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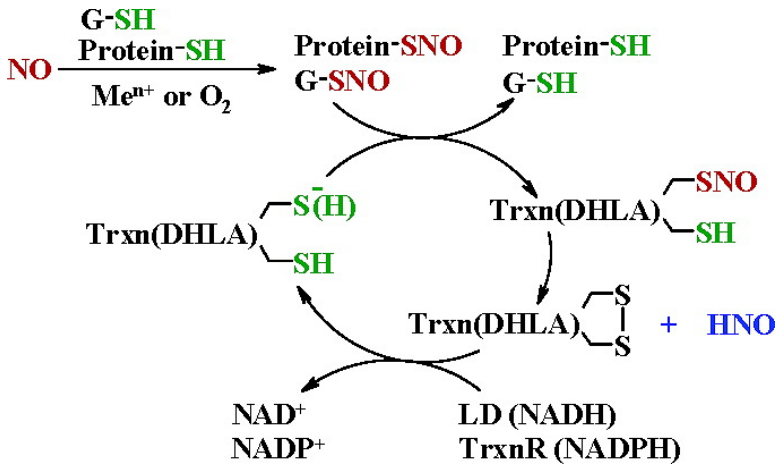

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Thioredoxin and Lipoic Acid Catalyze the Denitrosation of Low Molecular Weight and Protein *S*-Nitrosothiols

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Abstract: The nitrosation of cellular thiols has attracted much interest as a regulatory mechanism that mediates some of the pathophysiological effects of nitric oxide (NO). In cells, virtually all enzymes contain cysteine residues that can be subjected to *S*-nitrosation, whereby this process often acts as an activity switch. Nitrosation of biological thiols is believed to be mediated by N_2O_3, metal−nitrosyl complexes, and peroxynitrite. To date, however, enzymatic pathways for *S*-denitrosation of proteins have not been identified. Herein, we present experimental evidence that two ubiquitous cellular dithiols, thioredoxin and dihydrolipoic acid, catalyze the denitrosation of *S*-nitrosoglutathione, *S*-nitrosocaspase 3, *S*-nitrosoalbumin, and *S*-nitrosometallothionenin to their reduced state with concomitant generation of nitroxyl (HNO), the one-electron reduction product of NO. In these reactions, formation of NO and HNO was assessed by ESR spectrometry, potentiometric measurements, and quantification of hydroxylamine and sodium nitrite as end reaction products. Nitrosation and denitrosation of caspase 3 was correlated with its proteolytic activity. We also report that thioredoxin-deficient HeLa cells with mutated thioredoxin reductase denitrosate *S*-nitrosothiols less efficiently. We conclude that both thioredoxin and dihydrolipoic acid may be involved in the regulation of cellular *S*-nitrosothiols.

Introduction

Thioredoxin (Trxn) is a ubiquitous protein whose activity has been linked to cell growth, transcription factor regulation, DNA synthesis, and protein binding.[1−3] Trxn isozymes of all organisms contain a conserved -Cys-Gly-Pro-Cys- active site that is essential for the function of this class of proteins as general protein disulfide reductases.[1,2] Trxn-$(SH)_2$ regulates the activity of thiol-containing proteins via reduction of their S−S bonds to protein−$(SH)_2$ at the expense of its own oxidation to Trxn-$(S)_2$. This, in turn, is reduced back to Trxn-$(SH)_2$ by the NADPH-dependent thioredoxin reductase (TrxnR). The Trxn/TrxnR system is also involved in detoxification of free radicals[4] and regeneration antioxidant compounds, such as ascorbic acid, selenium-containing substances, and ubiquinones.[5] Similar redox properties were reported for 6,8-dimercapto-octanoic acid (dihydrolipoic acid; DHLA; Scheme 1, **1**), which could be viewed as a low molecular weight mimetic of Trxn. DHLA is the reduced form of 5-[1,2]dithiolan-3-yl-pentanoic acid (lipoic acid, LA), an essential prosthetic group in the dihydrolipoyl transacetylase component of the α-ketoacid dehydrogenase complex in mitochondria. LA, found in micromolar concentrations in blood,[6,7] is used as a pharmacologically effective agent in treatment of diabetes mellitus and intoxications with heavy metals.[8] In cells, LA can be reduced to DHLA by lipoamide dehydrogenase (LD), glutathione reductase, and TrxnR.[9,10] DHLA is a potent antioxidant[11] and can reduce protein disulfides.[12]

In contrast to the reduction of disulfides and free radicals, the interactions of Trxn and DHLA with *S*-nitrosothiols are not well studied. In recent years, the nitrosation of cellular thiols has attracted much interest as a regulatory mechanism that mediates some of the pathophysiological effects of nitric oxide (NO). It is noteworthy that virtually all enzymes contain cysteine

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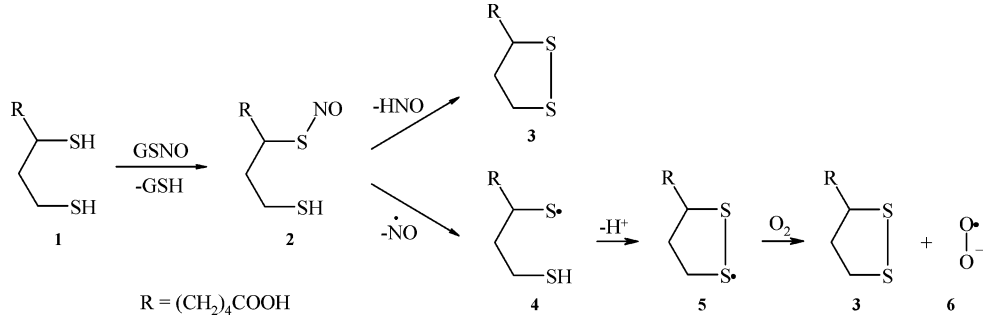
Scheme 1



residues that can be subjected to *S*-nitrosation, whereby this process often acts as an activity-switch.[13] Nitrosation of biological thiols is believed to be mediated by N_2O_3, metal−nitrosyl complexes, and peroxynitrite.[14,15] The catabolism of *S*-nitrosothiols, however, remains incompletely understood. Transition metal ions,[16] Trxn,[17] protein disulfide isomerase (PDI),[18] and alcohol dehydrogenase (ADH)[19,20] were reported to catalyze the denitrosation of *S*-nitrosoglutathione (GSNO) back to glutathione (GSH) with generation of either NO (metal ions; Trxn; PDI) or hydroxylamine (ADH). To date, enzymatic pathways for *S*-denitrosation of proteins have not been identified.

The denitrosation of GSNO by DHLA (Scheme 1, **1**) and Trxn has been studied by three research groups, which proposed conflicting reaction mechanisms. Arnelle and Stamler reported that DHLA reduces GSNO to GSH and nitroxyl (HNO) via the intermediate formation of HS-DHLA-SNO (**1** → **2** → **3**),[21] whereas Petit et al. suggested that HS-DHLA-SNO decomposes to NO and the *S*-centered radical **4**.[22] The latter has been shown to undergo a rapid intramolecular ring closure to **5**, which readily interacts with oxygen to form LA and superoxide anion radical ($O_2^{-\bullet}$).[23,24] However, Petit et al. observed that both LA and DHLA are formed from **2**, suggesting that the cyclization of **4** was balanced by its reduction by **5**, $O_2^{-\bullet}$ and/or HNO (**1** ← **4** → **5**).

