A Novel Class of Compounds that Increase CFTR Expression and Maturation in Epithelial Cells

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Nonstandard Abbreviations:
γ-glutamyl transpeptidase (GGT)
cysteine string proteins (Csp)
Cystic Fibrosis (CF)
cystic fibrosis transmembrane conductance regulatory protein (CFTR)
electrospray ionization (ESI)
endoplastic reticulum (ER)
ethyl nitrite (ENO)
GSNO diethyl ester (GNODE)
Hsc70 interacting protein (CHIP)
liquid chromatography/mass spectrometry (LC-MS)
nitric oxide (NO)
Small Interfering RNA (siRNA)
S-nitrosocysteinyl glycine (CGSNO)
S-nitrosoglutathione (GSNO)
S-nitroso-N-acetylcysteine (SNOAC)
Transepithelial resistance (TER)
wild type (wt)

Abstract

The endogenous bronchodilator, S-nitrosoglutathione (GSNO), increases expression, maturation and function of both the wild type and the ΔF508 mutant of the cystic fibrosis transmembrane conductance regulatory protein (CFTR). Though transcriptional mechanisms of action have been identified, GSNO appears also to have post-transcriptional effects on CFTR maturation. Here, we report that 1) GSNO is only one of a class of S-nitrosylating agents that, at low µM concentrations, increase ΔF508 and wild-type CFTR expression and maturation; 2) NO itself (at these concentrations) and 8-bromo-cyclic GMP are minimally active on CFTR; 3) a novel agent, S-nitroso-glutathione diethyl ester, bypasses the need for GSNO bioactivation by γ-glutamyl transpeptidase (GGT) to increase CFTR maturation; 4) surprisingly, expression—but not S-nitrosylation—of cysteine string proteins (Csp) 1 and 2 is increased by GSNO; 5) the effect of GSNO to increase full maturation of wild-type CFTR is inhibited by Csp silencing (si)RNA; and 6) proteins relevant to CFTR trafficking are SNO-modified, and SNO-proteins traffic through the endoplasmic reticulum (ER) and Golgi following GSNO exposure; and 7) GSNO alters the interactions of ΔF508 CFTR with Csp and Hsc70 in the ER and Golgi. These data suggest that GSNO is one of a class of S-nitrosylating agents that act independently of the classical NO radical/cyclic GMP pathway to increase CFTR expression and maturation. They also suggest the effect of GSNO is dependent on Csp and on intracellular SNO trafficking. We speculate that these data will be of relevance to the development of NO donor-based therapies for CF.

Cystic Fibrosis (CF) is a multisystem disease associated with mutations in the gene encoding the CF transmembrane conductance regulatory (CFTR) protein (Riordan, 1999). CFTR has several functions, but is classically regarded as an apical membrane Cl⁻ channel in epithelial cells. Its post-translational processing involves a complex and incompletely defined series of interactions with a variety of chaperones and co-chaperones that fold and glycosylate the protein and screen it for defects. The most common mutation associated with CF, ΔF508, results in a single amino acid deletion (Riordan, 1999; Givson et. al., 2003; Drumm, 1991). The majority of wild type (wt) CFTR—and virtually all ΔF508 CFTR—is degraded before reaching the cell surface (Drumm, 1991). Certain agents and conditions increase expression, maturation and (in the presence of cyclic AMP-stimulating agonists) function of ΔF508 CFTR. Therefore, there is an interest in identifying compounds with a favorable pharmacological profile that could have this effect in vivo, reversing the molecular defect and preventing disease progression (Ward and Kopito, 1994; Denning et al., 1992; Zeitlin et al., 2002). High-throughput screening has been used to identify compounds that, by augmenting ΔF508 CFTR trafficking, increase its functional expression on the surface of epithelial cells (Verkman, 2004).

S-Nitrosoglutathione (GSNO) is an endogenous bronchodilator and signaling molecule (Gaston et al., 1993) that enhances ventilation-perfusion matching (Snyder et al., 2002), increases ciliary beat frequency (Xue, 1996), has antimicrobial effects (Liu et al., 2001; Liu et al., 2004) and, in physiological low μM concentrations, increases expression, maturation and function of wt and ΔF508 CFTR in epithelial cells (Andersson et al., 2002; Howard et al., 2003; Zaman et al., 2001; Zaman et al., 2004; Chen 2006). The effect of GSNO at these low concentrations is partly transcriptional— through altered Sp1 and Sp3 expression and binding (Zaman et al., 2004)—and partly post-translational (Zaman et al., 2001). Concentrations 10-100

fold higher than those normally present in human airway lining fluid have the potential 1) to inhibit CFTR transcription (Zaman et al., 2004); and 2) produce excess nitric oxide (NO), leading both to tyrosine nitration associated CFTR degradation (Jilling et al., 1999) and to glutathionylation-mediated (i.e., oxidative) inhibition of wt CFTR activity (Wang et al., 2005). Of note, levels of NO and S-nitrosothiols are low in the CF airway, leading to interest in replacing physiological airway lining fluid concentrations of GSNO as a treatment for CF (Snyder et al., 2002; Grasemann et al., 1999).

Here, we have begun to study the post-transcriptional biochemistry by which these low μM concentrations of GSNO stabilize CFTR, showing that GSNO is only one of a class of agents that increase CFTR expression and maturation in polarized airway epithelial cells *in vitro*. The activity of these low μM concentrations of S-nitrosylating agents in augmenting ΔF508 CFTR maturation appears to be independent of their ability to generate NO (radical) or glutathione, suggesting that the mechanism of action involves transnitrosylation chemistry—as opposed to NO-mediated chemistry and/or oxidation (glutathionylation). One of these is a newly synthesized, cell-permeable agent, GSNO diethyl ester (GNODE), designed to bypass the requirement of GSNO for bioactivation by γ glutamyl transpeptidase (GGT). Surprisingly, GSNO appears to act—in part—by increasing expression of cysteine string protein (Csp) and increasing the association between Csp and CFTR in the ER and Golgi. We speculate that these data may be of importance to the development of NO donor-based therapies for CF.

