Published in final edited form as:

For Immunopathol Dis Therap. 2012 ; 3(2): 183–191. doi:10.1615/ForumImmunDisTher.2012006372.

A Conspectus of Cellular Mechanisms of Nitrosothiol Formation from Nitric Oxide

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Abstract

Although chemical mechanisms for the formation of nitrosothiol from •NO have been studied extensively “in the test tube”, surprisingly little is known regarding the mechanism(s) of how nitrosothiols are formed *in vivo*. This lack of understanding has hampered more general acceptance of the concept of cysteine nitrosothiol formation as a generally applicable, regulated, and functionally significant protein posttranslational modification (as opposed to multiple other •NO-induced thiol modifications). Here we provide a brief overview/summary of the cellular formation of nitrosothiols from •NO *via* two possible mechanisms involving oxygen or transition metals.

Keywords

nitric oxide; nitrogen monoxide; nitrosation; nitrosothiol; dinitrosyliron complexes; cytochrome c

I. Introduction: NITROSATION AND ITS BIOLOGICAL IMPORTANCE

Nitric oxide (nitrogen monoxide, •NO) reacts appreciably with only two types of biological targets, (1) transition metal ions and (2) other species that, like •NO, contain unpaired electrons (such as dioxygen, superoxide, and lipid radicals). In spite of this apparent simplicity, the subsequent reactions of the resulting higher nitrogen oxides are exceedingly complex, and we have only a superficial knowledge about which of the many reactive species that are formed “in the test tube” are most important in the biological milieu. The primary reason for our ignorance is that most of these species are highly reactive and therefore exist only transiently. Thus, we are forced to rely on observation of the “footprints” that these species leave as an indication of their existence and effects.

One type of reaction that definitely occurs whenever •NO is produced biologically is nitrosation[†]. Formally, this reaction can be viewed as the attack of the one-electron oxidation product of •NO, nitrosonium (NO⁺), on a nucleophile. Although free NO⁺ does not exist at neutral or even moderately acid pH,² it can be transferred from one species to another. In this regard, it is similar to the proton, which also does not exist in a free state

biologically (in aqueous solution it exists associated with water molecules)³ but is certainly transferred from one species to another.

It has been amply verified that exposure of cells and tissues to •NO results in nitrosation, specifically the nitrosative attack of sulfur and nitrogen. Nitrosation of thiol results in formation of nitrosothiol (RSNO); nitrosation of amine results in nitrosamine ([R,R']NNO). The nitrosamine formed from a primary amine is unstable and undergoes deamination, thus converting a primary amine to an alcohol. Nitrosamines formed from secondary amines can be potent carcinogenic agents; in addition, nitrosation of the exocyclic amine group of guanine results in its conversion to xanthine and formation of abasic sites. Nitrosation of amines could therefore be an important reaction biologically.⁴ In the case of thiols, it has been proposed that formation of nitrosothiol from critical thiol residues may act as an important post-translational modification of proteins, similar in principle to phosphorylation⁵ (although aspects of this proposal have been questioned).^{6,7} In addition, cell and tissue injury can occur due to accumulation of nitrosative products resulting from exaggerated •NO synthesis, termed nitrosative stress.⁸ Thus, nitrosation is an important biological consequence of •NO formation.

In this brief overview, we provide a summary of the chemical and biochemical principles and biological applications of RSNO formation from •NO[‡].

II. NITROSATION FROM •NO: AN OXIDATIVE CHEMISTRY

Contrary to what is sometimes stated in the literature^{12,13} the letters “NO” in the chemical formula for nitrosothiol (RSNO) do not denote nitric oxide. Although in biological systems nitrosothiols can be formed from thiols upon exposure to •NO, and •NO can be formed from nitrosothiols, the functional group in RSNO is a nitroso group covalently bound to the thiol sulfur atom (RS-N=O). The following balanced equation shows RSNO formation from •NO requires a one-electron oxidation:

Thus, either thiol or •NO must first be oxidized, so the central question in understanding cellular mechanisms of RSNO formation from •NO becomes what is the oxidant that accepts the electron.