Similar to reaction **2** → **4**,[17] Nikitovic and Holmgren reported that Trxn catalyzes the denitrosation of GSNO with generation of NO via the intermediate formation of HS-Trxn-SNO.[17] On the other hand, the amounts of NO_2^- formed in this reaction system were 40−50% of the theoretical yield ($NO + O_2 \rightarrow N_2O_3$; $N_2O_3 + H_2O \rightarrow NO_2^-$), whereas NO_3^-, a breakdown product of peroxynitrite, was not found ($O_2^{-\bullet} + NO \rightarrow ONOO^- \rightarrow NO_3^-$).[17] This incomplete balance suggests that any HNO, potentially formed in the reaction, was undetected. The discrimination between reductive/heterolytic (**2** → **3** + HNO) versus homolytic (**2** → **4** + NO) breakdown of the S−N bond of both **2** and HS-Trxn-SNO is important with regard to the markedly different (bio)chemical properties of NO and HNO.[25]

In the present study, we evaluated if Trxn and DHLA (i) reduce *S*-nitrosothiols with concomitant generation of NO and/or HNO and (ii) catalyze the denitrosation of *S*-nitrosoproteins. Since *S*-nitrosation can modulate the activity of numerous enzymes,[13] the identification of catalytic pathway(s) for protein *S*-denitrosation is of principal importance.

Experimental Section

Reagents. All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO). The solutions used in the experiments were prepared in deionized and Chelex-100-treated water or potassium phosphate buffer. Sodium trioxodinitrate was either purchased from Calbiochem, Inc. (La Jolla, CA) or synthesized as described in ref 26. Thioredoxin (recombinant, expressed in *Escherichia coli*) and TrxnR (*E. coli*) were purchased from Sigma; qualitatively similar results were obtained with TrxnR (*E. coli*) purchased from Calbiochem (San Diego, CA).

HPLC Analysis of GSNO, GSH, and NH_2OH. Isocratic separations were achieved at a flow rate of 1 mL per min with C-18 reverse phase columns (Alltima 4.6 mm × 250 mm, 5 μ; Alltech Associates, Inc.; Deerfield, IL). Electrochemical and UV detection were carried out with an LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN) and an SPD-M10Avp photodiode array detector (Kyoto, Japan), respectively. A mobile phase consisting of 50 mM phosphate buffer (pH = 3.0; adjusted with glacial acetic acid), 0.1 mM EDTA, and either 5% or 10% methanol (v/v) was used to quantitate GSH (electrochemical oxidation at + 0.95 V) or GSNO (λ_{max} = 335 nm). NH_2OH was quantified by HPLC−UV (mobile phase, 30% (v/v) methanol) after its derivatization with 4-hydroxy-3-methoxybenzaldehyde (vanillin (VAN), 2 mM) to vanillin oxime (VANO, pH 6.0; incubation time, 5 min at 60 °C).

ESR Spectroscopy. Reactions were carried out for 15 min at room temperature in Chelex 100-treated phosphate buffer (25 mM, pH 7.4) containing DTPA (100 μM) and myoglobin (50 μM). Additions of GSNO (300 μM), lipoic acid (50 μM), LD (0.04 units/mL), NADH (600 μM), and diethylamine NONOate were made as indicated in Figure 3f (Inset). Thereafter, aliquots of 150 μL were frozen in liquid nitrogen, and ESR spectra were recorded at liquid nitrogen temperature with a JEOL-RE1X spectrometer (Kyoto, Japan). Spectrometer settings were the following: field center, 320.0 mT; sweep width, 25 mT; field modulation, 0.5 mT; amplitude - either 4000 (spectra 3,4) or 1000 (spectrum 1); microwave power, 10 mW; time constant, 0.1 s; and sweep time, 4 min.

ESR spin trapping experiments were carried out with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, 0.12 M) at room temperature. ESR spectrometer settings were the following: field center, 335.094 mT; microwave power, 5 mW; sweep time, 2.0 min; time constant, 0.3 s;

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modulation width, 0.2 mT. ESR spectra simulation was performed with a computer program created by Philip D. Morse, II, and Richard Reiter (ESR Simulation System 2.01, Scientific Software Services, IL). The hyperfine splitting constants (in gauss) used for simulation of the spectra of the DMPO/α-hydroxyethyl radical, DMPO/•SG and DMPO/•S-DHLA-SH nitroxides were as follows: ($a_N = 15.80$; $a_H = 22.80$), ($a_N = 15.1$; $a_H = 16.00$) and ($a_N = 15.25$; $a_H = 16.60$), respectively.[24,27]

Analysis of *S*-Nitrosothiols and NO. *S*-Nitrosothiols were analyzed either spectrophotometrically ($\lambda_{max} = 335$ nm) or after their photolytic or Cu^+-catalyzed breakdown to NO (Figures 1e, 2d and 3b,e). The latter species was determined by the DAF2 assay (HPLC−FL analysis of DAF2 triazole;[28] Figure 5b), potentiometrically (Figure 1e, 2c, and 5f), or with a Sievers model 280 NO analyzer (Sievers Inc., Boulder, CO; reacton solution, 50 mM ascorbic acid and 100 μM $CuCl_2$; Figure 5c). Potentiometric measurements of NO were performed with NO-specific electrode (World Precision Instruments, Sarasota, FL).

***S*-Nitrosation of Proteins.** Reactions were carried out in 0.1 M phosphate buffer (pH 7.4) containing 0.1 mM EDTA. Caspase 3 (5 μM; human; recombinant, *E. coli*; Calbiochem, Inc. San Diego, CA) and reduced metallothionein (20 μM, from rabbit liver; Sigma, Inc. St. Louis, MO) were *S*-nitrosated for 30 min at 37 °C by GSNO (90 μM and 400 μM, respectively). Casp-SNO and MT-SNO were isolated via ultrafiltration (Vivaspin 20 filters; Vivascience, Inc. Edgewood, NY) and size-exclusion chromatography (Sephadex G10; column, 1 cm × 45 cm), respectively. Alb-SNO was prepared as described previously.[29] Caspase activity was determined by using Enzcheck Caspase 3 assay kit (Molecular Probes, OR).