Materials And Methods

Reagents. Reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated. SNOs were made as previously described (Gaston et al., 1993). Glutathione diethyl ester was purchased from Bachem (King of Prussia, PA); ethyl nitrite (ENO) was from Aldridge (Milwaukee, WI). An anaerobic solution of 1.77 mM NO was prepared as previously described (Beckman et al., 1996).

Cell Culture. A549 and CFPAC-1 cells were from ATCC (Manassas, VA). CFBE 41o$^-$ cells were kindly provided by Dr. D. Gruenart. A549 cells were grown in Ham's F12K medium (Zaman et al., 2004; Gaston et al., 1998) and CFPAC-1 cells in Iscove's medium with 10% (v/v) fetal calf serum and 1% (v/v) penicillin/streptomycin (Life Technologies, Gaithersburg, MD) (Zaman et al., 2001; Zaman et al., 2004). CFBE 41o$^-$ cells were seeded at 0.5 - 1 x 10^6 cells/cm^2 and grown on 0.4 μm pore filters (Transwell; Corning, NY) at an air-liquid interface. Transepithelial resistance (TER) was measured by volt-ohm meter (Millicell; Millipore, Bedford, MA) (Hentchel-Franks et al., 2004). Experiments were done at a stable TER of >300 ohm x cm^2, indicating establishment of tight junctions (Hentchel-Franks et al., 2004).

Chemical Methods. SNOs were assayed in cysteine and CuCl as described (Gaston et al., 1998; Gaston et al., 2003). GNODE was assayed by liquid chromatography/mass spectrometry (LC-MS) using a Waters C8 column (2.1 × 150) and 2695 HPLC system (Milford, MA) in a gradient of 0.1% formate (initial composition, 0.1% formate; methanol increased to 100% over 30 min) followed by electrospray ionization (ESI) MS in a Finnigan LCQ system (San Jose, CA) in full scan positive ion mode. Proteins isolated by anti-CFTR immunoprecipitation from each cell compartment were injected into an Argon stream in a reflux chamber containing 1m cysteine saturated with CuCl as previously described (Mannick, 2001).

SNO content was determined using a GSNO standard curve, and data were expressed relative to protein concentration (Lowry).

Immunoblotting. This was performed as previously described (Zaman et al., 2001; Zaman et al., 2004). Cell extracts in 1% NP-40 lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 μM leupeptin, 1 μM aprotinin and 1 μM pepstain, 1 μM PMSF and 2 μM Na$_2$VO$_4$ (Boehringer Mannheim, Indianapolis, IN) were sheared through a 25-gauge needle, fractioned on a 6% SDS polyacrylamide gel in 1X electrode buffer (25 mM Tris, 192 mM glycine, 0.1% SDS at pH 8.3), transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in Tobin Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3), blocked in Tris buffered saline-Tween 20 (TBS-T=10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 5% nonfat dried milk at pH 8.0) and probed (45 min, RT) with 1:1000 of either anti-CFTR R-domain-specific monoclonal antibody (R and D Systems, Minneapolis, MN) or anti-CFTR monoclonal antibody (Chemicon, Temecula, CA). Blots were washed, incubated (30 min) with 1:2,000 HRP-conjugated anti-mouse antibody (Pierce, Rockford, IL) in TBS-T (30 min) and visualized using Hyperfilm (Amersham Pharmacia Biotech). Blots were stripped and re-probed with α- tubulin antibodies (Santa Cruz, CA; 1:5000).

Isolation of cytosol, endoplasmic reticulum and Golgi. Polarized CFBE41o- cells before and after 2, 4, and 6 hours of exposure to 10 μM GSNO were rinsed twice with ice cold PBS and lightly trypsinized, then pelleted by centrifugation at 5000 rpm for 10 min. The pellet was washed twice with ice cold PBS and then suspended in 50 mM Tris-HCl, pH 8.0, containing 0.5 M sucrose. After the homogenate underwent centrifugation (5000 rpm; 10 min; 4° C) the supernatant was transferred to ultracentrifuge tubes. The cytosol was clarified centrifugation at (30,000 rpm; 60 min at 4°C). 2.3M sucrose stock was added to the clarified supernatant to make

the final concentration 1.25M sucrose. A sucrose density step gradient was performed in an ultracentrifuge tube using a paired gradient (by weight) with a balance tube. Then, the organelles were separated by density by centrifugation on the gradient (30,000 rpm; 2 hours; 4°C). Finally, the off-white bands of Golgi membrane and endoplasmic reticulum membrane were harvested (4°C) with a Pasteur pipette.

Immunoprecipitation. Cystosolic, endoplasmic reticulum and Golgi fractions were transferred to pre-chilled Eppendorf tubes. Ten µl of primary CFTR antibody (anti-CFTR clone L12B4 and anti-CFTR clone M3A7, mouse monoclonal antibodies: Upstate, NY) were added to each fraction and incubated over night at 4°C with gentle shaking. To avoid nonspecific binding, to supernatant antibody mixtures were added 70 µl of Protein A (Boehringer Mannheim, Indianapolis, IN) and incubated for another 4 hours. Then, samples underwent centrifugation (1 min) and proteins not bound to the beads were washed twice with RIPA buffer. Proteins were then eluted from beads by incubation with 100 µl sample buffer at room temperature with continuous mixing for one hour. Samples were divided into two aliquots: for SNO assay and the other set for immunoblotting.

Small Interfering RNA (siRNA) knockdown of Csp. siRNA sequences were synthesized and then identified (using MALDI-TOF spectrometric analysis) by Qiagen (Qiagen Inc., Valencia, California). siRNA duplexes were > 90% pure as measured by HPLC analysis. Csp siRNA with antisense r(UAC UUG UCG UAG AUG UUC C)dTdT and sense r(GGA ACA UCU ACG ACA AGU A)dTdT, was designed to correspond to target region 386 to 405 and target sequence AAG GAA CAT CTA CGA CAA GTA. Human airway epithelial (A549) cells expressing wt CFTR were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, California) with 20 and 50 nM of Csp siRNA. Scrambled Csp siRNA was used as a control.

Cells were lysed after 48 hours of posttransfection and expression level were assessed by Western blot analysis with anti-Csp antibody (Chemicon International, Temecula, California).