III. OXYGEN AS THE OXIDANT

Historically, by far the most studied reaction of •NO is its reaction with dioxygen (O₂) in the gas phase. This reaction was in fact utilized by some of the founders of modern chemistry (including Scheele, Priestley, Cavendish, Lavoisier, and Dalton) to perform the original determinations of the oxygen content of the atmosphere.¹⁴ In addition to the gas phase, the overall reaction (called the autoxidation of •NO) also occurs in aqueous media, with identical overall stoichiometry:

This reaction is an oxidation of nitrogen, as illustrated by the concept of oxidation number.¹⁵ The oxidation number for the nitrogen in •NO is +2, since the molecule is uncharged and the

[‡]Once formed from •NO or nitrite, the nitroso group in nitrosothiol is subject to often extensive (and perhaps specific) transfer to different thiol acceptors via a process called transnitrosation;^{9–11} this exciting phenomenon is not covered here.

oxidation number for oxygen (the more electronegative atom) is -2 . In nitrite, the nitrogen oxidation number is $+3$ because the oxidation number for oxygen is -4 (2×-2) and the molecule carries a net -1 charge. Importantly, the nitrogen oxidation number in nitrosothiol is $+3$; thus, the nitrogen in nitrite is in the oxidation state “ready” for RSNO formation.

The autoxidation reaction of $\bullet\text{NO}$ has been one of the most intensively studied of all chemical reactions since the first kinetic study was published nearly 100 years ago.¹⁶ It is generally agreed that this multistep reaction involves first the formation of the oxidizing radical nitrogen dioxide ($\bullet\text{NO}_2$)[§]:

which reacts reversibly with remaining (excess) $\bullet\text{NO}$ to form nitrous anhydride (N_2O_3):

N_2O_3 hydrolyzes rapidly (which is also a reversible reaction):

There are two mechanisms whereby RSNO formation can occur from $\bullet\text{NO}$ autoxidation. First, N_2O_3 is a potent nitrosating agent because it can be considered a “carrier” of nitrosonium (formally, though certainly not accurately, “nitrosonium nitrite, NO^+NO_2^- ”). This is an example of oxidation of $\bullet\text{NO}$ because both nitrogens in N_2O_3 are in the $+3$ oxidation state, acceptable to form RSNO:

The second mechanism involves one-electron oxidation by $\bullet\text{NO}_2$ of thiol (more accurately, thiolate anion) to form the thiyl radical ($\text{RS}\bullet$) followed by radical–radical recombination with $\bullet\text{NO}$:

Which process is dominant is determined by the recognition that both involve $\bullet\text{NO}_2$, the former involving reaction with $\bullet\text{NO}$ and the latter reaction with thiol. It has been predicted that, due primarily to the much higher cellular concentration of thiol compared to $\bullet\text{NO}$, under biologically relevant conditions reaction of $\bullet\text{NO}_2$ with thiol will outcompete reaction with $\bullet\text{NO}$; thus, RSNO formation will occur primarily by the one-electron radical mechanism as opposed to N_2O_3 ,^{7,18,19} although, as has been demonstrated, it can be difficult to discriminate between the two experimentally because of the rapid reversibility of reaction 4.²⁰

Thus, exposure of $\bullet\text{NO}$ to thiols in the presence of O_2 results in RSNO formation *in vitro*, and this is commonly cited as a potential mechanism for RSNO formation *in vivo*. There are, however, three major complications regarding the relevance of this mechanism to the

[§]Or another oxidizing intermediate, distinct from $\bullet\text{NO}_2$ ¹⁷

universality of data interpretation supporting biological protein nitrosation as a signaling mechanism. First, it has been shown both theoretically and experimentally that nitrosation is a quite minor thiol modification quantitatively under aerobic conditions; oxidation of thiol is much more extensive, thus raising the possibility that RSNO formation may not be the functionally significant thiol modification (although could be an accurate marker).^{7,21,22} Second, cellular RSNO formation from •NO is increased with decreasing O₂ concentration¹⁹ and actually occurs in the absence of O₂, in both mitochondria²³ and also cells.^{8,24,25} Third, the kinetics of this reaction has been thoroughly studied, and the rate of reaction is exponential and extremely slow at physiologically relevant •NO concentrations, with an initial half-life as long as 50 hours and a rate of 50 fM/s (at [•NO] = 10 nM).²⁶ This conclusion is also strengthened by considering that as little as 0.02% of total NO (as judged by nitrite formation) yields RSNO in cells²⁷ and that cells consume •NO by an O₂-dependent mechanism that is much more rapid than the •NO/O₂ autoxidation by a process (of whatever nature) that does not produce measurable RSNO.²⁸ This is perhaps tempered somewhat by the demonstration that hydrophobic environments such as membranes (although not proteins)²⁰ accelerate this reaction approximately 30-fold;²⁹ however, the targets of this accelerated autoxidation are in the aqueous compartments immediately adjacent to the membrane as opposed to the membrane interior.³⁰ A proposal that •NO reacts directly with thiol and produces a product that is subsequently oxidized in the presence of an electron acceptor such as O₂³¹ is not supported by recent data²⁰ (with the possible exception of cytochrome c, see below).

