that is capable of oxidizing and chlorinating a broad spectrum of biomolecular species (9–11). Respiratory tract secretions from CF patients are known to contain abundant quantities of active MPO (12,13). MPO can catalyze protein tyrosine oxidation and produce significant levels of 3-nitrotyrosine, dityrosine and 3-chlorotyrosine in CF (13), thus along with HOCl representing a potent potential source of respiratory tract cytotoxic injury (13,14).

Not surprisingly, proposed therapeutic strategies for CF have included administrations of oral or aerosol delivered antioxidant substances, especially glutathione (GSH) and *N*-acetylcysteine (NAC) (15–22). Many of the existing limited therapeutic antioxidant trials have administered a “fixed dose” of oral or aerosolized antioxidant without quantitative consideration of the magnitude of respiratory tract oxidative processes. In the present study we have used MPO activity in CF respiratory tract secretions as a biomarker of respiratory tract “oxidants” and tested various thiol antioxidants utilized as possible therapeutic strategies in CF for their effectiveness in inhibiting MPO activity *ex vivo*. The primarily focus of this study was on the interactions of GSH and NAC with MPO contained in the complex matrix of CF sputum, as these antioxidant thiols have been widely studied as therapeutic agents (19) and are being used in several clinical trials that are currently in progress in CF patients. We also determined the levels of the long-lived MPO-derived *N*-chloramines in CF sputum and defined the capacity of NAC and GSH to scavenge these species. Finally, we demonstrate that GSH is capable of modulating the pro-inflammatory effects of CF airway secretions in a respiratory epithelial cell model using NF-κB activation, a key regulator of the inflammatory-immune system (23), as a reporting marker of sputum bioactivity. The results herein provide a biochemical construct to further understand the reactions that GSH and NAC undergo in the CF airway, and provide a platform for the development of novel and more efficacious thiol-based therapeutics for the treatment of CF.

Materials and Methods

Subjects

Studies were performed with approval from the Office of Human Research Protection/ Institutional Review Board (IRB) of the University of California, Davis. All subjects gave informed consent. Inclusion criteria included the ability of the patient to spontaneously produce sputum. Exclusion criteria included any use of glutathione (GSH), *N*-acetyl cysteine (NAC), or ascorbic acid supplementations in the 28 days before recruitment. Freshly expectorated sputum specimens were collected from 21 adult CF patients. Table 1 presents the demographics of the 21 patients (7 male, 14 female; ages 19–58). These patients represent a typical spectrum of the 75 patients being followed in the UC Davis Adult CF clinic.

Sputum processing

Freshly obtained sputum specimens were weighed and diluted in equal amounts of phosphate buffered saline (pH 7.0) (w/v). The samples were kept in a shaker for 30 mins at 37°C followed by centrifugation at high speed (14000 rpm, 4°C, 15 mins). The upper clear layer (sol-phase) was aliquoted and stored at −80°C until further analyses. It was not possible to perform all assays on all CF sputum obtained due to limitations of sputum quantity and availability.

Analysis of sputum MPO activity

The aliquoted CF sputum sol-phase was further diluted and heme peroxidase activity assessed with 3, 3′, 5, 5′-tetramethylbenzidine (TMB) as a substrate (24) against a standard curve using purified human MPO (EMD Biosciences, La Jolla, CA). It should be recognized that this assay is not specific for MPO. However, the vast majority of heme peroxidase activity in CF sputum is due to MPO (13,25).

Analysis of sputum N-chloramine levels

The basal CF sputum *N*-chloramine levels were analyzed in 25 μl of sol-phase using iodide to catalyze the oxidation of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (26). The readings were extrapolated against a standard curve using known concentrations of *N*-chloro-taurine.

Ex vivo antioxidant treatments

Diluted sol-phase CF sputum was incubated with glutathione (GSH) or *N*-acetylcysteine (NAC) at different concentrations (1 nM – 100 mM). MPO activities were compared to untreated controls and the half maximal MPO inhibitory concentration (IC_{50}) of each antioxidant was determined.

Similarly, to investigate the effect of GSH and NAC on sputum *N*-chloramine levels, a known concentration of antioxidants (IC_{50} obtained from the above experiment) was incubated with 25μl of CF sputum sol-phase and compared to untreated controls.

Cell Culture and treatments

Human bronchial epithelial cells (HBE1), a papilloma virus-immortalized cell line (27), were plated on standard six-well culture plates (BD Falcon, San Jose, CA) at 2×10^4 cells/well in serum-free bronchial epithelial cell basal medium (BEBM)/Dulbecco's modified Eagle's medium (DMEM) (1:1) supplemented with various growth factors including bovine pituitary extract (50 μg/ml), gentamycin (50 μg/ml), insulin (5 μg/ml), hydrocortisone (0.1 μM), epidermal growth factor (30 μg/ml) and transferrin (5 μg/ml) (13). When cells attained 80–85% confluency, they were starved for 6 hrs by replacing media with BEBM/DMEM (1:1) medium devoid of growth factors. Following growth factor deprivation, cells were treated with sol-phase of CF sputum (10 μl/ml of BEBM/DMEM (1:1) medium supplemented with growth factors) and harvested at 30 and 60 minutes after treatment for analysis of NF-κB activation. Nuclear and cytosolic protein fractions were separated using Pierce protein-extraction kit as per vendor's protocol (Thermo Scientific, Rockford, IL) and stored at −80°C until further analyses. In another set of cells, GSH (1mM) was added along with sol-phase of CF sputum (10 μl/ml of BEBM/DMEM (1:1) medium supplemented with growth factors) and processed similarly. For untreated condition, same amount of phosphate buffered saline (PBS), without CF sputum solphase was added to cells. HBE1 cells treated with 20 μg/ml tumor necrosis factor-α (TNF-α) served as positive controls for analysis of NF-κB activation by western blot technique (23).

Western blot for analysis of NF-κB activation

Nuclear and cytosolic fractions of cells were separated on 4–20% gradient precast protein gels (Thermo Scientific, Rockford, IL) (40 μg protein per well) and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking the membranes in 5% milk (in PBS-Tween 20 solution), they were probed for p65 and IκB-α proteins by incubating with p65 (1:500; Santa Cruz Biotechnology, CA) and IκB-α antibody (1:500; Santa Cruz Biotechnology, CA) respectively. NF-κB activation was monitored by nuclear translocation of p65 and degradation of cytosolic I B-α, subunits of NF-κB (28). Antibodies against β-actin (1:5000; Abcam Inc., Cambridge, MA) and p84 (1 μg/ml; Abcam Inc., Cambridge,

MA) proteins were used as loading controls for cytosolic and nuclear proteins respectively. Horseradish peroxidase (HRP) conjugated goat anti-mouse (Abcam Inc., Cambridge, MA) for p84 and goat anti-rabbit secondary antibody (Abcam Inc., Cambridge, MA) for p65, IκB-α and β-actin were used and detected by enhanced chemiluminescence (ECL) western blotting kit (GE Healthcare, Amersham, UK).

