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Mechanisms of *S*-nitrosothiol formation and selectivity in nitric oxide signaling

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Abstract

Although it is widely accepted that *S*-nitrosation occurs *in vivo*, questions remain regarding *S*-nitrosation as a signaling mechanism. The chemistry of *S*-nitrosation includes NO oxidation to N₂O₃ followed by reaction with thiolates, radical recombination of NO and thiyl radicals, and transition metal catalyzed pathways. Once formed, nitrosothiols can be transferred between small molecule or protein thiols through transnitrosation reactions. The pathways that lead to selective *S*-nitrosation of only a subset of cellular cysteines remain largely unknown. Selectivity may be conferred through colocalization with NOS isoforms, protein-protein interaction driven transnitrosation reactions, regulation of *S*-nitrosoglutathione levels, or directed denitrosation of protein nitrosothiols.

Introduction

Nitric oxide (NO) is synthesized by three mammalian nitric oxide synthase isoforms: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS). The primary NO receptor is the soluble isoform of guanylate cyclase (sGC), and NO captured by the heme cofactor of sGC activates sGC to produce the secondary messenger cyclic GMP (cGMP) [1]. However, cGMP-independent NO signaling pathways have been reported and attributed to *S*-nitrosation of structurally and functionally important cysteine residues. Dysregulation of *S*-nitrosation is implicated in the pathology of a variety of disorders including asthma, hypertension, cancer, and neurodegeneration [2]. Although it is widely accepted that *S*-nitrosation occurs *in vivo*, the mechanism(s) by which nitrosothiols are formed and removed are unclear. This review focuses on potential mechanisms by which cellular nitrosothiols are formed and how selectivity for individual cysteine residues might be conferred.

Nomenclature

S-nitrosation is a post-translational modification in which a thiol is converted to a nitrosothiol. Although nitrosation and nitrosylation are often used interchangeably, nitrosation is the proper term to describe formation of a nitroso group (R-NO), which involves a one-electron oxidation from the NO radical. Although “nitrosylation” was originally coined to suggest an analogy to phosphorylation (the transfer of the phosphoryl group), nitrosylation is chemically misleading. The nitrosyl group is formally a NO radical,

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and the nitroso group (not the nitrosyl group) is transferred during nitrosation reactions. Nitrosylation instead refers to the coordination of the nitrosyl group with a transition metal. Therefore, we utilize *S*-nitrosation to describe the conversion of a thiol to a nitrosothiol.

Mechanisms of nitrosothiol formation

S-nitrosation requires a one-electron oxidation of the initial addition complex between NO and a thiol. In the absence of an electron sink (*e.g.* under anaerobic conditions and in absence of transition metals), a nitrosothiol will not form. Nitrosothiol formation can take place through NO autooxidation to N₂O₃, radical recombination between NO and a thiyl radical, and transition metal catalyzed pathways (Figure 1). Once formed, nitrosothiols can be transferred to small molecule or protein thiols through transnitrosation reactions.

NO autooxidation to N₂O₃

Aerobic solution decomposition of NO generates N₂O₃ in a reaction that is second order in NO and first order in O₂ (Figure 2). N₂O₃ is highly reactive with water (forming NO₂⁻) or with thiolates to produce nitrosothiols and NO₂⁻ [3]. However, kinetic modeling has predicted that physiological NO concentrations (1 μM) result in femtomolar N₂O₃ concentrations [4]. As N₂O₃ is formed from radical recombination of NO and NO₂, NO must compete with the rapid reaction of NO₂ radical with cellular thiols and other reductants present at concentrations far exceeding NO concentrations. Furthermore, cellular *S*-nitrosation occurs and is sometimes enhanced under anaerobic conditions [5] suggesting that O₂ is not required for *S*-nitrosation. Therefore, the biological relevance of the N₂O₃ pathway might be limited to a small subset of cellular compartments with properties that favor reaction with N₂O₃.

