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REVIEW

Biological nitric oxide signalling: chemistry and terminology

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Biological nitrogen oxide signalling and stress is an area of extreme clinical, pharmacological, toxicological, biochemical and chemical research interest. The utility of nitric oxide and derived species as signalling agents is due to their novel and vast chemical interactions with a variety of biological targets. Herein, the chemistry associated with the interaction of the biologically relevant nitrogen oxide species with fundamental biochemical targets is discussed. Specifically, the chemical interactions of nitrogen oxides with nucleophiles (e.g. thiols), metals (e.g. hemeproteins) and paramagnetic species (e.g. dioxygen and superoxide) are addressed. Importantly, the terms associated with the mechanisms by which NO (and derived species) react with their respective biological targets have been defined by numerous past chemical studies. Thus, in order to assist researchers in referring to chemical processes associated with nitrogen oxide biology, the vernacular associated with these chemical interactions is addressed.

Abbreviations
PUFA, polyunsaturated fatty acids; sGC, soluble guanylate cyclase

Introduction

Due to the discovery of endogenous nitric oxide (NO) generation in mammalian cells, the chemical biology of NO has been an important research topic for several decades (for an initial description see, Wink and Mitchell, 1998; for more recent reviews, see Thomas *et al.*, 2008; Toledo and Augusto, 2012) (note: 'nitric oxide' is the common name for the molecule NO. The systematic name for NO is nitrogen monoxide. However, in the current literature, the use of 'nitric oxide' predominates and will be used exclusively herein). The biological effects of nitric oxide and derived nitrogen oxide species (this is a term that collectively refers to all nitrogen oxides including NO_2, N_2O_3, NO_2^-, NO_3^-, etc.) are a result of their fundamental chemical reactions with specific biological targets. The remarkably versatile and unique chemistry of NO and related nitrogen oxides (at times collectively referred to as 'reactive nitrogen species') is ideal for mediating numerous signalling networks. Indeed, cellular and physiological processes have exploited the novel and specific chemistry of NO with a variety of biological targets to evolve and develop signalling systems such as the regulation of vascular tone. Aberrant production of NO, often via misregulation of NO biosynthesis or problems associated with its availability, can lead to pathophysiological conditions. In such cases, problems with spatial and temporal NO generation can lead to disease states. The accurate description of NO chemistry and derived species with biological targets not only provides important insight into mechanisms of action but also provides guidance in the development of potential pharmacological agents/strategies to treat different diseases.

The fundamental chemistry of NO has been examined extensively. Importantly, these chemical studies involving the reactions of NO with inorganic and organic molecules have provided a framework for explaining the physiology and pathophysiology of NO. However, important aspects to consider when extrapolating purely chemical studies to an understanding of biological systems are potential differences in conditions, especially reactant concentrations and environment. Previous chemical studies have also provided a

lexicon for describing distinct chemical processes. Therefore, it is important to be consistent and specific when referring to chemical phenomena in biological systems since a seemingly minor change in word usage can imply a significant difference in the chemistry. That is, use of proper chemical terms is vital to providing a rigorous description of chemical processes associated with NO biology. Improper use of terms/descriptors can be misleading and may misrepresent the intimate mechanisms of NO biology. Unfortunately, much of the current literature associated with nitrogen oxide biology/physiology uses a mixture of chemical terms to describe biological phenomena that is at times confusing, misleading and/or chemically incorrect. In this review, the biologically relevant chemistry of NO is described with an emphasis on the terminology and nomenclature of these processes.

Nitric oxide

A simple Lewis dot depiction of NO shows that it has one unpaired electron (Figure 1). Formal convention often requires that free radicals such as NO be depicted as '·R' to clearly indicate the presence of the single unpaired electron. Herein, this convention has not been adopted for several reasons. First, a more rigorous understanding of the nature of the unpaired electron (by molecular orbital theory) indicates that it is not localized on either the nitrogen or oxygen but is delocalized over both atoms (although shared unevenly towards the nitrogen nucleus). Second, in the absence of any charges, it is clearly implied that NO has an unpaired electron thus it need not be shown explicitly. And finally, a similar molecule, O_2, actually has two unpaired electrons and few attempts to depict this in text are seen (i.e. O_2 is rarely depicted as $\cdot O_2 \cdot$).