Inhibition kinetics of MPO-dependent TMB oxidation by thiol antioxidants

GSH and NAC at different concentrations (0.1–1 μM) were incubated with a known amount of purified human MPO (1.5 nM) and MPO activity assessed with TMB as a substrate at varied concentrations (20–180 μM). From the resulting data, Lineweaver-Burk graphs were plotted between reciprocal values of velocity of reaction (1/V) and TMB substrate concentrations (1/[TMB]).

Statistical analyses

All data are presented as means ± SEM. Spearmann's correlation coefficient was used to measure correlation coefficient between samples. Statistical tests were performed using GraphPad Prism software (San Diego, CA). P value ≤ 0.05 was considered as significance in each analysis.

Results

Sputum MPO levels inversely correlate with lung function

We observed an inverse correlation between CF sputum MPO levels and lung function as assessed by percent predicted forced expiratory volume in 1 second (FEV_1 % predicted) (n=21, r = −0.59, P= 0.004) (Fig 1A), supporting earlier studies in a younger cohort of patients (12). However, we did not observe any significant correlation between plasma CRP levels, a marker of systemic inflammation, and lung function in CF patients (Fig 1B). Sol-fractions of sputum from CF patients contained high levels of MPO activity, consistent with our previous studies (13,25). No specific investigations were performed that would further characterize outliers and no attempts were made to determine intra-subject variability of the sputum MPO levels. However, although sputum MPO levels in CF can be expected to exhibit sharp rises during acute clinical exacerbations (29), during periods of clinical stability sputum MPO levels in individual CF patients have been reported to be reasonably reproducible (30). Based on the MPO activity, the present group of CF patients could be empirically grouped into low, moderate and high levels of MPO (Fig 2), representing 27, 33 and 38% of the patients, respectively. We believe that this may represent a clinically useful index of the degree of CF respiratory tract inflammation. We did not observe any significant correlation between CF sputum MPO levels and sputum *N*-chloramine levels (Fig 3).

Inhibition of MPO activity by GSH and NAC is dependent upon sputum MPO concentration

Fig 4A and 4B shows the IC_{50} of the thiol antioxidants, GSH and NAC respectively, based on their inhibition of sputum MPO activity. Diluted CF sputa having varied ranges of MPO (0.3 nM–2.2 nM) were used. The data illustrate that the higher the MPO activity in CF sputum, the higher the thiol antioxidant concentrations needed to inhibit MPO activity as measured by TMB oxidation.

GSH and NAC inhibit MPO by different mechanisms

To gain insight into the mechanism(s) by which GSH and NAC inhibited the 1-electron oxidation reactions of MPO, we next performed kinetic experiments with varied substrate (TMB) and inhibitor (NAC/GSH) concentrations and generated double reciprocal plots of reaction velocity (1/V) and TMB concentration (1/[TMB]). The results for GSH and NAC

are shown in Fig 5. Inhibition of MPO-catalyzed oxidation of TMB by GSH was observed to occur by competitive mode (Fig 5A). However, NAC displays a non-competitive mode of inhibition of MPO as demonstrated by data from reciprocal plots of velocity of reaction (1/V) against TMB concentrations (1/[TMB]). When the slope of each curve from Fig 5A and 5B was plotted against different concentrations of GSH and NAC, a K_i of 0.08 and 0.1 μM was obtained, respectively.

Sputum N-chloramines are reduced by GSH and NAC

A known concentration of GSH or NAC (2 μM, as derived from data shown in Fig 6A and 6B) was incubated with 25 μl of CF sputum sol-phase for 30 min. Fig 6A and 6B shows CF sputum *N*-chloramine levels before and after treatment with GSH and NAC respectively. GSH treatment lowered *N*-chloramine levels by 62 ± 11%, where as NAC treatment reduced *N*-chloramine levels by 80 ± 11%.

CF sputum mediated NF-κB activation is blocked by GSH in HBE1 cells

As shown in Fig 7A, CF sputum (sol-phase) induces nuclear translocation of NF-κB subunit, p65 and degradation of cytosolic IκB-α suggesting induction of NF-κB pathways in HBE1 cells. Of note, p65 nuclear protein levels in CF sputum treated cells (Fig 7A) were seen more than TNF-a (positive control) treated cells (Fig 7B) at 60 minutes. This suggests a 'prolonged' NF-κB activation in response to CF sputum treated cells. It was interesting to note that GSH (1mM) along with CF sputum (solphase) when incubated with HBE1 cells was seen to inhibit p65 nuclear translocation (Fig 7C). Collectively, these results suggest that sol-phase of CF sputum alone can activate NF-κB proteins but this activation can be ameliorated by GSH, suggesting possible scavenging of 'NF-κB sensitive' oxidants such as *N*-chloramines, in CF sputum (31,32) or by other redox-sensitive mechanisms (33). Due to the complex biochemical composition of CF sputum known to contain a number of well-known inducers of NF-κB, we did not further pursue the identification of the biomolecular species responsible or the upstream mechanisms of NF-κB activation.

Discussion

MPO and CF disease severity

CF patients demographically described in Table 1 are representative of a typical cross section of adult patients with CF. These CF patients had a wide distribution of sputum MPO levels (Fig 2) that were inversely correlated to their FEV_1 levels (Fig 1A). In contrast, although other investigators have observed a negative correlation between plasma C-reactive protein (CRP), a frequently utilized systemic biomarker of inflammation, with lung function (FEV_1) in (34,35), we did not observe a significant correlation in this relatively small cohort of relatively stable adult outpatients with CF (Fig 1B). Our data indicate that systemic inflammatory markers (CRP) do not necessarily reflect the degree of inflammation in the CF airway.

Our observations with MPO were consistent with the reports of other investigators relating MPO levels to pulmonary function decrements in CF patients as a result of increased respiratory tract inflammatory processes (12,29,36–40). Of note, the −463GA MPO promoter polymorphism, in which the GG phenotype is found to result in increased MPO gene expression in leukocytes, has been reported to be associated with a more aggressive loss of pulmonary function in CF (41). Interestingly, this latter study also suggests a proinflammatory role of MPO in the early phase of CF, where severity of the disease was associated with higher expressing GG phenotype in the absence of documented bacterial infection (41).

Elevated oxidants in CF respiratory tract lining fluid (42), along with increased amounts of various MPO-derived oxidants and the abundant proteolytic processes accompanying inflammatory processes (13,43), are important considerations in the pathobiology of CF respiratory tract injury. The results provided herein further support the notion that MPO may play a causative role in CF lung disease, and that current thiol antioxidant therapeutics (GSH and NAC) can influence the activity of MPO by different mechanisms. Additionally, our findings reveal that MPO activity may be an important parameter for determining the dose of thiol antioxidant required to achieve a therapeutic benefit.

Implications for thiol-based antioxidant therapeutic strategies in CF

Aerosolized “antioxidant therapy” represents a current therapeutic approach to counteract oxidative stress in CF airways (42). Several clinical trials using aerosolized antioxidants in CF patients have achieved beneficial effects (15–17,22), although many others have not been as successful (19). Interestingly, although the positive studies have reported improvements in lung function, few changes have been observed in markers of oxidative stress (16). Surprisingly, most of the aerosolized antioxidant CF clinical trials have not reported the pre-/post-treatment sputum MPO levels/activity, inspite of MPO being one of the most abundant pro-oxidant constituents in CF respiratory tract lining fluids.

