A Genetic Analysis of Nitrosative Stress

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ABSTRACT: Nitrosative stress is induced by pathophysiological levels of nitric oxide (NO) and *S*-nitrosothiols (e.g., *S*-nitrosoglutathione, GSNO) and arises, at least in significant part, from the nitrosylation of critical protein Cys thiols (*S*-nitrosylation) and metallocofactors. However, the mechanisms by which NO and GSNO mediate nitrosative stress are not well understood. Using yeast *Saccharomyces cerevisiae* strains lacking NO- and/or GSNO-consuming enzymes (flavohemoglobin and GSNO reductase, respectively), we measured the individual and combined effects of NO and GSNO on both cell growth and the formation of protein-bound NO species. Our results suggest an intracellular equilibrium between NO and GSNO, dependent in part on cell-catalyzed release of NO from GSNO (i.e., "SNO-lyase" activity). However, whereas NO induces multiple types of protein-based modifications, levels of which correlate with inhibition of cell growth, GSNO mainly affects protein *S*-nitrosylation, and the relationship between *S*-nitrosylation and nitrosative stress is more complex. These data support the idea of multiple classes of protein−SNO, likely reflected in divergent routes of synthesis and degradation. Indeed, a significant fraction of protein *S*-nitrosylation by NO occurs in the absence of O_2, which is commonly assumed to drive this reaction but instead is apparently dependent in substantial part upon protein-bound transition metals. Additionally, our findings suggest that nitrosative stress is mediated principally via the *S*-nitrosylation of a subset of protein targets, which include protein SNOs that are stable to cellular glutathione (and thus are not metabolized by GSNO reductase). Collectively, these results provide new evidence for the mechanisms through which NO and GSNO mediate nitrosative stress as well as the cellular pathways of protein *S*-nitrosylation and denitrosylation involving metalloproteins, SNO lyase(s) and GSNO reductase.

Nitric oxide (NO)[1] exerts its cellular influence largely through nitrosylation of protein transition metal prosthetic groups, as in the case of guanylate cyclase, and of cysteine thiol side chains (*S*-nitrosylation) in proteins of most or all functional classes (*1, 2*). NO groups may also be covalently bound to cysteine thiol in peptides, in particular glutathione (to form GSNO), and to transition metals as heme−NO or dinitrosyl non-heme iron complexes; these metal nitrosyls can serve as sources of NO^+ equivalents for protein *S*-nitrosylation (*1, 2*). There is as yet no unifying scheme that encompasses the transactions between NO and its substrates, and between NO-bearing species, which govern the disposition of NO groups in the cellular milieu.

In the model eukaryote, yeast *Sacchromyces cerevisiae*, flavohemoglobin (FHb; encoded by the *yhb1* gene) and GSNO reductase (GSNOR; encoded by the *sfa1* gene) efficiently metabolize NO and GSNO, respectively, and serve as the principal cellular mechanisms for inactivation of these species (*3, 4*). Accordingly, we have shown that FHb deletion sensitizes yeast cells to NO, which coincides with increased protein *S*-nitrosylation. In the human pathogens *Cryptococcus neoformans* (*5*) and *Salmonella typhimurium* (*6*), FHb mutants have attenuated virulence in a mouse model of infection, and virulence is restored in mice lacking inducible NO synthase (iNOS) (*5, 6*). Deletion of both FHb and GSNOR further decreases virulence of *Cryptococcus neoformans* (*5*). Similarly, it has recently been shown that genetic deletion of GSNOR decreases virulence of *Steptococcus pneumoniae* (*7*). In yeast, we have shown that GSNOR deletion results in increased sensitivity to nitrosative stress induced by exogenously applied GSNO (*3*). In mice, elimination of GSNOR is coupled to an increase in SNO-protein levels that is greatly exaggerated under conditions of iNOS upregulation (e.g., bacterial infection, sepsis) and linked to tissue injury and death (*8*).

Although previous studies address the individual roles of FHb and GSNOR in nitrosative stress resistance and the modulation of protein−SNO levels, they do not address the conjoint roles of these enzymes in governing the cellular equilibrium between NO and GSNO. Here, we utilized genetic deletion of FHb and/or GSNOR, in combination with administration of relatively low levels of NO or SNO, to modulate selectively the proportional distribution of NO congeners and to elucidate the interrelationships between NO-bearing low molecular weight species and SNO-proteins as well as the redox (O_2, transition metals, glutathione)

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[1] Abbreviations: NO, nitric oxide; SNO, *S*-nitrosothiol; GSNO, *S*-nitrosoglutathione; SNCEE, *S*-nitrosocysteine ethyl ester; GSNOR, *S*-nitrosoglutathione reductase; FHb, flavohemoglobin; DEANO, diethlamine NONOate; DETA−NO, diethyltriamine NONOate; DFO, deferoxamine.

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requirements that govern physiological protein *S*-nitrosylation. The overall scheme for cellular protein *S*-nitrosylation that emerges from our observations should be of broad utility in analysis of the mechanisms that generate nitrosative stress and may also apply generally to analysis of physiological signal transduction that is mediated or modulated by NO in eukaryotic cells.

