Concentration-dependent effects of endogenous *S*-nitrosoglutathione on gene regulation by specificity proteins Sp3 and Sp1

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The activities of certain nuclear regulatory proteins are modified by high concentrations of S-nitrosothiols associated with nitrosative stress. In the present study, we have studied the effect of physiological (low μM) concentrations of the endogenous S-nitrosothiol, GSNO (*S*-nitrosoglutathione), on the activities of nuclear regulatory proteins Sp3 and Sp1 (specificity proteins 3 and 1). Low concentrations of GSNO increased Sp3 binding, as well as Sp3-dependent transcription of the cystic fibrosis transmembrane conductance regulatory gene, *cftr*. However, higher GSNO levels prevented Sp3 binding, augmented Sp1 binding and prevented both *cftr* transcription and CFTR (cystic fibrosis transmembrane conductance regulator) expression. We conclude that low concentrations of GSNO favour Sp3 binding to 'housekeeping' genes such as *cftr*, whereas nitrosative stress-associated GSNO concentrations shut off Sp3-dependent transcription, possibly to redirect cellular resources. Since low micromolar concentrations of GSNO also increase the maturation and activity of a clinically common CFTR mutant, whereas higher concentrations have the opposite effect, these observations may have implications for dosing of S-nitrosylating agents used in cystic fibrosis clinical trials.

Key words: cystic fibrosis, cystic fibrosis transmembrane conductance regulator, *S*-nitrosoglutathione, S-nitrosylation, specificity protein 1.

INTRODUCTION

Sp3 (specificity protein 3) and Sp1 are members of a family of proteins that are important for the regulation of TATA-less genes [1–2]. Proteins in this family are structurally similar and bind to GC-rich DNA sequences. The conserved DNA-binding domain comprises three zinc fingers close to the C-terminus and a serine/threonine and glutamine-rich domain in the N-terminal region [1,3]. Sp1 is rapidly degraded in a process that can be prevented by proteasome inhibitors. Sp3 and Sp1 commonly compete for transcriptional regulatory effects and transactivation by these factors can be differentially modified by high levels of oxidative stress [4–5].

The activities of nuclear factor κB and HIF1 (hypoxia-inducible factor 1), nuclear regulatory proteins involved in inflammation and in the cellular response to hypoxia, are modified by endogenous SNOs (S-nitrosothiols) at concentrations of the order of 10–100 μM [6,7]. These SNO levels are higher than those ordinarily measured in healthy mammalian tissues [8,9], but may be present in disease states. In the present study, we have investigated the effect of physiological airway levels of the endogenous S-nitrosylating agent, GSNO (*S*-nitrosoglutathione) [9] (here defined to be $\leqslant 10$ μM) on the activity of Sp3 and Sp1. Those proteins may be involved more in the regulation of gene expression under physiological conditions than under stress. We report that physiological concentrations of GSNO increase Sp3 binding, as well as downstream transcription, in a manner that is (i) inhibited by MMA (mithramycin A), (ii) inhibited by the γGT (γ glutamyl transpeptidase) blocker, acivicin, and (iii) partially reversed by DTT (dithiothreitol). On the other hand, higher GSNO levels, those associated with inflammation and nitrosative stress, prevent Sp3 binding and augment Sp1 binding, inhibiting transcription. These observations suggest that physiological concentrations of GSNO may favour baseline Sp3 binding to GC-rich regions of genes for certain proteins expressed under baseline conditions, whereas nitrosative stress-associated GSNO concentrations shut off Sp3-dependent transcription, redirecting cellular resources to the stress response.

Physiologically low concentrations of GSNO and other SNOs increase maturation and function of ΔF508 CFTR [cystic fibrosis (CF) transmembrane conductance regulator], the mutant protein most commonly associated with CF [10–12]. Paradoxically, high micromolar concentrations of 'NO donors', similar to those observed *in vivo* under conditions of nitrosative stress [13–14], inhibit expression of wild-type CFTR, at least partially by signalling increased proteasomal degradation through tyrosine nitration [15]. Although regulation of CFTR expression is primarily post-translational, cell-specific levels of CFTR are partly dictated by the genomic sequence upstream of the *cftr* transcription initiation region [16–19]. Analysis of *cftr*, particularly the promoter region, reveals the following characteristics of a housekeeping gene: (i) it is TATA-less, (ii) transcription is initiated at multiple transcription start sites, (iii) the promoter region has a high GC content, and (iv) the proximal region of the *cftr* promoter contains many potential Sp3- and Sp1-binding sites [16,17]. Therefore we hypothesized that the dose-dependent paradoxical effects of GSNO on Sp3/Sp1 DNA binding could be relevant to the dose-dependent paradoxical effects of GSNO on CFTR expression.

CFTR is an apical membrane protein, which functions as a cAMP-regulated chloride channel and is expressed on many

Abbreviations used: BHK cells, baby hamster kidney cells; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, *S*-nitrosoglutathione; γGT, γ glutamyl transpeptidase; HIF1, hypoxia-inducible factor 1; Hsp, heat-shock protein; MMA, mithramycin A; NEM, *N*-ethylmaleimide; oxyHb, oxyhaemoglobin; SNO, S-nitrosothiol; Sp1, specificity protein 1.

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types of epithelial cells under baseline conditions [20,21]. It is folded and glycosylated in the endoplasmic reticulum. Degradation accounts for nearly 100 % of newly synthesized ΔF508 CFTR [21]. However, ΔF508 CFTR protein can form functional chloride channels if expressed on the cell surface of *Xenopus oocytes* [22] or mammalian cells [11,12,23], suggesting that augmented maturation and cell-surface expression could be of benefit to CF patients. Its maturation, expression and function can be increased when cells are cultured or in the presence of GSNO [10–12]. In addition to its effects on ΔF508 CFTR expression, GSNO has other potential benefits in the CF airway [24], including bronchodilatation, increased ciliary motility, inhibition of amiloride-sensitive sodium transport, antimicrobial effects and stimulation of neutrophil apoptosis [9,24,25]. Airway levels of GSNO are lower than normal in patients with mild CF [26]. In the present study, we show that GSNO increases both Sp3 and Sp1 binding to DNA and transcription of *cftr* at physiological concentrations, but inhibits Sp3 binding and *cftr* transcription at nitrosative stress levels. These observations suggest a novel cell-signalling paradigm: physiological levels of GSNO may promote physiological homoeostasis, whereas nitrosative stress levels may signal a change from the expression of housekeeping genes to the expression of stress-response genes. Additionally, these observations may have implications for the development of new treatments for CF.