While not entirely surprising, data obtained from reacting sputum from CF patients with thiol antioxidants (Fig 4A and B) shows that higher sputum MPO levels require higher concentrations of antioxidants (GSH and NAC) to inhibit MPO activity (Fig 4A and 4B). Of interest, it was necessary to dilute CF sputum (sol-phase) samples approximately 2000 times in order to accurately quantify MPO activity. The *ex vivo* antioxidant incubations were also carried out in these diluted samples. Hence, using this dilution factor, the actual concentration of antioxidants to be theoretically administered to patients, with respect to MPO inhibition, may require respiratory tract lining fluid levels up to 50 mM to effectively inhibit the highly abundant MPO contained in CF respiratory tract secretions. Griese *et al* reported an increase in alveolar GSH levels and improved lung function, but not in biomarkers of oxidative state, after administering aerosolized GSH to CF patients (three times daily, doses of 300/450 mg GSH for 14 days) (16). At an inhaled dose of 450 mg, bronchoalveolar lavage (BAL) fluid taken 1 hr after the completion of the 14 day trial, GSH levels were 15.59 ± 7.18 Nmol/L as compared to baseline values of 3.83 ± 1.83 Nmol/L. Interestingly, oxidized GSH levels (GSSG) were 16.32 ± 4.27 Nmol/L as compared to baseline levels of 1.24 ± 0.33 Nmol/L (16). Our current data suggest that to effectively inhibit MPO-catalyzed reactions, GSH may need to be administered at much higher levels to achieve respiratory tract lining fluid concentrations of 1–50 mM (Fig 4A) in order to directly influence MPO activity. This might partially explain why the previous studies did not observe any changes in oxidative stress biomarkers in CF patients after administering aerosolized GSH (16).

A GSH pro-drug, NAC, is occasionally used for inhalation in CF patients but has significant side effects secondary to its acidic nature (pK_a 2.2) which can induce bronchospasm. NAC has also been administered orally in CF patients to increase circulating GSH (44). Administration of high doses of NAC orally (0.6–1.0 g, three times daily for 4 weeks) increased neutrophil GSH content, decreased neutrophil influx in CF airways, decreased sputum elastase activity, and decreased sputum IL-8 levels; observations that were attributed to decreased airway inflammation (21). However, there was no improvement in lung function, as assessed by FEV_1, possibly due to the relatively short duration of treatment. Of note, there are no clear data on GSH/GSSG levels in CF respiratory tract secretions after a high dose oral NAC administration and/or how NAC might modify the “oxidative stress” of CF airways.

As previously reviewed by Nash *et al*, either oral or nebulized (aerosolized) thiol derivatives have failed to show significant beneficial effects in CF disease (19). As observed in our *ex vivo* studies, similar to GSH, the higher the sputum MPO levels, the higher the concentration of NAC required to inhibit MPO activity (Fig 4B), and as for the case of GSH and NAC, a concentration 1–50 mM would appear to be required to inhibit the actions of the high quantities of MPO in the CF airway. Additionally, a significant portion of MPO may be trapped in the gel-phase of the sputum. Hence, one of the major questions still needing to be addressed (and a limitation of the current study) is how administered antioxidants interact with the gel-phase, as most of the proposed therapeutic antioxidants are water soluble and may have difficulty penetrating the highly viscous gel phase of sputum.

Proposed mechanisms of action of thiol antioxidants in CF sputum

***N*-Chloramines and their scavenging by GSH and NAC—**As illustrated in Fig 8A, *N*-Chloramines are formed by MPO-catalyzed formation of HOCl and subsequent chlorination of amines (45). Somewhat surprisingly, we did not observe a significant correlation between sputum MPO levels and sputum *N*-chloramine levels (Fig 3). In fact, a negative correlation between *N*-chloramines and MPO in CF sputum has been previously observed (46). *N*-Chloramines are selectively reactive with specific molecular targets (ie. sulfur-containing amino acids) that makes them fairly labile in airway secretions and may not necessarily be expected to parallel MPO levels. *Ex vivo* incubation of CF sputum with GSH and NAC were able to substantially reduce sputum *N*-chloramine levels (Fig 5). *N*-chloramines are known to react with GSH, thus leading to GSH depletion (47). Comparing the two antioxidants, NAC appeared to be more potent in reducing *N*-chloramine levels as compared to GSH (eg. 80 ± 11% vs 62 ± 11%). Peskin and Winterbourn have reported that NAC (k= 46 ± 7 $M^{-1}sec^{-1}$) was approx. 2.5 times less reactive than GSH (115 ± 14 $M^{-1}sec^{-1}$) with taurine chloramine (48). However, in our data, NAC seemed to be more effective in scavenging *N*-chloramines in CF sputum. This potentially could be due to the smaller size of NAC as compared to GSH, that may allow NAC to gain entry to *N*-chloramines located in sterically-hindered sites within proteins. The reaction of thiol antioxidants with N-chloramines is illustrated in Fig 8A, and may represent one pathway by which thiol antioxidant therapeutics alter the inflammatory and oxidative processes occurring in the CF lung.

The capacity of GSH to inhibit CF sputum-mediated p65 nuclear translocation in lung epithelial cell culture model (Fig 7) may demonstrate the ‘beneficial effect’ of GSH supplementation in controlling NF-κB mediated inflammatory-immune responses (33). *N*-chloramines are known to react with GSH (47), and may represent one possible mechanism by which NF-κB was activated by CF sputum (49). In the present study, GSH was able to scavenge CF sputum *N*-chloramines (Fig 6A) and also block CF sputum mediated p65 nuclear translocation (Fig 7). Of note, a previous study using significantly higher concentrations of *N*-chloramines has shown inhibition of NF-κB activity (50). Future studies are needed to further interrogate the substances in CF sputum that serve to modulate NF-κB activity in underlying respiratory tract cells, as well as dose-response relationships between the capacity of *N*-chloramines to modulate NF-κB activation and inhibition.

Scavenging of MPO-derived radicals by NAC and GSH—The present results indicate that the thiol antioxidants NAC and GSH can inhibit the MPO system in CF respiratory tract secretions. This could occur at a number of potential mechanistic points. First, it is theoretically possible that GSH and NAC could decrease MPO activity by simple scavenging of H_2O_2; that is, decreasing the substrate available to MPO. However, at pH 7.4, the second-order rate constants for reaction of NAC and GSH with H_2O_2 are very slow (0.16 and 0.87 $M^{-1}s^{-1}$, respectively) (51) and can’t compete with the very fast reaction of H_2O_2

with ground state ferric MPO ($k = 2.6 \times 10^7$ $M^{-1}s^{-1}$) (52). Thus, it is unlikely this mechanism can explain the present results.