EXPERIMENTAL PROCEDURES

Construction of sfa1 yhb1 Double Mutant. The haploid yeast strain Y190 and its isogenic *sfa1* (*sfa1*Δ*::KanMX2*) and *yhb1* (*yhb1*Δ*::KanMX2*) mutant strains have been described previously (*3, 4*). To construct *sfa1 yhb1* double mutants, the entire open reading frame of the *YHB1* gene in the haploid *sfa1*Δ*::hphMX* strain was replaced by the KanMX2 cassette as described for the *yhb1* single mutant (*2*). The resultant clones were selected on yeast extract/peptone/dextrose (YPD) plates supplemented with G418 (200 μg/mL) and hygromycin (200 μg/mL). Replacement of the YHB1 gene by KanMX2 was confirmed by PCR amplification of a YHB1-KanMX2 fusion fragment using primers HMP1512se (*3*) and Kan560as (5′-GCTACCTTTGCCATGTTTCA-3′). PCR detection of wild-type, *YHB1* and *SFA1* genes was performed as previously described (*3, 4*), and detection of the *sfa1*Δ*::hphMX* construct was performed using the primers FDH754se and Hgh594as (5′-CCGATGCAAAGTGCCGATAA-3′). GSNO reductase (GSNOR) activity was assayed as described previously (*3*).

NO Consumption. Overnight cultures were washed with phosphate-buffered saline, pH 7.4 (PBS) containing 0.1 mM EDTA (PBS/EDTA) and resuspended at $OD_{600} \sim 1$. Four microliters of a saturated aqueous NO solution was added to 4 mL of a stirred solution of yeast, and NO consumption was measured using an amino-30 electrode (Innovative Instruments, Inc., Tampa, FL). Nitric oxide concentrations were determined using a standard curve generated by addition of sodium nitrite to a solution of 0.1 M potassium iodide in 0.1 M sulfuric acid.

Growth Inhibition. Mid-log phase yeast ($OD_{600} = 0.4-0.6$ following overnight culture) was diluted to an OD_{600} of ~0.05 and cultured aerobically at 30 °C in YPD medium (yeast extract/peptone/dextrose) supplemented with diethyltriamine NONOate (DETA−NO; Cayman Chemical, Ann Arbor, MI) (2 mol of NO/mol of DETA−NO; $t_{1/2}$ of NO release ~ 40 h) or GSNO. Cell growth was measured after 10 h as OD_{600} (*3*).

Measurement of SNO-Lyase Activity. *S*-Nitrosocysteine ethyl ester (50 mM) was added at a final concentration of 50 μM to a stirred solution of yeast ($OD_{600} \sim 7$ in 4 mL of PBS containing 0.1 mM DTPA (PBS/DTPA)), and NO formation was measured as described above. For measurements of NO release from GSNO, yeast cells were permeabilized as described previously (*9*). Briefly, cells were resuspended at OD_{600} in 0.1 M MOPS pH 6.5 containing 0.1% digitonin. Yeast were incubated at 30 °C for 30 min and washed 3× with PBS/DTPA. Cells were heat inactivated at 95 °C for 10 min in PBS/DTPA.

Measurement of Aerobic Nitrosylation. Mid-log phase yeast cells were diluted to $OD_{600} \sim 1$, exposed to DETA−NO (3 mM) for 1 h or GSNO (5 mM) for 2 h at 30 °C, pelleted and washed with PBS, resuspended in lysis buffer (PBS containing 20 mM DTPA, 0.5 mM EDTA, 0.1% NP-40 and 1 mM PMSF) and disrupted with a bead beater (Biospec). Fractions containing only high molecular weight species (protein) were generated by centrifugation of lysates though Bio-Gel P-6 (Bio-Rad), and fractions containing only low-mass species comprised the flow-through from lysates spun through a 5 kDa cutoff ultrafiltration membrane. Photolyzable NO species (including XNOs and SNOs) were measured by photolysis-chemiluminescence (*10*) (Nitrolite photolysis unit and TEA 510 analyzer, Thermo Orion) and differentiated by addition of $HgCl_2$. Specifically, XNOs were quantified as the signal that survived the treatment of samples with 2.5 mM $HgCl_2$ or 2 mM *p*-chloromercuriphenyl sulfonic acid for 10 min at room temperature, and SNOs (mercury-sensitive signal) were quantified as the difference between total NO and XNO (*11*). Data were normalized with respect to protein content of the whole-cell lysate. Protein−NO complexes (SNO plus XNO) were also quantified by subtracting the low-mass from the total photolyzable NO in the lysate.

Anaerobic vs Aerobic Nitrosylation. Yeast were grown overnight in YPD medium ($OD_{600} \sim 1$), washed with PBS/DTPA and resuspended at $OD_{600} \sim 40$. For anaerobic treatments, cell suspensions were sparged with N_2 in sealed vials for 30 min, then treated for 30 min at 30 °C with 100 μM diethylamine NONOate (DEA−NO; Cayman Chemical, Ann Arbor, MI) (1.5 mol of NO/mol of DEA−NO; $t_{1/2}$ of NO release ~ 9 min under these conditions). Following 4 washes with PBS/DTPA (N_2-sparged for anaerobic treatment, and carried out in a glovebox maintained at <1 ppm O_2), cells were lysed with a bead beater in lysis buffer and protein S(X)NO was separated by gel filtration and quantified as above.