MATERIALS AND METHODS

Cell culture and reagents

A549 (type II alveolar epithelial) and *Drosophila* SL2 cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.). A549 cells were grown in Ham's F12K medium with 10 % (v/v) foetal calf serum and 1 % (v/v) penicillin–streptomycin (Life Technologies, Gaithersburg, MD, U.S.A.). *Drosophila* SL2 cells (Schneider cells) were cultured in Schneiders *Drosophila* media (Life Technologies) with 10 % foetal calf serum and 1 % penicillin–streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5 % carbon dioxide and air and passaged at confluence approximately every 4 days. GSNO was prepared by acid nitrosation followed by neutralization in 10 mM PBS as described previously [8] and stored at − 80 °C until use.

Preparation of Hb

Purified, stripped and free Hb A_o (1 mM; Apex Bioscience, Durham, NC, U.S.A.) was dialysed against 2 % (w/v) borate (0.5 mM EDTA, pH 9.2) overnight at 4 °C; methaemoglobin < 2 % was confirmed spectrophotometrically by the Winterbourn [27] method. This method, performed at room temperature (25 °C), produces oxyHb (oxyhaemoglobin). To render 1 aliquot of Hb βcys^{93} unavailable for transnitrosation reactions, 0.5 ml of Hb was incubated with 13.3 μl of 50 mM NEM (*N*-ethylmaleimide) in 100 % ethanol for 10 min at 20 °C (Hb/NEM ratio 100:1). The reaction was stopped by rapid transfer of the mixture to a Sephadex G-25 column equilibrated with 10 mM PBS (pH 7.40); [Hb] was measured after conversion into cyanomethaemoglobin and adjusted to 200 μM. All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise mentioned.

Nuclear extracts

Nuclear extracts were prepared as described previously [6,28]. Briefly, cells were rinsed twice with cold PBS (pH 7.4) and lightly trypsinized and pelleted by centrifugation at 5000 ***g*** for 5 min. The pellet was washed twice with PBS and then suspended in lysis buffer A (10 mM Tris/HCl, pH 7.5, containing 1.5 mM $MgCl_2$, 10 mM KCl, and freshly supplemented with 1 mM DTT, 1 μM PMSF, 2 μM Na_3VO_4, 2 μM leupeptin, 1 μM aprotinin and 1 μM pepstatin). The cell suspension was homogenized and nuclei were pelleted by centrifuging at 12 000 ***g*** for 5 min at 4 °C. The pellet was resuspended in buffer C [20 mM Tris/HCl, pH 7.5/0.42 mM KCl/20 % (v/v) glycerol/1.5 mM $MgCl_2$ and freshly supplemented with Na_3VO_4, DTT, PMSF, leupeptin, aprotinin and pepstatin at concentrations as used for buffer A]. The lysate was rotated for 30 min in a cold room and centrifuged for 30 min at 16 000 ***g***. The protein concentration in the supernatant was determined by the BCA Protein Assay kit (Pierce, Rockford, IL, U.S.A.) and nuclear extracts were frozen and stored at − 80 °C for further use.

EMSA (electrophoretic mobility-shift assay)

We performed EMSA as described previously [6,28]. Briefly, the Sp1 and Sp3 consensus oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were end-labelled with [γ-^{32}P]-ATP (NEN Life Science Products, Boston, MA, U.S.A.) using T4 kinase (New England Biolabs, Beverly, MA, U.S.A.). Radiolabelled probe (1 μl; activity 30 000–80 000 c.p.m./μl) was added to the mixture, and the samples were incubated for 20 min at room temperature; 5 μg of nuclear protein was used per reaction. The binding buffer contained 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 50 mM KCl, 1 μM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5 % glycerol and 1 μg of poly(dI/dC). For supershift assay, 4 μg of monoclonal anti-Sp1/Sp3 antibody (Santa Cruz Biotechnology) was added 20 min after the addition of the probe and the samples were incubated for an additional 30 min at 25 °C. For the unlabelled competition experiments, 20- and 100-fold molar-excesses of unlabelled oligonucleotides were added to the reaction mixture. DNA–protein complexes were separated from unbound probe on native 6 % (v/v) polyacrylamide gels at 195 V for 4 h. The gels were transferred to Whatman MM filter paper and vacuum-dried at 80 °C for 2 h and exposed to Kodak film for 12–15 h at − 80 °C.

Western-blot analysis

Western-blot analysis was performed as described previously [6,10]. Briefly, whole cell extracts were prepared in 1 % Nonidet P40 (Sigma) lysis buffer containing 50 mM Tris/HCl (pH 8.0), 1 % Nonidet P40, 150 mM NaCl, 2 μM leupeptin, 1 μM aprotinin, 1 μM pepstain, 1 μM PMSF and 2 μM Na_2VO_4 (Boehringer Mannheim, Indianapolis, IN, U.S.A. and Roche Diagnostics, Mannheim, Germany). Insoluble material was recovered and sheared by passage through a 25-gauge needle. Protein from whole cells was quantified by the BCA Protein Assay kit. Protein (25 μg) was fractionated on a 6 % SDS/polyacrylamide gel in 1× electrode buffer (25 mM Tris/192 mM glycine/0.1 % SDS, pH 8.3). The fractioned protein was transferred on to pure nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using an electrophoretic transfer cell with Tobin Transfer buffer (25 mM Tris/192 mM glycine/20 % methanol, pH 8.3). Blots were blocked in Tris-buffered saline/Tween 20 (TBS-T: 10 mM Tris/HCl/150 mM NaCl/0.05 % Tween 20, pH 8.0), containing 5 % (w/v) non-fat dried milk and probed with a 1:1000 dilution of anti-CFTR antibodies (anti-hCFTR, R-domain-specific monoclonal; R & D Systems, Minneapolis, MN, U.S.A.) in TBS-T containing 5 % (w/v) non-fat milk for 45 min at room temperature. Blots were washed and incubated for 30 min with a 1:2000 dilution of horseradish

