Published in final edited form as:

Org Biomol Chem. 2013 July 21; 11(27): . doi:10.1039/c3ob40809d.

Glutathione and thioredoxin type 1 cooperatively denitrosate HepG2 cells-derived cytosolic S-nitrosoproteins

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Abstract

In this study, we present experimental evidence that glutathione acts in concert with human thioredoxin type 1 in the denitrosation of cytosolic S-nitrosoproteins (PSNOs) from HepG2 cells.

INTRODUCTION

Over the past two decades, the S-nitrosation and denitrosation of cellular proteins have attracted considerable interest as signalling processes that are similar to protein phosphorylation and dephosphorylation. Many proteins contain thiol groups that can be subjected to reversible trans-S-nitrosation (Scheme 1), whereby nitrosation of critical thiol groups often plays the role of an activity switch that regulates cellular functions.¹⁻⁴

The facile SH-to-SS and SH-to-SNO interconversions underlie enzymatic reactions, protein folding and trafficking, signaling by reactive oxygen and nitrogen species, and detoxification pathways (Ref. 5 and the references therein). However, the participation of sulfhydryls in dynamic equilibria has made the assessment of the ratio between their redox forms a significant challenge. The multitude of proteins that can be subjected to S-nitrosation, the similar reactivity of SH and SNO functions, and the short half-life of S-nitroso proteins (PSNOs) in biological milieu are all factors that complicate the analysis of S-nitrosative posttranslational protein modifications.

To date, a total of over 500 experimentally verified sites of S-nitrosation have been reported throughout over 300 proteins,^{1, 6} out of an estimated total of 84,000 proteins in human cells before posttranslational modification.⁷ The number of cysteine residues in proteins positively correlates with the complexity of the organisms, with humans having at least one cysteine residue in approximately 90 % of proteins.⁸

Protein thiols represent a larger active redox pool than glutathione (GSH).⁹ The normal intracellular concentration of GSH is in the mM range, while the concentrations of PSNOs and S-nitrosoglutatione (GSNO) are in the low nM range.^{10, 11} The total content of S-nitrosothiols in various tissues can be pharmacologically increased in vivo to μM concentrations,¹¹ thus inducing a dynamic equilibrium between thiol-containing proteins, PSNOs, GSNO, metal centers and GSH.

The general mechanisms of thiolate/disulfide and thiolate/*S*-nitrosothiol interchange reactions are described by reversible bimolecular nucleophilic substitution and addition–elimination reactions.^{12–14} GSNO is often used in up to mM concentrations to trans-*S*-nitrosate proteins. However, the reverse reaction (PSNOs + GSH) has received little attention. In cells, the mM concentrations of GSH versus nM levels of GSNO indicate a marked shift of the equilibrium between thiols and *S*-nitrosothiols in favor of the reduced, SH state of the native proteins (Scheme 1).

We recently found that human thioredoxin type 1 (Trxn) catalyzes the denitrosation of a large group of PSNOs.^{15, 16} In this study, we present experimental evidence that GSH acts in concert with Trxn in the denitrosation of cytosolic *S*-nitrosoproteins (PSNOs) from HepG2 cells.

RESULTS

In order to assess the denitrosation of a complex mixture of PSNOs by GSH and Trxn, we isolated cytosolic proteins from HepG2 cells (Scheme 2). Following a group separation, proteins with molecular masses over 10 kDa (PSH fraction) were separated for further *S*-nitrosation, while low molecular mass (LMM) compounds and proteins < 10 kDa were discarded (Electronic Supplementary Information; ESI). The latter fraction included cellular reductants and transition metal complexes, which can mediate the breakdown of PSNOs. Next, we trans-*S*-nitrosated PSH with GSNO to obtain, after removal of GSH and the excess of GSNO via ultrafiltration through a 10 kDa cut-off filter, a fraction of PSNOs that was relatively stable in buffered aqueous solutions. In previous studies, we have shown data indicating that this fraction is enriched with PSNOs with molecular masses ranging from 10 kDa to 80 kDa.¹⁵ A search in the Human Protein Reference Database (www.hprd.org) for cytosolic proteins in this molecular mass range returns over 10,000 entries.

Trxn is a ubiquitous protein present in cellular cytosol at concentrations of 10 - 50 μM .^{17, 18} In the presence of TrxnR and NADPH, Trxn acts as a protein disulfide reductase,¹⁹ protein denitrosylase,^{15, 16} and GSNO reductase.^{16, 20} Trxn contains a -Cys-Gly-Pro-Cys- motif that is essential for its enzyme activity. Cysteines 32 and 35 in the active site of Trxn (Trxn(SH)₂) can reduce SNO functions on substrate proteins with concomitant disulfide ring closure to Trxn(S)₂ and release of the reduced, SH form of the substrate protein. In turn, Trxn(S)₂ is converted back to Trxn(SH)₂ by the NADPH-dependent TrxnR. Denitrosation of *S*-nitrosothiols by Trxn leads to formation of nitroxyl (HNO; Scheme 3), which is readily reduced by thiols (including by Trxn(SH)₂) to hydroxylamine.^{16, 21, 22} Similarly, thioredoxin type 2 has been shown to catalyze the denitrosation of *S*-nitrosoproteins in mitochondria.²³