RESULTS

Yeast Genetic Models for the Study of Protein S-Nitrosylation. We employed previously generated *yhb1*- and *sfa1*-deficient yeast strains, and in addition constructed the double knockout *yhb1 sfa1* strain (Figure S1A in the Supporting Information), to analyze the roles of NO and GSNO in nitrosative stress as well as the speciation of protein−NO conjugates under nitrosative stress conditions. Whereas the wild-type and *sfa1* strains rapidly consumed NO (Figure 1A), this new strain, like the *yhb1* mutant, was largely deficient in NO-consuming activity (compared to buffer alone). Furthermore, *yhb1 sfa1* yeast had no apparent growth defects compared to the wild-type under normal conditions or under oxidative stress induced by exposure to H_2O_2 (Figure S1B in the Supporting Information), as is the case for deletion of FHb or GSNOR alone (*3, 4*).

Effects of Nitrosative Stressors on Growth of Yeast Mutant Strains. Differential effects of FHb and/or GSNOR deletion on nitrosative stress induced by NO or GSNO should be indicative of the forms of NO that convey biological activity. Thus, we first examined the effects of NO and of GSNO on the growth of wild-type vs mutant yeast strains. As reported previously (*4*), treatment with the NO donor DETA−NO significantly retarded growth of the *yhb1* mutant vis-à-vis wild-type cells (Figure 1B). In contrast, the effects of DETA−NO were indistinguishable between the *sfa1* mutant and the wild-type (Figure 1C), and the effects of combined mutation of *yhb1*

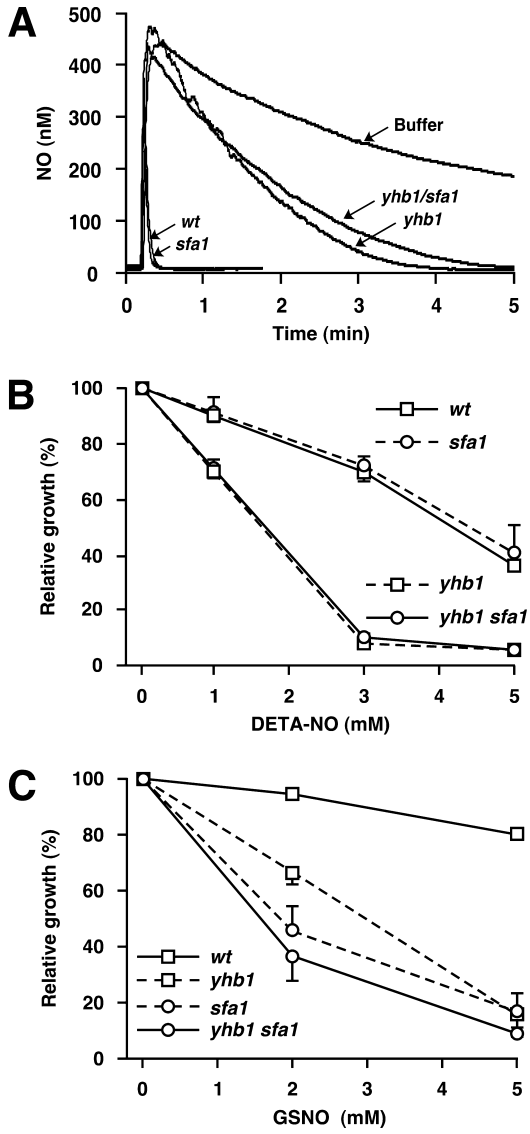

FIGURE 1: NO consumption by and growth of wild-type and mutant yeast cells under nitrosative stress. (A) Consumption of aqueous NO (~1−2 μM) was measured with an NO-electrode in control buffer (PBS/EDTA) or in buffer containing OD_{600} ~ 1.0 of wild-type (Y190), isogenic *sfa1* (lacking GSNOR), *yhb1* (lacking FHb) or *sfa1 yhb1* double mutant cells. (Note that traces from wild-type and *sfa1* yeast are essentially indistinguishable.) (B and C) Mid-log phase wild-type and mutant strains were cultured for 10 h in the presence of various concentrations of (*B*) the NO donor DETA−NO or (*C*) GSNO. Traces in A are representative of 2−3 independent experiments. Cell growth was measured as OD_{600}, and values (mean ± SD) were derived from 2−3 independent experiments.

and *sfa1* were indistinguishable from those resulting from mutation of *yhb1* alone. Accordingly, GSNOR does not confer protection against NO under these conditions. GSNOR does, however, confer protection against SNO-induced nitrosative stress, as evidenced by the retardation of growth of the *sfa1* strain as compared to wild-type in the presence of GSNO (Figure 1C). Finally, consistent with the generation by GSNO of both SNO- and NO-induced stress, the effects of GSNO on the *sfa1 yhb1* double-knockout were greater than the effects of GSNO on either of the single-knockout strains (Figure 1C).

