PERSPECTIVE

Is It Go or NO Go for *S*-Nitrosylation Modification-Based Therapies of Cystic Fibrosis Transmembrane Regulator Trafficking?

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ABSTRACT

Nitric-oxide synthases (NOS) are abundant in the respiratory epithelium and generate the NO radical, which can activate guanylate cyclase, react with superoxide, or modify proteins by *S*-nitrosylation (SNO) of Cys thiols. There is increasing appreciation that SNO modification is analogous to phosphorylation, because both signaling mechanisms modulate a wide range of cellular functions. Zaman et al. (p. 1435) in this issue report on the capability of *S*-nitrosoglutathione (GSNO) to increase the expression, trafficking, and function of mutant and wild-type cystic fibrosis transmembrane regulator (CFTR). The CFTR is a cAMP-regulated chloride channel that functions to regulate salt and water content in glands and ducts of secretory epithelia. GSNO is a low molecular weight SNO (*S*-nitrosothiol) formed during oxidation of NO. The authors use GSNO as a lead compound to restore mutant CFTR function. Earlier contradictory reports that GSNO decreased CFTR function by oxidative modification (glutathionylation) may now be explained by high concentrations of GSNO associated with decreased CFTR transcription and disruption of CFTR function. Zaman et al. show that at physiologic concentrations, GSNO and the constitutively active *S*-nitroso-glutathione diethyl ester stimulate CFTR transcription through SP1 and SP3 and promote normal trafficking. The mechanism behind rescue from the degradative pathway relies on increasing the expression of cysteine string proteins and SNO modification of chaperones involved in mediating CFTR transit through the endoplasmic reticulum and Golgi apparatus.

Why all the excitement surrounding nitric oxide, glutathione, and *S*-nitrosoglutathione (GSNO)? NO is a diffusible gas with a high affinity for hemoglobin and potent vasodilator properties through stimulation of cyclic GMP (Stamler et al., 1997). Exogenous NO is an approved treatment for pulmonary hypertension of the term newborn (Moya et al., 2002). Endogenous NO is formed from any of three isoforms of nitric-oxide synthase (NOS). NO combines with an electron acceptor such as oxygen (O_2) to form 2 NO_2, a relatively slow reaction when NO is nanomolar and oxygen is micromolar. Continued reaction of NO_2 with NO generates N_2O_3 or $^+ON \cdot NO_2^-$, which combine with an SH residue on a protein to make SNO protein and HNO_2 (Fig. 1A) (Gow et al., 1997). *S*-Nitrosylation is catalyzed by enzymes containing motifs that create partial electron acceptors. During synthesis of low molecular weight SNOs, such as GSNO from glutathione, NO is transferred from one molecule to another. In turn, enzymes exist to decompose or catabolize GSNO and other low molecular weight SNOs (Fig. 1B). Mutations in one such enzyme, copper zinc superoxide dismutase, are associated with accelerated GSNO catabolism and familial amyotrophic lateral sclerosis (Selverstone et al., 2005). The enzyme γ-glutamyl transferase (γGT) catalyzes the metabolism of GSNO to form glutamate and *S*-nitrosocysteinyl glycine (Hogg et al., 1997), and when γGT is absent, recovery from hypoxia is blunted.

Oxidative stress and protein oxidation were once considered markers of cell dysfunction and injury. However, oxi-

ABBREVIATIONS: GSNO, *S*-nitrosoglutathione; SNO, *S*-nitrosylation; eNOS, endothelial nitric-oxide synthase; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; HIF-1, hypoxia-inducible factor 1; GST, glutathione transferase.

dants are produced during the course of numerous signaling events, and it is questionable whether the word "stress" is accurate anymore (Paolicchi et al., 2002). Cysteine residues are the most reactive amino acids in proteins. Individual thiols become very reactive as pK_a decreases. A number of fates are possible from disulfide bridge formation to *S*-thiolation or *S*-glutathionylation (Fig. 1C). Zaman et al. (2006) show that *S*-nitrosylation promotes rescue of a common protein-folding mutation in CFTR responsible for most cases of classic cystic fibrosis.

Role of NO Biochemistry in Disease

NO-related diseases can be caused by either too much or too little NO. Vascular NO is generated from NOS_3 or eNOS. It is not surprising that disturbances in NO lead to cardiovascular illness. Polymorphisms in eNOS have been identified in introns, exons, and the eNOS promoter. These variations have sometimes been associated with hypertension, coronary artery disease and cardiac failure (Casas et al., 2004). However, other studies fail to make the link (Spence et al., 2004). The complexity of NO metabolism, the cellular milieu, and even environmental insults clearly affect the phenotype.