Cell Experiments. TrxnR activity in wild-type HeLa cells and HeLa cells transfected with cysteine-to-serine mutant expression plasmid (mTrxnR) was determined as described in ref 30. TrxnR and mTrxnR cells (250,000 per plate) were treated with SNCEE (100 μM) for 30 min at 37 °C. Thereafter, the cells were washed with 3 × 2 mL of PBS, and intracellular *S*-nitrosothiols were determined by the DAF2 assay. Imaging of intracellular *S*-nitrosothiols was performed by fluorescence microscopy following the biotin switch method,[31,32] which consists of the following steps: (i) nitrosation of cellular proteins, (ii) alkylation of free thiols in permeabilized cells, and (iii) conversion of SNO functions to SH groups by ascorbate in the presence of a thiol-labeling reagent. Briefly, TrxnR and mTrxnR HeLa cells were incubated for 30 min in SNCEE (0.1 mM)-containing MEM, washed with PBS (3 × 5 mL), and then fixed with 2% paraformaldehyde.[32] Thereafter, the cells were washed with 0.1 M Tris buffer (pH 7.4) containing 137 mM NaCl and permeabilized with 0.2% Triton × 100. Thiols in permeabilized cells were alkylated with 0.2 M methylmethanethiosulfonate (MMTS; Calbiochem, Inc. San Diego, CA) for 30 min, and then the cells were washed with Tris buffer and treated with 0.1 mM 2-((5(6)-tetramethylrhodamine)amino)methylmethanesulfonate (MTSEA-TAMRA; Calbiochem, Inc.) and 1 mM ascorbate for 1 h. Cellular *S*-nitrosothiols were visualized using a Nikon fluorescence imaging system equipped with Nikon ECLIPSE TE 200 microscope and digital camera (Hamamatsu CCD; C4742-95-12NRB).

Data Analysis. Results are given as mean ± S. D. ($n = 4-6$).

Results and Discussion

Denitrosation of GSNO by DHLA and Trxn. Since Trxn and DHLA are dithiols with similar redox properties,[33,34] we reinvestigated their interactions with GSNO with focus on the redox form of NO as an important reaction product. To minimize the occurrence of transition metal ion-catalyzed decomposition of GSNO and/or oxidation of HNO to NO, all buffers were filtered through a Chelex 100-containing column. In the presence of Trxn, TrxnR, and NADPH, GSNO was stoichiometrically reduced to GSH (Figure 1a, b). GSNO was not consumed to any significant extent when Trxn was omitted from the reaction system, indicating that TrxnR did not directly interact with the nitrosothiol (Figure 1c). Maximal rate of GSNO reduction was attained with 6−10 μM Trxn (Figure 1d), well within the concentration range of this protein in cells (5−50 μM).[35,36] No changes in the rate of GSNO denitrosation by Trxn were

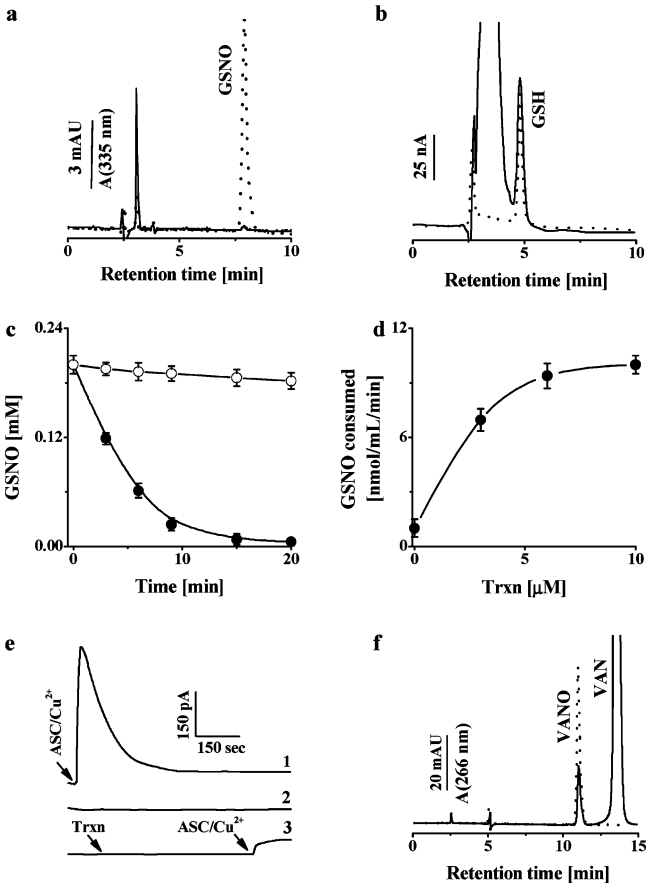

Figure 1. Trxn denitrosates GSNO. Experiments were carried out in 0.1 M phosphate buffer at 37 °C. Desferrioxamine, Trxn, TrxnR, and NADPH were used at concentrations of 100 μM, 10 μM, 5 μM (15 units/mL) and 0.4 mM, respectively (Panels a−e). (a, b) HPLC-monitored reduction of GSNO to GSH by the Trxn/TrxnR/NADPH system. Chromatograms of GSNO (0.2 mM) prior to the addition of Trxn/TrxnR/NADPH and after completion of the reaction are presented with dashed and solid lines, respectively (a); incubation time, 30 min. Chromatograms of GSH accumulated in the course of the reaction and of a standard solution of GSH (0.2 mM) are presented with solid and dashed lines, respectively (b); incubation time, 30 min. (c, d) Kinetics of reduction of GSNO by Trxn in the presence of TrxnR and NADPH. Open circles, GSNO (0.2 mM), TrxnR and NADPH; closed circles, plus Trxn. Results are mean values ± SE ($n = 3$). (e) Potentiometric measurements of NO in aqueous solutions of GSNO. Decomposition of GSNO (2 μM) to NO was initiated by addition of ascorbic acid (0.1 mM) and $CuCl_2$ (5 μM) before (traces 1) and after (trace 3) addition of Trxn, TrxnR and NADPH; trace 2 - GSNO, no additions. (f) Formation of NH_2OH in phosphate buffer containing Trxn and GSNO. TrxnR, NADPH and Trxn (0.2 mM; reaction volume, 0.02 mL) were incubated for 15 min at 37 °C; then GSNO (0.4 mM) was added, and the reaction solution was further incubated for 20 min. At the end of the incubation, NH_2OH was quantified by HPLC−UV after its derivatization with VAN to VANO. With dashed lines is presented the chromatogram of a standard solution of 120 μM VANO.

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observed in the absence of desferrioxamine or oxygen (data not shown), implying that this process was not dependent on catalysis by transition metal ions.[16] Since catalytic amounts of Trxn were required for the reduction of GSNO to GSH, we speculated that the reaction proceeded via the intermediate formation of HS-Trxn-SNO. The nitrosation of one SH group in vicinal thiols leads to the formation of unstable mono-*S*-nitroso thiols that were suggested to decompose to either disulfides and HNO[21] or nitric oxide (NO) and thiyl-centered radicals.[17,22] To discriminate between these two mechanisms, we assessed the generation of both NO and HNO as potential reaction end products. Addition of ascorbic acid and $CuCl_2$ to a solution of GSNO expectedly triggered generation of NO, as evidenced by potentiometric assay, via Cu^{1+}-dependent reduction of the nitrosothiol to GSH (Figure 1e, 1; ref 16). When GSNO was incubated with Trxn/TrxnR plus NADPH, release of NO was not observed (Figure 1e, 3). Notably, subsequent addition of ascorbic acid and $CuCl_2$ did not elicit release of NO, indicating that the source of releasable NO was exhausted. These data indicated that potentiometrically silent HNO, rather than NO,[17] was produced semiquantitatively in the presence of Trxn/TrxnR plus NADPH. Formation of HNO most likely followed the reaction sequence GSNO + $Trxn(SH)_2$ → HS-Trxn-SNO → $Trxn\text{-}S_2$ + HNO.