Yeast Cell-Dependent "SNO-lyase" Activity. It was noteworthy that a significant degree of protection during exposure to GSNO is provided by FHb, as evidenced by the retarded

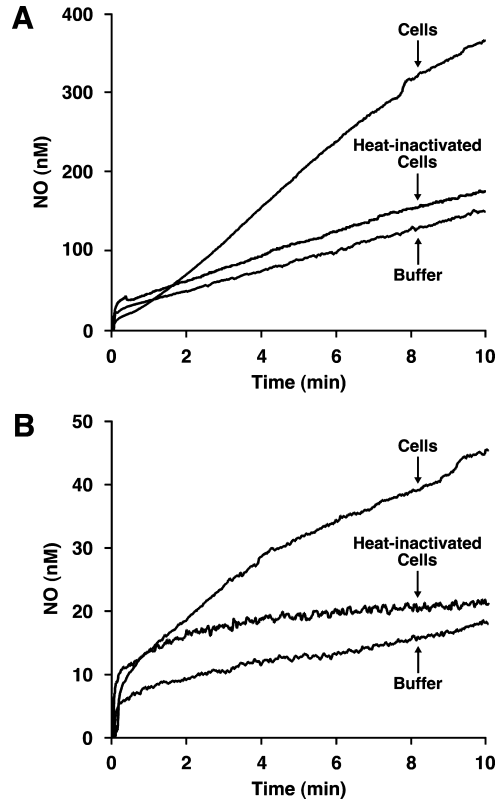

FIGURE 2: Cell-catalyzed SNO-lyase activity. 50 μM CysNO ethyl ester (A) or GSNO (B) was added to buffer (PBS/DTPA) alone, or buffer containing *sfa1 yhb1* double mutant cells or heat-inactivated cells, and NO release was measure with an NO-electrode. Traces are representative of 2−3 independent experiments.

growth of *yhb1* mutants relative to wild-type cells. Thus, GSNO appeared to serve as a significant cellular source of bioactive NO, consistent with previous descriptions of cell-mediated homolysis or reductive cleavage of SNO ("SNO-lyase" activity) in bacterial and mammalian cells, which was revealed initially by the production of NO from exogenous SNO in both cell lysates (*12*) and intact cells (*13*−*15*), and more recently by the stimulus-induced generation of intracellular NO from endogenous *S*-nitrosothiol (*16*).

We examined directly the production of NO from *S*-nitrosothiol by yeast using an NO-sensitive electrode. Although we could not detect cell-mediated NO release from GSNO with intact yeast (not shown), consistent with a slow rate of GSNO uptake, NO release from the cell-permeable SNO, *S*-nitrosocysteine ethyl ester (SNCEE), was greatly accelerated in the presence of yeast (Figure 2A). In addition, digitonin-permeabilized yeast (in which proteins are *S*-nitrosylated by GSNO much more readily than in intact yeast (unpublished observations)) potentiated NO release from GSNO (Figure 2B). Boiling of cells greatly diminished cell-catalyzed SNO-lyase activity with either substrate, suggestive of a protein- or enzyme-dependent activity (Figure 2A,B). Both SNCEE and to a lesser extent GSNO appeared to release NO in the absence of cells, suggesting that the decomposition of SNO to NO in the extracellular milieu may also generate bioactive nitric oxide.

Differential Protein Nitrosylation in Mutant Strains. The distinguishable roles of FHb and GSNOR in protection from nitrosative stressors indicates that NO and GSNO may mediate their effects through different levels or types of protein-based modifications, which can include *S*-nitrosyla-

