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S-Nitrosylating Agents: A Novel Class of Compounds That Increase Cystic Fibrosis Transmembrane Conductance Regulator Expression and Maturation in Epithelial Cells

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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 seems 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 micromolar 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*-nitrosoglutathione diethyl ester, bypasses the need for GSNO bioactivation by γ-glutamyl transpeptidase 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; 6) proteins relevant to CFTR trafficking are SNO-modified, and SNO proteins traffic through the endoplasmic reticulum (ER) and Golgi after 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 classic NO radical/cyclic GMP pathway to increase CFTR expression and maturation. They also suggest that 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 typically 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 cochaperones 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 (Drumm et al., 1991; Riordan, 1999; Gibson et al., 2003). The majority of wild-type (wt) CFTR—and virtually all ΔF508 CFTR—is degraded before reaching the cell surface (Drumm et al., 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 (Denning et al., 1992; Ward and Kopito, 1994; Zeitlin et al., 2002). High-throughput screening has been used to identify

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ABBREVIATIONS: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulatory protein; wt, wild-type; GSNO, *S*-nitrosoglutathione; GNODE, *S*-nitrosoglutathione diethyl ester; GGT, γ-glutamyl transpeptidase; ENO, ethyl nitrite; SNO, *S*-nitrosothiol; ER, endoplastic reticulum; MS, mass spectrometry; Csp, cysteine string protein; siRNA, small interfering RNA; CGSNO, *S*-nitrosocysteinyl glycine; SNOAC, *S*-nitroso-*N*-acetylcysteine; CHIP, carboxyl terminus Hsc70 interacting protein; ER, endoplasmic reticulum.

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 et al., 1996), has antimicrobial effects (Liu et al., 2001, 2004) and, in physiological (low micromolar) concentrations, increases expression, maturation, and function of wt and ΔF508 CFTR in epithelial cells (Zaman et al., 2001, 2004; Andersson et al., 2002; Howard et al., 2003; Chen et al., 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- to 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) to 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). It is noteworthy that 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 (Grasemann et al., 1999; Snyder et al., 2002).

In this work, we have begun to study the post-transcriptional biochemistry by which these low micromolar 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 micromolar concentrations of *S*-nitrosylating agents in augmenting ΔF508 CFTR maturation seems 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). To our surprise, GSNO seems 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 described previously (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 described previously (Beckman et al., 1996).

Cell Culture. A549 and CFPAC-1 cells were from the American Type Culture Collection (Manassas, VA). CFBE 41o$^-$ cells were kindly provided by Dr. D. Gruenert (University of California, San Francisco, CA). A549 cells were grown in Ham's F-12K medium (Gaston et al., 1998; Zaman et al., 2004) and CFPAC-1 cells in Iscove's medium with 10% (v/v) fetal calf serum and 1% (v/v) penicillin/streptomycin (Invitrogen, Carlsbad, CA) (Zaman et al., 2001, 2004). CFBE41o$^-$ cells were seeded at 0.5–1 × 10^6 cells/cm^2 and grown on 0.4-μm pore filters (Transwell; Corning Glassworks, Corning, NY) at an air-liquid interface. Transepithelial resistance was measured by volt-ohm meter (Millicell; Millipore Corporation, Billerica, MA) (Hentchel-Franks et al., 2004). Experiments were done at a stable transepithelial resistance of >300 ohm · cm^2, indicating establishment of tight junctions (Hentchel-Franks et al., 2004).

Chemical Methods. SNOs were assayed in cysteine and CuCl as described previously (Gaston et al., 1998, 2003). GNODE was assayed by liquid chromatography/mass spectrometry (MS) using a C8 column (2.1 × 150) and 2695 high-performance liquid chromatography system (Waters, 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 MS in an LCQ system (Thermo Electron, Waltham, MA) 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 1 M cysteine saturated with CuCl as described previously (Fang et al., 1998; Mannick et al., 2001). SNO content was determined using a GSNO standard curve, and data were expressed relative to protein concentration (Lowry et al., 1951).