Another possible mechanism by which NAC and GSH could modulate MPO one-electron peroxidase activity is by direct reaction with either compound I or compound II of MPO. However, because of their structure and charge state, NAC and GSH are poor substrates for reaction with both compound I and II of MPO ($k = 10^1$–10^2 $M^{-1}s^{-1}$) (53). In fact, the reaction of our substrate molecule TMB is 4–5 orders of magnitude faster with MPO compound I and II (3.6×10^6 and 9.4×10^5 $M^{-1}s^{-1}$) (54), compared to NAC and GSH. Given the vast excess of TMB substrate in the biochemical assays, and its much faster reaction with MPO, it is unlikely that NAC and GSH inhibit MPO activity by directly reacting with compounds I and II of MPO.

Having excluded scavenging of H_2O_2 and largely negating the direct reactions with MPO, one reasonable explanation is that NAC and GSH exert their ‘inhibitory’ effects on MPO by scavenging radical species produced by MPO (in the present case TMB substrate radicals) (Fig 8B). There is precedence for such reactions (55,56), and conjugates of GSH with TMB have been previously identified in horseradish peroxidase-catalyzed reactions (57). Taken together, scavenging of intermediate TMB radicals, or adduction of benzoquinonediimine intermediates, is one likely mechanism by which NAC and GSH alter MPO reactions occurring in CF sputum.

Competitive inhibition of MPO by GSH: A previously unrecognized interaction —Detailed kinetic analyses in our study have revealed that NAC and GSH inhibit MPO by two independent mechanisms. On the one hand, NAC inhibits MPO by a non-competitive mechanism (Fig 5B), suggesting an interaction/reaction of NAC with the MPO-TMB radical complex not at the TMB binding site (ie. consistent with the radical scavenging mechanism in Fig 8B). In contrast, GSH is a competitive inhibitor of MPO. Kinetic analyses shown in Fig 5A reveal that GSH increases the apparent K_m for TMB as substrate for MPO, and this strongly suggests that GSH combines reversibly with MPO at or near the active site. Previous studies have revealed that GSH is a poor substrate for MPO (58), and that the oxidation of GSH by MPO is largely dependent upon the formation of HOCl (59). This, taken together with our data presented herein, suggests that GSH serves to inhibit binding and oxidation of MPO substrates without actually participating in the catalytic peroxidase cycle. While there are no data that we are aware of in the literature to document a binding site for GSH on MPO, there is precedence for a binding interaction between GSH and lactoperoxidase (60). Additionally, the microsomal heme protein prostaglandin E synthase-1 binds GSH and utilizes it as a cofactor in its catalytic cycle (61). It remains possible that a specific binding site for GSH exists at, or near, the substrate binding site/active site in MPO. Structural interrogation of the active site of MPO, combined with computational docking models, and binding experiments are needed to further understand the previously unrecognized interaction of MPO with GSH. Lastly, structure-activity relationships of diverse GSH derivatives remain future goals for potentially developing novel inhibitors of MPO for use in the treatment of CF.

Conclusions: The major aim of this investigation was to explore the effect of *ex vivo* antioxidants on MPO activity in CF respiratory tract secretions. It must be noted that CF sputum is a rich source of inflammatory biomarkers, containing abundant amounts of oxidants, proteases, bacterial products, chemokines, cytokines, etc (43,62). It is thus very challenging to ‘single-out’ a molecule in CF sputum that could be contributing to the detrimental effect in respiratory tract epithelial cell NF-κB activation (63). As reported here, possible inhibition of NF- B activation processes in HBE1 cells by GSH-mediated consumption of *N*-chloramines remains speculative as other mechanisms cannot be

excluded. Equally likely would be the GSH scavenging of other endogenously produced oxidants in CF sputum or even bacterial-derived oxidants such as pyocyanin (64). Different methods of CF sputum processing also significantly influence several parameters analyzed (62,65). Based on the present study, we suggest that using fresh respiratory tract secretions collected from CF patients and using these fluids to characterize interactions of these secretions on airway epithelial cells represents a useful simulation of pathobiological events occurring in the airways of CF patients. Finally, we suggest that strategies designed to therapeutically address “oxidative stress” in CF patients may need to take into consideration the overall magnitude of MPO in CF respiratory tract secretions.

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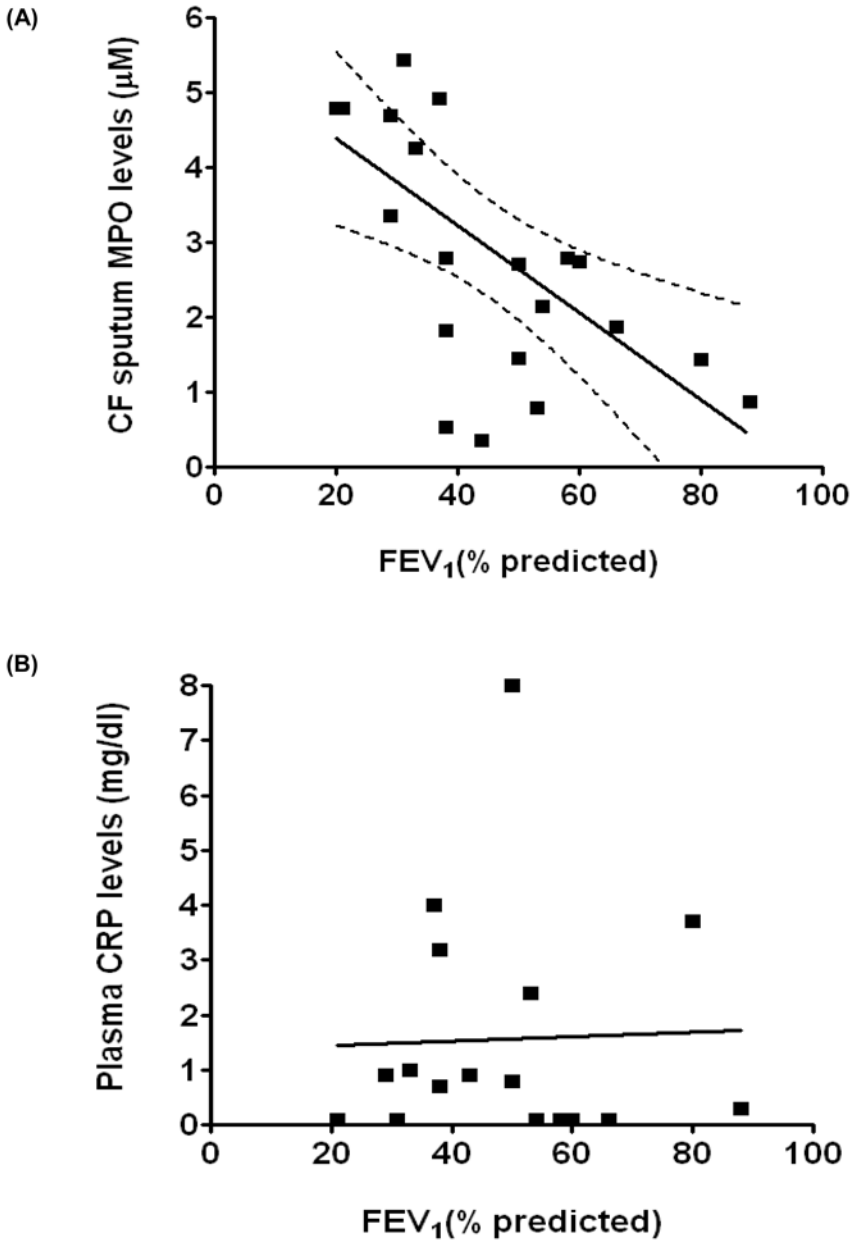

Figure 1. Sputum MPO but not plasma CRP levels in CF patients correlate with lung function

(A) An inverse correlation between CF sputum MPO levels and FEV_1 (% predicted) (n=21, r=−0.59, P=0.004) was observed. (B) No correlation was observed between plasma CRP levels and FEV_1 (% predicted) (n=17, r=0.033, P=0.9). Spearmann's correlation coefficient was used to measure correlation coefficient between samples.