Finally, these concepts reveal that any process that accomplishes one-electron oxidation of thiol to thiyl radical will, in the presence of •NO, result in RSNO formation (Reaction 8). This includes, for example, •NO₂ generation from peroxynitrite or hydroxyl radical³² and also protein radical exchange with tyrosyl radical.³³ However, generation of one-electron oxidants in general requires oxygen, and some data demonstrate that under some conditions cellular RSNO formation can occur without O₂.^{8,24,25} Nevertheless, under some conditions, and in some microenvironments, RSNO formation may thus be a ubiquitous (and inevitable) outcome and also perhaps a quantitative indicator of oxidative stress in the presence of •NO.³² This could have important consequences regarding both nitrosative stress and also the possibility that RSNO levels could act as “sensors” of the copresentation of reactive oxygen species and •NO.

IV. TRANSITION METAL(S) AS THE OXIDANT

The other major candidate as electron acceptor for RSNO formation from •NO is transition metals:

It is important to point out that biological systems go to a great deal of effort to “shield” transition metals, most especially iron and copper, because the “free” metal[¶] possesses potent activity in reaction with partially reduced oxygen species to produce highly oxidizing and damaging reactive species. Thus, most (but not all) metal ions are bound as prosthetic groups in proteins or bound in storage proteins, with only limited ability to coordinate •NO.

As with oxygen, it is theoretically possible that metals could initially oxidize either thiol or •NO. Not surprisingly, because of the well-documented affinity of •NO for transition metals, most studies of metal-mediated RSNO formation from •NO have involved as the first step

[¶]Transition metals are never truly “free”, and are most accurately described as “loosely bound”.

complexation of •NO with metal to form metal nitrosyl. Classic inorganic chemistry studies have documented the electrophilicity (propensity to attack nucleophilic groups such as thiolates) of the nitrosyl group in some (but not all) metal nitrosyls,³⁴ as exemplified by the well-known reaction of the iron-nitrosyl nitroprusside with thiols to form sulfur-containing adducts; however, in this case, transfer of the nitroso group to thiol, forming nitrosothiol, does not occur to an appreciable extent.³⁵

Vanin *et al.*³⁶ provided *in vitro* evidence for the formation of nitrosothiol (glutathione) from •NO mediated by iron ions as judged by enhancement upon metal addition and attenuation with iron chelator, although surprisingly ferrous, not ferric, iron was utilized. Other studies reported similar thiol nitrosation from •NO in the presence of Cu²⁺, with formation of reduced metal (Cu⁺) (glutathione^{37, 38}, albumin³⁸, and hemoglobin³⁸). Inoue *et al.*³⁷ showed that the copper-containing protein ceruloplasmin can also accomplish this chemistry. In the case of ceruloplasmin, the mechanism appears to involve first formation of copper-nitrosyl followed by nitroso group transfer to thiol, while the reaction with albumin involves copper-thiol complexation followed by reaction with •NO. The physiological relevance of these results, however, is not clear. These results were obtained with reactions occurring either *in vitro* or extracellularly, and “free” copper is maintained at very low levels biologically. Also, this reaction would be confined mainly to the plasma and liver, since these are the major locations of ceruloplasmin.

In terms of *cellular* mechanisms of metal-catalyzed nitrosothiol formation, the first such claim was the proposal in 1996 of heme-catalyzed nitrosation from •NO of cysteine β93 of hemoglobin.³⁹ The specific chemical mechanism of electron transfer that was proposed was reduction of ferriheme upon complexation to •NO with nitroso group transfer to the cysteine thiol,⁴⁰ formally equivalent to a well-known chemical phenomenon called reductive nitrosylation.⁴¹ However, the physiological relevance of this reaction is not clear, and aspects of this overall hypothesis have been challenged.⁴² In addition, this specific mechanism is confined to a single cell type, the erythrocyte. There is *in vitro* evidence that other cellular hemoproteins catalyze RSNO formation from •NO *via* reductive nitrosylation, including myoglobin, neuroglobin, nitrophorin, and cytochrome c;⁴¹ however, evidence for significant activity in cells has been reported only for cytochrome c, as described below.

Another potential cellular pool of transition metal capable of acting as a redox partner for nitrosothiol formation from •NO is the chelatable (or labile) nonheme iron pool, which has been shown to be the major species responsible for production of highly reactive oxygen species *via* chemical mechanisms including the Fenton reaction.⁴³ Binding of •NO to this cellular iron pool and prevention of this chemistry has been proposed.^{44,45} We previously reported that this iron pool is quantitatively responsible for the formation of a species visible by electron paramagnetic resonance (EPR) spectroscopy that yields a “g=2.04” signal that has been extensively observed *in vitro* and *in vivo* resulting from either exogenous or endogenous •NO exposure.⁴⁶ This signal has been shown to arise from a dinitrosyliron complex (DNIC) involving coordination of two nitrosyls and two other (most commonly thiol) ligands, resulting in a species in which the nitrosyl groups possess substantial nitroso character (Fig. 1).⁴⁷ Cellular DNICs have been shown to be the most abundant cellular nitrogen oxide adduct when exposed to •NO.⁴⁸ It is thus reasonable to surmise that formation of RSNO from this species might be accomplished by a type of transnitrosation. This was first proposed in 1994 by Mülsch⁴⁹ and has been demonstrated *in vitro* with model DNIC and various thiols.^{36,50–54}