Immunoblotting. This was performed as described previously (Zaman et al., 2001, 2004). Cell extracts in 1% Nonidet P-40 lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 μM leupeptin, 1 μM aprotinin, 1 μM pepstatin, 1 μM phenylmethylsulfonyl fluoride, and 2 μM Na_2VO_4 (Roche, Indianapolis, IN) were sheared through a 25-gauge needle, fractioned on a 6% SDS polyacrylamide gel in 1× electrode buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS at pH 8.3), transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) in Tobin transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3), blocked in Tris-buffered saline/Tween 20 (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, and 5% nonfat dried milk at pH 8.0) and probed (45 min, room temperature) with 1:1000 of either anti-CFTR R-domain-specific monoclonal antibody (R&D Systems, Minneapolis, MN) or anti-CFTR monoclonal antibody (Chemicon, Temecula, CA). Blots were washed, incubated (30 min) with 1:2000 horseradish peroxidase-conjugated anti-mouse antibody (Pierce, Rockford, IL) in Tris-buffered saline/Tween 20 (30 min) and visualized using Hyperfilm (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were stripped and reprobed with α-tubulin antibodies (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA).

Isolation of Cytosol, Endoplasmic Reticulum, and Golgi. Polarized CFBE41o$^-$ cells before and after 2, 4, and 6 h of exposure to 10 μM GSNO were rinsed twice with ice-cold phosphate-buffered saline and lightly trypsinized, then pelleted by centrifugation at 5000 rpm for 10 min. The pellet was washed twice with ice-cold phosphate-buffered saline 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 by centrifugation (30,000 rpm; 60 min at 4°C). Sucrose stock (2.3 M) was added to the clarified supernatant to make the final concentration 1.25 M 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 h at 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 prechilled Eppendorf tubes. Ten microliters of primary CFTR antibody (anti-CFTR clones L12B4 and M3A7, mouse monoclonal antibodies; Upstate Biotechnology, Lake Placid, NY) were added to each fraction and incubated overnight at 4°C with gentle shaking. To avoid nonspecific binding, 70 μl of protein A (Boehringer Mannheim) was added to supernatant antibody mixtures, which were then incubated for another 4 h. Samples underwent centrifugation (1 min), and proteins not bound to the beads were washed twice with radioimmunoprecipitation assay

buffer. Proteins were then eluted from beads by incubation with 100 μl of sample buffer at room temperature with continuous mixing for 1 h. Samples were divided into two aliquots: one for SNO assay and the other for immunoblotting.

Small Interfering RNA Knockdown of Csp. siRNA sequences were synthesized and then identified (using matrix-assisted laser desorption ionization/time-of-flight spectrometric analysis; QIAGEN Inc., Valencia, CA). siRNA duplexes were >90% pure as measured by high-performance liquid chromatography 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, CA) with 20 and 50 nM Csp siRNA. Scrambled Csp siRNA was used as a control. Cells were lysed after 48 h after transfection, and expression level were assessed by Western blot analysis with anti-Csp antibody (Chemicon International).