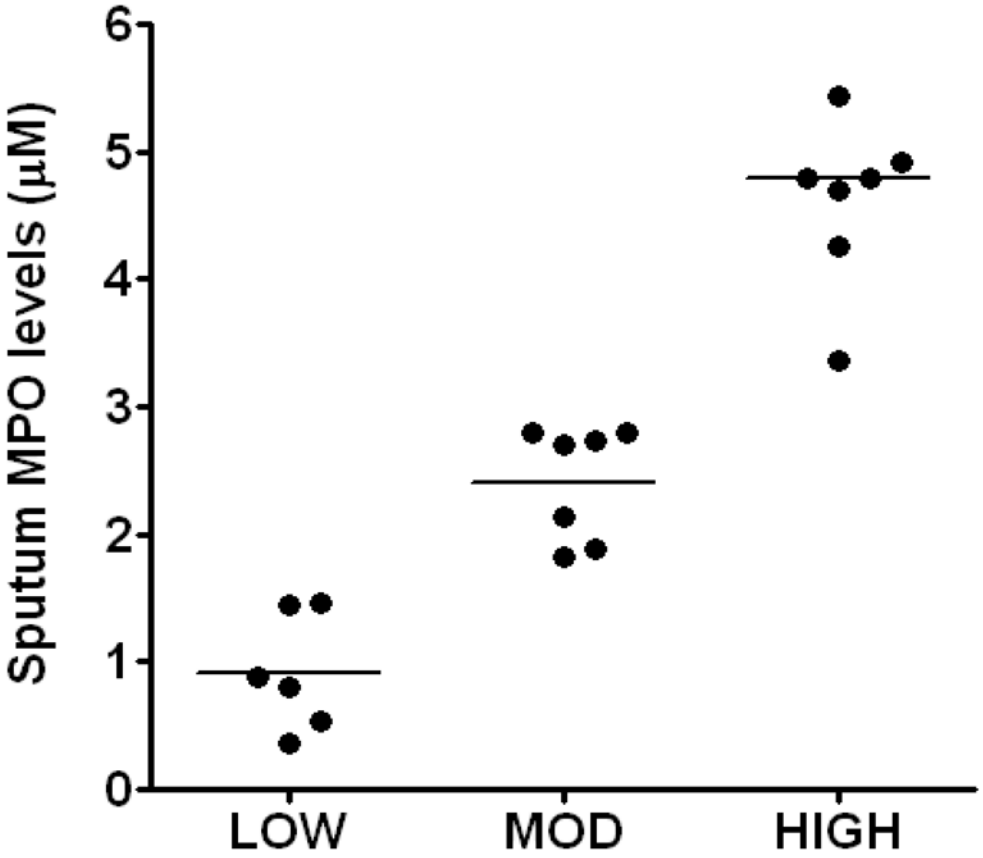

Figure 2. Sputum MPO levels in CF patients

CF patients had varied levels of sputum MPO levels and were categorized into low, moderate and high groups comprising 27%, 33% and 39% of total patients. Values are presented as mean ± SEM.

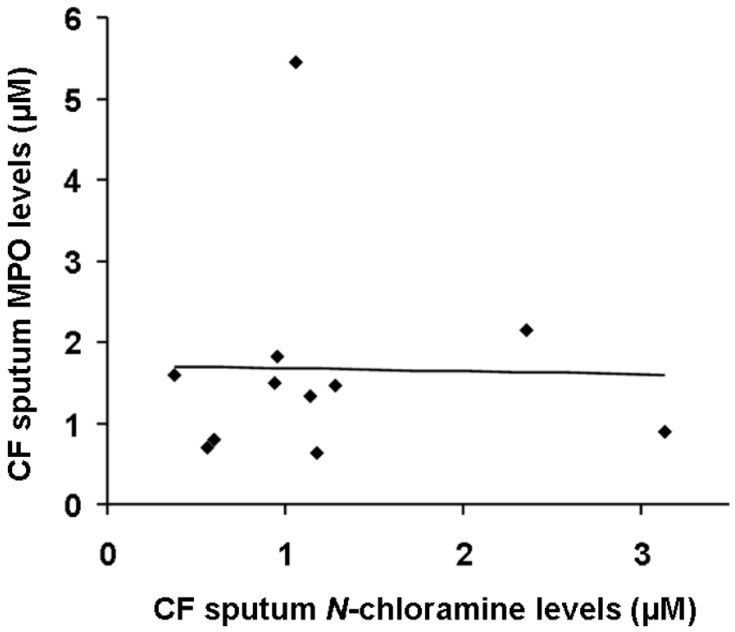

Figure 3. Correlation between sputum MPO and *N*-chloramine levels in CF patients

No correlation was observed between sputum MPO and chloramine levels (n=9, r=0.0005). Spearmann's correlation coefficient was used to measure correlation coefficient between samples.