The first evidence for such a mechanism in cells was provided by Kim *et al.*, who reported that cells with relatively high amounts of nonheme iron (e.g., the rat hepatocyte) which exhibit more intense g=2.04 signals upon treatment with •NO donor (as compared to cells

with lower amounts of nonheme iron) also showed increased RSNO levels. In addition, evidence was presented that caspase-3 is inactivated through nitrosation of an essential thiol in its active site.⁵⁵ We showed that the extent of cellular RSNO formation correlates with the intensity of the DNIC signal (both species being localized to large molecular weight species), including using an iron chelator that specifically binds to the chelatable iron pool;^{**} we also showed that cellular RSNO formation from •NO does not involve O₂,²⁴ an observation which has been reported elsewhere.⁸ At this point, however, the evidence for this mechanism is correlative as opposed to causal.

Recently, it has been demonstrated that oxidized cytochrome c accomplishes efficient nitrosation of glutathione (GSH) from •NO to form nitrosoglutathione (GSNO) which can then accomplish nitrosation of proteins.^{56,57} In cells, protein nitrosation from GSNO (and *vice versa*) is a well-established phenomenon with important consequences.^{8,9} The mechanism seems to involve an initial complex of GSH with ferricytochrome c followed by reaction of the complex with •NO and formation of GSNO plus reduced cytochrome c. Most importantly, evidence was presented that this is an important mechanism in cells: cytochrome c null embryonic mouse cells display significant (approximately 75%) decrease in RSNO upon treatment with •NO donor and immunodepletion of cytochrome c in cell extracts significantly (although partially, 40%) decreases RSNO formation from •NO.⁵⁷ In addition, treatment of cells with the mitochondrial inhibitor antimycin A increases cellular RSNO formation, consistent with an effect due to increased cytochrome c oxidation as a result of upstream inhibition of electron flow.⁵⁷

V. CONCLUSIONS

Although nitrosative mechanisms from •NO resulting from reaction with O₂ and/or O₂^{•−} are commonly cited as the main candidate for RSNO formation biologically, there is little evidence for this conclusion and good reason to suspect it is a minor contributor (if at all). There is growing evidence for the involvement of transition metals, with some metalloproteins contributing under specific conditions and in specific *in vivo* locations (hemoglobin, ceruloplasmin). Two widely distributed species that may be involved are cytochrome c (with participation of glutathione) and the chelatable iron pool, and there may be specific conditions where one or the other of these two species plays a dominant role. In any event, there is as yet very little evidence for a selective, specific mechanism of thiol nitrosation from •NO, as might be expected for a physiologically specific signaling mechanism. One exciting possibility is that some degree of selectivity/specificity is imparted by nitrosation/denitrosation involving specific transnitrosation mechanisms, such as thioredoxin.⁵⁸ This could suggest that the only function for •NO is in a relatively nonselective generation of a heterogeneous collection of cellular nitrosothiols, which provides a “substrate pool” of RSNO (with glutathione perhaps playing a key role) that is utilized for further specific protein-mediated transnitrosation mechanisms.⁵⁹ The important question, however, then becomes: *Assuming a biological action of signaling protein nitrosation is in response to •NO, what is the mechanism whereby the extent of nitrosation reflects modulations in levels of •NO?* The answer to this question can only be provided by understanding the cellular mechanism(s) of nitrosation from •NO, which is at a very early stage. One intriguing possibility, for which there is at present only *in vitro* evidence, is that nitric oxide synthase(s) is an immediate target of nitrosation upon stimulation to produce •NO, although the mechanism is unclear.⁶⁰

^{**}Inhibition of cellular RSNO formation by metal chelators has also been reported elsewhere.^{8, 25}

Acknowledgments

This review was supported by NIH grant CA131653 (to J.R.L.).

ABBREVIATIONS

DNIC	dinitrosyliron complexes
EPR	electron paramagnetic resonance
GSH	glutathione
GSNO	S-nitrosoglutathione
[R,R']NNO	nitrosamine
RSNO	nitrosothiol

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FIGURE 1.

General structure of a dinitrosyliron complex (DNIC). In cells, the two ligands (L) are typically protein cysteine thiols.