Analysis of *S*-Nitrosylated Proteins. Cells were treated with 0.1, 5, or 10 μM GSNO or buffer (4 h), 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). In brief, 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). Biotinylated proteins were washed in neutralization buffer (20 mM HEPES, pH 7.7, 10 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100), and then purified using Streptavidin-Agarose beads and eluted with β-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, ~20:1) at 37°C overnight. Aliquots corresponding to approximately 1 μg of digested protein were loaded onto a 10-cm self-packed C18 (5–15 μm YMC ODS-A; Waters) 360-μm (o.d.) × 100-μm (i.d.) 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 self-packed C18 (5 μm YMC ODS-AQ) 360-μm (o.d.) × 50-μm (i.d.) 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; B, 0.1% HOAc/70% MeCN) of 0 to 60% B in 80 min and 60 to 100% B in 10 min. The eluted peptides were electrosprayed directly into a linear quadrupole ion trap mass spectrometer (LTQ; Thermo Electron) at a flow rate of 50 to 100 nl/min. The LTQ was operated with a "top 10" data-dependent analysis method consisting of a repeated data acquisition cycle of 1 full mass spectrum (MS; *m/z* 300-2000) followed by 10 tandem MS spectra corresponding to fragmentation mass spectra of the top 10 most abundant ions from the MS. For peptide identification, the tandem MS spectra were searched with the SEQUEST algorithm against the nonredundant human protein database from the NCBI (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health). To obtain high-confidence peptide sequence matches, first the search results were filtered using SEQUEST match scoring criteria whereby peptides were retained if 1) the cross-correlation score (XCorr) was greater than or equal to 3.0 (+3 ion), 2.5 (+2), and 2.0 (+1), 2) the scoring rank (RSp) was less than or equal to 10, and 3) 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 Student's *t* test. Welch-Satterthwaite analysis was also 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, 2001; Zaman et al., 2004). However, not all agents had an equal effect in causing full CFTR maturation [180 kDa; rank order, GSNO > *S*-nitroso-cysteinyl glycine (CGSNO) = *S*-nitroso-*N*-acetylcysteine (SNOAC) > 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

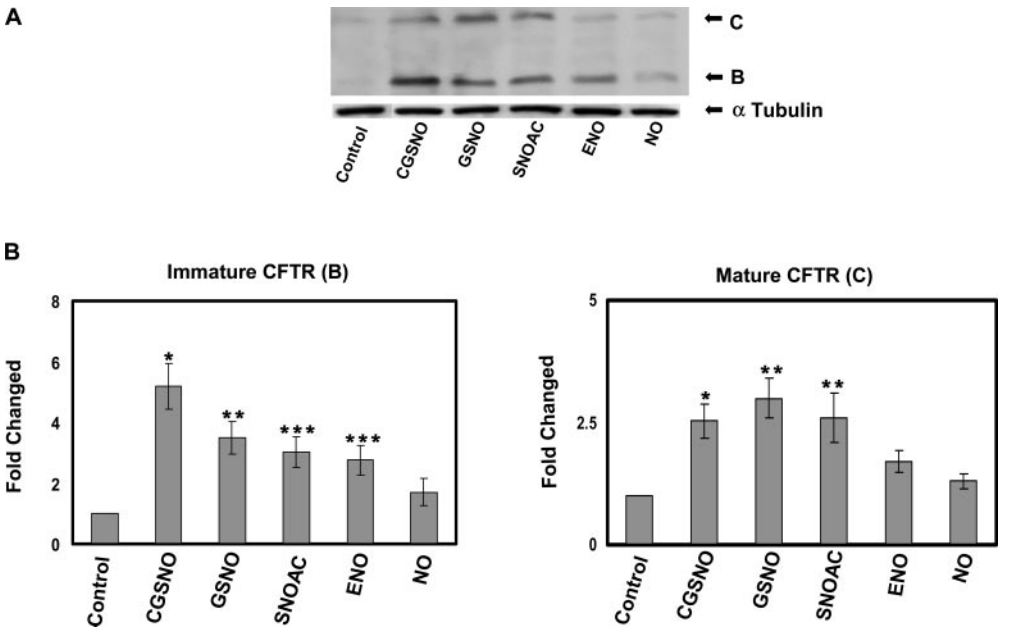

Fig. 1. Different *S*-nitrosylating agents increase wild-type CFTR maturation in human airway epithelial cells. Western blot analysis of CFTR was performed on whole-cell extracts from A549 cells that had been in the absence or presence of different *S*-nitrosylating agents at a concentration of 10 μM (4 h.) (A). Lane 1, control; lane 2, CGSNO; lane 3, GSNO; lane 4, SNOAC; lane 5, ENO; lane 6, 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 (*, $P < 0.001$; **, $P < 0.003$; ***, $P < 0.005$).