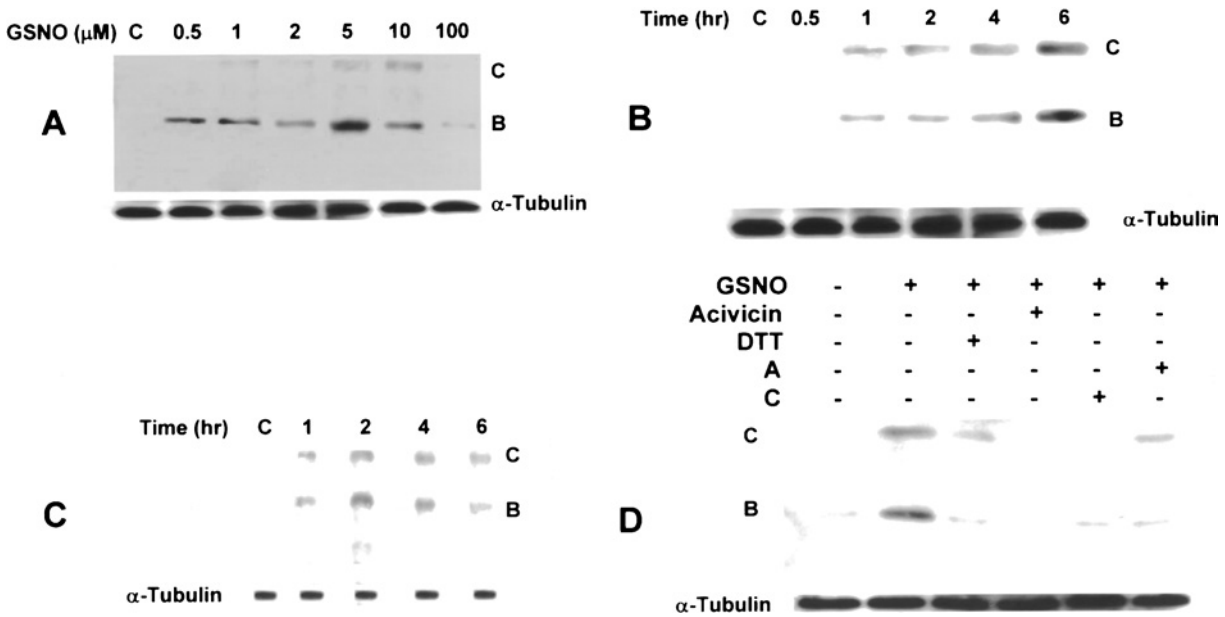

Figure 1 Low concentrations of GSNO increase, whereas higher concentration inhibit the expression of wild-type CFTR in A549 cells

(**A**) Western-blot analysis was performed on whole cell extracts from A549 cells treated with various concentrations of GSNO for 6 h. Note that wild-type CFTR expression is increased by 10 μM GSNO, but inhibited by 100 μM GSNO. CFTR bands B (160 kDa) and C (180 kDa) are indicated. The membrane was stripped and re-probed with anti-α tubulin to verify equal protein loading. (**B**) GSNO (10 μM) also increases the expression of mature, wild-type CFTR as a function of time. Western-blot analysis was performed on cells after incubation with 10 μM GSNO for different periods of time. (**C**) Time-course analysis of the effects of 100 μM GSNO (1, 2, 4 and 6 h) on CFTR expression and maturation was performed on whole cell extracts from A549 cells. (**D**) GSNO induction of CFTR maturation is inhibited by acivicin and reversed by DTT. Western-blot analysis was performed on whole cell extracts from A549 cells treated with 10 μM GSNO in the presence of 100 μM acivicin for 6 h or 200 μM DTT during the last 30 min of the 6 h incubation period. GSNO-induced maturation of wild-type CFTR is both transcriptional and translational. 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 10 μM GSNO for a total of 6 h; A, actinomycin D; C, cycloheximide. Consistent with our previous findings, the effect of GSNO was more post-transcriptional than transcriptional, although both levels of regulation were observed; 25 μg of protein was loaded on to each lane for each experiment.

peroxidase-conjugated anti-mouse antibody (Pierce) in TBS-T containing 5% non-fat dried milk for 30 min. Blots were washed as described previously and then once in TBS (Bio-Rad Laboratories). CFTR protein was visualized by enhanced chemiluminescence (ECL®; Amersham Biosciences, Piscataway, NJ, U.S.A.) using Hyperfilm (Amersham Biosciences).

Northern-blot analysis

mRNA from A549 cells was extracted from each treatment group using an RNA easy kit (Qiagen, Valencia, CA, U.S.A.). After 4 h, cells were washed and grown for 48 h in complete media to the indicated treatment and mRNA was extracted according to the manufacturer's instructions. For each sample, 5 μg of mRNA was electrophoresed in a 1% agarose and formaldehyde gel, and transferred to a positively charged nylon membrane (Promega, Madison, WI, U.S.A.) by capillary blotting. The membrane was hybridized using Quick Hyb according to the manufacturer's instructions (Stratagene, La Jolla, CA, U.S.A.). The ^{32}P-labelled CFTR probe consisted of the 1.5 kb *Eco*RI–*Eco*RI fragment of human CFTR cDNA (kindly provided by Dr A. Edelman, Institut National de la Santé et de la Recherche Médicale, France, and Dr J. R. Riordan, Johnson Medical Research, Mayo Foundation and Mayo Clinic, Scottsdale, AZ, U.S.A.) labelled by random priming. The membrane was also hybridized with a human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA probe (Ambion, Austin, TX, U.S.A.). After washing, the blots were exposed to Kodak film for 24–48 h at -80 °C. The mRNAs were quantified by densitometric scanning of the autoradiograms and the amounts of mRNA were normalized to those of GAPDH.