The increased hydrophobicity of protein interiors or membranes was proposed to enhance rates of *S*-nitrosation 30-fold [6] due to favorable hydrophobic partitioning of NO and O₂. While nitrosothiol formation near membranes or within proteins could take place, *S*-nitrosation would have to proceed faster than diffusion. N₂O₃ reacts with glutathione (GSH) to form *S*-nitrosoglutathione (GSNO) at a rate of $6.6 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, which is orders of magnitude slower than diffusion [4]. Conversely, thiol deprotonation is disfavored in hydrophobic environments and a thiolate is required for reaction with N₂O₃ [7]. These two opposing effects must be balanced for hydrophobic phase-catalyzed *S*-nitrosation. Due to the second-order dependence on NO concentrations, N₂O₃ formation could also be favored near NOS isoforms (see “Direct interaction or colocalization with nitric oxide synthases”).

Thiyl radical recombination with NO

A second mechanism of nitrosothiol formation involves radical recombination of NO with thiyl radicals, yielding nitrosothiols [8]. Among the proposed mechanisms of nitrosothiol formation, only thiyl radical recombination is fast enough ($1\text{--}3 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ [8]) to compete with NO binding to heme ($>1.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for sGC [9]). In isolated systems containing only NO, O₂, and a thiol, thiyl radical formation proceeds through radical abstraction by a NO₂ radical [3] (Figure 1). However, thiyl radicals can be formed by a variety of mechanisms *in vivo* and formation of thiyl radicals by NO-independent mechanisms would shift nitrosothiol formation from second-order to first-order in NO concentration and be favored at low O₂ concentrations (Figure 2). Kinetic modeling suggested that nitrosothiol formation occurs exclusively through radical recombination as opposed to reaction with N₂O₃ [10]. However, GSH was the only thiol included in the kinetic model and protein thiols may favor other mechanisms of *S*-nitrosation.

Transition metal catalyzed nitrosothiol formation

A third mechanism is the direct addition of NO to a thiol to form a thionitroxyl radical (RSNOH•) followed by a one-electron oxidation to form a nitrosothiol (Figure 1). While it has been proposed that electron acceptors (e.g. O₂ or a transition metal) could oxidize the intermediate thionitroxyl radical, kinetic studies argue against this reaction contributing to nitrosothiol formation in solution [11]. However, the direct addition mechanism could be operative in an enzyme active site. Indeed, cytochrome *c* (Cyt *c*) was recently proposed to synthesize GSNO. In the proposed mechanism, initial weak binding of GSH to ferric Cyt *c* is followed by reaction with NO to form GSNO• (Figure 2), which then reduces the heme to the ferrous state to form GSNO. This reaction is energetically favorable [12] and was found most efficient at the nanomolar NO concentrations expected during NO signaling [13]. However, Cyt *c* is localized to the mitochondrial intermembrane space and GSNO does not cross the cell membrane [14] and might not cross the outer mitochondrial membrane to the cytosol (Figure 3). Therefore, *S*-nitrosation by Cyt *c* might be limited to the mitochondrial intermembrane space. Nonetheless, depletion of Cyt *c* in cells or a whole cell lysates resulted in a decrease in protein *S*-nitrosation [15] consistent with an *in vivo* role for Cyt *c*.

Cu²⁺ and Fe³⁺ can catalyze nitrosothiol formation through transient oxidation of NO to NO⁺ followed by reaction with a thiol. This reaction mechanism was proposed for GSNO formation by ceruloplasmin [3]. However, this reaction must be concerted, as NO⁺ released into solution would immediately react with water to form NO₂⁻. A more recent study showed that NO₂⁻ is the major product of the reaction of NO with ceruloplasmin, and depletion or supplementation of ceruloplasmin in plasma failed to alter plasma nitrosothiol levels [16]. A similar mechanism of nitrosothiol formation involves the binding of NO to ferric heme. The Fe³⁺-NO will possess significant Fe²⁺-NO⁺ character and can react with a thiol resulting in nitrosothiol formation and heme reduction (Figure 2). This has been proposed for ferric hemoglobin, but this process is unlikely to occur *in vivo* as the concentration of ferric hemoglobin is low and NO much more rapidly reacts with both oxy- and deoxyhemoglobin [12].

