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S-Nitrosoglutathione Increases Cystic Fibrosis Transmembrane Regulator Maturation

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Endogenous *S*-nitrosoglutathione (GSNO) is known to increase the expression of certain proteins at concentrations present in the normal human airway. We hypothesized that GSNO would increase expression and maturation of the cystic fibrosis transmembrane conductance regulator (CFTR). Cells expressing ΔF508 and wild type CFTR were exposed to GSNO and analyzed for expression and maturation by Western blot analysis. Physiologically relevant concentrations of GSNO resulted in dose- and time-dependent increases in expression. The GSNO-induced increases were eliminated by cycloheximide, suggesting a post-transcriptional effect. Unlike proteasome inhibitors, GSNO resulted in an increase CFTR maturation. The GSNO effect could be reversed by dithiothreitol and inhibited by acivicin, a γ glutamyl transpeptidase inhibitor. These observations suggest that GSNO leads to maturation of mutated ΔF508 CFTR, a process associated with restoration of CFTR function. Because endogenous levels of GSNO are low in the cystic fibrosis (CF) airway, these results raise the possibility that GSNO replacement therapy could be an effective treatment for CF.

***Key Words:* *S*-nitrosoglutathione; cystic fibrosis; cystic fibrosis transmembrane conductance regulator; S-nitrosylation.**

Cystic fibrosis (CF) is caused by inherited mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel expressed in epithelial cells. CFTR is synthesized and glycosylated in the endoplasmic reticulum (ER). However, most of the CFTR protein (~80%) does not mature in the ER, but is polyubiquitinated and degraded immediately following its synthesis in a ubiquitin-dependent manner (1, 2). Inhibition of CFTR degradation *in vitro* using proteasomal inhibitors has demonstrated that CFTR is ubiquinated. However, coordinate increases in the maturation of the CFTR have not been seen.

One of the most common mutations of the CF gene, a deletion of three nucleotides encoding the amino acid phenylalanine at residue 508 of the CFTR (ΔF508), interferes with maturation of CFTR (3–5). At least 90% of patients with CF have at least 1 ΔF508 allele. Interestingly, both wild type and ΔF508 mutant CFTRs are initially synthesized as a 140 kDa core glycosylated primary translation product associated with cytosolic chaperones Hsp70, Hsc70, and calnexin (6). The misfolded ΔF508 CFTR molecules appear to remain associated with these molecular chaperones until degraded, whereas correctly folded molecules lose these chaperones and mature. The presence of the ΔF508 mutation increases the inefficiency of CFTR folding; however it is not thought to change the kinetics or the ubiquitin dependence of its degradation. Moreover, degradation of ΔF508 CFTR is indistinguishable from the processes involved in the degradation of wild type immature CFTR (4, 5). Of note, if permitted to mature, ΔF508 CFTR is believed to be functional as (i) ΔF508 molecules can be detected at the cell surface of Xenopus oocytes (7) or mammalian cells (8) when cultured at reduced temperatures (20–30°C), and 2) ΔF508 maturation can be increased by treating cells with glycerol (9, 10), 4-phenylbutyrate (11), CPX (12), or trimethylamine oxide (10). Given the fact that mutant CFTR is functional, strategies that increase the maturation of the CFTR will be a potential benefit to a majority of patients that have CF.

S-nitrosoglutathione (GSNO) is present in μM levels in the extracellular fluids of the human lung and brain (13, 14). It has been shown to relax smooth muscle (13), improve airway ciliary motility (15), inhibit airway epithelial amiloride sensitive sodium transport (16),

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activate calcium-dependent airway epithelial chloride transport (17), promote neutrophil apoptosis, and inhibit microbial replication (18, 19); each of these functions being of potential benefit to patients with CF-related lung disease (20). However, the levels of GSNO are low in the cystic fibrosis airway (20). Here, we demonstrate that GSNO also increases CFTR maturation.