To further verify this mechanism, we attempted to detect HNO, which is a highly reactive species that often maintains low, submicromolar steady-state concentrations.[37] In semi-neutral aqueous solutions, HNO dimerizes to *cis*-hyponitrous acid (HO−N=N−OH; $k_{HNO} = 8 \times 10^6\ M^{-1}\ s^{-1}$)[38] which is unstable and decomposes to N_2O and H_2O.[39] Competitively, HNO can interact (in contrast to NO) with thiols to form unstable *N*-derived hydroxylamines that undergo thiol-dependent reduction to NH_2OH.[40] Hence, we attempted to detect NH_2OH as a reporter of HNO released during the denitrosation of GSNO. Preincubation of 0.2 mM $Trxn\text{-}S_2$ with TrxnR and 0.2 mM NADPH resulted in depletion of the latter and formation of $Trxn(SH)_2$ (data not shown), whereas subsequent addition of GSNO led to formation of NH_2OH (Figure 1f). This result further supports a reaction mechanism whereby $Trxn(SH)_2$ denitrosates GSNO with concomitant release of HNO. It is not clear why under similar reaction conditions Nikitovic and Holmgren observed a predominant generation of NO.[17] In ref 17, HNO release was monitored spectrophotometrically by following the reduction of metmyoglobin to its ferrous form under anaerobic conditions. Increases in absorbance at 542 and 580 nm, accompanied by decreases at 640 nm were considered indicative of the reduction of Fe(III) myoglobin to Fe(II) myoglobin and HNO release. Reactions were reported for phosphate buffers (pH 7.4) containing EDTA, metmyoglobin (40 μM), and equimolar concentrations (100 μM) of *E. coli* $Trxn\text{-}(SH)_2$ and GSNO. After an incubation of 25 min, the authors observed spectral changes consistent with the reduction of metmyoglobin ($\Delta_{542} \simeq 0.04$ and $\Delta_{580} \simeq 0.03$ au, respectively; Figure 4b in ref 17). However, qualitative and/or quantitative

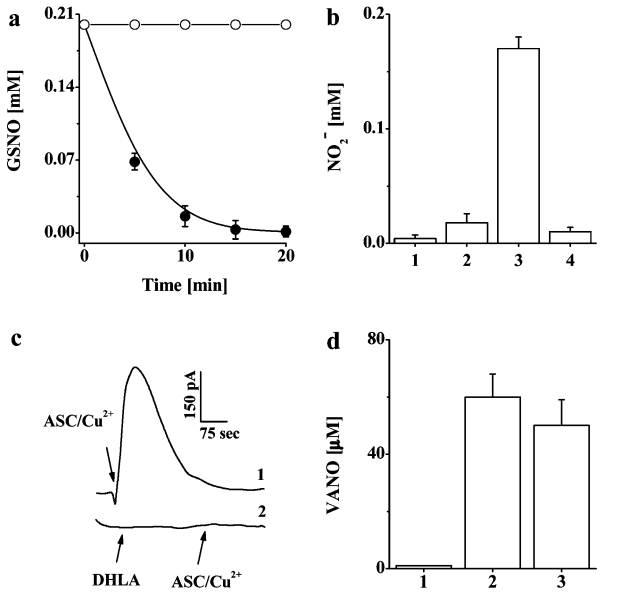

Figure 2. DHLA denitrosates GSNO to GSH and HNO. Experiments were carried out in 0.1 M phosphate buffer at 37 °C. (a) GSNO (0.2 mM) in the absence (open circle) and presence of DHLA (0.5 mM; closed circles). (b) Accumulation of nitrite in solutions of GSNO (0.2 mM) in the absence (1) and the presence of DHLA (0.5 mM; (2), $CuCl_2$ (5 mM; (3) and 10 μM Trxn/5 μM TrxnR/0.4 mM NADPH (4), respectively; incubation time, 30 min. (c) Potentiometric measurements of NO in aqueous solutions of GSNO. Decomposition of GSNO (2 μM) to NO was initiated by addition of ascorbic acid (0.1 mM) and $CuCl_2$ (5 μM) before (traces 1) and after (trace 2) addition of DHLA (20 μM). (d) Formation of NH_2OH in solutions of GSNO (0.2 mM) in the absence (1) and the presence of 0.5 mM DHLA (2) and 10 μM Trxn/5 μM TrxnR/0.4 mM NADPH (3); incubation time, 30 min.

correlations of the resulting spectrum with HNO are difficult because of the lack of assay validation via standard additions of HNO donors, such as sodium trioxodinitrate ($Na_2N_2O_3$; Angeli's salt) and NaNO. Nevertheless, Nikitovic and Holmgren concluded that "*there was little if any conversion of metmyoglobin to Fe(II)-nitrosyl metmyoglobin when incubated with Trxn(SH)₂ and GSNO thus showing that HNO was not released*". Several factors may complicate the analysis of HNO in this reaction system. Trace amounts of transition metal ions, introduced in the reaction milieu with buffers and/or metmyoglobin, can catalyze the oxidation of HNO to NO.[41] The use of metal chelators such as EDTA would minimize the breakdown of GSNO to GSH and NO[16] but may promote the oxidation of HNO via dissolving metal hydroxides and/or phosphates.[41] Furthermore, dimerization of HNO,[39] as well as its addition to GSNO[42] and reduction by $Trxn(SH)_2$ to NH_2OH,[40] can impair the sensitivity of the metmyoglobin assay. In the presence of thiols, the data presented in Figure 1f and 2d, as well as in references 21 and 40 suggest that NH_2OH is a more specific reporter of HNO than metmyoglobin. In our experiments, the lack of potentiometrically detectable NO (Figure 1e) and the insignificant accumulation of NO_2^- (Figure 2b) in solutions of GSNO and Trxn/TrxnR/NADPH indicate that the denitrosation process proceeded with semiquantitative generation of GSH (Figure 1a, b) and HNO.