Zn²⁺ ligation has also been proposed to activate thiols towards *S*-nitrosation. Selective *S*-nitrosation of Zn²⁺ coordinated cysteines has been observed in several proteins including iNOS/eNOS [17], Sirt1 [18], and XIAP [19]. *S*-nitrosation of these proteins generally results in Zn²⁺ loss and profound effects on protein function. O₂ was required for *in vitro* *S*-nitrosation of Zn²⁺-thiolate model complexes by NO consistent with N₂O₃ as the relevant nitrosating agent [20,21].

Transnitrosation

Transnitrosation is a reversible second-order reaction between a nitrosothiol and a thiol (Figure 1). Transnitrosation proceeds through nucleophilic attack of a thiolate anion at the nitrosothiol nitrogen resulting in a nitroxyl disulfide intermediate [22,23] or transition state [24] (Figure 2). The overall mechanism of transnitrosation resembles an S_N2 reaction. Factors that control transnitrosation selectivity are discussed under "Protein-protein interaction driven transnitrosation reactions".

Mechanisms of selectivity in *S*-nitrosation

In order for *S*-nitrosation to be a viable signaling mechanism the formation and decay of nitrosothiols must be tightly controlled. Several mechanisms might impart selectivity to nitrosothiol formation and degradation including cysteine reactivity, localization with NOS isoforms, transnitrosation reactions, control of GSNO concentrations, and denitrosation reactions.

Analysis of known sites of *S*-nitrosation

Some proteins may have a cysteine in a microenvironment that favors *S*-nitrosation. However, the majority of factors that activate a particular cysteine towards *S*-nitrosation will also activate this cysteine towards other cysteine post-translational modifications. Analysis of *S*-nitrosation sites has not yielded a dominant sequence motif [25–27], and factors including the pK_a value and nearby hydrophobicity were not predictive. Instead these analyses found that most *S*-nitrosation sites contained charged residues within 6–8 Å of the nitrosated cysteine [28,29]. Importantly, the majority of these charged residues were pointed away from the nitrosated cysteine indicating that they were not directly involved in the chemistry. In addition, *S*-nitrosation was enriched in surface-accessible sites [28], suggesting that these charged residues are important for protein-protein transnitrosation reactions.

The lack of a consensus motif for *S*-nitrosation is likely due to the many mechanisms leading to nitrosothiol formation (Figure 1). Indeed, genetic manipulation of NO and GSNO concentrations suggested that distinct proteins are *S*-nitrosated by NO oxidation pathways versus GSNO transnitrosation pathways [30]. Therefore, it may be possible to cluster *S*-nitrosation sites based on their mechanism of formation. To this end, several groups have developed machine-learning techniques to describe and predict the nitrosoproteome. These efforts have resulted in three web servers (SNOsite, GPS-SNO, and CPR-SNO) in which a user can input a protein sequence and generate predicted *S*-nitrosation sites [31–33]. In addition, a centralized database (dbSNO) of >3000 protein *S*-nitrosation sites curated from >219 research articles was recently made available [34].

Direct interaction or colocalization with nitric oxide synthases

There are several examples where interaction with NOS results in selective protein *S*-nitrosation due to increased concentration of nitrosating agents near the site of *S*-nitrosation (Figure 3). For example, the formation of an iNOS/cyclooxygenase-2 (COX-2) or iNOS/arginase-1 complex was necessary for COX-2 or arginase-1 *S*-nitrosation, respectively [17,35]. In cases where direct interaction does not occur, a scaffolding protein can facilitate protein *S*-nitrosation. For instance, COX-2 enables the association of cPLA₂α with iNOS resulting in cPLA₂α *S*-nitrosation [36].

Protein *S*-nitrosation may also be controlled through compartmentalization with NOS isoforms. eNOS is the only NOS isoform that is myristoylated/palmitoylated and targeted to specific intracellular membranes including the caveolae and Golgi apparatus (Figure 3). Therefore, colocalization with eNOS may represent a mechanism for local protein *S*-nitrosation even when direct interaction with eNOS does not occur [37]. Indeed, a general increase in protein *S*-nitrosation surrounding eNOS at the Golgi apparatus has been observed [38].