Cell transfection

A549 cells were seeded at a density of 5×10^5 cells/well plate and grown in complete medium for 48 h to achieve approx. 75% confluence. Transfection was performed using a mixture of 5 μl of LIPOFECTAMINE™ Plus reagent (Life Technologies)/1 μg of Sp1 construct (generously provided by Dr R. Tijan, University of California, Berkeley, CA, U.S.A., and Dr G. Suske, University of Marburg, Germany) in serum-free medium. After 4 h, cells were washed and grown for 48 h in complete medium before the indicated treatments. The transfected cells were washed three times with ice-cold PBS and mRNA was extracted using a RNA easy kit (Qiagen) as described above.

Statistical analysis

Means were compared by ANOVA followed by the Student *t* test. Results are expressed as means $\pm$ S.E.M. $P < 0.05$ was considered significant.

RESULTS

GSNO increases CFTR expression and maturation in A549 cells

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 in control and GSNO-treated A549 cells. At baseline, A549 cells expressed relatively little CFTR. However, treatment with physiological concentrations of GSNO resulted in dose-dependent increases in the expression and maturation of bands B and C (Figure 1A), whereas band A was not detected. It was noted that the relative

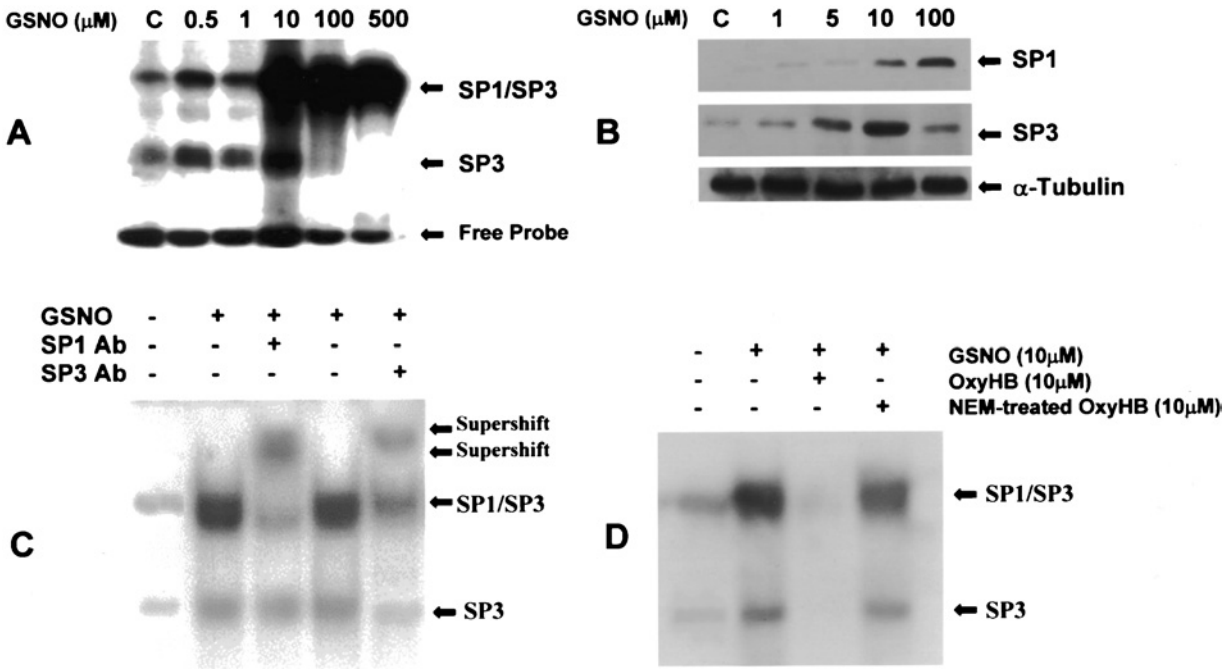

Figure 2 Effect of GSNO on Sp3/Sp1–DNA-binding activity in A549 cells

(**A**) EMSA using a consensus Sp3/Sp1 oligonucleotide as probe and nuclear extracts from control (lane 1) and GSNO-treated cells at concentrations of 0.5–500 μM (lanes 2–6). Physiological GSNO concentrations increased Sp3/Sp1–DNA binding. Supraphysiological GSNO concentrations inhibited Sp3 binding but augmented Sp1 binding. (**B**) Nuclear protein extracts were prepared from A549 cells treated with 1, 5, 10 and 100 μM GSNO for 6 h and then subjected to immunoblot analysis using anti-Sp1, Sp3 and α-tubulin antibodies. Molecular masses of Sp1 and Sp3 were 112 and 90–112 kDa respectively. Results are representative of three separate experiments. (**C**) The induction of Sp1/Sp3–DNA-binding activities by 10 μM GSNO was confirmed by supershift analysis. Antibodies recognizing Sp1 and Sp3 were added after the addition of radiolabelled oligonucleotide to the nuclear-binding reaction. Results are representative of three separate experiments; Ab, antibody. (**D**) EMSA was performed on nuclear extracts from A549 cells, with 5 μg of nuclear extracts from control and GSNO-treated cells (10 μM) in the presence or absence of 10 μM oxyHb, or oxyHb pretreated with 10 μM NEM for 4 h. Results are representative of three experiments.

density of bands B and C vary with GSNO concentration, probably reflecting different (transcriptional as well as post-transcriptional) mechanisms by which this expression is regulated by GSNO at different doses. For example, there is probably more post-translational stabilization, but less transcriptional up-regulation, with 10 μM GSNO when compared with 5 μM GSNO. In addition, 10 μM GSNO increased the expression and maturation of CFTR as a function of time. The expression level started increasing after 1 h with maximum induction of bands B and C occurring between 4 and 6 h (Figure 1B).