Similar to Trxn, DHLA readily denitrosated GSNO (Figure 2a). Measurements of NO_2^- (Figure 2b) and direct potentio-

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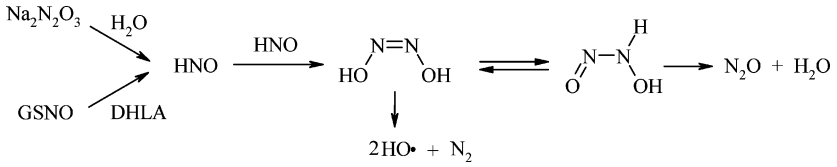
Scheme 2



metric analysis (Figure 2c) of the reaction systems containing either DHLA (Figure 2b, 2; Figure 2c) or Trxn (Figure 2b, 4) indicated that NO was not formed to any significant extent. In contrast, addition of $CuCl_2$ to GSNO-containing solutions caused a pronounced formation of NO_2^- (Figure 2b, 3), as reported previously.[16] The denitrosation of GSNO by DHLA was paralleled by accumulation of NH_2OH (Figure 2d), indicating the intermediate generation of HNO. Furthermore, the LA/LD/NADH system also catalyzed the reduction of GSNO (Figure 3, open circles), albeit not very efficiently; this was likely due

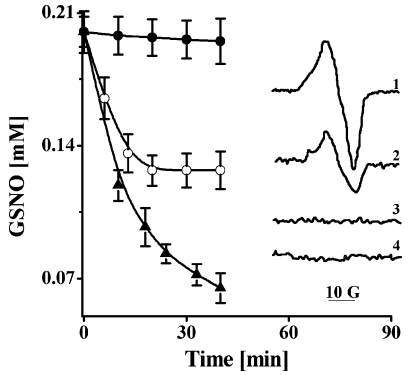
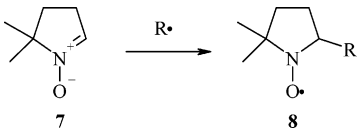


Figure 3. LA denitrosates GSNO in the presence of LD and NADH. Denitrosation of GSNO (0.2 mM) by LA (10 μM) for 30 min at 37 °C in 0.1 M phosphate buffer (pH 7.4) containing LD (0.1 U/mL), NADH (0.4 mM), and desferrioxamine (0.1 mM; open circles); plus GSH (5 mM; triangles); closed circles - GSNO, GSH, LD and NADH. Values are the mean ± SE ($n = 3$). (Inset) ESR spectrum 1 - solution containing Angeli's salt (0.2 mM) and myoglobin; spectrum 2 - GSNO, LA, LD, NADH and myoglobin; spectrum 3 - GSNO, LD, NADH and myoglobin; spectrum 4 - myoglobin, diethylamine NONOate, LA, LD and NADH. Specific concentrations and incubation times are indicated in Methods.

to inhibition of LD by HNO produced in the reaction. In support of this hypothesis, GSH potentiated the denitrosation of GSNO (Figure 3, triangles), presumably via reduction of HNO to NH_2-OH. To directly detect HNO formation we utilized its property to interact with ferric complexes yielding characteristic signals in ESR spectra.[40,43] Incubation of myoglobin with Angeli's salt, a commonly used donor of HNO, resulted in the appearance of a broad ESR spectrum with anisotropic *g*-values characteristic for nitrosomyoglobin (ref 44, Figure 3, ESR spectrum 1). A similar spectrum was observed in a reaction system consisting of LA, LD, GSNO, NADH, and myoglobin (Figure 3, ESR spectrum 2), whereas no ESR activity was observed in the absence of LA or when myoglobin was incubated with the NO donor diethylamine NONOate (Figure 3, Inset, traces 3 and 4, respectively). The latter indicates that under the experimental conditions used myoglobin-Fe^{III} preferentially interacted with HNO.

ESR Spin Trapping Analysis of the Denitrosation of GSNO by DHLA. In solutions of DHLA and GSNO, formation

Scheme 3



of several radical intermediates could be envisaged: (i) metal ions-catalyzed breakdown of GSNO and/or homolysis of ONS-DHLA-SH may lead to the generation of GS• and HS-DHLA-S•, respectively, and (ii) HNO dimerizes to HO−N=N−OH that can undergo a pH-dependent homolysis to N_2 and hydroxyl radical (•OH),[45] in parallel to its breakdown to N_2O and H_2O (Scheme **2**).[39]

Previous studies from our laboratory have demonstrated that the generation of •OH by HNO becomes significant in weakly acidified milieu (pH 4−6).[45,46] To assess the formation of radical species in aqueous solutions of GSNO and DHLA, we have conducted ESR experiments with 5,5-dimethyl-1-pyrroline *N*-oxide as a spin trap (DMPO, Scheme 3, **7**). Whereas the nitroxides DMPO/•OH, DMPO/•SG, and DMPO/•S-DHLA-SH (**8**) exhibit poorly distinguishable four-line ESR spectra, ethanol was used to discriminate •OH from the corresponding thiyl radicals.[27,45] Ethanol readily interacts with •OH to form the α-hydroxyethyl radical, whose adduct with DMPO exhibits a specific six-line ESR spectrum.[47]

Aqueous solutions of DMPO and ethanol containing either GSNO or DHLA did not exhibit any ESR activity (Figure 4A, spectra 1 and 2). However, the addition of $CuCl_2$ to a solution of GSNO and DMPO resulted in the appearance of the typical ESR spectrum of DMPO/•SG (Figure 4A, spectrum 3; in gauss, $a_H = 16.0$ and $a_N = 15.1$).[23,24] In contrast, the reduction of GSNO by DHLA at pH 7.4 was not paralleled by the formation of nitroxides with distinguishable ESR spectra, suggesting that a homolytic breakdown of OSN-DHLA-SH did not occur to any significant extent (Figure 4A, spectrum 4). In Figure 4A, trace 5 represents a computer simulation of the ESR spectra of DMPO/•SG (solid lines) and DMPO/•S-DHLA (dashed lines)

However, the denitrosation of GSNO by DHLA in acidic solutions containing ethanol was paralleled by a marked generation of DMPO/α-hydroxyethyl radical nitroxide (Figure 4B, 1 compared to A, 4: (a) pH 5.0, (b) pH 6.0). The kinetics of α-hydroxyethyl radical generation closely followed that of GSNO denitrosation (Figure 4B, 2). Under anaerobic conditions, the generation of α-hydroxyethyl radical was not inhibited, suggesting that metal ions and/or oxygen were not involved in this process (Supporting Information). Similarly, Angeli's salt, which hydrolyzes to HNO and NO_2^- at a constant rate in the pH interval of 4−8.5,[39] oxidized ethanol to α-hydroxyethyl

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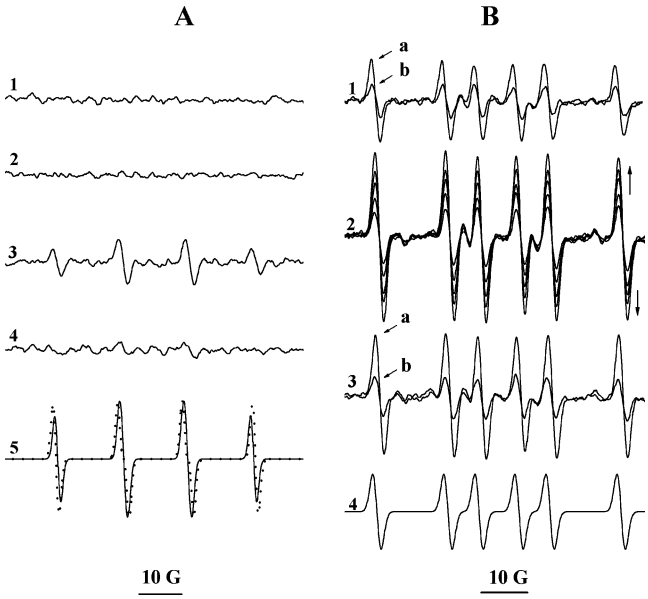