Analysis of S-nitrosylated proteins. Cells were treated with 0.1, 5 or 10 μM GSNO or buffer (4 hr) and proteins were extracted without reducing agents. S-Nitrosylated proteins were isolated from 100 μg of each extract by the biotin switch method (Jaffrey et al., 2001). Briefly, thiols were blocked using methyl methane thiosulfanate, SNO bonds were reduced using 1 mM ascorbate, and newly reduced thiols were biotinylated using N-[6-biotinamino)hexyl]-1'-(2'[pyridyldithioprotionamide biotin-HPDP (Pierce, Rockford, IL). Biotinylated proteins were washed in neutralization buffer (20 mM HEPES, pH 7.7, 10 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100), and then purified using Streptavidin-Agarose beads and eluted with ß-mercaptoethanol. Purified protein samples were dissolved in a 100 mM ammonium acetate buffer (pH 8.9) and digested with trypsin (Promega, Madison, WI) (protein:trypsin ca. 20:1) at 37°C overnight. Aliquots corresponding to approximately 1 μg of digested protein were loaded onto a 10-cm self-packed C18 (5-15μm YMC ODS-A, Waters, Milford, MA) 360-μm OD x 100-μm ID fused silica (Polymicro Technologies, Phoenix, AZ) precolumn. This column was washed with 0.1% acetic acid and then butt-connected with a teflon sleeve to a 10-cm long self-packed C18 (5μm YMC ODS-AQ) 360-μm OD x 50-μm ID analytical column with a pulled nanospray tip (1-2 μm orifice). Peptides were eluted using a high-performance liquid chromatograph (Agilent 1100, Agilent Technologies, Palo Alto, CA) and a binary solvent gradient (solvent A: 0.1%HOAc and B: 0.1%HOAc/70%MeCN) of 0-60%B in 80 minutes and 60-100%B in 10 minutes. The eluted peptides were electrosprayed directly into an LTQ linear quadruple ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) at a flow rate of 50-100 nL/min. The LTQ was operated with a "top10" data-dependent analysis method consisting of a

repeated data acquisition cycle of 1 full mass spectrum (MS; m/z 300-2000) followed by 10

MS/MS spectra corresponding to fragmentation mass spectra of the top 10 most abundant ions

from the MS. For peptide identification, the MS-MS spectra were searched with the SEQUEST

algorithm against the non-redundant human protein database from the NCBI (National Center for

Biotechnology Information, NLM, NIH). To obtain high-confidence peptide sequence matches,

first the search results were filtered using SEQUEST match scoring criteria whereby peptides

were retained if (a) the cross-correlation score (XCorr) was greater than or equal to 3.0 (+3 ion),

2.5 (+2) and 2.0 (+1), (b) the scoring rank (RSp) was less than or equal to 10, and (c) the

measure of the similarity of the top matches for any spectrum (DeltaCn) was greater than or

equal to 0.1. Second, selected peptide identifications were confirmed by manual inspection of

the data.

Statistical Methods. Multiple means were compared using Analysis of Variance, or

Kruskall-Wallis if not normally distributed, followed by pair-wise comparison using Students' t-

test. Welch-Satterthwaite analysis was used to compare means with heterogenous variance. $P <$

0.05 was considered significant.

Results

Different S-nitrosylating agents increase expression and maturation of wild-type

CFTR. Each agent increased mature wt CFTR bands (160 and 180 kDa; core-and fully-

glycosylated, respectively) relative to control. The dose of 10 µM was chosen based on previous

dose response studies using GSNO (Zaman, 2003; Zaman, 2004). However, not all agents had

an equal effect in causing full CFTR maturation (180 kD; rank order, GSNO> S-nitrosocysteinyl

glycine (CGSNO) = S-nitroso-N-acetylcysteine (SNOAC)> ethyl nitrite (ENO)> NO) (Fig. 1).

The effect on maturation was not related to the stability of the S-nitrosothiols: for example, the

half-life of SNOAC was 300-fold greater than that of CGSNO (Table 1).

Synthesis and characterization of S-nitrosoglutathione diethyl ester. Glutathione

diethyl ester underwent S-nitrosylation by equimolar reaction with sodium nitrite in 1N HCl.

The product was confirmed to be S-nitrosoglutathione diethyl ester (GNODE) by MS; this

product had a half-life of $3,020 \pm 610$ minutes at 37°C (also analyzed by MS; Table 1; Fig. 2).

Effect of S-nitrosylating agents on Δ F508 CFTR expression and maturation in

polarized airway epithelial cells. We screened different S-nitrosylating agents to evaluate their

activity in increasing expression and maturation of ΔF508 CFTR using polarized cultures of

human airway epithelial cells (CFB41o⁻). Transepithelial resistance, monitored daily, peaked at

939 ± 215 ohm-cm^{-2} after a median of 13 days. Cells were studied within four days of achieving

polarization. Of note, GSNO (10 µM) increased transepithelial conductance acutely (from 17.3

µAmps/cm^2 to 115.2 µAmps/cm^2; n = 3; P < 0.001) and decreased resistance (from 597 kOhms x

cm^2 to 175 kOhms cm^2) after 4 hours (n = 3; P < 0.003). As was the case with wt CFTR in

A549 cells, all S-nitrosylating agents studied increased expression and maturation of ΔF508

CFTR in polarized human epithelial cells (Fig. 3). Of note, the effect of GSNO was partially

inhibited by the inhibitor of GGT, acivicin, particularly with regard to core glycosylation (B

band) (Fig. 3). However, this GGT inhibition was overcome by CGSNO, the product of GSNO

cleavage by GGT (Fig. 3). The effect of acivicin was also overcome by GNODE (Fig. 3), which

is substantially more stable than CGSNO (Table 1). 8-Bromo cGMP was inactive, and free

radical NO was minimally active, suggesting that the mechanism of action is independent of the

classical free radical NO/cGMP pathway. Glutathione supplementation did not augment the

effect of ENO suggesting chemical decomposition of ENO in solution under these conditions;

indeed, further optimization may be possible in the future (Fig. 3).