To evaluate the mechanism by which GSNO mediates its effects on the CFTR, A549 cells were treated with 10 μM GSNO in the presence or absence of 100 μM acivicin, an inhibitor of γGT-mediated GSNO bioactivation [6,10,12,29,30]. Treatment with acivicin decreased GSNO-mediated induction of CFTR maturation in A549 cells (Figure 1D), consistent with our previous observations in CFPAC-1 (cystic fibrosis pancreatic adenocarcinoma cells 1) and ΔF508 CFTR-expressing BHK cells (baby hamster kidney cells) [10]. Furthermore, when A549 cells were treated with 200 μM DDT during the last 30 min of GSNO exposure, the effect of GSNO was partially eliminated (Figure 1D), indicating the involvement of thiol groups. To evaluate whether the GSNO-induced increases in CFTR were the result of transcriptional and/or translational mechanisms, cells were treated with 20 μg/ml actinomycin D or 50 μg/ml cycloheximide in the presence of 10 μM GSNO (Figure 1D). In these cells, unlike other cell lines examined [10], both actinomycin D and cycloheximide inhibited the induction of bands B and C, although the effect of cycloheximide was greater when compared with that of actinomycin D. This suggests that, in A549 cells, the effect of 10 μM GSNO on CFTR induction and expression is both transcriptional and translational. It was noted that 100 μM GSNO inhibited CFTR expression (Figure 1A); maximum CFTR expression occurred between 2 and 4 h; and after 4 h of treatment the expression of bands B and C rapidly decreased (Figure 1C).

Effects of GSNO on Sp3/Sp1–DNA binding in A459 cells

To determine whether the transcriptional response to GSNO in A549 cells could involve activation and binding of Sp3 and Sp1, EMSAs were performed on nuclear extracts using a radiolabelled oligonucleotide containing an Sp3/Sp1–DNA binding site. At physiological GSNO concentrations ($\leqslant$ 10 μM), maximum induction of Sp3 and Sp1 binding was observed for 6 h. However, at nitrosative stress-associated concentrations, Sp3 binding started to decrease (Figure 2A). Binding to Sp3 and Sp1 was specific, as the addition of Sp3 or Sp1 antibodies supershifted Sp3- and Sp1-binding activities (Figure 2C), and the addition of unlabelled oligonucleotides decreased Sp3/Sp1–DNA-binding activity dose-dependently (Figure 3A). In control experiments, we have shown that GSNO does not have any effect on Sp3/Sp1–DNA-binding activity in Sp-deficient *Drosophila* SL2 (Schneider) cell lines. EMSA was performed with 5 μg of nuclear extracts from control and GSNO at various concentrations (0.5, 1, 5, 10 and 100 μM) for 6 h, but no binding was observed (results not shown). Consistent with these observations, the addition of MMA, an inhibitor of transcription factors that bind to GC-rich regions [28,31], resulted in concentration-dependent inhibition of both Sp3/Sp1–DNA-binding activities (Figure 3B). Furthermore, GSH had no effect at

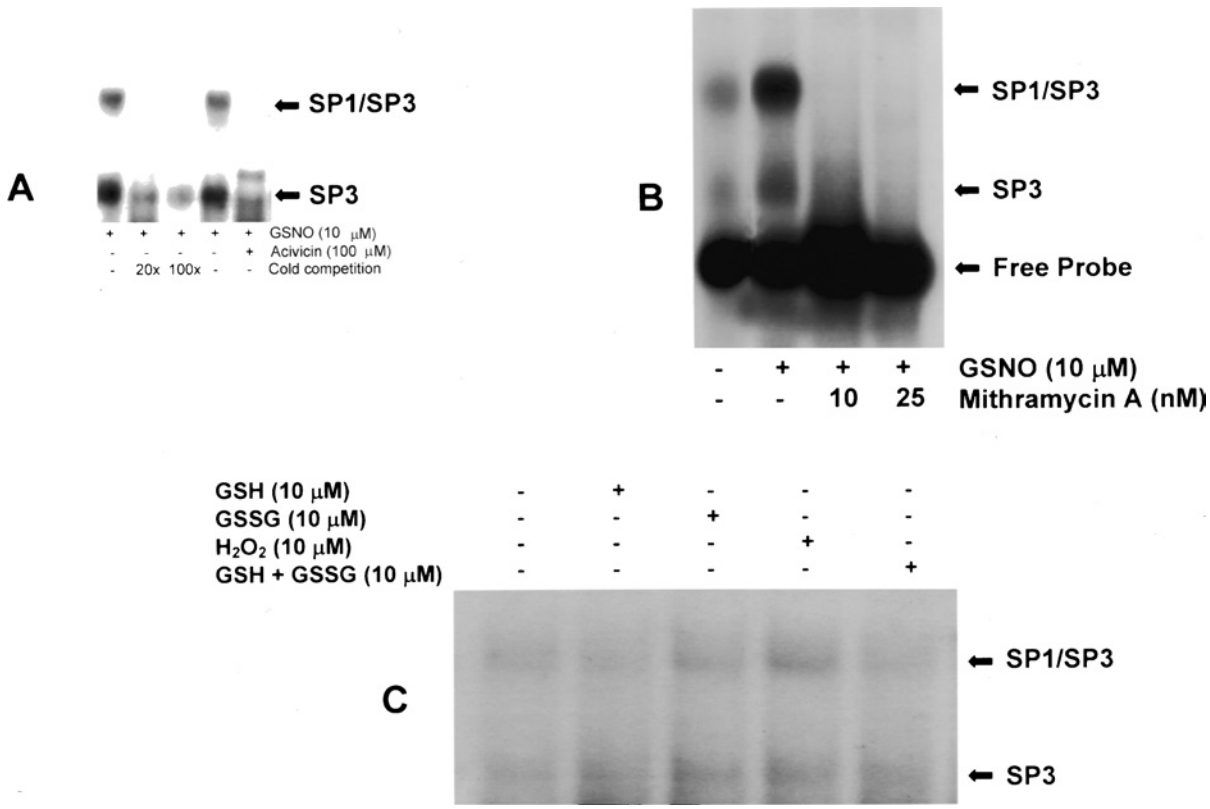

Figure 3 Effect of GSNO on Sp3/Sp1–DNA-binding activity is specific and is inhibited by acivicin and mithramycin