Figure 4. ESR spectra of DMPO nitroxides formed in solutions of GSNO and DHLA. Reactions were carried out at room temperature in 0.1 M phosphate buffer (panel A, pH 7.4; panel B, pH 5−6) containing 50 μM desferrioxamine and 400 mM ethanol. DMPO, GSNO, DHLA, $CuCl_2$ and Angeli's salt were used at concentrations of 0.12 M, 0.25 mM, 0.28 mM, 10 μM, and 0.3 mM, respectively. All ESR spectra were recorded after 4 min of incubation, except for the ESR spectra presented in B2, were the time interval between two consecutive ESR scannings was 2 min. (A) 1 - DMPO plus GSNO; 2 - DMPO plus DHLA; 3 - DMPO, GSNO and $CuCl_2$; 4 - DMPO, GSNO and DHLA; 5 - computer simulation of the spectra of DMPO/•SG (solid lines) and DMPO/S-DHLA-SH (dashed lines), respectively. (B) spectrum 1 - DMPO, GSNO and DHLA [(a) pH 5.0; (b) pH 6.0]; trace 2 represents the ESR-monitored kinetic of accumulation of DMPO/α-hydroxyethyl radical in a solution containing GSNO, DHLA and DMPO (pH 5.0); 3 - Angeli's salt [(a) pH 5.0; (b) pH 6.0]; 4 - computer simulation of the spectrum of DMPO/α-hydroxyethyl radical.

radical in a pH-dependent manner (Figure 4B, 3: (a) pH 5.0, (b) pH 6.0).[45] The experimental and computer-simulated spectra of DMPO/α-hydroxyethyl radical were in a good agreement (Figure 4B, 4). These data further support the notion that DHLA denitrosated GSNO with concomitant generation of HNO.

Denitrosation of *S*-Nitrosoproteins by DHLA and Trxn. We were further interested in determining the extent to which Trxn can interact with *S*-nitrosoproteins. To this end, we tested the ability of Trxn to denitrosate three representative *S*-nitrosoproteins: (caspase 3)-SNO (casp-SNO), metallothionein-SNO (MT-SNO), and albumin-SNO (Alb-SNO) (Figure 5a−d). Selection of these structurally, as well as functionally, different *S*-nitrosoproteins was based on recent studies, suggesting that the native sulfhydryl forms of these proteins undergo endogenous *S*-nitrosation.[48−50] Incubation with GSNO resulted in poly-*S*-nitrosation of caspase 3 and loss of its enzymatic activity, as previously reported;[49] both effects were reversed by addition of either Trxn/TrxnR/NADPH or dithiothreitol (DTT) (Figure 5a, b). Under the experimental conditions used, Trxn/Trxn/NADPH restored the catalytic activity of caspase 3 without complete denitrosation of the poly-*S*-nitrosated protein, suggesting that a thiol that is critical for the enzyme's activity

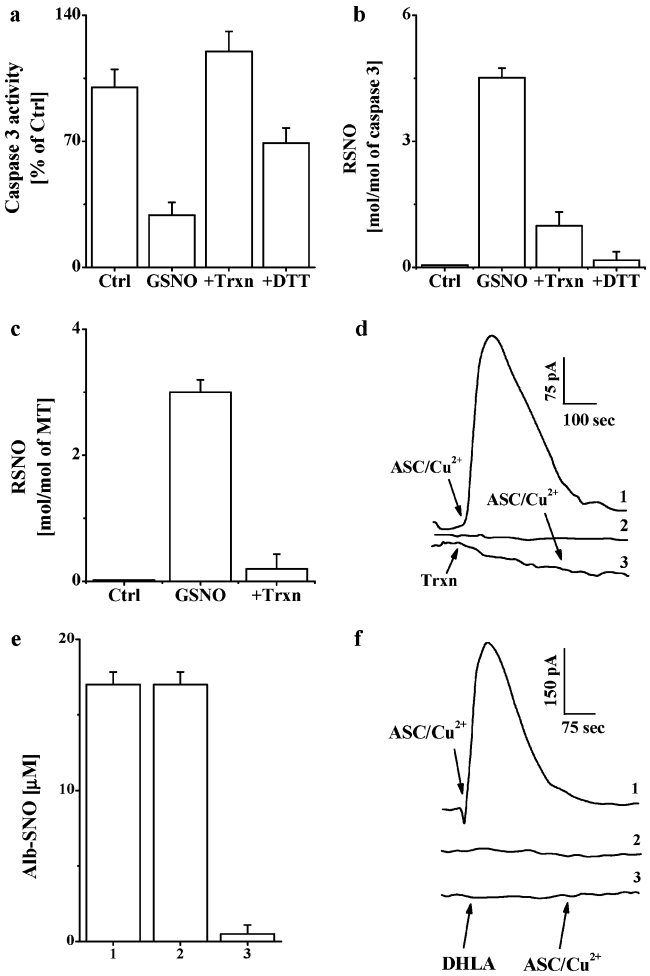

Figure 5. Trxn and DHLA denitrosates *S*-nitrosoproteins. Experiments were carried out in 0.1 M phosphate buffer at 37 °C. Desferrioxamine, Trxn, TrxnR, GSNO, NADPH, and DTT were used at concentrations of 100 μM, 10 μM, 5 μM (15 units/mL), 0.1 mM, 0.4 mM and 5 mM, respectively. Additional reaction specifics are included in Methods. (a) Effects of GSNO, Trxn/TrxnR/NADPH and DTT on the activity of caspase 3 (5 μM). (b) *S*-Nitrosation of caspase 3 (5 μM) by GSNO and denitrosation by Trxn/TrxnR/NADPH and DTT, respectively. (c) *S*-Nitrosation and denitrosation of metallothionein (2 μM) by GSNO and Trxn/TrxnR/NADPH, respectively. Data in panels a−c are presented as mean ± SE ($n = 3$). (d) Potentiometric measurements of NO in aqueous solutions of Alb-SNO. Decomposition of Alb-SNO (2 μM) to NO was initiated by addition of ascorbic acid (0.1 mM) and $CuCl_2$ (5 μM) before (traces 1) and after (trace 3) addition of Trxn, TrxnR and NADPH; trace 2 - Alb-SNO (2 μM), no additions. (e) Consumption of Alb-SNO (18 μM) in the absence (1) and the presence of 0.5 mM LA (2) and 0.5 mM DHLA (3), respectively (incubation time, 30 min). Changes in the concentration of Alb-SNO were monitored spectrophotometrically at 335 nm. (f) Potentiometric measurements of NO in aqueous solutions of Alb-SNO. Decomposition of Alb-SNO (2 μM) to NO was initiated by addition of ascorbic (0.1 mM) acid and $CuCl_2$ (5 μM) before (traces 1) and after (trace 3) addition of DHLA (0.1 mM).

was regenerated. Similarly, Trxn/TrxnR/NADPH *S*-denitrosated MT-SNO and Alb-SNO (Figure 5c, e), and the reduction of these nitrosothiols proceeded without release of NO (Figure 5d, f; data for MT-SNO not shown).