GSNO is a bronchodilator that is 2 log orders more potent than theophylline (Gaston et al., 1993). GSNO increases ciliary beat frequency, thereby improving mucociliary clearance. GSNO concentrations are low in airways affected by CF, and GSNO turnover is more rapid in asthmatic airways (Gaston et al., 2006). Thus, GSNO concentrations may be low in CF because of lower iNOS expression affecting synthesis, whereas in asthma, GSNO may be consumed faster and GSNO reductase is increased.

SNOs play a central role in hypoxic ventilatory drive (Lipton et al., 2001). Hypoxia-sensing cells in the carotid body stimulate neurons that project to NOS_1-rich neurons in the nucleus tractus solitarius. GSNO is a product of NOS_1 and, when processed by γ-glutamyl transpeptidase to *S*-nitroso-L-cysteine, increases minute ventilation. Systemic *N*-acetylcysteine therapy augments the hypoxic ventilatory drive and has been considered for use in preterm babies and patients with chronic obstructive pulmonary disease (Hildebrandt et al., 2002). High dose *N*-acetyl cysteine is under study to reduce inflammation in cystic fibrosis (Tirouvanziam et al., 2006). Oral *N*-acetyl cysteine was used in this phase I clinical trial to replenish the low levels of glutathione present in CF airways, thereby reducing neutrophil burden. It is noteworthy that there may be more than one positive effect from this supplement, because *N*-acetyl cysteine also seems to interfere with epithelial sodium channel surface expression and function and reduces the excessive sodium absorption complicating CF disease in vitro (Rochat et al., 2004). Aerosolized *N*-acetylcysteine is under development as an airway mucolytic (App et al., 2002) in part based on a long history of use in the gastrointestinal tract to relieve distal intestinal obstruction syndrome.

SNO signaling in red blood cells in the pulmonary microcirculation affects pulmonary vascular tone and ventilation to perfusion matching (Moya et al., 2001). Hemoglobin is *S*-nitrosylated at high partial pressure of oxygen, whereas NO groups are preferentially released at low partial pressure of oxygen (Jia et al., 1996). Vasodilation by red blood cells is proportional to the magnitude of hypoxemia. Patients with sickle cell disease have low levels of SNO-Hgb and abnormal transfer of NO from Hgb to the anion exchanger 1 (Pawloski et al., 2001). The defect worsens in acute chest syndrome and is absent in those who escape vaso-occlusive crises. Inhaled NO therapy during acute chest syndrome has been studied (Atz and Wessel, 1997).

Genetic Variation

Gene and protein expression is regulated by SNO reactions. Hypoxia-inducible factor 1 (HIF-1), SP1, SP3, nuclear factor κB (NFκB), and OxR have been well studied. Physiologic concentrations of SNOs tend to sustain transcription of normal physiologic genes, whereas stress elevation or excess exogenous levels induce stress response genes and proteins (Marshall et al., 2000). HIF-1 is a dimer and in response to hypoxia, the α subunit is stabilized, binds to the β subunit, and induces transcription of hypoxia-related genes. SNOs stabilize the HIF-1α in normoxia (Palmer et al., 2000). HIF-1 and NFκB increase NOS2, which feeds back and inhibits NFκB-mediated transcription via the P50 subunit and the IκB. In the example under consideration today, GSNO levels that enhance CFTR expression and maturation also seem to be levels considered "low" in airways affected by CF. Higher doses than those administered in the study would possibly reduce transcription of the very gene whose protein expression was being up-regulated.

When reactive oxygen species and reactive nitrogen species overwhelm cellular antioxidant levels, proteins progressively become modified or damaged by processes that include carbonylation, *S*-thiolation, or *S*-glutathionylation. *S*-Glutathionylation may inactivate a protein; however, it is reversible when the redox balance is restored. *S*-Glutathionylation modifies the activity of cyclic AMP-dependent protein kinase, creatine kinase, and protein phosphatase 2a (Rao and Clay-
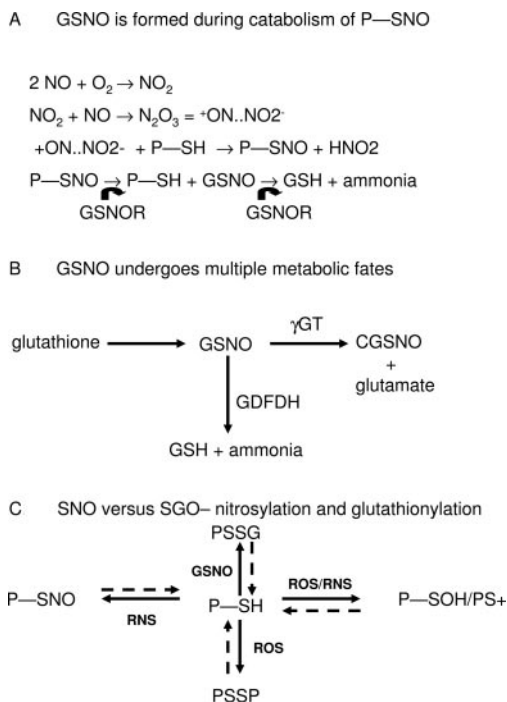