S-nitrosylation by equimolar reaction with sodium nitrite in 1 N HCl. The product was confirmed to be GNODE by MS; this product had a half-life of 3020 ± 610 min at 37°C (also analyzed by MS; Table 1; Fig. 2).

Effect of *S*-Nitrosylating Agents on ΔF508 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. It is noteworthy that GSNO (10 μM) increased transepithelial conductance acutely (from 17.3 to 115.2 μAmps/cm^2; $n = 3$; $P < 0.001$) and decreased resistance (from 597 to 175 kΩ · cm^2) after 4 h ($n = 3$; $P < 0.003$). 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). It is noteworthy that the effect of GSNO was partially inhibited by acivicin, an inhibitor of GGT, 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 classic 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 h), we expected Csp siRNA to mimic GSNO in increasing fully mature (C Band) CFTR (Zhang et al., 2002). However, treatment with Csp siRNA did not mimic the effect of GSNO (Fig. 4), which was unexpected. Csp protein levels at baseline were low but relatively stable. 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 liquid chromatography/MS analysis of whole-cell and membrane fraction biotin-substituted SNO proteins after treatment with 0.1, 1, 5, and 10 μM GSNO (4 h). *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, Hsp90, peroxiredoxins, and tropomyosin.

We then studied the regulation of the effect of GSNO on Csp. We were surprised to find that the effect of GSNO to increase Csp was inhibited by 15-min pretreatment with cycloheximide, but not with 2-h pretreatment with actinomycin D (Fig. 5), suggesting 1) that Csp mRNA is relatively stable and 2) that 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 h after exposure to 10 μM GSNO. GSNO increased the CFTR-associated SNO content of the ER and Golgi at 2 and 4 h; by 6 h, the SNO proteins were completely absent from the ER and had passed through to the Golgi (Fig. 6A). The principal protein modified was a 70-kDa heat shock precursor relevant to CFTR and Csp trafficking (Fig. 6B).

GSNO caused increased association of Csp with CFTR in the ER at 4 h and maximally increased Csp interaction in the Golgi at 6 h (Fig. 6B). In parallel, GSNO caused increased association of Hsc70 with CFTR-Csp in the ER, at 2, 4, and 6 h (Fig. 6C).

TABLE 1
Half-life of *S*-nitrosylating agents (21% O_2, 37°C)
Data are presented as mean ± S.D.

Compound	Half-Life
	min
S-Nitrosocysteine	2.2 ± 0.26
S-Nitrosocysteinyl glycine	4.5 ± 0.34
S-Nitrosoglutathione	441 ± 28
S-Nitroso-*N*-acetyl cysteine	1310 ± 31
S-Nitrosoglutathione diethyl ester	3020 ± 610

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 patients with CF have at least one copy of the most common abnormal CFTR allele, ΔF508. This mutation encodes for a protein that is potentially functional but is misfolded and normally degraded in the ER (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 micromolar concentrations increase expression, maturation, and function of ΔF508 CFTR (Zaman et al., 2001, 2004; Andersson et al., 2002; Howard et al., 2003). We report here 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 seem to be low both in CF and in asthma (Gaston et al., 1998; Grasemann et al., 1999; Dweik et al., 2001). 3) It relaxes airway 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; Jain et al., 1998; Fortenberry et al., 1999; Li et al., 2000; Liu et al., 2004). 4) It increases wild-type CFTR function (Chen et al., 2006). 5) Finally, levels of GSNO present in the normal airway increase ΔF508 expression, maturation, and function (Zaman et al., 2001, 2004; Andersson et al., 2002; Howard et al., 2003); dose-response experiments suggest that concentrations of 5 to 10 μM are optimal for this effect (Zaman et al., 2001, 2004). These low micromolar levels of GSNO seem to affect CFTR primarily through a post-transcriptional mechanism, although there seems also to be a modest transcriptional effect through augmented Sp3 expression and binding (Zaman et al., 2004). It is noteworthy that our transmembrane resistance data are consistent with those of Howard et al. (2003), 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 classic activation of guanylate cyclase; 2) a different "NO donor" effect of GSNO involving nitration of tyrosine residues, an effect demonstrated in the