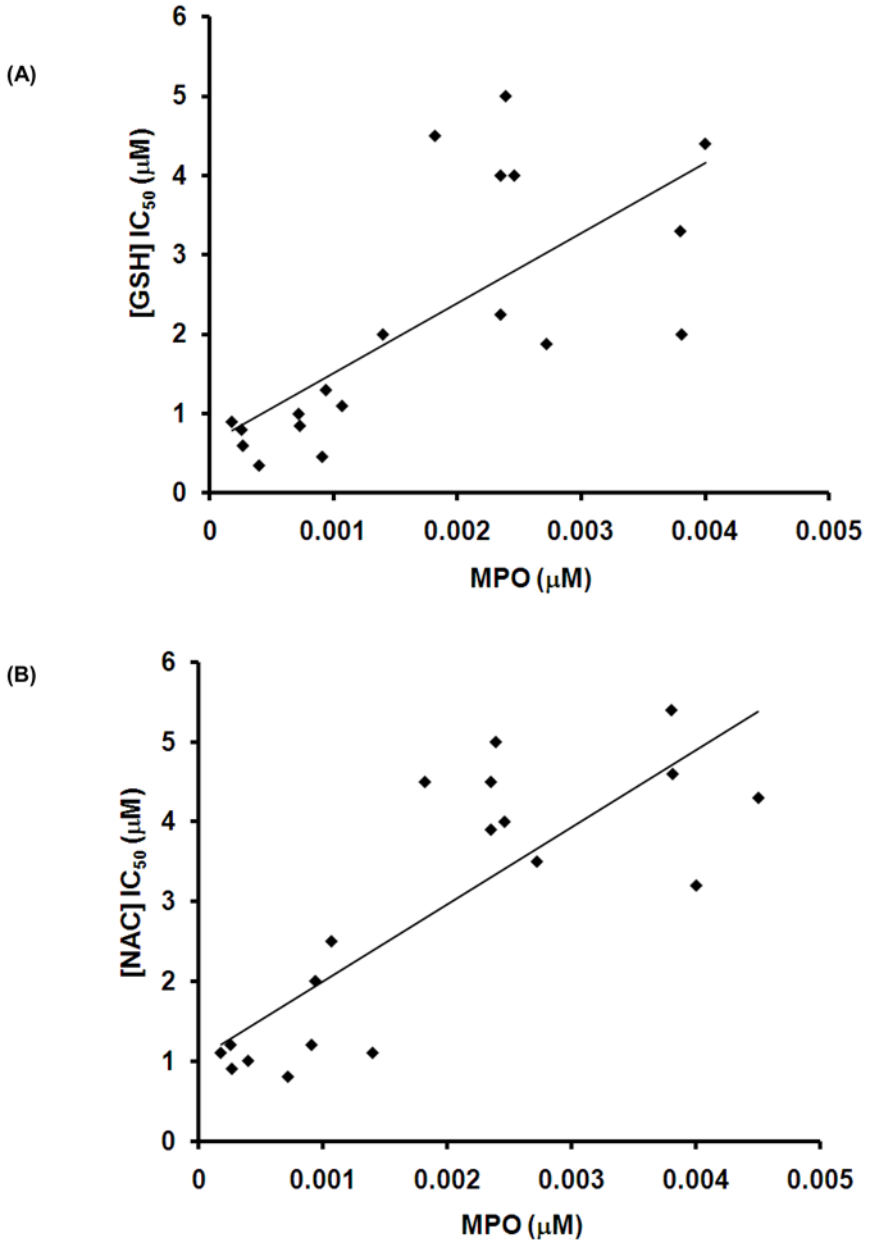

Figure 4. Inhibition of CF sputum MPO activity by GSH and NAC as represented by IC_{50} values

CF sputum was incubated with different concentrations of antioxidants and MPO-activity measured as described in Materials and Methods. MPO levels were determined from the standard graph using purified human MPO. The measurements were compared to untreated samples which served as controls. Antioxidant IC_{50} values of individual sputum samples were plotted against respective MPO levels. The figure suggests that the higher the MPO levels, the more the antioxidant needed for MPO inhibition.

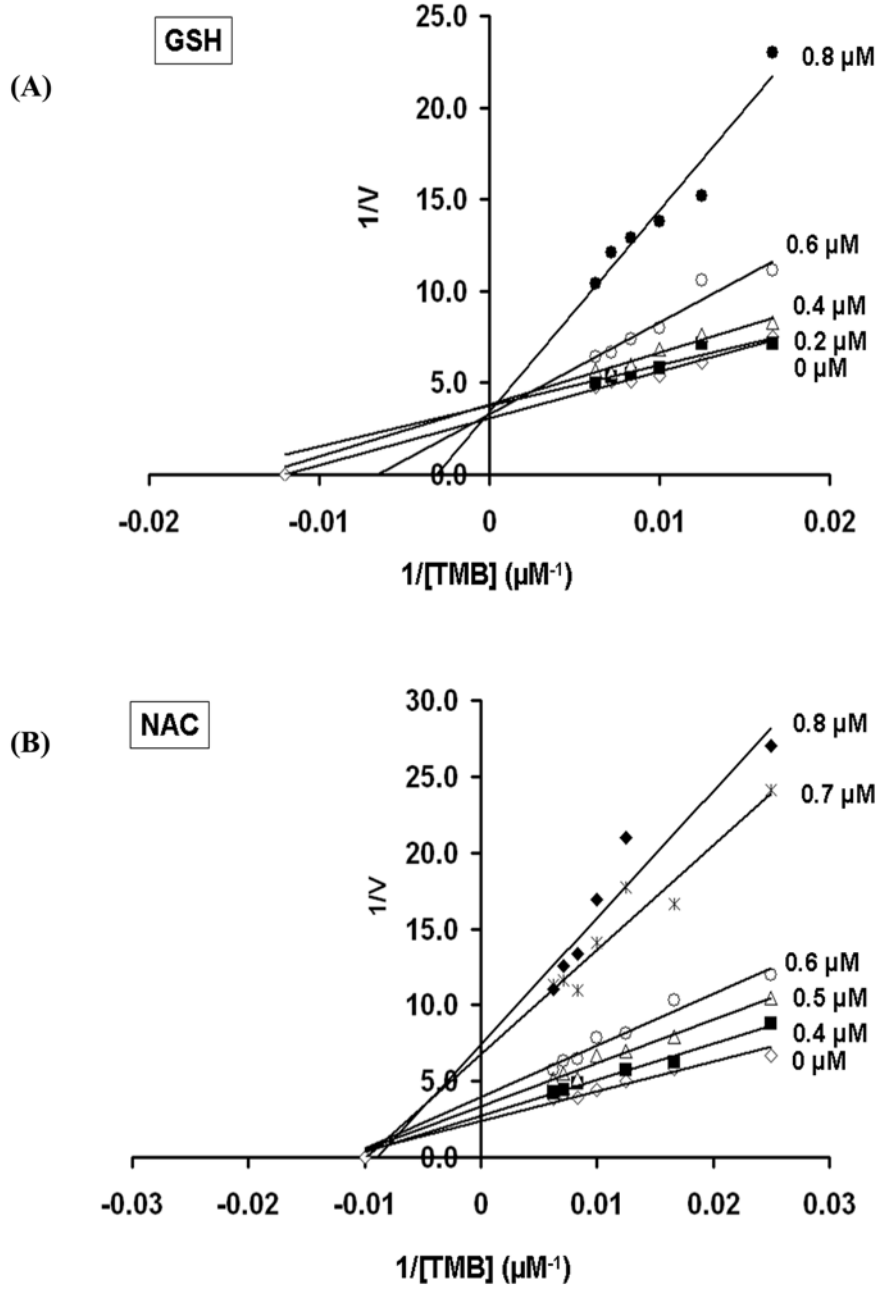

Figure 5. GSH and NAC inhibit MPO by distinct mechanisms

Double reciprocal plot of initial rate of MPO-catalyzed oxidation of TMB vs. its concentration in the presence of **(A)** 0, 0.2, 0.4, 0.6 or 0.8 μM GSH and **(B)** 0, 0.4, 0.5, 0.6, 0.7 or 0.8 μM NAC. Inhibition of MPO-catalyzed oxidation of TMB by GSH was observed to occur by competitive mode, whereas NAC showed a non-competitive mode of inhibition as demonstrated by data from reciprocal plots of velocity of reaction (1/V) against TMB concentrations (1/[TMB]).