(**A**) EMSA was performed on nuclear extracts from A549 cells after 6 h of incubation with 10 μM GSNO either in the presence of competition with unlabelled probe (20 and 100 times) or in the presence of 100 μM acivicin. Competition with an unlabelled probe in lanes 2 and 3 demonstrated that this interaction is specific. Moreover, the effect of GSNO on Sp1 and Sp3 induction was prevented by 100 μM acivicin, an inhibitor of γGT-mediated GSNO bioactivation (lane 5; lanes are counted from left to right). (**B**) MMA inhibits GSNO-induced Sp3 and Sp1 binding in A549 cells. EMSA was performed on nuclear extracts from A549 cells after 6 h incubation with 10 μM GSNO in the presence of 10 and 25 nM MMA. Results are representative of three experiments. (**C**) GSH at a concentration of 10 μM for 6 h had no effect on Sp1/Sp3–DNA-binding activity. Moreover, GSSG and H_2O_2 at 10 μM had minimal effect. EMSA was performed with 5 μg of nuclear extracts from A549 cells treated with 10 μM GSH, GSSG and H_2O_2 for 6 h.

10 μM on Sp1–DNA or Sp3–DNA binding activity and similarly, GSSG and H_2O_2 at a concentration of 10 μM for 6 h had a minimal effect on Sp1/Sp3–DNA-binding activity (Figure 3C). These results suggest that GSNO at a concentration of 10 μM does not produce its effects through non-specific redox mechanisms.

To determine whether the effects of GSNO on Sp3 and Sp1 binding were mediated by effects on protein–DNA binding (i.e. through modification of a zinc finger site) and/or on protein stabilization (as for HIF-1), the effects of increasing concentrations of GSNO on Sp3 and Sp1 protein expression were studied. Sp3 and Sp1 protein expressions were altered by GSNO in a dose-dependent manner, with maximum induction occurring between 10 and 100 μM GSNO for Sp1 and between 5 and 10 μM GSNO for Sp3 (Figure 2B). However, (i) Sp3 expression was still detectable in the presence of 100 μM GSNO, although DNA binding was completely inhibited; and (ii) there was Sp1–DNA binding at 1 μM GSNO, although Sp1 expression was minimal. These observations suggest that GSNO increases Sp1 binding, and inhibits SP3 binding, by a mechanism that is partially independent of its effect on protein expression.

To examine the biochemical signalling process by which GSNO might affect Sp3/Sp1–DNA-binding activity, the role of GSNO as an NO donor was assessed by measuring the ability of oxyHb and oxyHb pretreated with NEM to block the Sp3/Sp1–DNA-binding effects of GSNO. A549 cells were treated with 10 μM GSNO in the presence and absence of dimeric (non-allosterically regulated) oxyHb untreated or pretreated with NEM to block the β93 cysteine thiol. OxyHb eliminated the ability of GSNO to induce Sp3/Sp1–DNA-binding activity, whereas NEM-treated oxyHb had no effect ($n = 3$ each; Figure 2D). Note that acivicin also inhibited the effect of GSNO on Sp3/Sp1–DNA-binding activity (Figure 3A), whereas it did not inhibit the release of NO from GSNO. Furthermore, 1 mM 8-Br cGMP, a cell-permeable cGMP mimic (downstream in the classical NO-guanylate cyclase pathway), did not affect Sp1 or Sp3 bindings (results not shown). Taken together, these observations suggest that the effect of GSNO on Sp3/Sp1–DNA-binding activity may not be dependent on the extracellular generation/diffusion of NO radical.

Effect of GSNO on CFTR mRNA in A549 cells

We next studied whether the activation of Sp3 and Sp1 induced by GSNO would cause a downstream effect on CFTR mRNA levels. GSNO at a concentration of 10 μM increased the amount of CFTR mRNA, whereas 100 μM GSNO decreased the amount (Figure 4A). Addition of 100 μM acivicin or 25 nM MMA for 30 min, followed by treatment with 10 μM GSNO for 6 h, decreased CFTR mRNA (Figure 4A). Northern-blot analysis, after transfection of A549 cells with Sp1, demonstrated that the amounts of CFTR mRNA in Sp1-overexpressing cells treated with GSNO were greater than those without GSNO (Figure 4B).

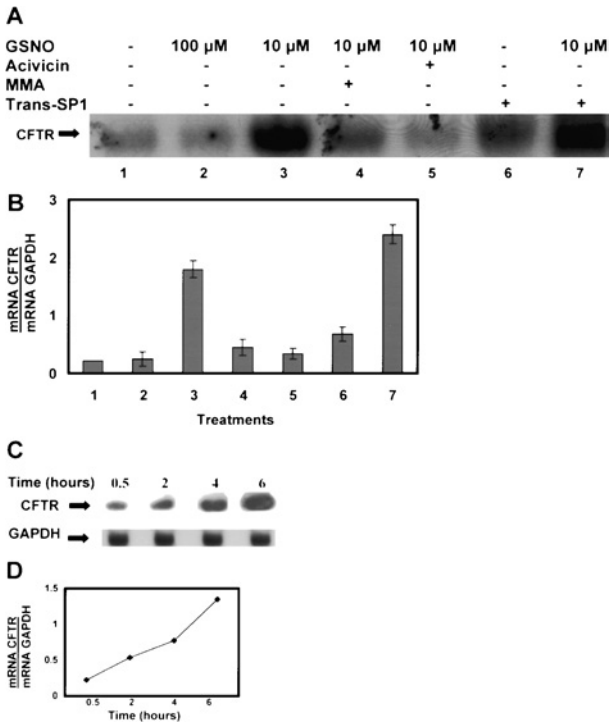

Figure 4 Northern-blot analysis of CFTR mRNA in A549 cells after treatment with GSNO