Both iNOS and eNOS are selectively *S*-nitrosated at the Zn²⁺-tetrathiolate site [17]. In all cases involving NOS in protein *S*-nitrosation, it is unclear if *S*-nitrosation is due to transnitrosation with *S*-nitrosated NOS or due to an increase in the local concentration of nitrosating agents (Figure 3). Transnitrosation with *S*-nitrosated NOS would represent an elegant mechanism for controlled and compartmentalized nitrosothiol formation and reconcile the apparent paradox between the high diffusability of NO with the localized protein *S*-nitrosation observed in close proximity to NOS [38]. Furthermore, transnitrosation from NOS *S*-nitrosated at the Zn²⁺-tetrathiolate (or potentially *N*-nitrosated at the tetrahydrobiopterin cofactor [39]) to GSH would allow for controlled GSNO formation [40]. There is evidence that NOS auto-*S*-nitrosation is a controlled mechanism. All three mammalian NOS isoforms contain a hydrophobic tunnel between the heme cofactor and the

Zn^{2+} -tetrathiolate. This tunnel was proposed to provide a hydrophobic cavity for generation of nitrosating agents such as N_2O_3 and shuttle these nitrosating agents to the Zn^{2+} -tetrathiolate. Detailed kinetic analyses were consistent with the ability of NO generated at the iNOS heme cofactor to *S*-nitrosate the Zn^{2+} -tetrathiolate without leaving iNOS [40].

Protein-protein interaction driven transnitrosation reactions

Structural analysis of protein *S*-nitrosation suggested that protein-protein interaction driven transnitrosation is a major mechanism imparting selectivity to protein nitrosothiol formation [28,29]. Several factors control the rate of transnitrosation including steric hindrance of the nitrosothiol or attacking thiolate, the $\text{p}K_a$ of the attacking thiol, and the electrophilicity of the nitrosothiol. However, the $\text{p}K_a$ of the attacking thiol is unlikely to determine which thiols are stably *S*-nitrosated, since a thiol with a low $\text{p}K_a$ is both a better nucleophile and a better leaving group during transnitrosation. Instead low $\text{p}K_a$ thiols might catalyze transnitrosation reactions through two subsequent transnitrosation reactions with a nitrosothiol intermediate analogous to a ping-pong mechanism with a covalent enzyme intermediate. This will increase the rate of transnitrosation and also impart specificity through specific protein-protein interactions. Both glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and thioredoxin 1 (Trx1) have been shown to participate in protein-protein interaction driven transnitrosation reactions [18,25,41,42].

Although transnitrosation is reversible, the reaction can be driven in one direction if *S*-nitrosation results in a conformational change rendering the nitrosothiol inaccessible or if *S*-nitrosation results in translocation of the protein to a different cellular compartment. GAPDH *S*-nitrosation at C152 results in GAPDH binding to Siah1, which contains a nuclear localization sequence and translocation of the GAPDH/Siah1 complex to the nucleus occurs [43] (Figure 3). Once in the nucleus, GAPDH-C152-SNO transnitrosates Sirt1, histone deacetylase 2 (HDAC2), and DNA-dependent protein kinase (DNA-PK) [18]. GAPDH *S*-nitrosation at C247 has also been observed [25,28,44,45], but the transnitrosation targets of GAPDH-C247-SNO have not been identified. In addition, the mechanism of GAPDH *S*-nitrosation at either C152 or C247 is unknown. GSNO is often used to selectively *S*-nitrosate GAPDH at C152 *in vitro* and docking analysis suggested good affinity for GSNO near C152 [29]. Alternatively, GAPDH was shown to form a complex with NOS isoforms [46] and might be directly transnitrosated by *S*-nitrosated NOS. Interestingly, treatment of cells with interferon- γ and oxidized low-density lipoprotein resulted in selective *S*-nitrosation of C247, whereas treatment with interferon- γ and lipopolysaccharide resulted in selective *S*-nitrosation of C152 [44].