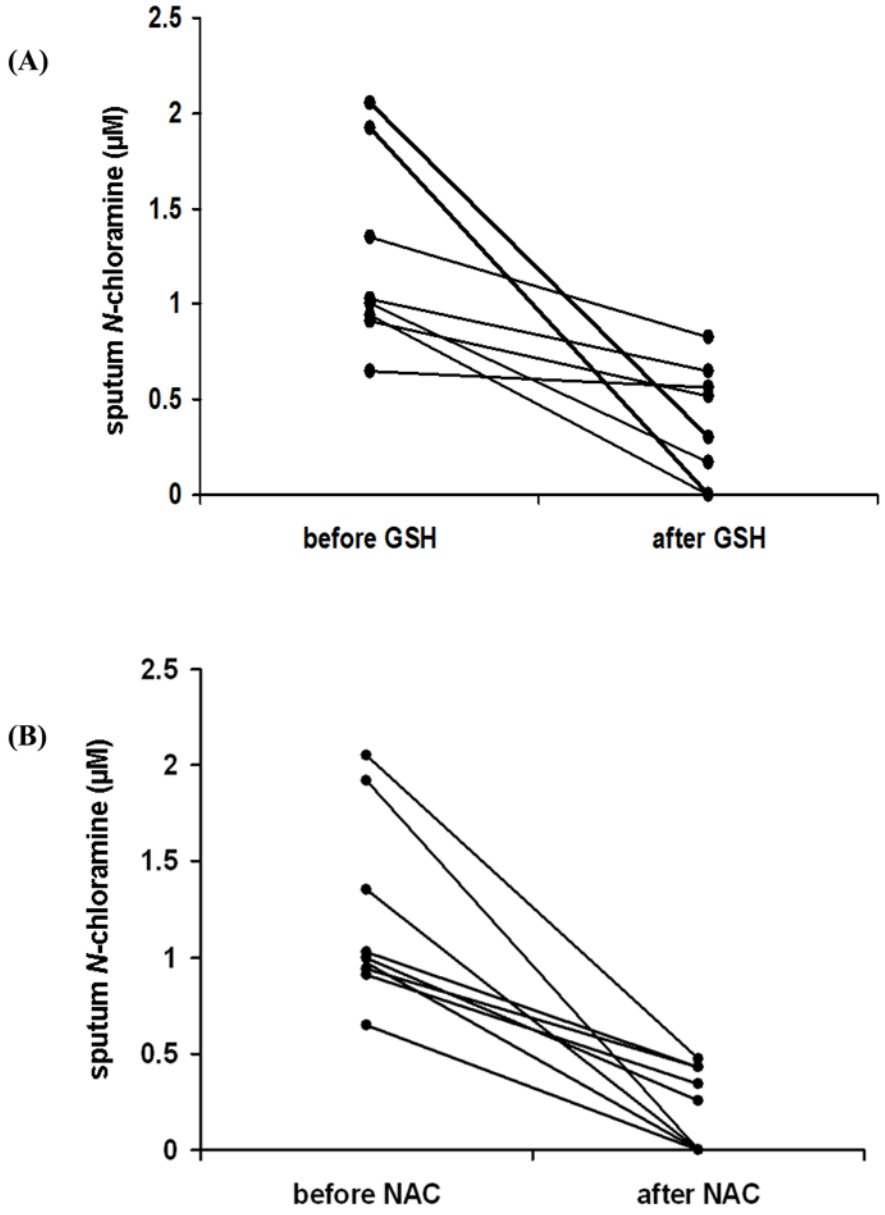

Figure 6. GSH and NAC scavenge *N*-chloramines in CF sputum

Sol-phase of CF sputum was incubated with/without 2 μM of GSH or NAC for 30 minutes and chloramine levels measured as described in Materials and Methods. *N*-Chloramine levels were measured by a standard curve using hypochlorous acid. **Fig 6A and B** shows sputum *N*-chloramine levels before and after treatment with GSH or NAC, respectively. GSH treatment lowered *N*-chloramine levels by 62 ± 11 %, whereas NAC treatment lowered *N*-chloramine levels by 80 ± 11%.