Effect of GSNO on regulation of CFTR by Csp. We considered cysteine string protein

a possible target for inhibition by GSNO because a cysteine modification preventing

palmitoylation would prevent membrane association, which could permit full CFTR maturation

(Zhang et al., 2002). Therefore, when A549 cells were transfected with 20 and 50 nM siRNA

duplexes specific for Csp (48 hr) we expected Csp siRNA to mimic GSNO in increasing fully

mature (C Band) CFTR (Zhang et al., 2002). Unexpectedly, however, treatment with Csp

siRNA did not mimic the effect of GSNO (Fig. 4). Csp protein levels at baseline were low but

relatively stable. Remarkably, GSNO increased Csp 1 and Csp 2 expression, and Csp siRNA—

but not scrambled Csp siRNA—prevented the GSNO-induced increase in 1) Csp expression; and

2) CFTR maturation (P < 0.005; Fig. 4).

We next sought to determine whether Csp could be S-nitrosylated in this cell system. We

performed LC/MS analysis of whole cell and membrane fraction biotin substituted SNO-proteins

following treatment with 0.1, 1, 5 and 10 μM GSNO (4 hr). S-nitrosylated Csp and CFTR were

not identified. However, other proteins were S-nitrosylated by GSNO that were of interest with

regard to CFTR trafficking; these included Hsp27, an Hsc70 precursor, Hsp 90, peroxiredoxins and tropomyosin.

We then studied the regulation of the effect of GSNO on Csp. Surprisingly, the effect of GSNO to increase Csp was inhibited by 15 min pretreatment with cycloheximide, but not with 2 hrs pretreatment with actinomycin D (Fig. 5), suggesting that 1) Csp mRNA is relatively stable; and 2) the effect of GSNO to increase Csp expression is post-transcriptional.

Effects of GSNO on SNO content and CFTR-Csp-Hsc70 interactions in the endoplasmic reticulum and Golgi. Extracts were separated into endoplasmic reticulum Golgi and cytosol fractions from polarized CFBE41o⁻ cells before and 2, 4 and 6 hours after exposure to 10 μM GSNO. GSNO increased the CFTR-associated SNO content of the endoplasmic reticulum and Golgi at 2 and 4 hours; by 6 hours, the SNO proteins were completely absent from the ER and had passed through to the Golgi (Figure 6A). The principal protein modified was a 70 kDa heat shock precursor relevant to CFTR and Csp trafficking (Figure 6B).

GSNO caused increased association of Csp with CFTR in the endoplasmic reticulum at 4 hours and maximally increased Csp interaction in the Golgi at 6 hrs (Figure 6B). In parallel, GSNO caused increased association of Hsc70 with CFTR-Csp in the endoplasmic reticulum, at 2, 4 and 6 hrs (Figure 6C).

Discussion

Background. Decreased CFTR expression in epithelial cells is associated with the abnormal transmembrane Cl^- and Na^+ transport as well as immune effects associated with clinical CF (Gibson et al., 2003). Despite major advances in understanding the molecular basis of CF—and in antimicrobial and nutritional therapy for the disease—the life expectancy of patients with CF remains approximately half that of the general population (Cystic Fibrosis Foundation, 2004). Ninety percent of CF patients have at least one copy of the most common abnormal CFTR allele, ΔF508. This mutation encodes for a protein that is potentially functional, but that is mis-folded and normally degraded in the endoplasmic reticulum (Gibson et al., 2003). Evidence that certain agents, such as 4-phenylbutyrate, cause ΔF508 CFTR to be expressed on the surface of epithelial cells and to function (in the presence of cAMP-activating agonists) (Zeitlin et al., 2002) has led to interest in the development of compounds that could be of use to restore ΔF508 CFTR function in vivo. GSNO may be one such agent: low μM concentrations increase expression, maturation and function of ΔF508 CFTR (Andersson et al., 2002; Howard et al., 2003; Zaman et al., 2001; Zaman et al., 2004). Here, we report that GSNO is but one of a class of compounds, S-nitrosylating agents, that increase CFTR expression and maturation.

GSNO and CFTR. GSNO itself has several unique features that make it appealing as a potential therapy for CF. 1) It is an endogenous compound, normally present in the airways (Gaston et al., 1993). 2) Airway levels appear to be low both in CF and in asthma (Grasemann, et al.; Gaston et al., 1998; Dweik et al., 2001). 3) It relaxes airways smooth muscle, augments ventilation/perfusion matching, increases ciliary beat frequency, inhibits amiloride-sensitive Na^+ transport, promotes inflammatory cell apoptosis, and has antimicrobial effects (Gaston et al., 1993; Fortenberry et al., 1999; Li et al, 2000; Liu et al., 2004; Jain et al., 1998). 4) It increases

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wild type CFTR function (Chen, 2006). 5) Lastly, levels of GSNO present in the normal airway

increase ΔF508 expression, maturation and function (Zaman et al., 2001; Andersson et al., 2002;

Howard et al., 2003; Zaman et al., 2004); dose-response experiments suggest that concentrations

of 5-10 μM are optimal for this effect (Zaman, et al. 2001; Zaman, et al. 2004). These low μM

levels of GSNO appear to affect CFTR primarily through a post-transcriptional mechanism,

although there appears also to be a modest transcriptional effect through augmented Sp3

expression and binding (Zaman et al., 2004). Of note, our transmembrane resistance data are

consistent with those of Howard, et al., who have shown that GSNO increases short-circuit Cl^-

current in ΔF508 expressing cells; however, there are also other possible explanations for this

effect of GSNO on transmembrane potential.