(**A**) GSNO induced the expression of CFTR mRNA. A549 cells were treated with 10 and 100 μM GSNO for 6 h; then mRNA was prepared, fractionated on 1 % agarose gel, transferred on to nylon membranes and hybridized with CFTR cDNA probe. A Northern-blot autoradiograph, representative of three independent experiments, is shown. Also, A549 cells were treated with 100 μM acivicin or 25 nM MMA for 30 min and subsequently treated with 10 μM GSNO for 6 h. mRNA was then analysed by Northern blot. A549 cells were transfected with 0.4 μg/well of CMV (cytomegalovirus) Sp1 expressor vector, or with 1.0 μg of CMV empty vector, in 12-well plates for 6 h. mRNA was extracted and subjected to Northern-blot analysis to detect CFTR mRNA levels. (**B**) mRNAs were quantified by densitometry and the amount of mRNA were normalized to those of GAPDH. Results are expressed as ratio of arbitrary units. Bars 1–7 represent the following conditions: 1, control; 2, 100 μM GSNO; 3, 10 μM GSNO; 4, 10 μM GSNO + 100 μM acivicin; 5, 10 μM GSNO + 25 nM MMA; 6, transfected Sp1; 7, 10 μM GSNO + transfected Sp1. (**C**) At 10 μM, GSNO increased the expression of CFTR, but not GAPDH, mRNA as a function of time. (**D**) Time-dependent increase in *cftr* mRNA by 10 μM GSNO. mRNAs were quantified by densitometry and the amounts of mRNA were normalized to those of GAPDH. Results are representative of three separate experiments and expressed as ratios of arbitrary units.

Furthermore, GSNO caused a rapid increase in steady-state levels of CFTR mRNA in a time-dependent manner (Figure 4C) and continued its effect for up to 6 h of treatment (Figure 4D), whereas the constitutively expressed GAPDH mRNA was stable under all conditions. These results indicate that the effect of GSNO on CFTR mRNA is dose- and time-dependent.

DISCUSSION

The endogenous S-nitrosylating agent, GSNO, increases the expression and maturation of CFTR in ΔF508 mutant homozygous pancreatic cells (CFPAC-1), ΔF508-transfected BHK cells, wild-type CFTR-transfected CFPAC-1 cells (CFPAC-1LJ6), BHK-wild-type transfected cells [10] and mIMCD3 (mouse inner medullary collecting duct cells) infected with ΔF508-recombinant adenovirus [12]. Additionally, GSNO increases the cell-surface expression and function of ΔF508 CFTR in mIMCD3-ΔF508 cells [12] and ΔF508 CFTR homozygous human airway epithelial cells [13]. Wild-type CFTR is expressed at low levels in many cell types; and *cftr* mRNA is detected in epithelial and non-epithelial cells [16]. Consistent with this relatively widespread mRNA expression, the 5′-flanking region of *cftr* has the characteristics of a housekeeping-gene promoter [16,17] and has a GC-rich Sp-binding site [16]. In the present study, we show that (i) physiological concentrations of GSNO favour the transcription of wild-type *cftr* in A549 airway epithelial cells in a time- and dose-dependent manner; (ii) this transcriptional effect of GSNO is mediated, partially, by activation of the transcription factors, Sp3 and Sp1; and (iii) at higher concentrations, GSNO actually inhibits wild-type *cftr* transcription and Sp3 binding.

We have shown previously that expression and activity of another transcription factor, HIF1, is increased by GSNO under normoxic conditions [6]. Additionally, activity of the inflammation-associated transcription factor, nuclear factor κB, is affected by S-nitrosylation chemistry [7]. Our current observations show that GSNO signalling also affects Sp3- and Sp1-dependent gene transcription, and that this transcriptional regulation by GSNO varies with GSNO concentration: physiological and nitrosative stress-associated concentrations have opposite effects on Sp3 and Sp1 expression and activity in parallel with their effects on Sp3- and Sp1-dependent gene transcription. Specifically, low-micromolar GSNO levels, similar to those in normal airway lining fluid and brain [9–10], augment Sp3-binding activity, whereas higher concentrations seen under conditions of nitrosative stress inhibit Sp3 binding and activity.

GSNO concentrations are regulated metabolically by synthetic and catabolic enzymes [14,29,30,32]. Furthermore, SNO concentrations are differentially regulated in different tissues and cell compartments [33]. Although activation of neuronal, endothelial and inducible nitric oxide synthase isoforms can each substantially increase SNO formation, SNO levels in a particular tissue are not a reflection of the activation of one type of SNO as opposed to another [49]. We suggest that levels of GSNO ordinarily maintained in the airway may support transcription of housekeeping genes in airway epithelial cells, partially through augmented Sp3- and Sp1-dependent transcriptional effects.

Enhanced Sp3–DNA binding in the presence of physiological concentrations of GSNO is associated with increased Sp3 expression. This may result, partially, from inhibition of Sp3 ubiquitination and proteosomal degradation. A similar effect has been proposed for the stabilization of the α subunit of HIF1, although higher levels (10–100 μM) of GSNO appear to be required [6]. At levels of GSNO associated with nitrosative stress, Sp3 binding and transcription of *cftr* are shut off, at least partially through inhibition of Sp3 expression. Clearly, there is an additional mechanism: Sp3 expression persisted even when there was no Sp3 binding at 100 μM GSNO.