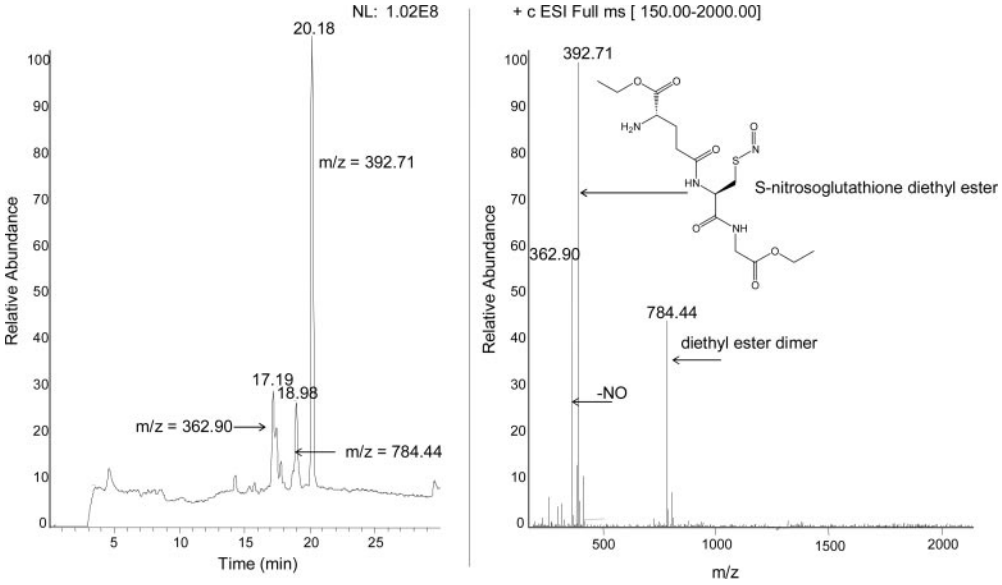

Fig. 2. Product confirmation. *S*-Nitrosoglutathione diethyl ester (GNODE). Left, LC chromatograph of GNODE (full scan, *m/z* = 150-2000). GNODE was detected as *m/z* = 392.71 at retention time (R_t) 20.18 min. The two smaller peaks *m/z* 362.90 (R_t = 17.19) and *m/z* = 784 (R_t = 18.98) were identified 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.