S-Nitrosylation signaling. As with all agents that improve ΔF508 CFTR expression, the

post-transcriptional mechanisms by which GSNO acts on ΔF508 CFTR are not known. That is to

say, the exact targets of GSNO are not known. In general terms, the possibilities include protein

modification caused by: 1) homolytic cleavage of GSNO to form NO, with subsequent classical

activation of guanylate cyclase; 2) a different "NO donor" effect of GSNO involving nitration of

tyrosine residues, an effect demonstrated in the presence of higher concentrations of NO to

accelerate wt CFTR degradation—and therefore somewhat unlikely to be relevant to the

augmentation of CFTR expression caused by physiological concentrations of GSNO—(Xue et

al., 1996); 3) glutathionylation of CFTR or proteins relevant to CFTR trafficking (Wang et al.,

2005); and 4) S-nitrosylation—or transfer of a NO^+ equivalent to the cysteine thiolate—of

proteins relevant to CFTR trafficking. Our results favor S-nitrosylation as a mechanism because:

1) several different S-nitrosylating agents are active, and the activity of these agents is not related

to either the rate at which they form NO or their ability to serve as glutathione donors; 2) the

only common feature of all of these agents is that they have the ability to cause cysteine S-nitrosylation (with the exception of NO itself, which requires the presence of an electron acceptor-such as oxygen or a metalloprotein-to have this effect [Gaston, 2003]); 3) ENO, which does not readily evolve NO (33), is more active than NO; 4) the cell-permeable, cGMP-mimetic, 8-Br cGMP is inactive; 5) the effect of GSNO is reversed by DTT, which readily reduces SNO bonds (Howard et al., 2003; Zaman et al., 2004); 6) both tyrosine nitration—which decreases rather than increases wt CFTR expression (Jilling et al., 1999)—and glutathione-associated mechanisms require higher concentrations of GSNO (Jilling et al., 1999; Wang et al., 2005), concentrations that can also inhibit cftr transcription (Zaman et al., 2004); and 7) low μM concentrations of these agents S-nitrosylate proteins of potential relevance to CFTR trafficking in airway epithelial cells.   Overall, these data are consistent with accumulating evidence suggesting that NO^+ transfer reactions may represent an important signaling mechanism relevant to health and disease (Liu et al., 2004; Gaston et al., 2003; Moya et al., 2001; Lipton et al., 2001).

S-Nitrosylation targets in airway epithelial cells. Several proteins relevant to CFTR trafficking contain reduced thiols that may be targets for S-nitrosylation. These include cysteine string protein (Csp) (Zhang et al., 2002), ubiquitin ligases—such as the carboxy terminus of Hsc70 interacting protein (CHIP) (McDonough and Patterson, 2003; Meacham et al., 2001)—and CFTR itself (Wang et al., 2005; Chen et al., 2004; Choglay et al., 2001). Csp's were of interest to us because in Xenopus oocytes, Csp expression prevents full CFTR maturation–though it enhances partial maturation–(Zhang et al., 2002) and Csp contains a cysteine-rich "string", the palmitoylation of which is important for membrane attachment (Chamberlain and Burgoyne, 1997). We hypothesized that GSNO might modify Csp cysteines,

preventing membrane association and allowing CFTR maturation. We report that 1) GSNO does not S-nitrosylate Csp; 2) GSNO actually increases Csp expression, primarily post-transcriptionally; and 3) inhibition of new Csp translation using siRNA prevents the ability of GSNO to increase full CFTR maturation, suggesting that the effect of GSNO to increase Csp expression is critical to its mechanism of action in CFTR.

Zhang and coworkers (Zhang et al., 2002) have found that Csp overexpression stabilizes CFTR Band B—but does not permit maturation to Band C—in Csp and CFTR-cotransfected Xenopus oocytes. These data may be understood in the context of the Csp co-chaperone model proposed by Zhang and coworkers, extended as follows. First, our immunoprecipitation data confirm that Csp associates with CFTR in the ER. Of note in this regard, 50 nM Csp siRNA treatment decreased CFTR Band B (Fig. 4), consistent with the Csp over-expression data of Zhang and coworkers suggesting that the association may favor core glycosylation (Zhang et al., 2002). Next, another protein interacts with CFTR and Csp. The immunoprecipitation and proteomic data (Figures 5 and 6) suggest that this may be Hsc70, consistent with previous work (Zhang et al., 2002; Chamberlain and Burgoyne, 1997; Cyr, 2005). In the absence of GSNO, this interaction may lead to ubiquitination and degradation. Indeed, Csp initiates activation of Hsc70 ATPase activity (Braun et al., 1996; Chamberlain and Burgoyne, 1997) which can augment interaction with CHIP (Meacham et al., 2001) to lead to CFTR degradation. In the presence of GSNO, however, we propose that this degradation is inhibited, allowing increased Csp to continue to stabilize CFTR. Hsc70 has a single critical cysteine in its ATP binding domain. Inhibition of ATP binding inhibits Hsc70 activation (Wilbanks, McCay, 1998), but we propose that this could be a mechanism by which GSNO modification of the Hsc70 cysteine (Figure 6) allows Csp binding to augment folding without leading to degradation (Braun et al., 1996;

Chamberlain LM and Burgoyne RD (1997)). Additional work will be required on each step of this proposed mechanism. Of note, our data suggest that Csp mRNA and protein are quite stable in these cells.

S-Nitrosothiol metabolism in the airway epithelium. The metabolism of GSNO involves specific synthetic and catabolic pathways (Gaston et al., 1993; Liu et al., 2001; Grasemann et al., 1999; Que et al., 2005). Its catabolism in the asthmatic airway epithelium, for example, appears to be accelerated (Gaston et al., 1998; Dweik et al., 2001; Que et al., 2005); and levels are low in the CF airway, perhaps because of lack of substrate (Grasemann et al., 1999; Gao et al., 1999; Kelley and Drumm, 1998). GSNO catabolism has the potential paradoxically to increase GSNO bioactivity (for example, through GGT) and/or to decrease its duration of action (Zaman et al., 2001; Lipton et al., 2001; Que et al., 2005). Therefore, we have developed GSNO analogs that may act independently of GGT bioactivation and may be resistant to intracellular catabolism. Here, we report that GNODE is active in the presence of the GGT inhibitor, acivicin, and has a stability profile comparable to that of GSNO.

Therapeutic potential of GSNO and other S-nitrosylating agents. With regard to the development of NO donor-based therapies for CF, it is important to stress that different doses— and different donors—can have opposing effects. We and others have shown that low μM levels of GSNO and other S-nitrosylating agents can, under certain circumstances, increase CFTR expression, maturation and function (Zaman et al., 2001; Andersson et al., 2002; Chen et al., 2006; Howard et al., 2003; Zaman et al., 2004). However, higher doses of NO itself can augment CFTR breakdown, by causing tyrosine nitration or oxidation (Jilling et al., 1999). Further, high μM or mM concentrations of GSNO can both inhibit cftr transcription—through Sp

1 (Zaman et al., 2004)—and wt CFTR function—through cysteine glutathionylation (Wang et al., 2005).