EXPERIMENTAL METHODS

Materials. The compounds used in the experiments were obtained from the following: Pepstatin A (Boehringer Mannheim Corp, Indianapolis, IN), leupeptin and aprotinin (Roche Diagnostics, Mannheim, Germany). Electrophoresis reagents were from Bio-Rad, (Hercules, CA). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Cell lines. The CFPAC-1 cells expressing the mutant ΔF508-CFTR (20) were obtained from the American Type Culture Collection (Mannasas, VA). CFPAC-1 pLJ6 cells expressing full-length wt-CFTR cDNA (22) were obtained from Centro de Genetica Humana, Instituto National de Saude Dr. Ricardo Jorge, Lisboa, Portugal. CFPAC-1 and CFPAC-1pLJ6 cells were cultured in Iscove's Modified Dulbecco's Medium, 10% fetal calf serum (v/v), 1% (w/v) L-glutamine, and 1% (v/v) penicillin/streptomycin, 0.5% (v/v) Fungizone (GIBCO BRL). The medium for CFPAC-1pLJ6 also contained 500 μM methotrexate. Stable transfectants of baby hamster kidney fibroblast (BHK) expressing wild type CFTR (BHK-wt) or the ΔF508 mutant (BHK-ΔF508) cells were generated by transfecting cells with the respective cDNA-pNUT vector (23) using LIPOFECTIN (GIBCO BRL) according to the manufacturer's instructions. After 10 days under selective medium (1:1 mixture of F12 and DMEM media containing 10% (v/v) fetal calf serum and 500 μM methotrexate), clones were selected and propagated in the same medium, followed by Western blot to assess CFTR expression levels. All cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide and air.

Western blot analysis of CFTR. Whole cell extracts were prepared in 1% NP-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 1% NP40, 150 mM NaCl, 2 μM leupeptin, 1 μM aprotinin, and 1 μM pepstatin, 1 mM DTT, 1 μM PMSF, and 2 μM Na_3VO_4). Insoluble material from NP-40 was recovered and sheared by passage through a 25-gauge needle. Protein was quantitated by the Lowry assay by using protein assay kit (Sigma Chemical Co., St. Louis, MO). One hundred micrograms of protein was fractionated on a 6% SDS polyacrylamide gel in 1X Electrode Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS at pH 8.3). The fractionated protein was transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) using an electrophoretic transfer cell with Tobin Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol at pH 8.3). Blots were blocked in Tris buffered saline-Tween 20 (TBS-T) (TBS-T = 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dried milk. Blots were probed with a 1:1000 dilution of anti CFTR antibodies (anti-hCFTR, R-Domain Specific monoclonal, R&D Systems, Minneapolis, MN) in TBS-T containing 5% nonfat dried milk for 45 min at room temperature. Blots were washed several times in TBS-T, incubated for 30 min with a 1:2000 dilution of (HRP)-conjugated anti-mouse antibody (Bio-Rad, Hercules, CA) in TBS-T containing 5% nonfat dried milk for 30 min. Blots were washed as previously described then once in TBS. CFTR protein was visualized by enhanced chemiluminescence (ECL, Amersham) using Hyperfilm (Amersham Pharmacia Biotech).

Cytotoxicity assay. Cell viability was quantified by the release of the cytosolic enzyme lactate dehydrogenase (LDH). To test cell viability in the presence of GSNO, 1×10^6 cells were plated in each well of a 12-well plate. One day after plating, the medium was changed and media containing the appropriate concentrations of GSNO were added. After the appropriate time, cells were washed with cold PBS and lysed with Triton X-100 in 0.1 M potassium phosphate buffer at pH 7.0. The cell viability index was generated by measuring the cell-associated LDH-activity (Promega, Madison, WI).

Statistics. Means were compared by ANOVA followed by *t*-test or Rank Sum testing if nonparametric. Data are presented as mean ± SEM. $P < 0.05$ was considered significant.

RESULTS

GSNO Increases CFTR Maturation and Expression

Western blot analysis was used to examine the ability of *S*-nitrosoglutathione (GSNO) to increase the expression and maturation of the CFTR. Whole cell extracts were made from CFPAC-1, CFPAC-1pJL6, BHK-wt, and BHK-ΔF508 cells grown in the absence or presence of increasing concentrations of GSNO (Fig. 1). Three CFTR forms corresponding to the unglycosylated immature CFTR (band A), the glycosylated core CFTR (band B), and the glycosylated mature CFTR (band C) were examined. GSNO treatment of CFPAC-1 cells (ΔF508 homozygous) resulted in dose dependent increases in the expression of bands A and B (Fig. 1A). More importantly, GSNO treatment resulted in the appearance of fully mature CFTR (band C). In CFPAC-1pLJ6 cells, GSNO resulted in little change in band A yet dose dependent increases of both the bands B and C (Fig. 1B). In both cell types, the increase in CFTR expression was evident as low as 1 μM GSNO with maximum expression seen between 10 and 100 μM. There was a tendency for C-band density (mature glycosylated) to be increased to a greater extent in the wt cells than in the ΔF508 expressing cells, but this difference was not significant (Fig. 1B). *S*-nitrosoglutathione-induced increases in CFTR expression and maturation in BHK-wt cells were similar to that seen in CFPAC-1pLJ6 cells, whereas, BHK-ΔF508 resembled the CFTR in CFPAC-1 cells (Fig. 1B). Taken together, GSNO increases the expression and maturation of the CFTR.