Denitrosation of PSNOs (24.5 nmol *S*-nitrosothiols/mg protein) and GSNO by Trxn (5 μM) in the presence of TrxnR (0.08 μM (1 unit/mL)) and NADPH (0.4 mM) is shown in Figures 1 and 2A. *S*-nitrosothiols were quantified following their Cu⁺-catalyzed breakdown to thiols and nitric oxide with concomitant chemiluminescence measurements of the latter in the gas-phase using a Sievers Nitric Oxide analyzer (ESI). At given time points (arrows), aliquots from the reaction solution were assessed for PSNOs content. The denitrosation of PSNOs required the presence of the complete enzymatic system, proceeded at relatively high initial rates, and stopped after an approximately 50% decrease of the total content of *S*-nitrosothiols. After the first 12 min of incubation, a second addition of Trxn, TrxnR and NADPH (closed-head arrows) failed to re-initiate the process, indicating that the remaining PSNOs were not substrates of Trxn. In contrast, the Trxn/TrxnR/NADPH system (but not GSH; 5 mM) fully denitrosated GSNO (Fig. 2A).

Incubation of PSNOs for 10 min at 25 °C with 0.5 – 5 mM GSH led to an approximately 80 % decrease in the total content of PSNOs (Fig. 2B). Longer incubations of PSNOs with GSH did not enhance the denitrosation process ($t_{\text{incubation}} = 30$ min; data not shown). In these experiments, quantification of PSNOs required separation of proteins from GSH and GSNO (ultrafiltration through 10 kDa cut-off filters, followed by 3 washing cycles with 0.2 mL of phosphate buffer containing 0.1 mM EDTA) as the total content of *S*-nitrosothiols in the system consisting of thiol-containing proteins, PSNOs, GSH and GSNO was constant. After the washing procedures, the content of GSNO in the final protein fraction was less than 1 % from the total content of *S*-nitrosothiols, as assessed by reverse phase HPLC with electrochemical detection (ESI). The Trxn (5 μM)/ TrxnR (0.08 μM)/NADPH (0.4 mM) system, however, fully denitrosated the sub-fraction of PSNOs (5.15 nmol *S*-nitrosothiols/mg protein) that was stable in the presence of GSH (Fig. 3B). Similarly, sequential treatment of PSNOs with Trxn, TrxnR and NADPH (open-head arrows) and then with GSH (2 mM; closed-head arrows) led to complete denitrosation of the *S*-nitrosothiols (Fig. 3C). Thus, while GSH and Trxn exhibited differential substrate selectivity for PSNOs, all PSNOs were denitrosated by GSH plus Trxn, TrxnR and NADPH.

DISCUSSION

The main finding of this study is the cooperative denitrosation of PSNOs by Trxn and GSH.

The incomplete denitrosation of PSNOs by GSH suggests that, following the trans-*S*-nitrosation of PSH by GSNO, conformational changes in some proteins restrict access to their SNO groups. Data indicating that intracellular GSH mediates the denitrosation of *S*-nitrosoproteins in rat spinal cord slices have been recently presented by Romero and Bizzozero,²⁴ while Paige et al. have assessed the denitrosation of over 100 *S*-nitrosoproteins by GSH.²⁵ In the latter study, the authors classified PSNOs as “stable” or “unstable”, with 10 *S*-nitrosoproteins found to be stable in the presence of mM concentrations of GSH.

The Trxn-catalyzed denitrosation of the sub-fraction of PSNOs that was stable in the presence of GSH could be explained with Trxn-PSNOs interactions which exposed sterically hindered SNO functions in PSNOs to the active site of Trxn. The importance of such interactions has been recently addressed by Mitchell et al., who found that, in contrast to a wild type *S*-nitroso-Trxn, an *S*-nitroso-Trxn mutant that poorly binds to caspase 3 failed to trans-*S*-nitrosate the protease.²⁶

Our model is limited to reactions of trans-*S*-nitrosation in a system consisting of multiple thiol-containing proteins. In cells, the denitrosation of GSNO by alcohol dehydrogenase (ADH),²⁷ as well as denitrosation of GSNO and PSNOs by complexes of transition metals²⁸ and reductants such as ascorbic acid,²⁹ may further accelerate the clearance of *S*-nitrosothiols. Furthermore, variations of PSNOs and thereby in the substrate-specificity of Trxn with respect to cell type are not taken into consideration.