For Sp1, this additional mechanism for enhanced DNA binding appears to be particularly relevant. For example, there is a strong Sp1 binding at 1 μM GSNO, although the expression is very weak. Furthermore, the expression is greater with 100 μM when compared with that at 10 μM GSNO, although DNA binding is comparable. This increase in Sp1 binding probably occurs through a post-translational cysteine S-nitrosylation, since it is (i) reversed by DTT, (ii) inhibited by acivicin, and (iii) not mimicked by 8-Br cGMP [6,7]. Identification of the target cysteine is an active area of investigation. This could involve S-nitrosylation of cysteine residues in the Sp3 and Sp1 zinc finger-binding domain [34,35].

Specifically for CFTR, our observations regarding Sp3 and Sp1 may be of importance in the light of recent evidence that high concentrations of NO donors inhibit wild-type CFTR expression. Our results suggest that this inhibition has a transcriptional component in A549 cells. In other cells, NO release, peroxynitrite formation, tyrosine nitration and augmented proteasomal

degradation are of central importance to the mechanism by which high-micromolar and -millimolar concentrations of NO donors decrease CFTR expression [15]. Consistent with all of these observations, high concentrations of inhaled NO are not of clinical benefit in CF, whereas low concentrations of inhaled GSNO improve clinical status of the patient [24,36].

Bioactivities of GSNO may be mediated by covalent thiol modifications, whereby proteins undergo post-translational modification through S-nitrosylation [37]. For these bioactivities, the more the NO radical released from GSNO, the less active the GSNO becomes [30,38]. We have observed that the effects of physiological GSNO concentrations on Sp3 and Sp1 binding and on CFTR expression are inhibited by preventing GSNO cleavage to a more cell-permeable SNO, *S*-nitroso-cysteinyl glycine [29,30], are reversed by DTT and are not mimicked by 8-Br cGMP. Furthermore, the effects are inhibited by dimeric oxyHb, on which the β93 cysteine is available for transnitrosation, but not by oxyHb on which this cysteine is blocked by NEM [39]. These observations suggest these bioactivities are mediated by transnitrosation from an intact S–NO bond, rather than by homolytic breakdown of GSNO to form NO radical. Thus if homolytic loss of NO from GSNO were affecting Sp3 and Sp1 binding, the effect would be prevented by NEM-treated oxyHb because the reactive iron centre is still available to scavenge NO radical. OxyHb and NEM-treated oxyHb differ only in that the thiol is blocked. Thus the scavenging effect of NEM-untreated oxyHb is most probably due to a thiol–thiol transfer, removing the bioactive nitrosonium equivalent from GSNO. This nitrosonium, in turn, would not be available to modify Sp1 and Sp3 protein activity.

Our observations that 10 μM GSNO has effects quite distinct from relatively inactive GSH, GSSG and H_2O_2 (10 μM), suggests that the mechanism of the GSNO effect may be different from previously reported redox modulation of Sp1 and Sp3 binding [28]. Notably, regulation of SNO signalling in association with Sp3 migration from one cell compartment to another, as has been demonstrated previously for caspases 3 and 9 [33], is possible.

Inhibition of the effects of GSNO on CFTR expression and maturation by cycloheximide suggests that there is a post-transcriptional effect of GSNO on CFTR in addition to the Sp3- and Sp1-mediated effect. Indeed, in many cells, this post-transcriptional effect appears to dominate [10]. The details of this mechanism are not clear. Most of the translated CFTR, both ΔF508 and wild-type, is degraded during a complex trafficking process [10,40]. Potential GSNO targets include the increased expression of the CFTR chaperone, Hsp70 (heat-shock protein 70), as well as S-nitrosylation of transcriptional regulators, ubiquitin ligases, cysteine proteases and/or cysteine string protein [40,41]. Specifically, GSNO can cause a cGMP-independent increase in Hsp 70 levels in hepatocytes [42], and increased Hsp 70 enhances the CFTR core glycosylation [15]. However, high doses (250–500 μM) are required, which may actually decrease with CFTR expression, while the physiological levels are inactive [42], and similar up-regulation has not been identified in non-hepatic cell lines [43]. With regard to proteolysis, S-nitrosylation-mediated inhibition of a ubiquitin ligase is possible, but may be non-specific, and degradation of CFTR is not known to involve a cysteine protease.

ΔF508 CFTR is at least partially functional when high-level expression and/or maturation cause it to be present on the cell membrane [44–46]. Both exposure to reduced temperature and treatment with chemical chaperones can result in increased surface expression of functional ΔF508 CFTR [46]. Recently [47], efforts have been made to alter the interaction of ΔF508 CFTR with the chaperone proteins through the application of the small-chain fatty acid derivatives such as 4-phenylbutyrate or sodium butyrate. The therapeutic benefit of these exogenous compounds is being studied [47].

Replacement of physiological levels of GSNO in the CF airway may also be appealing as a treatment to improve ΔF508 CFTR expression [10,12,24]. Airway levels of GSNO are low in the broncho-alveolar lavage fluid of patients with mild CF [26], perhaps because of decreased airway epithelial NO synthase expression [29], decreased glutathione levels [48] and/or increased GSNO catabolism [24]. Importantly, GSNO has several effects that are of potential benefit in the CF airway in addition to its effect on ΔF508 CFTR expression. These include bronchodilation, increased ciliary motility, inhibition of amiloride-sensitive sodium transport, augmentation of neutrophil apoptosis and antimicrobial effects [9,24,38]. Indeed, replacement of airway GSNO is well tolerated and improves oxygenation in CF patients [24]. To this list of CFTR independent and post-transcriptional CFTR effects of GSNO that may be of therapeutic importance, we now add an additional, transcriptional, effect. However, our results add an important caveat with regard to dosing S-nitrosylating agents in therapeutic development. Specifically, whereas physiological levels of GSNO in the airway may be of benefit, higher levels inhibit *cftr* transcription.

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