The optimal time necessary for GSNO to enhance the expression of the CFTR was also evaluated. Whole cell extracts were made from CFPAC-1, CFPAC-LJ6, BHK-wt and BHK-ΔF508 cells treated with 10μM GSNO for increasing amounts of time. In CFPAC-1pLJ6 cells, GSNO-induced increases began at 2 h and with maximum induction of the bands B and C occurring between 4 and 6 h (Fig. 2). Similar results were seen with from CFPAC-1, BHK-wt, and BHK-ΔF508. Taken together, the data suggests that GSNO results in a dose and time dependent increase in CFTR maturation.

The Effects of GSNO on CFTR Maturation and Expression Are Posttranscriptional

To evaluate whether the GSNO-induced increases in the CFTR are the result of transcriptional or post-

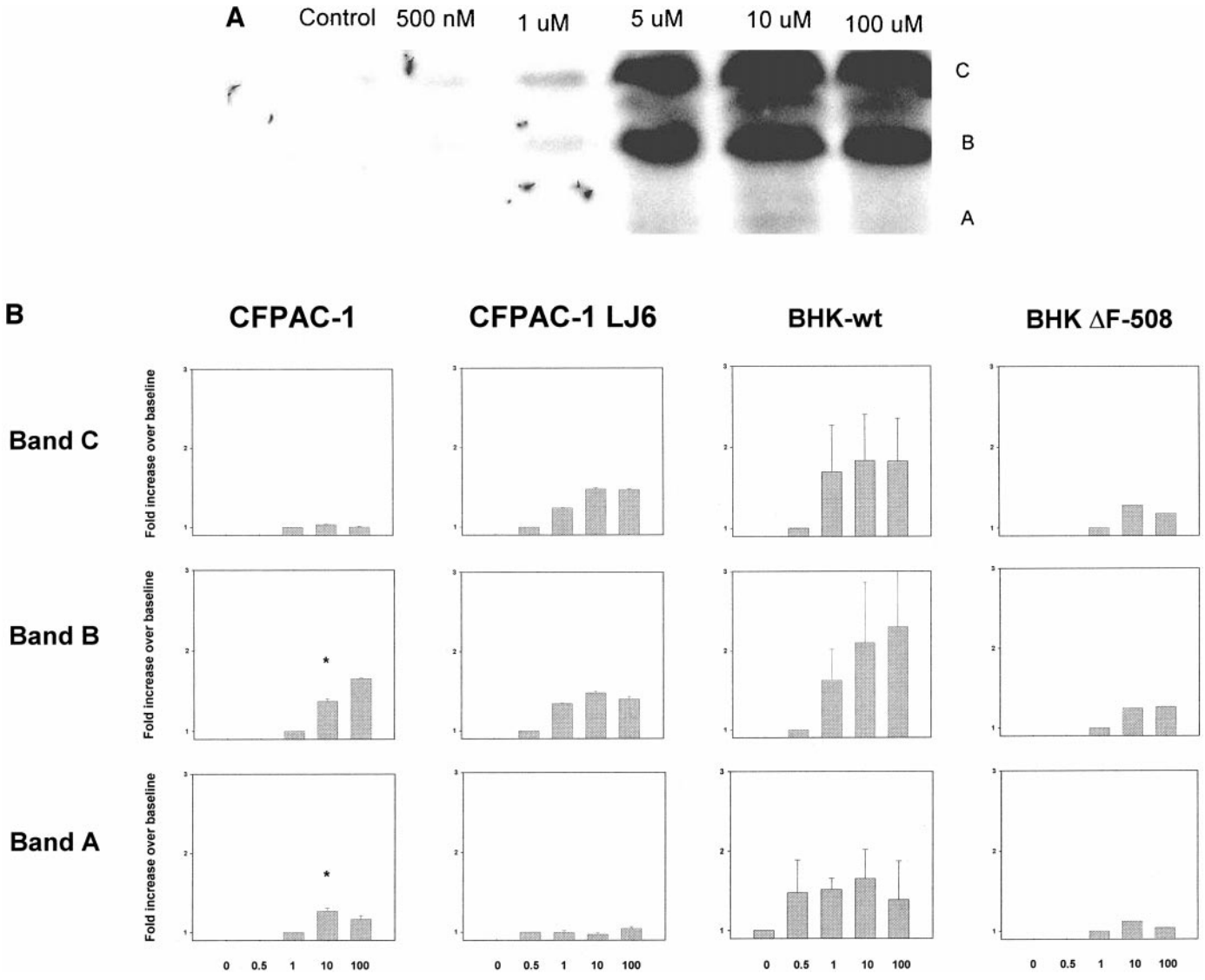

FIG. 1. GSNO increases the expression of mature ΔF508 and wt CFTR in a dose dependent manner. (A) Western blot analysis was performed on whole cell extracts (100 μg) made from CFPAC-1 cells treated with various concentrations of GSNO for 4 h. CFTR bands A, B, and C, are indicated. (B) The relative changes in unglycosylated immature (band A), core glycosylated (band B), and glycosylated mature (band C) in CFPAC-1 (ΔF508/ΔF508), CFPAC-1LJ6 (wt/ΔF508), BHK-wt, and BHK-ΔF508 cells upon GSNO treatment are illustrated in the presented histograms. Fold increases were measured from the initial appearance of the respective band being measured. Data represent the mean + SEM. Data was obtained from three independent experiments with CFPAC-1 