Thioredoxin is another important nexus of *S*-nitrosation signaling. Thioredoxin 1 (Trx1) contains five conserved cysteines: two redox-active catalytic cysteines (C32 and C35) and three non active-site cysteines (C62, C69, and C73). *S*-nitrosation of all three non active-site cysteines of Trx1 has been observed by GSNO, but more recent analyses showed that the redox state of the active-site cysteines is a critical factor in controlling GSNO transnitrosation [25,47]. Specifically, Trx1 is selectively transnitrosated by GSNO at C73 when the active-site cysteines (C32 and C35) are oxidized to a disulfide (oTrx1) [25,47] (Figure 3). Active-site reduced (rTrx1) was found to be selectively transnitrosated at C62 [47], although others could not detect rTrx1 *S*-nitrosation [25].

The redox dependent transnitrosation of Trx1 suggests the intriguing possibility that Trx1 may transnitrosate distinct proteins targets depending on the redox state of the cell. While the possibility that rTrx1-C62-SNO selectively transnitrosates distinct protein targets has not been explored, a recent proteomics study identified 47 putative transnitrosation targets of oTrx1-C73-SNO including GAPDH [25], suggesting crosstalk between the Trx1 and GAPDH transnitrosation pathways (Figure 3). oTrx1-C73-SNO is also known to selectively

transnitrosate the active-site cysteine of caspase-3 (C163) [41,42]. Importantly, the direct interaction of Trx1 and caspase-3 (Casp3) is essential for transnitrosation as a Trx1 mutant that lost the ability to bind Casp3 was unable to transnitrosate Casp3 [41,42]. Downstream, Casp3-C163-SNO was found to transnitrosate the X-linked inhibitor of apoptosis (XIAP) [19], and disruption of the Casp3/XIAP interaction abolished XIAP *S*-nitrosation.

Regulation of GSNO concentrations

Since GSNO participates in transnitrosation reactions with proteins, modulation of GSNO levels should directly affect levels of protein *S*-nitrosation. Several enzymes have been reported to degrade GSNO *in vitro* including protein disulfide isomerase, xanthine dehydrogenase/oxidase, superoxide dismutase, and glutathione peroxidase [48]. However, only two enzymes have been shown to degrade GSNO and affect protein *S*-nitrosation *in vivo*: GSNO reductase (GSNOR) and carbonyl reductase 1 (CBR1) (Figure 1 and 3).

GSNOR (a.k.a. alcohol dehydrogenase class III or glutathione-dependent formaldehyde dehydrogenase) catalyzes the NADH-dependent reduction of GSNO to form the intermediate, GSNHOH, which rearranges to form glutathione sulfinamide (GSNH₂) [49,50] (Figure 1, 2, and 3). GSNOR does not reduce *S*-nitrosocysteine, *S*-nitrosohomocysteine, *S*-nitroso-*N*-acetylpenicillamine, or protein nitrosothiols [51,52]. GSNOR knockout mice have markedly increased levels of both GSNO and *S*-nitrosated proteins [48], and GSNOR inhibitors are currently being developed to treat lung and cardiovascular disorders, asthma, and other inflammatory diseases [53,54].

CBR1 catalyzes the NADPH-dependent reduction of GSNO. CBR1 is specific for GSNO, as the reduction of *S*-nitrosocysteine was not catalyzed by CBR1. In cell lysates CBR1 was shown to account for 30% of the NADPH-dependent GSNO reductase activity [55]. Although CBR1 and CBR3 possess 72% sequence identity, only CBR1 is capable of GSNO reduction [56]. CBR1 and GSNOR possess similar K_M values for GSNO (~30 μ M), but GSNOR exhibits a 5-fold greater k_{cat} value [49,55]. The NADH-dependence of GSNOR and NADPH-dependence of CBR1 suggests that the NADH/NAD⁺ and NADPH/NADP⁺ ratios may influence the relative GSNO reduction rates of GSNOR versus CBR1.