In summary, these data suggest that low µM concentrations of GSNO and related compounds increase ΔF508 expression and maturation through cysteine S-nitrosylation as opposed to NO/cGMP-based or oxidative chemistry. After cell exposure to GSNO, specific proteins relevant to CFTR trafficking are S-nitrosylated as thiol-bound NO that fluxes through the ER and Golgi. Additionally, we have found that GSNO increases airway epithelial cell Csp expression, and that this increase appears to be necessary for GSNO to stimulate full CFTR maturation. We speculate that these observations may contribute to the development of new therapies for CF.

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FOOTNOTES

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Figure Legends

Figure 1. Different S-nitrosylating agents increase wild type CFTR maturation in human airway epithelial cells. Western blot analysis of CFTR was performed on whole cell extracts from A549 cells in the absence or presence of different S-nitrosylating agents at a concentration of 10 μM (4 hrs.) (A). Lanes: 1-Control; 2- S-nitrosocysteinylglycine (CGSNO); 3- S-nitrosoglutathione (GSNO); 4- S-nitroso-N-acetyl-cysteine (SNOAC); 5- Ethyl nitrite (ENO); 6- Nitric oxide (NO, prepared anaerobically from a saturated solution in deaerated water). The membrane was stripped and re-probed with α tubulin to verify that equal amounts of protein were loaded. The optical densities of the bands were quantified by densitometry (B). Representative of three separate experiments. The effects of CGSNO, GSNO, SNOAC and ENO on B and C band expression were greater than control; ($*P < 0.001$; $**P < 0.003$ and $***P < 0.005$).

Figure 2. Product confirmation: S-nitrosoglutathione diethyl ester (GNODE). Left: LC chromatograph of GNODE (full scan m/z = 150 to m/z = 2000). GNODE was detected as m/z = 392.71 at retention time 20.18 minutes. The two smaller peaks m/z 362.90 (R_t = 17.19) and m/z = 784 (R_t = 18.98) were identified as glutathione diethyl ester and the diethyl ester dimer respectively. Right: Full Scan (m/z 150-2000) mass spectrum of the LC trace shown at left.

Figure 3. Different S-nitrosylating agents increase ΔF508 CFTR expression and maturation in polarized human bronchial epithelial (CFBE41o⁻) cells. Western blot analysis of CFTR was performed on whole cell extracts from CFBE41o⁻ cells in the absence or presence of different S-nitrosylating agents at concentrations of 10 μM (A). Lanes: 1-Control; 2-Nitric oxide (NO, prepared anaerobically from a saturated solution in deaerated water); 3- S-nitrosoglutathione

(GSNO); 4- S-nitroso-N-acetyl-cysteine (SNOAC); 5- ethyl nitrite (ENO); 6- S-nitrosocysteinylglycine (CGSNO); 7- S-Nitrosoglutathione diethyl ester (GNODE); 8-ENO and 100 μM GSH; 9,10,11-GNODE, SNOAC and GSNO in the presence of the γ glutamyl transpeptidase inhibitor, acivicin; 10) 8-Bromo cyclic GMP (100 μM).   The membrane was stripped and re-probed with α tubulin to verify that equal amounts of protein were loaded. Blots were scanned and densitometry was performed for quantification (B). Fifty μg of protein was loaded to each lane (n=2 each). The effects of GNODE, SNOAC and GSNO on C band were greater than control (*P < 0.005; **P < 0.01 and ***P < 0.003).

Figure 4.   Inhibition of Csp translation with siRNA prevents the GSNO-induced augmentation of both core-and full-CFTR glycosylation. Western blot analysis of Csp (A) and CFTR (B) levels after transfection with siRNA duplexes specific for Csp. A549 cells were transfected with Csp siRNA duplexes and analyzed 48 hr after transfection. The cells were lysed and 50μg of protein was loaded onto an SDS-PAGE gel. Western blot analysis was performed using anti-Csp antibody.   The membrane was stripped and re-probed with α tubulin to verify that equal amounts of protein were loaded. (C) Blots were scanned, and densitometry was performed for quantification. The effect of GSNO on the expression of mature and immature CFTR was greater in the absence of Csp siRNA and in the presence of scrambled RNA than in the presence of either dose of siRNA, and was greater than the effect of RNA alone (*P < 0.005 and **P < 0.01 by Kruskal-Wallis testing followed by Welch-Satterthwaite pairwise comparison with negative control and with GSNO and siRNA). Data represent the average of two independent experiments.

Figure 5. GSNO increases Csp expression through a dose-dependent, post-translational

mechanism. A. Immunoblot of Csp levels at various concentrations of GSNO for 6 h. Csp1 and

Csp2 are indicated. The membrane was stripped and re-probed with anti-α tubulin to verify

equal amount protein loading. Relative Csp expression increased significantly by 10 μM GSNO

(P < 0.01) B. Csp expression is inhibited by cycloheximide but not by actinomycin D. Western

blot analysis was performed on whole cell extracts from A549 cells grown in the presence of 20

μg/ml actinomycin D for 2 hr or 50 μg/ml cycloheximide for 15 min before the addition of 5 μM

GSNO for a total of 4 hr. A, actinomycin D, C, cycloheximide. Fifty μg of protein was loaded

on to each lane for experiment.   The membrane was stripped and re-probed with α tubulin to
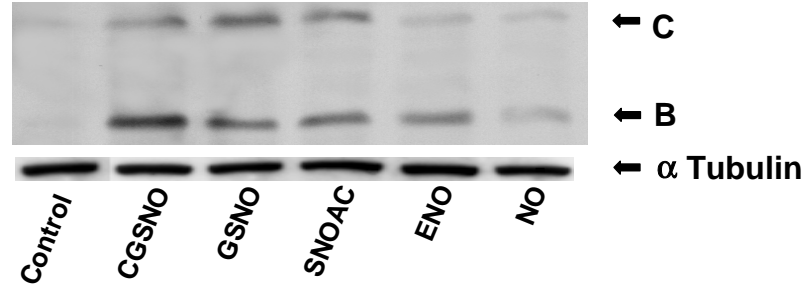
verify that equal amounts of protein were loaded.