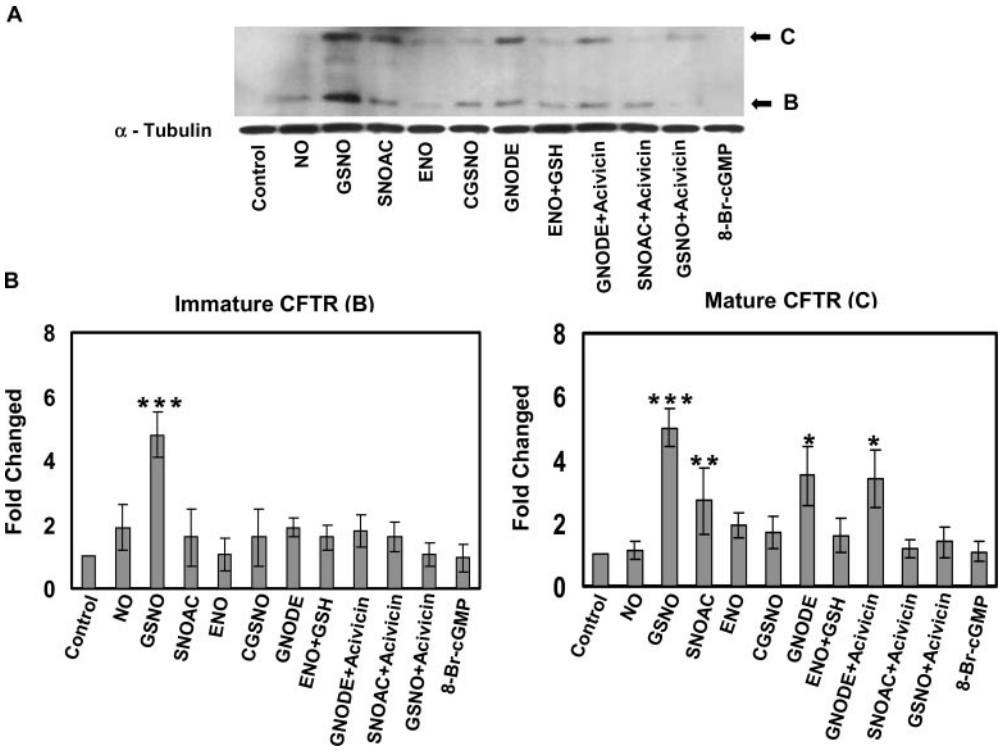

Fig. 3. Different *S*-nitrosylating agents increase ΔF508 CFTR expression and maturation in polarized human bronchial epithelial (CFBE41o$^-$) cells. Western blot analysis of CFTR was performed on whole-cell extracts from CFBE41o$^-$ cells that had been (4 h) in the absence or presence of different *S*-nitrosylating agents at concentrations of 10 μM (4h) (A). Lane 1, control; lane 2, NO (prepared anaerobically from a saturated solution in deaerated water); lane 3, GSNO; lane 4, SNOAC; lane 5, ENO; lane 6, CGSNO; lane 7, GNODE; lane 8, ENO and 100 μM GSH; lane 9, GNODE; lane 10, SNOAC; and lane 11, GSNO; each (lanes 9–11) in the presence of the GGT inhibitor acivivin; lane 12, 8-bromocyclic GMP (100 μM). The membrane was stripped and reprobed with α-tubulin to verify that equal amounts of protein were loaded. Blots were scanned, and densitometry was performed for quantification (B). Fifty micrograms of protein was loaded to each lane (n = 2 each). The effects of GNODE, SNOAC, and GSNO on C band were greater than control (∗, $P < 0.005$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.003$).

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 [except for NO itself, which requires the presence of an electron acceptor—such as oxygen or a metalloprotein—to have this effect (Gaston et al., 2003)]; 3) ENO, which does not readily evolve NO, is more active than NO; 4) the cell-permeable, cGMP mimetic 8-Br cGMP is inactive; 5) the effect of GSNO is reversed by dithiothreitol, 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 micromolar 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 (Lipton et al., 2001; Moya et al., 2001; Gaston et al., 2003; Liu et al., 2004; Gaston et al., 2006).

***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 carboxyl terminus of Hsc70 interacting protein (CHIP) (Meacham et al., 2001; McDonough and Patterson, 2003)—and CFTR itself (Choglay et al., 2001; Chen et al., 2004; Wang et al., 2005). Cysteine string proteins were interesting to us because in *X. laevis* 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 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 *X. laevis* oocytes. These data may be understood in the context of the Csp cochaperone model proposed by Zhang et al. (2002), 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 overexpression data of Zhang et al. (2002) in suggesting that the association may favor core glycosylation. Next, another protein interacts with CFTR and Csp. The immunoprecipitation and proteomic data (Figs. 5 and 6) suggest that this may be Hsc70, consistent with previous work (Chamberlain and Burgoyne, 1997; Zhang et al., 2002; Cyr, 2005). In the absence of GSNO, this interaction may lead to degra-