Fig. 1. Synthesis and catabolism of GSNO. A, GSNO is formed during catabolism of *S*-nitrosylated proteins from the activity of GSNO reductase. B, metabolic fates of GSNO are cell and tissue-dependent. C, proteins can undergo modification by *S*-nitrosylation or glutathionylation.

ton, 2002). Hsp27 is a target for *S*-glutathionylation, which modulates its chaperone activity (Eaton et al., 2002). Diseases that have *S*-glutathionylation as a feature include Friedrich's ataxia, HIV, and diabetes types 1 and 2. Whether increasing levels of these proteins are markers of disease development or whether early intervention during oxidative stress would be protective (Giustarini et al., 2004) is not known.

Given the relatively modest number of genes identified by the human genome project, it is comforting to find that our genetic makeup is also heavily influenced by polymorphic variation, gene splicing, exon shuffling, multimeric protein subunit combinations, and post-translational modifications. In addition to phosphorylation, glycosylation, methylation, acetylation, and *S*-nitrosylation (Arnelle and Stamler, 1995), glutathionylation can occur on proteins otherwise modified by one or more of the other reactions. The addition of a glutathione to a cysteine residue adds a net negative charge and thus has the potential of interfering in quaternary structure or in function.

Glutathione transferases form a superfamily of isozymes that contain polymorphic variation (McIlwain et al., 2006). Certain of them are overexpressed in tumors and thus have become drug targets. The human GSTs have been divided into three families: cytosolic, mitochondrial, and membrane bound. The cytosolic forms are further subdivided into seven classes based on sharing over 60% homology: Alpha, Mu, Omega, Pi, Sigma, Theta, and Zeta. The highly conserved N-terminal domain contains a catalytically active tyrosine, cysteine, or serine residue. This residue interacts with the thiol group of glutathione. The five Alpha genes are expressed mainly in the liver and participate in GSH-dependent detoxification of carcinogens (Coles and Kadlubar, 2005). Aberrant expression is associated with ovarian and colorectal cancers (McIlwain et al., 2006). Five genes in the Mu class have been implicated in bladder cancer. Polymorphisms are distributed along racial lines. The GSTM1 null phenotype is associated with increased risk of lung, colon, and bladder cancers. Several groups have suggested that the homozygous GSTM1-null genotype is associated with more severe CF lung disease, possibly as a result of decreased glutathione concentrations and reduced anti-oxidants (Acton and Wilmott, 2001; Merlo and Boyle, 2003). The Theta class of two subfamilies, TT1 and TT2, is associated with increased risk of head, neck, and oral cancers. The epidemiological linkage of polymorphisms in GST and cancer incidence or response to chemotherapy is tantalizing, and at least one investigational drug targeted to inhibiting activity of a GST is in development (McIlwain et al., 2006).

Therapeutic Pipeline for Cystic Fibrosis

Where does this work fit into the investigational drug pipeline for CF? None of the available, FDA-approved therapies for CF addresses the fundamental defect in CFTR expression and function. More than 1000 different CFTR mutations have been identified, and they can be grouped according to the nature of the defect. The ΔF508 mutation is the most common, and it has two defects, a reduced chloride conductance because of a reduced open time for chloride and premature ubiquitination and degradation from the endoplasmic reticulum. To "correct" this mutation, it may be necessary to block the ΔF508 from entering the proteasome,

encourage its transit through the Golgi apparatus to the plasma membrane, and slow recycling to the endosomal compartment. Can GSNO or *S*-nitroso-glutathione diethyl ester correct the fate of ΔF508 or all of the class II mutations? Zaman et al. (2006) suggest that ΔF508 is interacting with cysteine string proteins and other SNO-modified proteins as it is chaperoned along the maturation pathway. Zhang et al. (2006) knocked down cysteine string protein expression with RNA interference and observed increased maturation of CFTR. Cysteine string proteins seem to block exit from the endoplasmic reticulum in a mechanism independent of the classic NO radical/cGMP pathway and oxidation/glutathionylation. This is consistent with the observations that inhaled NO has not developed into a useful therapy for CF. Low concentrations of GSNO may be useful alone or in combination with other strategies to block CFTR from entering the aggresome or the proteasome. Additional correction of the chloride conductance defect in ΔF508 ("potentiation") is likely to be vital to full correction of CFTR activation of the outwardly rectifying chloride channel and CFTR inhibition of sodium reabsorption through the epithelial sodium channel.

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