Figure 6. GSNO exposure results in S-nitrosothiol trafficking through, and altered ΔF508 CFTR

association of proteins in, the endoplasmic reticulum and Golgi. A. S-nitrosothiol trafficking of

proteins associated with CFTR through the ER and Golgi (isolated by anti-CFTR IP) at various

times following exposure to 10 μM GSNO. B. Effects of GSNO on CFTR-Csp interactions in

the cytosol, ER and Golgi in polarized human bronchial epithelial CFBE41o- cells: Immunoblots

of Csp from cytosolic, endoplasmic reticulum, and Golgi fractions after immunoprecipitation

with anti-CFTR antibody. C. Immunoblots of Hsc70 from cytosolic, endoplasmic reticulum, and

Golgi fractions after IP with anti-CFTR antibody. Fifty μg of protein was loaded on to each lane

for experiment. Csp1 and Csp2 are indicated; c-cytosol; ER-endoplasmic reticulum; G-Golgi.

Table I. Half-life of S-Nitrosylating Agents (21% O_2, 37°C)

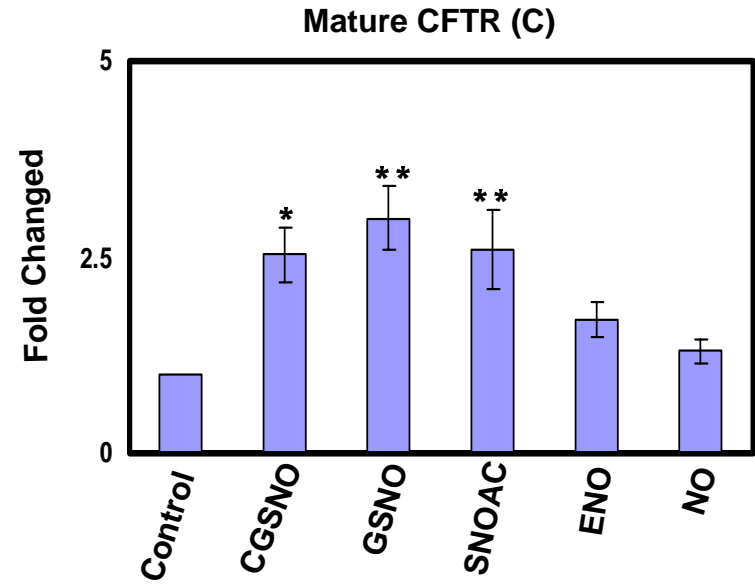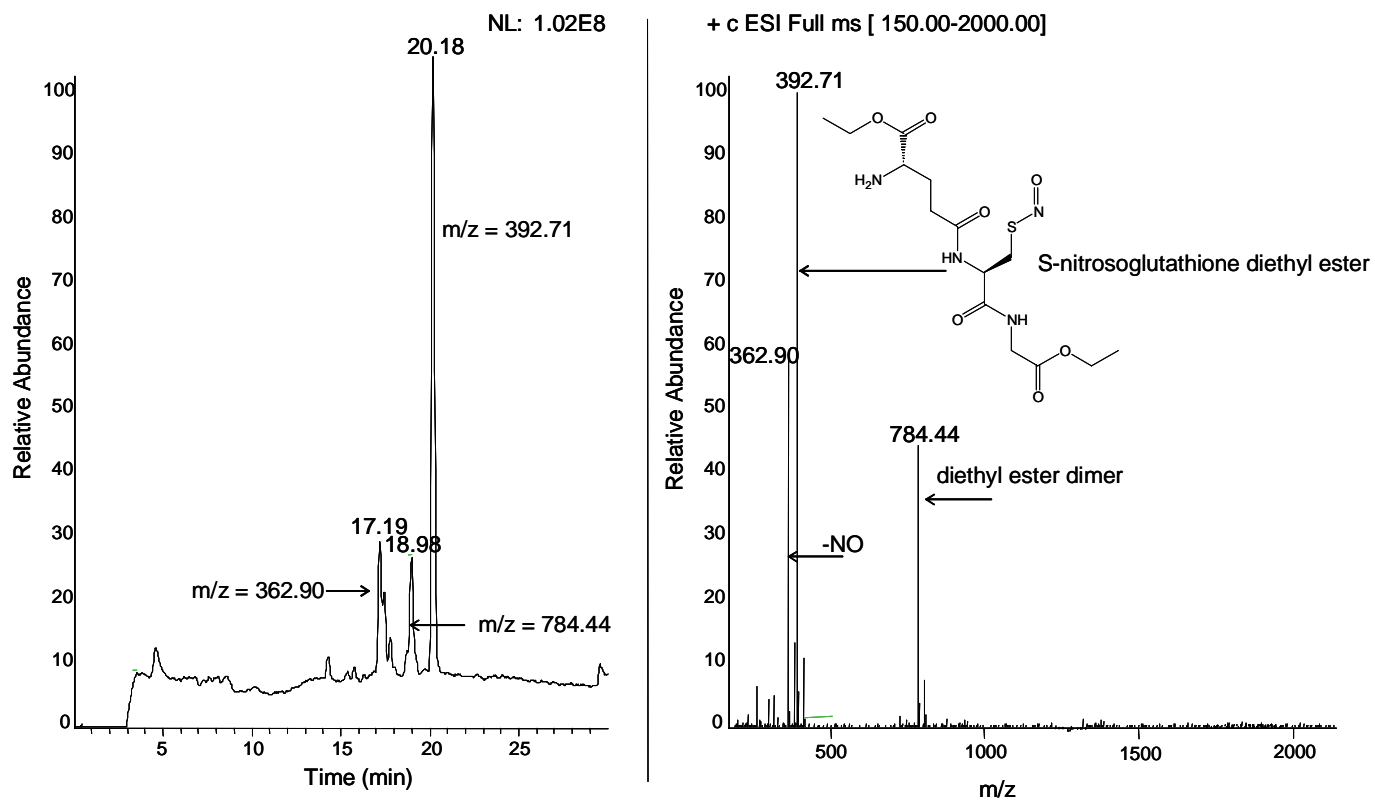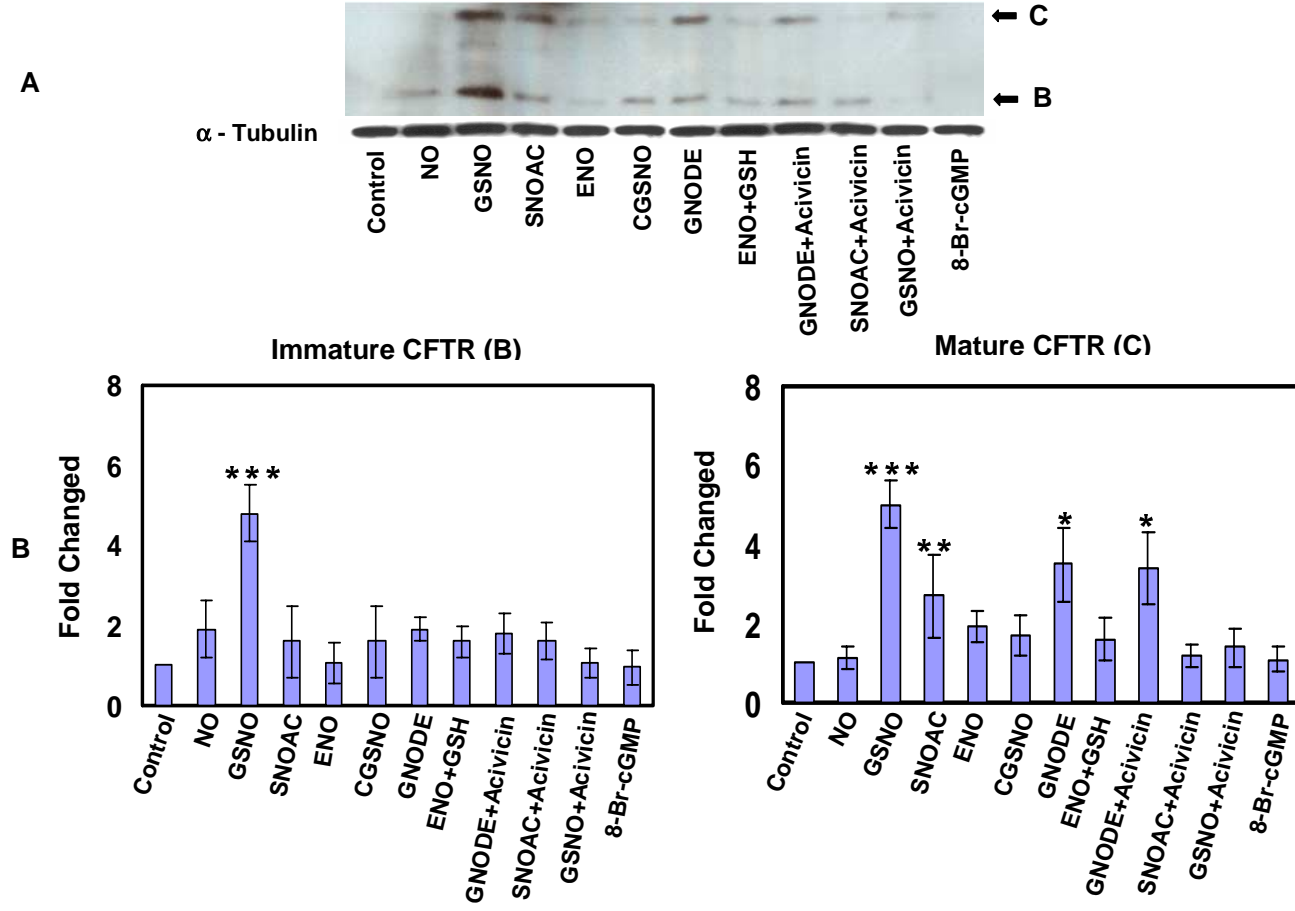| Compound	Mean Half-life (min)	Standard Deviation
S-Nitrosocysteine	2.2	+/- 0.26
S-Nitrosocysteinyl glycine	4.5	+/- 0.34
S-Nitrosoglutathione	441	+/- 28
S-Nitroso-N-acetyl cysteine	1,310	+/- 31
S-Nitrosoglutathione diethyl ester	3,020	+/- 610