Although there is an internationally agreed upon nomenclature (International Union of Pure and Applied Chemistry) for both organic and inorganic compounds, common names become widely used when the formal system is cumbersome. Common names are widespread in the nitrogen oxide literature as is use of NO rather than ·NO. Here, we are attempting to clarify the nomenclature rather than remake or formalize it. Regardless, that NO has an unpaired electron is one of its most important properties. Most organic molecules have all their electrons paired in either bonds or in non-bonding orbitals (e.g. lone pairs). Such molecules repel a magnetic field and thus are called diamagnetic. In contrast, unpaired electrons are attracted to magnets, leading to paramagnetic species. The rates of reactions between diamagnetic and paramagnetic molecules are often limited by high kinetic barriers due to spin forbiddenness. Thus, NO tends to diffuse readily through the medium of a cell until encountering other paramagnetic species.

At first glance, it appears that NO would either readily gain or lose an electron in order to have both atoms obey the octet rule, which states that for molecules containing main group elements, each nucleus should be surrounded by eight bonding and/or lone pair electrons so as to adopt an inert gas electron configuration. Reduction to NO^- or HNO would pair the lone electron while oxidation to NO^+ would result in loss of this electron. In both cases, a Lewis structure can be drawn whereby both atoms possess an octet. However, NO is a stable molecule without a high propensity to be reduced or oxidized or even to dimerize in a biological system. The reasons for the stability of NO as a paramagnetic species are numerous and are covered elsewhere (e.g. see Fukuto *et al.*, 2012). The important consideration is that although NO is a free radical, it is remarkably unreactive. This characteristic is critical for NO to function as a signalling molecule. In most cases, NO is produced in one cell for the purpose of eliciting a response in a neighbouring cell. This is possible due to the neutral charge and low reactivity of NO with diamagnetic species (as are most organic molecules). In biological systems, NO primarily reacts with other paramagnetic molecules as well as with transition metals. The impact of this chemistry is described below.

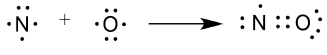
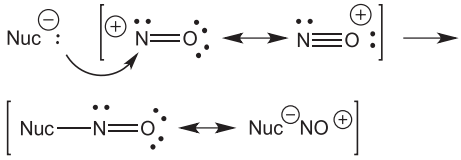


Figure 1
Lewis dot depiction of NO.

Nitrosation versus nitrosylation

Before proceeding further with a discussion of nitrogen oxide chemistry, it is worthwhile to first address some basic nomenclature associated with this chemistry. Especially important and a significant problem with the vernacular of NO chemical biology lies in the use of the terms 'nitrosation' and 'nitrosylation'. These terms have very specific chemical definitions. Therefore, defining these terms (and their proper usage) provides important descriptors of detailed mechanisms that allude to important pharmacological and physiological insights.

The term 'nitrosation' refers specifically to chemical reactions involving the addition of a nitrosonium ion (NO^+) to a nucleophilic group, such as an amine or thiolate. Heteronuclear diatomic molecules such as NO^+ or NO have a strong tendency to react with electron donor species at the less electronegative atom, in this case nitrogen. The product can be described as a hybrid of both ionic and covalent resonance structures, with the latter formed from electron pair donation from the nucleophile to the NO^+ (**Reaction 1**). The importance here is that the product has partial characteristics of both resonance structures.



(1)

In aqueous conditions, NO^+ has an appreciable lifetime only under highly acidic conditions, and thus is generally not considered to be biologically relevant as an independent entity. Instead, nitrosation involves NO^+ donors (species with significant 'NO^+-like' character), formed by processes described in more detail below. Thus, the term nitrosation should be used when referring to chemical or biochemical processes that involve reactions of electrophilic NO^+ donors with nucleophilic centres.

The related term 'nitrosative stress' refers to the indiscriminate nitrosation of biological nucleophiles that can lead to cell death and/or pathophysiological conditions. Nitrogen oxides such as NO_2 and $ONOO^-$ are potent oxidants (*vide infra*) rather than nitrosating agents and toxicity associated with these species should not be referred to as nitrosative stress. It should be noted, however, that although NO_2 itself is not a nitrosating agent, since it cannot directly participate in nitrosation chemistry, it can serve as a precursor to nitrosating species (*vide infra*). Therefore, 'nitrosative stress' should not be used as a general descriptor of all NO-mediated cellular stresses but rather used when referring specifically to stresses associated with nitrosation chemistry. Recently, nitrosation events in cellular signalling have been recognized as a new mechanism in cellular regulation (e.g. Marozkina and Gaston, 2012). 'Nitrosative signalling' is a non-toxic event that is part of a signal cascade to elicit a specific biological response. In cancer, for example, nitrosative stress resulting from an immune response leads to tumour eradication (e.g. Stuehr and Nathan, 1989). In contrast, nitrosative signalling in cancer cells leads to increased metastasis, proliferation and chemoresistance resulting in poor outcome in patients (e.g. Glynn *et al.*, 2010; Ridnour *et al.*, 2012; Switzer *et al.*, 2012). Therefore, biological nitrosation chemistry can result in either a stress or a signalling pathway that is both context and concentration dependent.