Catabolism of *S*-Nitrosothiols in TrxnR-Deficient Cells. We further assessed the interaction of Trxn with intracellular *S*-nitrosothiols using Trxn/TrxnR wild-type HeLa cells (TrxnR) and HeLa cells transfected with a cysteine-to-serine mutant expression plasmid (mTrxnR) that rendered cells with dysfunctional TrxnR and impaired Trxn activity.[30]

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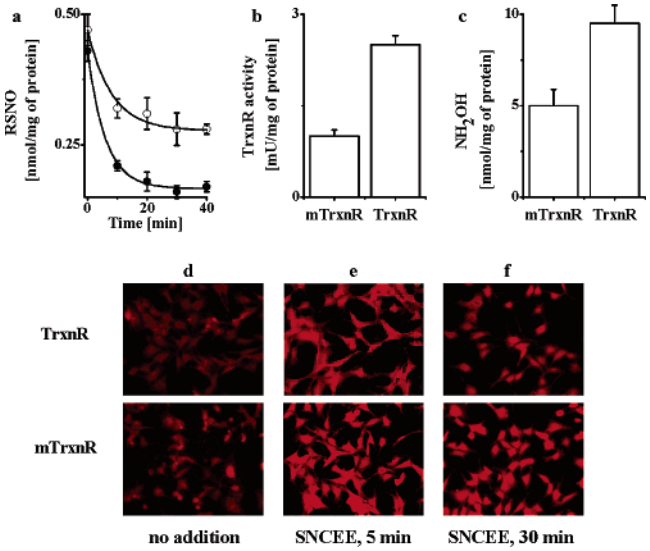

Figure 6. Trxn/TrxnR system catabolizes intracellular *S*-nitrosothiols. (a) Time course of the consumption of *S*-nitrosothiols in TrxnR (closed circles) and mTrxnR (open circles) HeLa cells loaded with SNCEE. *S*-nitrosothiols were determined by the DAF-5 assay. b- Analysis of Trxn/TrxnR activity in whole extracts of TrxnR and mTrxnR HeLa cells. (c) Quantification of NH_2OH in SNCEE-loaded TrxnR and mTrxnR HeLa cells. All data are representative of three independent experiments (mean ± SE; $n = 3$). (d−f) MMTS/MTSEA-TAMRA-stained intracellular *S*-nitrosothiols before (d) and after treatment of TrxnR and mTrxnR cells with SNCEE (e - 5 min; f - 30 min).

We confirmed that mTrxnR cells exerted 2.5-fold lower TrxnR activity than the wild-type HeLa cells (Figure 6b, ref 30). We then used *S*-nitroso-L-cysteine ethyl ester (SNCEE),[51] a membrane-permeable reagent, to *S*-nitrosate thiols in both types of cells. The treatment resulted in a marked accumulation of *S*-nitrosothiols in cells and allowed us to follow the time course of their *S*-denitrosation (Figure 6a, d−f). At the dose used, SNCEE did not cause any significant toxicity, as evidenced by the trypan blue exclusion test (data not shown). Clearance of intracellular *S*-nitrosothiols (Figure 6a, d−f; red stains) occurred at an increased rate and more completely in the TrxnR versus mTrxnR HeLa cells. The decay of *S*-nitrosothiols was paralleled by accumulation of NH_2OH that was more robust in the wild-type cells than in mTrxnR cells (Figure 6c). Thus, deficiency in the Trxn/TrxnR system caused inhibition of *S*-nitrosothiol decomposition in cells. Notably, the background intracellular level of *S*-nitrosothiols in wild-type TrxnR HeLa cells was approximately 2-fold lower than in mTrxnR cells (Figure 6a, f), indicating that decreased Trxn activity was associated with less effective catabolism of intracellular *S*-nitrosothiols. The denitrosation potential of mTrxnR cells was most likely due to incomplete inhibition of TrxnR activity (Figure 6b), as well as to the presence of additional pathways of *S*-denitrosation. It could be also speculated that the interrupted catabolism of intracellular *S*-nitrosothiols after 20 min of incubation reflected inhibition of the enzyme system(s) catalyzing this process (Figure 6a).

Biological Implications of the Catabolism of *S*-Nitrosothiols by Trxn and DHLA. While virtually all enzymes contain critical cysteine residues whose *S*-nitrosation acts as an activity-switch, *S*-nitrosation and denitrosation of cellular thiols have been suggested to be a fundamental posttranslational protein modification that is similar to protein phosphorylation and dephosphorylation, respectively.[13] However, the identification of specific reaction pathways of *S*-(de)nitrosation of cellular thiols has proven to be difficult, in part because most *S*-nitrosothiols are short-lived metabolites with a half-life ranging from minutes to hours.[52,53]

Measurements of NO_2^-, a stable end product of NO oxidation, have demonstrated that cells produce considerable amounts of this species. Primary mouse macrophages stimulated with inflammatory agents produce NO_2^- at a rate of 70 nmol/mg protein/h, limited only by L-arginine availability.[54] Similarly, vascular smooth muscle cells treated with the peptide hormone relaxin generate NO_2^- at a rate of 50 nmol/mg protein/h for a period of 24 h.[55] In the presence of oxygen, NO undergoes oxidation to N_2O_3 that readily nitrosates thiols. In a model system, Kharitonov et al. reported that, in the presence of physiological concentrations of GSH and O_2, almost all of N_2O_3 generated from NO is consumed to form GSNO.[56] In cells, however, NO competitively interacts with transition metals to form metal−nitrosyl complexes; some of these complexes can act as *S*-nitrosating agents.[57,58] Since GSH is the most abundant cellular thiol (15−30 nmol/mg protein), formation of GSNO is expected to parallel the activity of nitric oxide synthase. In fact, several studies have confirmed the formation of GSNO in biological systems: GSNO has been detected in rat brain (15 pmol of GSNO/mg protein),[59] rat liver cytosol and mitochondria (34 pmol of GSNO/mg protein,[60] or ∼3 nM GSNO[53]). It is noteworthy that GSNO accounts for a small fraction of the endogenously generated NO_2^-. Furthermore, GSNO levels appear to be insufficient to induce *trans*-*S*-nitrosation of protein thiols. Often, a concentration of at least 100 μM of GSNO is required for the in vitro *trans*-*S*-nitrosation of proteins. This suggests that the (de)nitrosation of critical thiols on enzymes may be GSNO independent, unless catalytic pathways for transfer of NO from GSNO and/or *S*-nitrosocysteine and *S*-nitrosohomocysteine to protein thiols exist in cells. Recently, Zhang and Hogg demonstrated that L-cystine enhances GSNO-dependent *S*-nitrosothiol uptake into cells, thus increasing intracellular *S*-nitrosothiol levels from ∼60 pmol/mg of protein to ∼3 nmol/mg of protein. The data obtained suggest that this process depends on the reduction of cystine to cysteine, which involves the xc-amino acid transport system, followed by formation and uptake of *S*-nitrosocysteine via the amino acid transport system L.[15,61] ADH,[19] Trxn,[17] and PDI[18] were found to catalyze the denitrosation of GSNO, whereas ADH does not catabolize *S*-nitrosocysteine and *S*-nitrosohomocysteine.[20] To