Figure 1
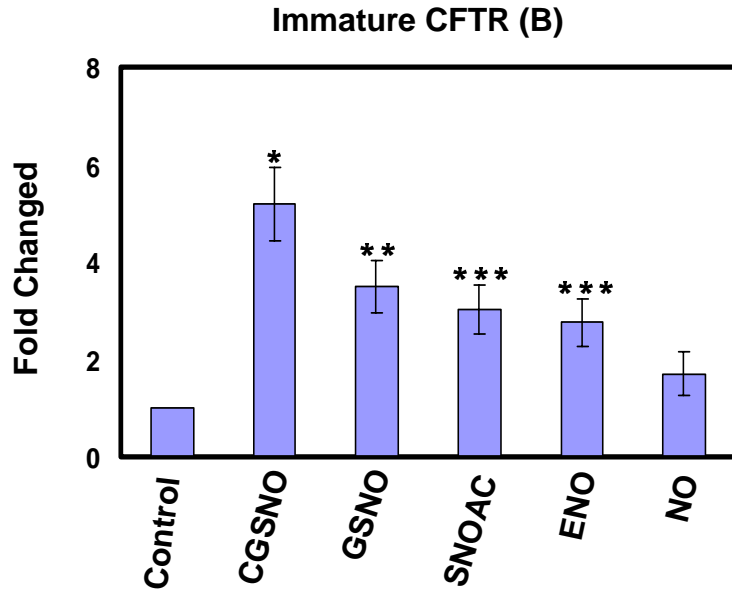
Figure 2

Figure 3.

Figure 4.

Figure 5.

Figure 6

A

B

Csp2
Csp1

C ER G   C ER G   C ER G   C ER G
0 h   2h   4h   6h

C

HSC70

C ER G   C ER G   C   ER G   C ER G
0 h   2h   4h   6h