Deconvolution of the data on the reverse reactions presented in scheme 1 provides a foundation for speculations about the end-products of the forward trans-*S*-nitrosative reactions. If a system consisting of thiol-containing proteins and GSH is exposed to catalytic amounts of GSNO, it is reasonable to expect that PSNOs that cannot be denitrosated by GSH will accumulate. It could be speculated that, irrespective of both the nature of the initial *S*-nitrosating species and the first target of “non-specific” protein nitrosation ($[\text{PSNOs}]_{\text{initial}}$), repeated cycles of trans-*S*-nitrosation will drive the ensemble of thiol-containing proteins, $[\text{PSNOs}]_{\text{initial}}$, GSH, and GSNO to a meta-stable equilibrium that is characterized by accumulation of PSNOs with SNO functions that are inaccessible for GSH

([PSNOs]_{final}). Introduction of Trxn, TrxnR and NADPH in this system will clear GSNO and [PSNOs]_{final} with concomitant generation of HNO (Scheme 3).

The complete denitrosation of PSNOs by GSH and Trxn is in agreement with observations that, under basal metabolic rate, cytosolic proteins are maintained by cellular reductants in their reduced, SH state. *S*-nitrosation of cellular thiols has been found to parallel the metabolism of glyceryl trinitrate (GTN). Janero et al. reported that pharmacological doses of GTN elicit, in the rate in vivo, extensive *S*-nitrosation throughout all major organs; minutes after treatment with GTN, *S*-nitrosothiols in tissues transiently increased from nM to μ M concentrations.¹¹ More recently, Sayed et al. have correlated the nitrate tolerance induced by GTN with *S*-nitrosation of soluble guanylyl cyclase.³⁰ At the level of a single enzyme, the cytosolic equilibrium between *S*-nitrosation and denitrosation is illustrated by our previous observation that exposure of apoptotic HepG2 cells to nitric oxide leads to inhibition of caspase-3, an executioner caspase that regulates apoptosis, and that, conversely, interruption of the nitrosative process is followed by a Trxn-dependent reactivation of caspase 3.¹⁵ *S*-nitrosocaspase 3 is stable in the presence of up to 10 mM GSH,³¹ but is readily denitrosated by Trxn, TrxnR and NADPH.^{15, 16}

Takagi et al. reported that brief exposures of endothelial cells to nitric oxide result in Trxn and Trxn mRNA induction,³² while Schulze et al. found that nitric oxide activates Trxn by impeding the expression of the thioredoxin-interacting protein, which is an inhibitor of Trxn function.³³ In contrast, extended exposure of endothelial cell to nitric oxide causes a marked decrease in Trxn and TrxnR protein mass with concomitant decrease in the catalytic activity of TrxnR but not of glutaredoxin or the cellular levels of GSH.³⁴ It is interesting to speculate that inhibition of TrxnR in cell subjected to nitrosative stress will lead to accumulation of [PSNOs]_{final}.

The denitrosation of PSNOs by GSH and Trxn are fast processes that can be viewed as both a defense mechanism and as a mechanism for fine tuning of cellular reactions. Sustained *S*-nitrosation of proteins has been associated with a number of pathological processes.³⁵⁻³⁷ On the other hand, rapid *S*-nitrosation of the cardiac ryanodine receptor³⁸ and its denitrosation by GSH³⁹ may affect the oscillations of cytosolic Ca²⁺ and thereby the frequency of muscle contractions.⁴⁰ Similarly, a transient *S*-nitrosation of the cytosolic phospholipase A₂ may enhance the biosynthesis of the short-lived prostaglandins,⁴¹ which exert a large array of physiological effects.⁴² Indeed, Salvemini et al. provided experimental evidence that the enhanced release of prostaglandins, which follows inflammatory mechanisms, is driven by nitric oxide.⁴³

Currently, the reactions that affect the cellular homeostasis of *S*-nitrosothiols are not well understood, and they require further study. Fast-labeling of SNO functions in proteins with 5,5-dimethyl-1-pyrroline *N*-oxide under photolytic conditions⁴⁴ may be a successful approach in this direction to elucidating the sequence of trans-*S*-nitrosative reactions in complex protein systems.

Conclusions

In conclusion, we present experimental evidence that GSH acts in concert with Trxn in the denitrosation of cytosolic *S*-nitrosoproteins from HepG2 cells, and that GSH and Trxn exhibit differential substrate specificity toward PSNOs. These findings provide a mechanistic framework for targeted identification and analysis of *S*-nitrosoproteins in cells subjected to nitrosative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chemiluminescence-monitored *S*-denitrosation of PSNOs by Trxn. A-PSNOs, TrxnR and NADPH. B- Plus Trxn. Closed-head arrows- second addition of Trxn, TrxnR and NADPH. Results are representative of three independent experiments where main values differed by less than 5%.

Figure 2. Denitrosation of GSNO by Trxn, Trxn and NADPH (A) and PSNOs by GSH (B). The data are presented as mean values of three independent experiments $\pm$ the standard error.

Figure 3. Sequential denitrosation of PSNOs by GSH and Trxn. A- PSNOs sub-fraction that was stable in the presence of GSH was obtained as indicated in Fig. 2B and then treated with TrxnR and NADPH. B- Plus Trxn. C- PSNOs were first treated with Trxn, TrxnR and NADPH (open-head arrows) and then with GSH (closed-head arrows). Results are representative of three independent experiments where main values differed by less than 5%.

Scheme 1.

Scheme 2.

Scheme 3.