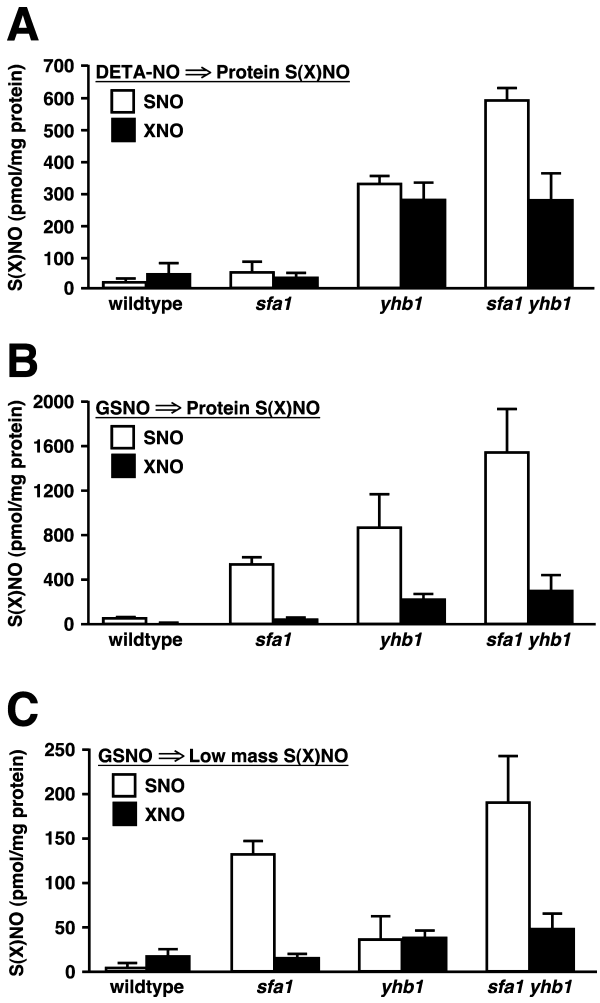

FIGURE 3: Nitrosylation in yeast cells under nitrosative stress conditions. Mid-log phase cells were cultured in room air in the presence of (A) DETA−NO (3 mM, 1 h); (B,C) GSNO (5 mM, 2 h). Protein−SNO (Hg^+-sensitive signal), protein−XNO (Hg^+-insensitive signal), low mass SNO (<5 kDa) and low mass XNO were measured by photolysis-chemiluminscence. Values (mean ± SD) were derived from a minimum of three independent experiments.

tion, *Fe*-nitrosylation and *N*-nitrosation. We therefore used the various deletion strains to assess the differential targeting of NO groups derived selectively from NO vs SNO under conditions identical to those under which growth inhibition was measured. We employed mercury-coupled photolysis-chemiluminescence to quantify the steady-state levels of protein-bound and low-mass nitroso/nitrosyl adducts (SNO, XNO) that formed after incubating mid-log phase cells with either GSNO or DETA−NO. Photolysis-chemiluminsecence identifies SNOs based on their selective decomposition by mercury(II) (*10*), although it cannot discriminate between the remaining photolyzable mercury-insensitive species (XNOs), which include metal-nitrosyls but perhaps also nitrosated amino acid side chains or nucleic acids (e.g., N−NO) (*10, 11*).

We first examined protein nitrosylation by NO donor. Following aerobic (room air) exposure to DETA−NO (3 mM; 1 h), wild-type cells had similar, low levels of protein SNO and protein XNO, which were altered only minimally in GSNOR-deficient cells (Figure 3A). These data are consistent with the growth assays and indicated that metabolism by FHb was sufficient to largely prevent both protein *S*-nitrosylation and the formation of protein XNOs. In contrast, protein SNOs were ~15-fold higher and protein XNOs ~10-fold higher in FHb-deficient cells than in wild-type cells. Since protein *S*-nitrosylation increased despite the presence of GSNOR, this pool of protein SNOs is evidently inaccessible to or relatively unreactive to cellular glutathione/GSNO (*17*). The deletion of both genes exerted a synergistic effect on protein *S*-nitrosylation without altering XNOs (Figure 3A): levels of SNO-protein in *sfa1 yhb1* cells after DETA−NO treatment exceeded the sum of SNO-protein in the *sfa1* and *yhb1* mutants by about 1.6-fold, consistent with the existence of a second subset of protein SNOs, which are in equilibrium with GSNO and therefore regulated by GSNOR. Even at doses of DETA−NO (1 mM) that were only partially cytostatic, the *sfa1 yhb1* and *yhb1* strains grew similarly, suggesting that these additional SNOs do not play an important role in nitrosative stress. Thus, among the pools of NO-derived species we are able to discriminate between, protein XNOs and GSH-inaccessible SNOs are evidently sufficient to elicit the maximal cytostatic effects of NO.

Incubation of yeast with GSNO (5 mM; 2 h) generated relatively low levels of protein SNO in wild-type yeast, which were increased significantly in each of the mutants (Figure 3B), consistent with their GSNO-dependent growth phenotypes. Modulation of protein *S*-nitrosylation by FHb confirms that GSNO can serve as an endogenous source of NO in addition to directly affecting *S*-nitrosylation. Under these conditions, SNO-protein levels were higher than those in NONOate-treated cells, reflecting different rate-limiting steps (GSNO uptake vs NO release, respectively). Because the level of SNO-protein in *sfa1 yhb1* cells was approximately equal to the sum of SNO-protein levels in *sfa1* and *yhb1* cells (Figure 3B), the functions of FHb and GSNOR in regulating protein *S*-nitrosylation by GSNO appear to be independent and additive, which indicates that NO derived from GSNO is not redistributed as low mass SNO. SNO levels were higher in the *yhb1* than *sfa1* cells, providing further evidence that major routes to protein−SNO via NO do not substantially involve transnitros(yl)ation by GSNO (transfer of NO^+ from SNO to thiolate) and thus are not significantly modulated by GSNOR. Only a minor increase in protein XNO was observed in *sfa1* vis-à-vis wild type cells. However, larger increases were observed in the FHb-deficient *yhb1* and *sfa1 yhb1* cells following incubation with GSNO (Figure 3B). Therefore, the formation of protein XNOs is predominantly NO-dependent but GSNO concentration-independent.