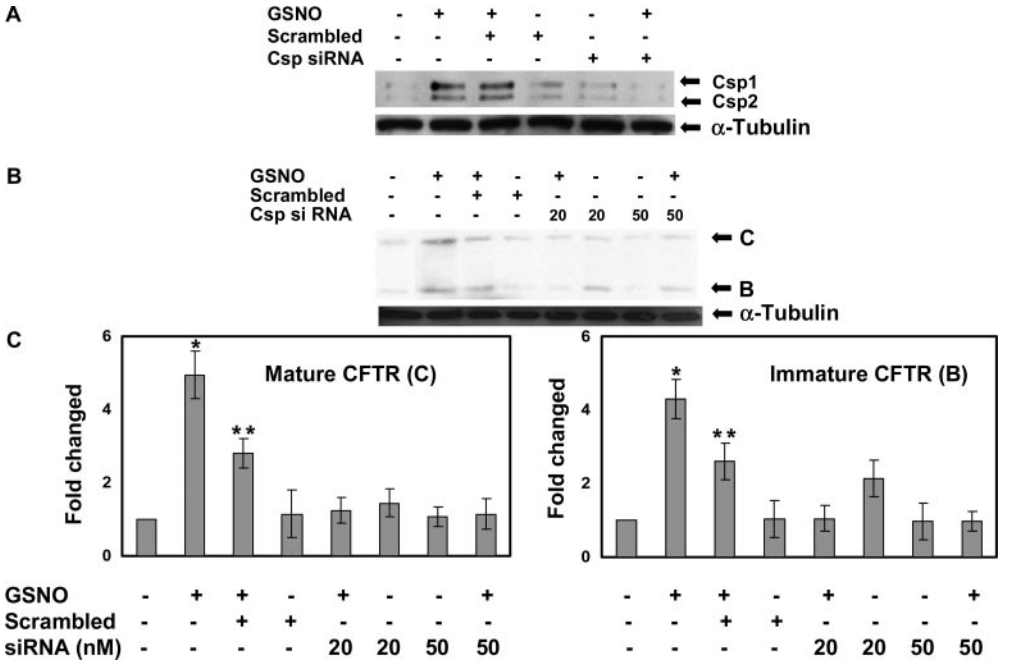

Fig. 4. Inhibition of Csp translation with siRNA prevents the GSNO-induced augmentation of both core-and full-CFTR glycosylation. Western blot analysis of Csp (A) and CFTR (B) levels after transfection with siRNA duplexes specific for Csp. A549 cells were transfected with Csp siRNA duplexes and analyzed 48 h after transfection. The cells were lysed and 50 μg of protein was loaded onto an SDS-polyacrylamide gel electrophoresis gel. Western blot analysis was performed using anti-Csp antibody. The membrane was stripped and reprobed 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 (*, $P < 0.005$; **, $P < 0.01$ 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.

dation. 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 and McCay, 1998), but we propose that this could be a mechanism by which GSNO modification of the Hsc70 cysteine allows Csp binding to augment folding without leading to degradation (Braun et al., 1996; Chamberlain and Burgoyne, 1997). Additional work will be required on each step of this proposed mechanism. It is noteworthy that 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; Grasemann et al., 1999; Liu et al., 2001; Que et al., 2005). Its catabolism in the asthmatic airway epithelium, for example, seems 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 (Kelley and Drumm, 1998; Grasemann et al., 1999; Gao et al., 1999). GSNO catabolism has the potential, paradoxically, to increase GSNO bioactivity (for example, through GGT) and/or to decrease its duration of action (Lipton et al., 2001; Zaman 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. We report here that GNODE is active in the presence of the GGT inhibitor acivicin and has a stability profile comparable with 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 micromolar levels of GSNO and other *S*-nitrosylating agents can, under certain circumstances, increase CFTR expression, maturation, and function (Zaman et al., 2001, 2004; Andersson et al., 2002; Howard et al., 2003; Chen et al., 2006). However, higher doses of NO itself can augment CFTR breakdown by causing tyrosine nitration or oxidation (Jilling et al., 1999). Furthermore, high micromolar or millimolar concentrations of GSNO can inhibit both 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 micromolar 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 fluxes through the ER and Golgi. In addition, we have found that GSNO increases airway epithelial cell Csp expression and that this increase seems 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.