Denitrosation of protein nitrosothiols

If *S*-nitrosation is a specific cellular post-translational modification, then regulated mechanisms to remove protein nitrosothiols (denitrosation) must exist. Implicit in the transnitrosation mechanism is denitrosation of the original nitrosothiol (Figure 1). Transnitrosation reactions involving GSH will result in GSNO formation, and denitrosation of GSH sensitive protein nitrosothiols can be driven to completion by GSNOR or CBR1. However, only a subset of cellular nitrosothiols are susceptible to transnitrosation by GSH [45,57] suggesting other mechanisms exist to denitrosate protein nitrosothiols. Although many proteins catalyze denitrosation *in vitro* [48], only thioredoxin has been shown to affect levels of cellular protein nitrosothiols.

Thioredoxin catalyzed denitrosation generates a free thiol at the original *S*-nitrosation site, active-site oxidized thioredoxin, and putatively NO⁻ [58] (Figure 2). Active-site reduced Trx1 is then regenerated through the action of thioredoxin reductase 1 (TrxR1). There is evidence that Trx1/TrxR1 catalyzes denitrosation of proteins such as eNOS [17], Casp3 [59,60], Casp8 [61], Casp9 [59], protein Tyr phosphatase 1B [59], GAPDH [59,62], and *N*-ethylmaleimide-sensitive factor [62] (Figure 2). In addition, recent proteomics studies identified >100 putative substrates of Trx1 denitrosation [28,63,64]. Trx1 denitrosation specificity might be determined by direct protein-protein interactions between Trx1 and *S*-nitrosated proteins. Compartmentalization might also regulate specificity in Trx catalyzed

denitrosation. For example, both the cytosolic Trx1 and mitochondrial Trx2 can catalyze protein denitrosation [59] suggesting these Trx isoforms may denitrosate distinct cytoplasmic or mitochondrial targets.

Trx1 possesses two seemingly opposed functions catalyzing both transnitrosation and denitrosation of proteins. This is best exemplified with Casp3 where different cysteine residues in Trx1 catalyze the transnitrosation (C73) or denitrosation (C32) of Casp3. Inhibition of TrxR results in an increase in protein *S*-nitrosation and Trx1 active-site oxidation (oTrx1) [25,57,59]. However, conversion of rTrx1 to oTrx1 will both inactivate the denitrosation by C32 and activate transnitrosation by C73. In either case an increase in protein *S*-nitrosation is expected. Further complicating matters is the observation that oTrx1-C73-SNO is denitrosated by rTrx1 [25,65] (Figure 3). Therefore, it is difficult to distinguish the transnitrosating and denitrosating activities of Trx1.

Outlook

It is becoming increasingly clear that NO signaling involves protein targets other than sGC. *S*-nitrosation is an attractive candidate for these cGMP-independent pathways, but several fundamental questions have yet to be resolved to establish *S*-nitrosation as a viable signaling mechanism and not simply a byproduct of nitrosative stress. How are nitrosothiols initially formed in the cell? What determines the specificity of *S*-nitrosation to individual cysteine residues in proteins? How are these processes regulated? This review has summarized how these critical questions are beginning to be addressed, but many details have yet to be uncovered.

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Highlights

- Nitric oxide can signal through *S*-nitrosation of cysteine residues.
- *S*-nitrosation can occur through NO oxidation to N₂O₃ or radical recombination.
- Cytochrome *c* was recently discovered to catalyze the synthesis of GSNO.
- Colocalization with NOS isoforms can confer selectivity to *S*-nitrosation.
- Another significant mechanism of selectivity is protein-protein transnitrosation.

Figure 1.

Potential mechanisms of protein nitrosothiol formation and degradation. Activation of nitric oxide synthases (NOS) results in NO production and auto-S-nitrosation. S-nitrosoglutathione (GSNO) or protein nitrosothiols can be generated from NO through either a transition metal catalyzed pathway, thiyl radical recombination, NO oxidation to N₂O₃, or transnitrosation with protein nitrosothiols such as NOS. Once formed, GSNO or S-nitrosated NOS can transfer nitrosothiols to other proteins through transnitrosation reactions. Degradation of nitrosothiols can occur through direct denitrosation of GSNO by the NAD(P)H-dependent enzymes GSNO reductase (GSNOR) or carbonyl reductase 1 (CBR1).