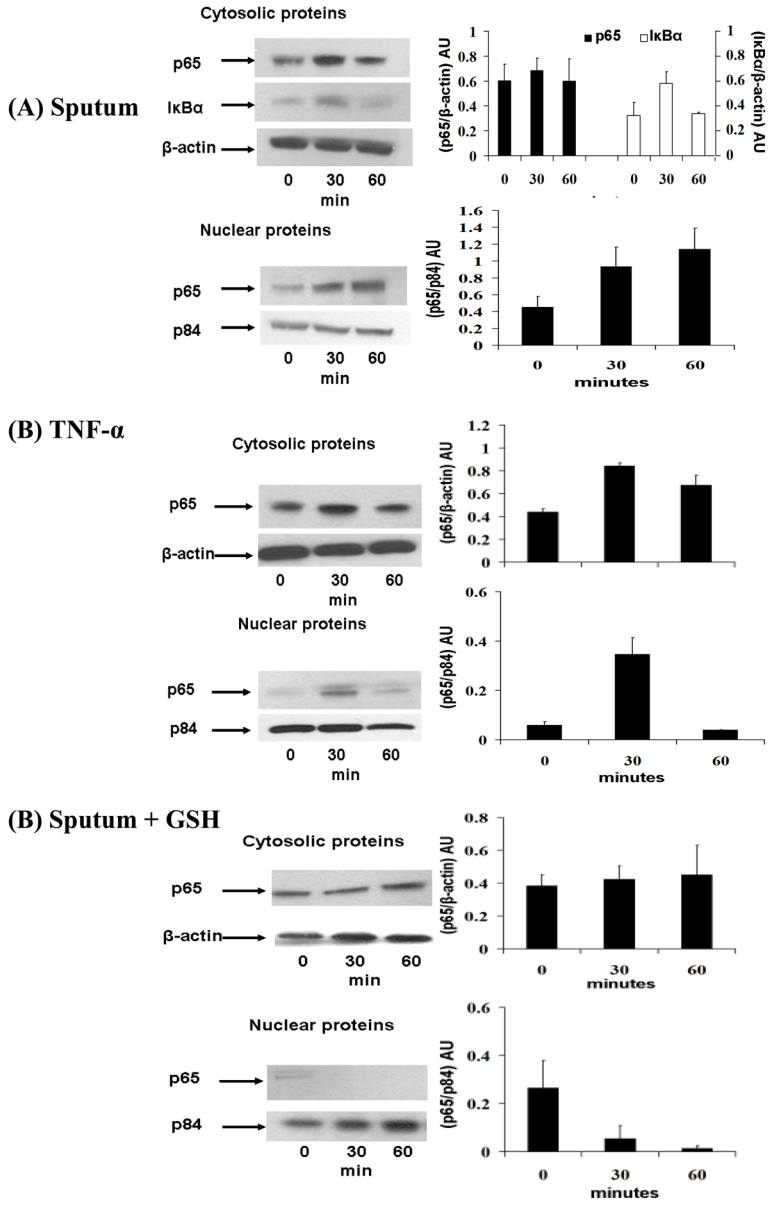

Figure 7. GSH inhibits CF sputum-induced NF-κB activation

HBE1 cells were starved of growth factors and treated with CF sputum (sol-phase) (10 μl/ml of media). Nuclear and cytosolic protein fractions were extracted at 0, 30 and 60 minutes. NF-κB activation was analysed by western blot assay. **(A)** Nuclear translocation of NF-κB subunit, p65 and degradation of cytosolic IκB-α suggesting CF sputum (sol-phase) mediated NF-κB activation. β-actin and p84 served as loading controls for cytosolic and nuclear protein fractions respectively. **(B)** Nuclear translocation of p65 in TNF-α treated (20 μg/ml) HBE1 cells which served as controls. **(C)** HBE1 cells when treated with CF sputum (sol-phase) along with GSH (1 mM) did not show p65 nuclear translocation suggesting inhibition of NF-κB activation. The blots shown are a representative of 3 independent experiments. Densitometric analysis was performed for each blot, and the quantitative data illustrated in graphs to the right of the figure.

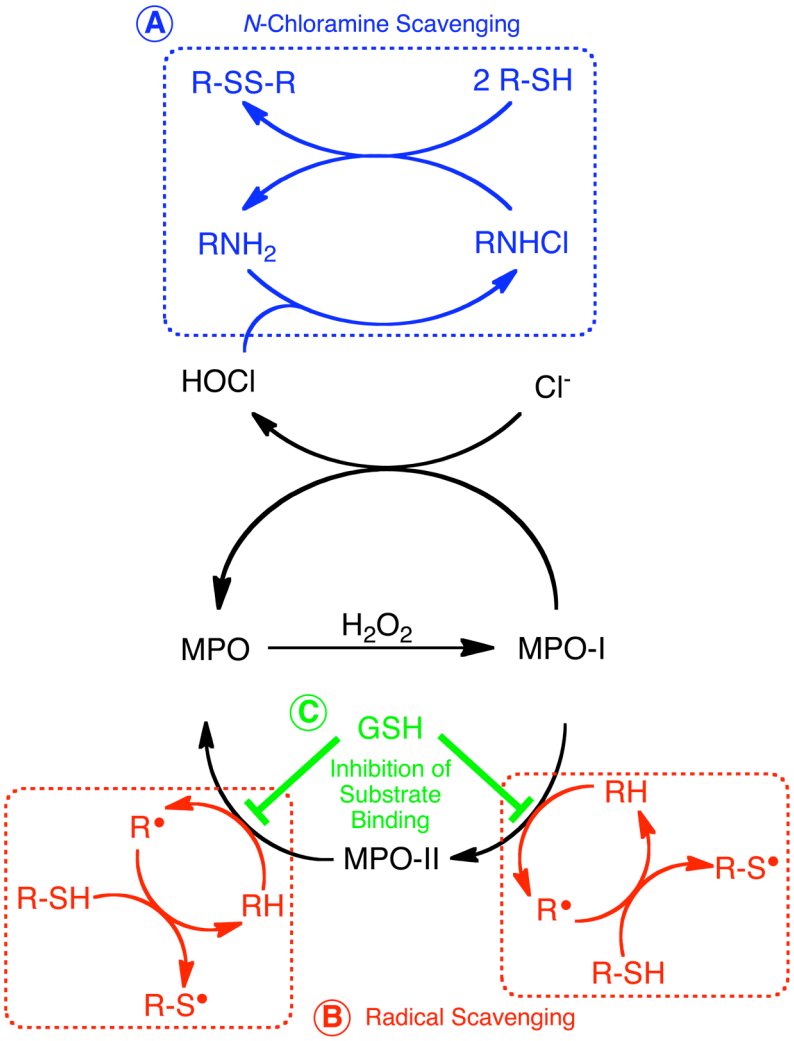

Figure 8. Proposed mechanisms by which GSH and NAC inhibit MPO-dependent reactions in the CF airway

(A) Scavenging of *N*-chloramines. **(B)** Scavenging of organic radicals produced by MPO (non-competitive inhibition). **(C)** GSH-dependent inhibition of substrate binding to MPO (competitive inhibition).

Table 1

Adult CF Patient Demographics.

Patient #, gender, age (yrs)	Sputum bacterial culture (type)†	FEV_1 (% predicted)	Mutation
1, M, 24	PA (m&n), MRSA, AF, Grp C BHS, Proteus, HI, AX, SP, SM, SA	38	ΔF508/Δ508
2, F, 58	PA (m&n), MRSA, Ser M, AX, Acaligenes sp.	43	ΔF508/Δ508
3, F, 35	PA (m&n), MSSA, AX, Acaligenes sp.	58	+/ΔF508
4, M, 22	PA (m&n), MRSA,	50	ΔF508/711+1 (G-T)
5, F, 27	SM, MSSA, AF	66	ΔF508
6, F, 39	PA (m&n), MRSA, MSSA	20	3659delC, 2789+5G->A
7, M, 45	PA (m&n), MSSA, MRSA	88	ΔF508
8, M, 26	PA (m&n), MRSA, HI, BC, AF, HF	33	ΔF508/ΔF508
9, F, 31	PA (m&n), MRSA, SA, HI	29	ΔF508, 1717-1G->A
10, F, 35	PA (m&n), HI, AX, MSSA, AF, Mould	60	ΔF508
11, F, 23	PA (m&n), MSSA	21	ΔF508/ΔF508
12, M, 23	PA (m&n), MRSA, AF, Mould	50	ΔF508/711+1 (G-T)
13, F, 36	PA (m&n), MSSA, AX, Mould, HI, AD	38	ΔF508
14, M, 26	PA (m&n)	54	ΔF508/ΔF508
15, F, 36	PA (m&n), MSSA, HI	53	ΔF508
16, F, 31	PA (m&n), AF	31	ΔF508/ΔF508
17, F, 48	PA (m), AF, SM, Ser M, AV, AD, mould	80	R117C, 3850-3T->G
18, M, 37	PA (m&n), MSSA, HI	29	ΔF508
19, F, 19	PA (m), MSSA, mould	38	ΔF508
20, F, 24	PA (m), SM, AF, HI	44	ΔF508/ΔF508
21, F, 30	PA (m), AF, HI	37	ΔF508/ΔF508

†PA (m/n) = Pseudomonas aeruginosa (mucoid/non-mucoid), MSSA = methicillin-susceptible Staphylococcus aureus, SM = Stenotrophomonas maltophilia, AF = Aspergillus flavus, HI = Haemophilus influenzae, MRSA = methicillin-resistant Staphylococcus aureus, Ser M = Serratia marcescens, AX = Achromobacter xylosoxidans, SP = Streptococcus pneumoniae, SA = Streptococcus agalactiae, BC = Burkholderia cepacia, AV = Aspergillus versicolor, AD = Acaligenes denitrificans, Grp C BHS = Grp C Beta Hemolytic Streptococcus