Low-mass SNO is thought to exist primarily in the form of GSNO in all eukaryotic cells (*3*), reflecting the high concentration of glutathione in comparison to other thiols. The quantitation of low mass SNOs from GSNO-treated cells would further help to discriminate between GSNO vs NO-mediated protein *S*-nitrosylation. Treatment of *sfa1* cells with GSNO resulted in a significant increase in the levels of low-mass SNO compared to the wild-type, in which levels were negligible (Figure 3C). The parallel increases in low-mass SNO and SNO-protein in *sfa1* cells indicate that, in the presence of a high NO-consuming activity, protein *S*-nitrosylation by GSNO occurs predominantly via transnitros(yl)ation. In contrast, whereas levels of protein SNO were greater in *yhb1* than in *sfa1* cells, the FHb-deficient strain had comparatively lower levels of low-mass SNOs, consistent

with metabolism by GSNOR (Figure 3C). The lack of correlation between the levels of low-mass and protein SNO in *yhb1* cells provides further evidence that, in the absence of FHb, NO derived from GSNO mediates protein *S*-nitrosylation directly. Note that the lower absolute levels of low-mass vs protein SNO in all mutant strains (including GSNOR-deficient cells) suggest that the equilibrium between these two nitrosothiol pools favors protein SNO. Collectively, these data indicate that protein SNO may form in significant part both directly from NO and via GSNO, whereas transnitros(yl)ation from SNO may contribute less to the formation of protein XNO than previously suspected.

Mechanisms of NO-Dependent Protein S-Nitrosylation. Our growth data and SNO measurements suggest that the reaction of NO with protein thiol (*5*) or thiyl radical (*51*) may contributes to nitrosative stress. In contrast to transnitros(yl)ative transfer of an NO^+ group by GSNO, protein *S*-nitrosylation by free radical NO (whether through a GSNO intermediate or direct reaction with protein thiol) requires a one-electron oxidation that can, in principle, be subserved by a number of electron acceptors, including transition metal ions and dioxygen (*1*). Although current dogma—which is based mainly on theoretical calculations and experiments with low molecular weight thiols *in vitro*—suggests that O_2 and perhaps reactive oxygen species (ROS) serve as the principal oxidants, *S*-nitrosylating reactions involving those species may be kinetically disfavored at physiological NO (and superoxide dismutase) concentrations (*18*), and empiric correlations between NO-dependent *S*-nitrosylation and O_2/ROS levels have not emerged in previous analyses (*19*).

We first examined the extent to which O_2 or ROS support protein *S*-nitrosylation in intact cells. Protein SNOs and XNOs under aerobic (room air) and anaerobic conditions were compared in *sfa1 yhb1* yeast exposed to the NO donor DEA−NO (100 μM; 30 min). Whereas DETA−NO (*20*) delivers a sustained flux of nitric oxide that recapitulates NO production by stimulated macrophages (*21*), the release of NO from DEA−NO (*20*) more closely resembles the rapid and transient production of NO by cells upon receptor stimulation, e.g. G protein-coupled receptors (*22*). Following DEA−NO treatment, levels of protein XNOs and of protein SNOs were roughly equal, under both aerobic and anaerobic conditions (Figure 4A). Strikingly, levels not only of XNO but also of SNO were generally comparable in anaerobic vs aerobic conditions (somewhat higher absolute yields of both XNO and SNO under anaerobic conditions probably reflect the extended half-life of NO in the absence of oxygen) (Figure 4A). Furthermore, incubation of cells with the radical scavenger 5,5-dimethylpyrroline-*N*-oxide (DMPO; 1 mM, 30 min pretreatment) prior to addition of DEA−NO had no effect on aerobic protein *S*-nitrosylation, suggesting that a route through thiyl radical was not significant under our experimental conditions (data not shown). These findings indicate directly that O_2/ROS are not required for protein *S*-nitrosylation *in situ*, consistent with a previous analysis in isolated mammalian mitochondria that demonstrated largely overlapping sets of *S*-nitrosylated proteins following administration of NO under aerobic or anaerobic conditions (*23*).

We further examined the extent to which redox-active metal ions (in particular, iron) promote protein *S*-nitrosylation by nitric oxide. Chelation of free iron (which is at low levels