Thioredoxin (Trx) can denitrosate protein nitrosothiols, a process that becomes catalytic through the activity of thioredoxin reductase (Trx).

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Figure 2.

Mechanisms of reactions discussed in this review. In the NO oxidation pathway NO first reacts with O_2 to form a peroxynitrite radical. The peroxynitrite radical then undergoes radical recombination with NO to form ONOONO (or other isomers) followed by homolytic cleavage to form two molecules of NO_2 radical. Radical recombination of NO and NO_2 radicals results in N_2O_3 formation, which then reacts with a thiolate (RS^-) to form a nitrosothiol (RSNO) and nitrite (NO_2^-). In the thiyl radical recombination pathway a thiyl radical is formed by hydrogen abstraction by another radical ($\text{X}^\bullet$). Radical recombination of the thiyl radical with NO forms a nitrosothiol. Several transition metal catalyzed pathways are possible. In one pathway GSH weakly associates with cytochrome *c*, and the bound GSH

attacks NO to form a GSNOH radical. The ferric heme of cytochrome *c* then accepts an electron from the GSNOH radical to form ferrous heme and GSNO. Reoxidation of cytochrome *c* completes the catalytic cycle. In another transition metal catalyzed pathway NO can bind to ferric heme, and the resulting $\text{Fe}^{3+}\text{-NO}$ complex (which possesses significant $\text{Fe}^{2+}\text{-NO}^+$ character) then reacts with a thiolate to form a nitrosothiol and ferrous heme. Once formed a nitrosothiol can be transferred through transnitrosation reactions in which a thiolate attacks the nitrogen atom of a nitrosothiol resulting in a nitroxyl disulfide intermediate or transition state that decays to a thiolate and a nitrosothiol. Denitrosation of nitrosothiols can proceed through several mechanisms. GSNOR and CBR1 catalyze hydride transfer from NAD(P)H to GSNO to form a GSNHOH intermediate that spontaneously rearranges to GSONH₂. Thioredoxin catalyzed denitrosation proceeds through transnitrosation of a thioredoxin active-site cysteine (C32). Subsequently, the other active-site cysteine (C35) eliminates the nitrosothiol intermediate at C32 to form active-site oxidized thioredoxin and nitroxyl (NO^-). The catalytic cycle is completed via thioredoxin reductase catalyzed NADPH-dependent reduction of the thioredoxin active-site disulfide.

Figure 3.

Potential transnitrosation signaling pathways. Nitric oxide synthase (NOS) activity results in NO formation and NOS auto-*S*-nitrosation. iNOS is cytoplasmic whereas eNOS can be palmitoylated/myristoylated and targeted to the caveolae. Direct interaction with iNOS or eNOS results in *S*-nitrosation of arginase-1, COX-2, cPLA₂α, and GAPDH among other proteins although it is unclear if NO, GSNO, or NOS *S*-nitrosation is the source of *S*-nitrosation. GSNO can be generated through a variety of pathways (see Figure 1), but it is unclear if cytochrome *c* (Cyt *c*) can contribute to the cytoplasmic GSNO pool as Cyt *c* is localized to the mitochondrial intermembrane space. Once formed, GSNO transnitrosates active-site reduced thioredoxin 1 (rTrx1) at C62 and active-site oxidized Trx1 (oTrx1) at C73. Although the downstream targets of rTrx1-C62-SNO are unknown, oTrx1-C73-SNO transnitrosates caspase-3 (Casp3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) among other proteins. *S*-nitrosated Casp3 can then transnitrosate X-linked inhibitor of apoptosis (XIAP). GAPDH is nitrosated at two different cysteines: C152 and

C247. GAPDH *S*-nitrosation at C152 recruits Siah1 binding resulting in translocation of the Siah1/GAPDH complex to the nucleus where GAPDH-C152-SNO transnitrosates Sirt1, HDAC2, and DNA-PK. The downstream transnitrosation targets of GAPDH-C247-SNO are unknown. Dashed arrows and question marks indicate areas where further research is particularly needed.

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