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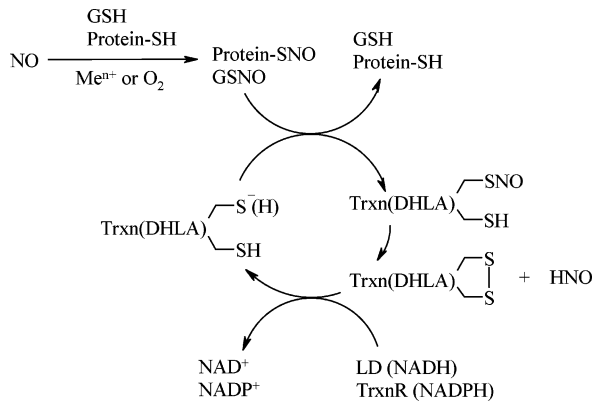
better understand the equilibrium between low molecular weight and protein *S*-nitrosothiols, further studies are needed to assess the potential of these enzymes to catalyze the denitrosation of *S*-nitrosoproteins, as well as to assess the catabolism of low molecular weight and protein *S*-nitrosothiols as a function of their cellular compartmentalization and tissue distribution.

Trxn is maintained in its active, reduced form by TrxnR that uses NADPH to reduce its S−S bridge between cysteines 32 and 35, respectively. The 3-D structure of Trxn is conserved through evolution and consists of five central stranded β-sheets externally surrounded by four α-helices.[62] Trxn's cysteines 32 and 35 are located in a protrusion of the protein between the β-2-strand and the α-2-helix.[62] The conserved active-site sequence and the 3-D structure of Trxn are common features of several Trxn-like proteins. These are proteins that either exclusively encode a Trxn domain or include thioredoxin domain as well as additional domains.[63] It is possible that Trxn-like proteins exert differential substrate selectivity in the redox regulation of disulfide- and/or *S*-nitrosothiol-containing proteins. The catalytic activity of Trxn is encompassed by the general mechanisms of cyclic disulfide/dithiol- and *S*-nitrosothiol/dithiol-type interactions. These interactions are described by either SN_2 nucleophilic substitution or addition−elimination interactions.[64] Strained cyclic disulfides are reduced at higher rates,[64] whereby a Brønsted relationship is followed in the values of pK_a of both the nucleophilic thiol and of the thiol being displaced.[65,66] Hydrophobic interactions between "attacking and central thiol" and "hard−soft acid−base behavior" have also been suggested to affect the kinetic profile of these reactions.[67,68] The redox-active S−S bridge in Trxn is surrounded by a hydrophobic molecular surface that is believed to represent the area involved in binding to TrxnR,[2] while cysteine 32, located in the active site of Trxn$(SH)_2$, exhibits a pK_a of 7.5.[69,70] Whereas most biological thiols have pK_a values in the range of 10 to 11, the reduction of accessible S−S and/or SNO functions in substrate proteins by Trxn-cysteine 32 would be kinetically favorable. However, variations in the steric hindrance of cysteine 32 in Trxn-like proteins may be a key factor in their substrate selectivity.

Haendeler et al. reported that overexpression of Trxn in endothelial cells activates eNOS, increases basal levels of endogenous *S*-nitrosothiols, and inhibits TNF-α-induced apoptosis.[71] Experiments with genetically manipulated cells that express cysteine 69-lacking Trxn suggested that these effects may reflect Trxn nitrosation in its redox inactive cysteine 69 to ONS-Cys(69)-Trxn, which, in turn, inhibited activators of apoptosis by delivering NO to their active sites.[71] These results were recently challenged by model experiments of Mitchell and Marletta who reported that GSNO (50 equiv relative to Trxn) preferentially nitrosated Trxn-S_2 in cysteine 73 without affecting cysteine 69. The authors proposed that ONS-Cys(73)-Trxn-S_2 acts as an inhibitor of caspase 3 via *trans*-*S*-nitrosation of this protease to ONS-Cys(163)-caspase 3, a reaction that may impede apoptosis.[72] Given these controversial data, it is important to elucidate whether ONS-Cys(73)-Trxn-S_2 undergoes autodenitrosation to HS-Cys(73)-Trxn-$(SH)_2$ in physiologically relevant conditions that include the presence of TrxnR and NADPH. In our experiments, the complete Trxn/TrxnR/NADPH system was able to fully reconstitute the activity of poly-*S*-nitrosated caspase 3.

Scheme 4



The data presented herein indicate that denitrosation of *S*-nitrosothiols by Trxn/TrxnR/NADPH and DHLA leads to the formation of HNO. Although HNO is a strong reductant,[73] it readily oxidizes thiols via the intermediate formation of *S*-derived hydroxylamines that can interact, intra- or intermolecularly, with a second thiol function to form disulfides and NH_2OH.[74] It is tempting to speculate that reduction of HNO to NH_2OH by GSH is the preponderant and final step in a cytoprotective process that prevents excessive *S*-nitrosation of cellular proteins. On the other hand, the formation of *S*-derived hydroxylamines reflects a nucleophilic addition of RS^- to HN═O (or HN^+-O^-), which suggests that low pK_a thiols which are deprotonated at physiological pH may interact with this species at rates higher than protonated thiols (including GSH). Examples of proteins containing low pK_a thiols are Trxn,[69,70] sulfiredoxin,[75] PDI,[76] RTEM-1 thiol β-lactamase,[77] papain,[78] and succinate dehydrogenase.[79]

Conclusions

In this work, experimental evidence is presented that Trxn and DHLA catalyze the denitrosation of *S*-nitrosothiols in both chemical and cellular systems. The predominant formation of HNO in this process (Scheme 4) is suggested by (i) the lack of potentiometrically detectable NO, (ii) the insignificant ac-

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cumulation of NO_2^-, (iii) the formation of NH_2OH, (iv) the formation of nitrosomyoglobin under conditions where HNO (but not NO) interacts with myoglobin, and (v) the generation of α-hydroxyethyl radical in acidic solutions of GSNO, DHLA and ethanol.

The denitrosation of proteins by the Trxn/Trxn system could be viewed as a novel and ubiquitous catabolic pathway whose pathophysiological significance remains to be elucidated. Recently, Ravi et al. reported that endogenous nitric oxide synthase activity or NO exposure leads to *S*-nitrosation and inhibition of endothelial nitric oxide synthase (eNOS), whereas the presence of Trxn/TrxnR significantly impedes the loss of eNOS activity. Hence, it is interesting to speculate that Trxn activity complements that of eNOS. However, further studies are needed to assess the specificity of Trxn toward *S*-nitrosated cysteine residues in various proteins.

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Supporting Information Available: ESR spectra of DMPO/α-hydroxyethyl radical nitroxide formed in aerobic and anaerobic aqueous solutions of GSNO, DHLA, DMPO, and ethanol; complete citations for refs 29, 30, and 63. This material is available free of charge via the Internet at http://pubs.acs.org.

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