and CFPAC-1 LJ6 cells, two independent experiments from BHK-wt, and one experiment for BHK-ΔF508. Significant differences in expression were seen between the CFPAC-1 and CFPAC-1 LJ6 cells indicated by the *. $P = 0.025$ for band A, $P = 0.047$ for band B.

transcriptional mechanisms, CFPAC-1 cells were treated with actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of translation, in the presence of GSNO (Fig. 3). Actinomycin D (15 μg/ml) had no effect on the induction of bands A, B, or C. On the other hand, cycloheximide (50 μg/ml) eliminated the induction of bands A, B, and C induced by GSNO. Similar results were obtained with CFPAC-pLJ6, BHK-wt, and BHK-ΔF508 cells. These results suggest that the effects of GSNO on CFTR induction and expression are post-transcriptional.

GSNO Increases CFTR Expression in the Presence of Proteasome Inhibitors

Both the wild type CFTR and Δ508CFTR have been shown to be degraded by the ubiquitin-proteasome pathway (6). To determine if the effects of GSNO are mediated through the inhibition of ubiquitin-dependent degradation, whole cell extracts were made from CFPAC-1 or CFPAC-1pLJ6 cells treated with GSNO in the absence or presence of the proteasome inhibitors, MG132 and clasto-lactocystine β lactone.

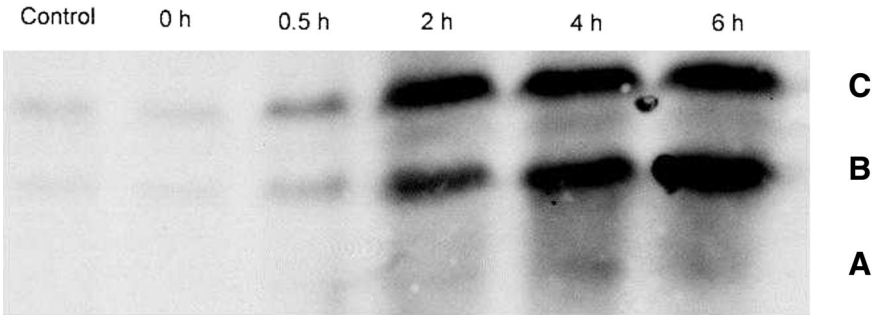

FIG. 2. GSNO increases the expression of mature CFTR as a function of time. Western blot analysis was performed on whole cell extracts (100 μg) made from CFPAC-pLJ6 cells treated with 10 μM GSNO as a function of time.