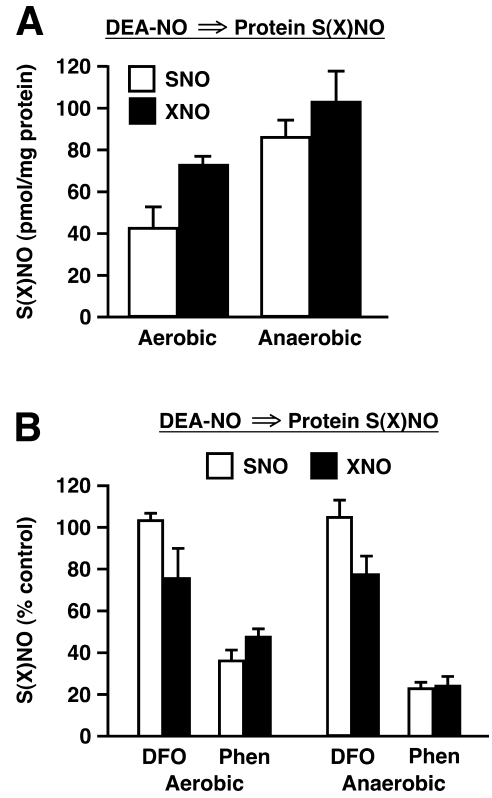

FIGURE 4: NO-dependent *S*-nitrosylation in yeast cells. (A) To compare aerobic and anaerobic protein nitrosylation, mid-log phase *sfa1 yhb1* yeast were exposed to DEA−NO (0.1 mM, 30 min) in room air or at <1 ppm O_2 (see Experimental Procedures). Protein−SNO and protein−XNO were measured by photolysis chemiluminescence. (B) Yeast was incubated with 1 mM deferoxamine (DFO) or 1,10-phenanthroline (Phen) for 30 min and treated with DEA−NO as in (A). Values (mean ± SD) were derived from a minimum of three independent experiments.

in yeast (*24*)) by deferoxamine (DFO; 1 mM, 30 min pretreatment) had no effect on protein *S*-nitrosylation by DEA−NO, although it did result in a small reduction in protein−XNO (Figure 4B). However, the divalent metal ion (e.g., Fe^{2+}, Cu^{2+}, Zn^{2+}) chelator 1,10-phenanthroline (1 mM, 30 min pretreatment) significantly reduced the formation of protein SNO and XNO (Figure 4B). This effect was accompanied by the formation of a low-mass, red compound visible in cell pellets and lysates, consistent with formation of the $Fe(phen)_3^{2+}$ complex. Under these conditions, total (low- and high-mass) NO complexes were also lower, demonstrating that these conditions did not simply favor a shift from high- to low-mass SNO or XNO. These results suggest that protein-bound transition metals (iron and perhaps copper) contribute significantly to protein *S*-nitrosylation by NO. The reduction in protein XNO (but not protein SNO) by DFO suggests that this chelator binds a pool of iron that binds NO but is not relevant for protein *S*-nitrosylation.

DISCUSSION

Our results provide the basis for a scheme of S(X)NO homeostasis and protein nitrosylation *in situ* under nitrosative stress (Figure 5). Notably, the finding that GSNOR does not influence the levels of protein XNOs indicates that GSNO does not directly transfer NO^+ to nonthiol (e.g., amine) centers, or more correctly, that the steady-state equilibrium governed by GSNOR strongly favors SNOs over NO

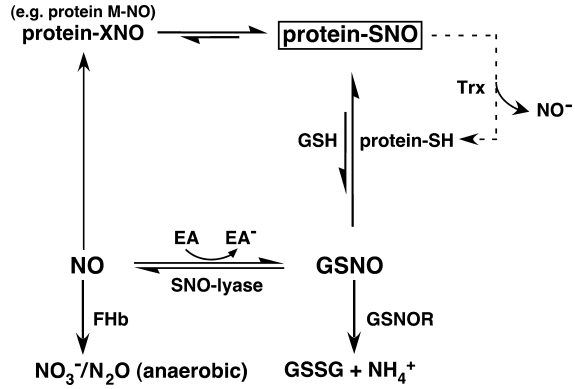

FIGURE 5: A scheme for the cellular mechanisms of (S)NO homeostasis that are implicated by genetic analysis, emphasizing distinct pathways of protein nitrosylation and denitrosylation. GSNO transnitros(yl)ates protein thiols (to form protein−SNO) but does not yield metal−NO (or other nucleophile−NO products), whereas NO reacts to form both protein−SNO and protein−XNO (thought to consist in large part of transition metal−NO (M−NO)). In addition, both the formation of GSNO from NO and the homolytic or reductive cleavage of GSNO to yield NO (i.e., SNO-lyase activity) subserve protein *S*-nitrosylation. Although *S*-nitrosylation formally requires an electron acceptor (EA), our results indicate that neither O_2 nor reactive oxygen species are required and implicate transition metal-nitrosyls (M−NO) as important intermediates in protein−SNO formation. In general, our findings suggest the existence of two principal classes of protein SNOs: those that are in equilibrium with GSNO (presumably solvent-accessible) and subject to regulation by GSNO reductase (GSNOR), and a second class that is unreactive with cellular GSH/GSNO and thus unaffected by GSNO metabolism (presumably solvent-inaccessible). Recent work also suggests that enzymatic protein denitrosylases, specifically thioredoxin (Trx), may act preferentially on this latter class of protein SNOs (*17*). In simple eukaryotes, flavohemoglobin (FHb) metabolizes NO to nitrate (aerobic) or dinitrogen oxide (anaerobic), and in all eukaryotes, GSNOR inactivates GSNO.

complexes with other nucleophiles and transition metals. These data are in agreement with the *in vitro* demonstrations that amine nitrosation by GSNO first requires NO release (*25, 26*) and that protein *S*-nitrosylation by endogenous (and exogenous) GSNO is strongly favored over formation of iron nitrosyls (*8, 27*). In contrast, GSNOR regulates *S*-nitrosylation by NO, indicating that GSNO is an intermediate in either production or metabolism of SNO derived from NO (note that GSNO may itself be formed as a product of NO group transfer to glutathione from SNO-protein (denitrosylation)).