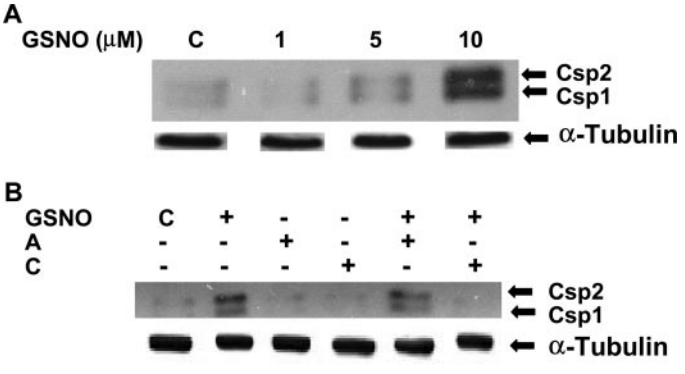

Fig. 5. GSNO increases Csp expression through a dose-dependent, post-translational mechanism. A, immunoblot of Csp levels at various concentrations of GSNO for 6 h. Csp1 and Csp2 are indicated. The membrane was stripped and re-probed with anti-α tubulin to verify equal amounts of protein loading. Relative 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 h or 50 μg/ml cycloheximide for 15 min before the addition of 5 μM GSNO for a total of 4 h. A, actinomycin D; C, cycloheximide. Fifty micrograms of protein was loaded onto each lane for experiment. The membrane was stripped and reprobed with α-tubulin to verify that equal amounts of protein were loaded.

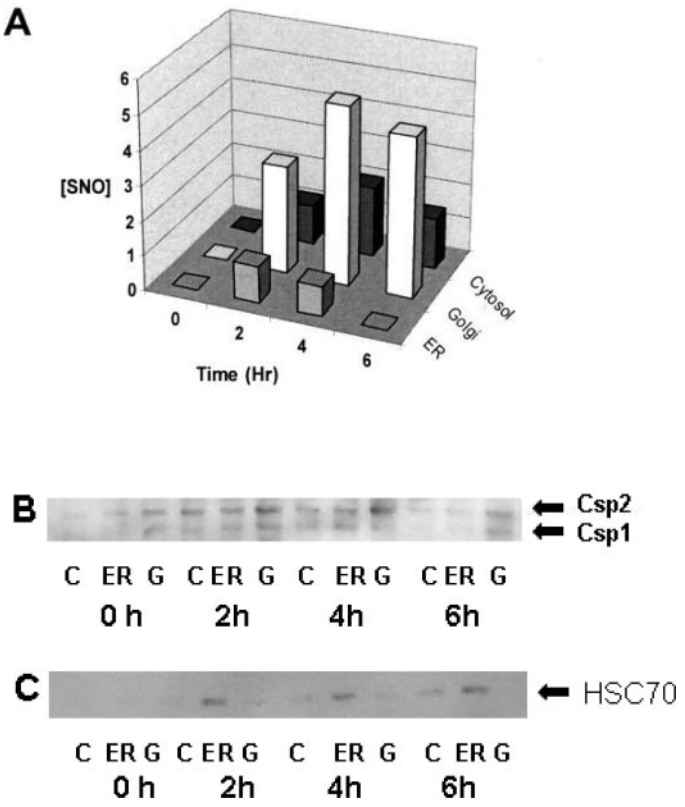

Fig. 6. GSNO exposure results in *S*-nitrosothiol trafficking through, and altered ΔF508 CFTR association of proteins in, the endoplasmic reticulum and Golgi. A, *S*-nitrosothiol trafficking of proteins associated with CFTR through the ER and Golgi (isolated by anti-CFTR IP) at various times after exposure 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 immunoprecipitation with anti-CFTR antibody. Fifty micrograms of protein was loaded onto each lane for the experiment. Csp1 and Csp2 are indicated; c, cytosol; ER, endoplasmic reticulum; G, Golgi.

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