The relative abundance of the bands A, B, and C forms were determined. Unlike the proteasomal inhibitors, GSNO resulted in an increase in bands B and C. Maturation was increased in the GSNO treated cells and further augmented in the presence of proteasomal inhibitors. Taken together, these results are consistent with the notion that the action of GSNO is mediated, at least in part, through ubiquitin-proteasome pathway.

Acivicin Blocks CFTR Maturation

To ensure that the GSNO-induced effects on the CFTR were mediated by GSNO, whole cell extracts were made from CFPAC-1 cells treated with 10 μM GSNO in the absence or presence of acivicin, an inhibitor of GSNO bioactivation (24). Treatment with acivicin significantly decreased GSNO induction of bands B and C whereas acivicin had little effect band A. Similar results were obtained with CFPAC-1pLJ6, BHK-wt, and BHK-ΔF508 cells. Taken together, acivicin effectively blocks the action of GSNO on CFTR maturation (Fig. 4).

Dithiolthreitol Alters the Effects of GSNO

To determine if the shift from immature unglycosylated form to core glycosylated and fully glycosylated mature CFTR levels by GSNO is dependent on the redox state of the cells, CFPAC-1 cells were treated with 200 μM dithiolthreitol (DTT) during the last 30 min of treatment with GSNO (Fig. 4). In extracts made from CFPAC-1 cells treated with GSNO with the addition of DTT during the last 30-min of incubation, the GSNO-induced increases in bands B and C were eliminated by treatment with DTT. Taken together, these observations suggest that the mechanism by which GSNO increases the level of bands B and C is mediated by reactivity with a thiol moiety. Similar results were obtained with CFPAC-1pLJ6, BHK-wt, and BHK-ΔF508 cells. Collectively, our data suggests that the effect of GSNO on CFTR maturation may be mediated through an S-nitrosylation reaction.

DISCUSSION

The cystic fibrosis transmembrane conductance regulator (CFTR) is an integral membrane protein localized to the apical surface of epithelial cells that functions to facilitate Cl^- transport. We have previously shown that the endogenous S-nitrosylating agent, GSNO, increases the expression of hypoxia inducible

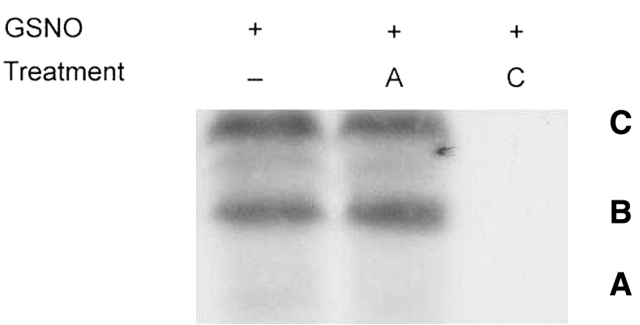

FIG. 3. GSNO-induced maturation of CFTR is posttranscriptional. Western blot analysis was performed on whole cell extracts (100 μg) made from CFPAC-1 cells grown in the presence of 15 μg/ml actinomycin D for 2 h or 60 μg/ml cycloheximide for 15 min prior to the addition of 10 μM GSNO for a total of 4 h. A, actinomycin D; C, cycloheximide.

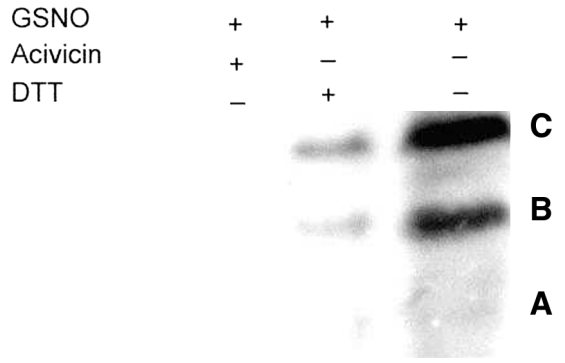

FIG. 4. GSNO induction of CFTR maturation is inhibited by acivicin and reversed by DTT. Western blot analysis was performed on whole cell extracts (100 μg) made from CFPAC-1 cells treated with 10 μM GSNO in the presence of 100 μM acivicin for 4 h or 200 μM DTT during the last 30 min of the 4 h incubation period.

factor-1 α and β (25). Here, we show for the first time that GSNO upregulates the expression and maturation of CFTR in CFPAC-1, CFPAC-1pLJ6, BHK-wt, and BHK-ΔF508 cells in a time- and dose-dependent manner. Increases in the core glycosylated and mature glycosylated forms of the CFTR were seen in cells expressing both wild type and ΔF508 mutant CFTR. Consistent with previous observations, the increase in maturation of the CFTR could not be mimicked with proteasome inhibitors. However, the effect could be effectively blocked by (i) cycloheximide, consistent with a posttranslational or cotranslational event; (ii) acivicin, an inhibitor of GSNO bioactivation; and (iii) DTT, a thiol modifying agent. Collectively, our data suggest that GSNO increases the maturation of the CFTR through an S-nitrosylation reaction.