The present findings and a previous analysis in mammalian mitochondria (*23*) demonstrate clearly that that O_2/ROS are not obligatory for protein *S*-nitrosylation *in situ,* although they do not rule out a role for *S*-nitrosylation, perhaps in hydrophobic compartments, by higher nitrogen oxides formed by reaction of NO with these species (*2*). Among possible mechanisms for oxygen-independent *S*-nitrosylation (*1*), there is good evidence that transition metals (largely iron and copper) can catalyze the one electron oxidation necessary for *S*-nitrosylation by NO (*28*−*34*). In particular, studies of invertebrate and mammalian hemoglobins and neuroglobin and of insect nitrophorin support the idea that *S*-nitrosylation may arise from intraprotein exchange of an NO^+ group between metal centers (e.g., heme iron) and thiol (*33*−*39*). Additionally, metal nitrosyl species serve as the source of NO^+ in the formation of GSNO by ceruloplasmin (*30*). Collectively, our findings suggest a substantial role for protein-bound non-heme iron (and perhaps also Cu^{2+}) in cellular, NO-dependent *S*-nitrosylation. The concomitant reduction by phenanthroline in both protein-bound XNO and SNO species may reflect an equilibrium between protein-bound dinitrosyl−iron complexes (DNICs) and protein *S*-nitrosothiols. DNICs are present constitutively in cells and their levels rise upon stimulation of NOS activity (*40, 41*), and these complexes are capable of NO^+ transfer chemistry (*28*). Catalysis of *S*-nitrosylation by transition metals would require redox cycling (i.e., oxidation) of the metal ion. In the case of ceruloplasmin, for example, O_2 serves as the electron acceptor; four NO molecules are oxidized for each molecule of H_2O formed (*30*). Nitrate, nitrite or even NO itself may conceivably serve as electron acceptors under anaerobic conditions, as exemplified in the case of Fe-catalyzed *S*-nitrosylation by DNIC (where nitroxyl anion (NO^-) formation may be coupled to donation of NO^+) (*42*).

The results of our analysis in yeast correlate well with and enhance current understanding of nitrosative stress in mammalian systems. In murine macrophages, GSNO replicates the effects of iNOS induction on protein *S*-nitrosylation and apoptosis (*11*). Consistent with these findings, GSNOR$^{-/-}$ mice have significantly elevated basal levels of SNO proteins and of iNOS-induced apoptosis in several tissues (*8*). Similarly, we found in yeast that cell growth was potently inhibited (without concomitant increases in nonthiol NO species, in particular protein−XNO) in *sfa1* mutant cells treated with GSNO. However, as we also found here (and observed previously *8, 11*), apoptosis (or cell cytostasis) and protein−SNO levels are not linearly related. The observations that a threshold level of *S*-nitrosylation is required to induce significant apoptosis (*11*) and that a ceiling is reached beyond which additional *S*-nitrosylation does not further inhibit cell growth (this work) may be explained by a preferential role in nitrosative stress of a limited set of SNO proteins. This concept also receives support from the findings that pro-apoptotic effects of NOS involve *S*-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that promotes its binding to Siah1 and transport to the nucleus, and that SNO-GAPDH both stabilizes nuclear Siah1 and independently initiates apoptosis (*43, 44*). Prevention of GAPDH *S*-nitrosylation is sufficient to protect from apoptosis (*45*), nothwithstanding *S*-nitrosylation of many other proteins. New methods for identifying SNO-proteins and of mapping sites of *S*-nitrosylation within individual targets on a proteomic scale (*46, 47*) should aid in the identification of SNO-sites implicated in nitrosative stress.

Our measurements of NO-dependent cell growth and protein nitrosylation further suggest that critical cellular targets of nitrosative stress include a subset of protein thiols (and possibly nonthiol protein side chains/metal cofactors) which are protected from reaction with glutathione (*17, 48*−*50*) and thus uninfluenced by GSNOR, since equivalent inhibition by NO was observed for both *yhb1* and *sfa1yhb1* cells, despite a large increase in protein SNOs in the double knockout. This is consistent with our recent discovery that SNO-caspase-3, which does not react with GSH, is enzymatically denitrosylated by thioredoxin (*17*), providing a mechanism for GSNOR-independent denitrosylation. It will therefore be important to determine the extent to which GSNOR (glutathione-dependent denitrosylation) and protein denitrosylases such as thioredoxin differentially regulate SNO

levels and protect against nitrosative stress. Finally, our findings also emphasize the need to elucidate more fully the role of metalloproteins and SNO lyases in SNO-based cellular signaling.

SUPPORTING INFORMATION AVAILABLE

Figure S1 describing construction of the yeast mutant strain deficient in both flavohemoglobin and *S*-nitrosoglutathione reductase. This material is available free of charge via the Internet at http://pubs.acs.org.

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