The mechanism by which ΔF508 CFTR maturation may be affected by GSNO is not known. The synthesis and maturation of CFTR is a complex and stepwise process that occurs in the endoplasmic reticulum (ER). Synthesis of new CFTR molecules begins on cytosolic ribosomes which are targeted to the Sec61 translocation complex in the ER membrane. Sec61 is a key component of the mammalian co-translational protein translocation system and has been proposed to function as a two way channel transporting protein both into the ER and back to the cytosol for degradation. Both wt and ΔF508 CFTR have been shown to interact with the Sec61 complex (26). In fact, Sec61-CFTR complexes are highest when CFTR degradation proceeds at the greatest rate (26). Thus, GSNO effects on CFTR-Sec61 association could increase maturation of the CFTR.

Assembly and maturation of the various domains of the CFTR is mediated by a number of cytosolic (hsp70, hsp40) and ER (calnexin) chaperones (6). Many of these chaperones facilitate protein folding in the endoplasmic reticulum and thus can distinguish correctly folded from misfolded proteins, the latter of which are then targeted for degradation. Interestingly, the ΔF508 misfolded CFTR protein appears to remain associated with these chaperones until degraded, whereas correctly folded molecules lose these chaperones and mature (6). Thus, GSNO could modify the interaction of the CFTR with a molecular chaperone resulting in increased maturation.

For reasons that are not clear, the majority of CFTR molecules are polyubiquitinated and rapidly degraded by the 26S proteasome. However, degradation of ΔF508 CFTR is indistinguishable from the processes involved in the degradation of wild type CFTR (4, 5). A number of the enzymes required for ubiquitination activation, especially ubiquitin activating enzyme (E1) and ubiquitin conjugating enzymes (E2) contain reactive thiol residues. Our data indicate involvement of an S-nitrosylation reaction. Thus, GSNO-induced alterations in CFTR ubiquitination may also result in increased CFTR maturation.

The above data indicate that physiological concentrations of GSNO increase the expression and maturation of both wild type and the ΔF508 CFTR. One could therefore hypothesize that any number of the steps in CFTR processing and maturation including association with chaperones, retrograde transport pathways, as well as ubiquitination could be targets of GSNO action. Our data demonstrate that GSNO increases core glycosylated and mature CFTR in wt cells and unglycosylated immature and core glycosylated CFTR in ΔF508/ΔF508 cells. Furthermore, in CFPAC-1 cells (ΔF508/ΔF508), GSNO induces the expression of fully mature CFTR whereas it augments CFTR expression in CFPAC-1 pLJ6 cells. This observation suggests the possibility that the effect of GSNO may be twofold; inhibiting the ubiquitination and retrograde transport as well as modulating chaperone interaction and productive folding of the CFTR. In this regard, GSNO may prove to be a useful pharmacological tool for evaluating CFTR maturation and trafficking.

The ability of GSNO to increase the maturation of the CFTR is a potentially important development in the treatment of CF. Unlike glycerol (9, 10), 4-phenylbutyrate (11), CPX (12) or trimethylamine oxide (10), GSNO is endogenously produced. In addition, GSNO is an endogenous bronchodilator (13) that improves airway ciliary motility (15), inhibits airway epithelial amiloride-sensitive epithelial sodium transport (16), activates calcium-dependent epithelial chloride transport (17), promotes neutrophil apoptosis (18, 19) and has antimicrobial activities (27); all properties of potential benefit to a CF patient. In addition, exogenous GSNO inhalation has been shown to increase oxygen saturation and is well tolerated in patients with CF (28). Taken together, these observations suggest the possibility that GSNO treatment could provide a useful strategy to increase the maturation of the CFTR with a benefit to the majority of CF patients who have a ΔF508 CFTR allele.

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