The term 'nitrosylation' traditionally refers to direct addition of NO to a reactant. The term originates from chemistry that describes the coordination of NO to a metal centre to form a metal nitrosyl complex (**Reaction 2**) (e.g. Ford *et al.*, 2005), with nitrosyl being the common name for NO when bound as a ligand (akin to 'carbonyl' being used to describe metal coordination complexes involving carbon monoxide, CO).

$$M + NO \rightarrow M\text{-}NO\ (M = \text{metal}) \quad (2)$$

It is worth noting that metal nitrosyls can also be formed via other chemistries. For example, they can be generated by reaction of a metal complex with acidified nitrite, although this reaction may involve a change in the oxidation state of the metal in a multistep process (e.g. Ford, 2010). The first characterized and probably the most important physiological receptor for NO in mammalian systems is the metalloenzyme soluble guanylate cyclase (sGC) (e.g. Ignarro, 1999). Coordination of NO to the ferrous heme of sGC is the quintessential example of nitrosylation in biology.

In biological systems, many post-translational modifications of macromolecules are described using the suffix '-ylation' such as 'phosphorylation', 'methylation' and 'sumoylation'. With respect to NO, descriptions of its biological actions have often used the term 'nitrosylation' to generally describe appending an NO group to macromolecules (akin to the terms above). From a purely chemical perspective, 'nitrosylation' is not nearly as general a term (*vide supra*). Indeed, the ubiquitous use of 'nitrosylation' can obscure the important chemical and biological mechanisms involved in nitrogen oxide signalling. Possibly the most prevalent, established and important macromolecular signalling phenomenon is protein phosphorylation, a process that is carefully regulated and controlled via a multitude of specific kinases and phosphatases that, in turn, are controlled in numerous ways. Therefore, the use of nitro'syl'ation to draw analogy to, for example, phosphorylation (and other established signalling systems) should be done with caution at this time since an analogous level of regulation established for these other signalling systems has not yet been demonstrated for 'nitrosylation'.

The terms 'nitrosation' and 'nitrosylation' are often used when describing the formation of S-nitrosothiols (RS-NO, *vide infra*). S-nitrosothiols can be formed by translocation of an NO^+ group from one sulfur to another (**Reaction 3**).

$$RS\text{-}NO + R'SH \rightarrow RSH + R'S\text{-}NO \quad (3)$$

This process has been referred to as 'transnitrosylation' (e.g. Anand and Stamler, 2012). Since the chemistry of this transfer likely involves nucleophilic attack of a thiol at the electrophilic nitrogen atom of the S-nitrosothiol (Singh *et al.*, 1996; Wong *et al.*, 1998; Houk *et al.*, 2003), this process is more appropriately termed transnitrosation (assuming the mechanism described above is being implied).

Transfer of a nitrosonium ion is presumably an important mechanism for modification of protein thiols. For example, Mitchell and Marletta (2005) have shown that the thiol redox protein thioredoxin can, with some specificity, transfer a nitrosonium species from one of its cysteines to the active site cysteine of capase-3. Thus, transnitrosation may in fact be a significant component of cellular signalling (e.g. Martinez-Ruiz and Lamas, 2007). Given the pre-existence of the terminology, their individual importance to nitrogen oxide signalling and the lack of similarity to other enzymatically controlled post-translational modifications, following the suggestion by Ford *et al.* (2005), we recommend that nitrosation be reserved exclusively for reactions involving transfer of NO^+ and nitrosylation for direct addition of NO.

Formation of S-nitrosothiols

It has become increasingly clear that modification of both low molecular weight thiols and thiol-containing proteins occurs as a result of the biosynthesis of NO, and there are several possible chemical mechanisms for their generation. As alluded to in the above section, NO itself does not react with thiols or thiolates under biological conditions (or at the very least, this is a very slow process that generally precludes biological relevance) (Pryor *et al.*, 1982). Modification of thiols by NO requires either prior oxidation of NO (to give an NO^+-donor) or of the thiol (to give a thiyl radical, RS·).

Oxidation of NO

One-electron oxidation of NO to give free NO^+ is highly unfavourable. In fact in aqueous systems, NO^+ only has an appreciable lifetime in very strong acid. In purely chemical systems, NO^+ can be formed by addition of nitrite (NO_2^-) to ~4 M sulfuric acid, but such conditions are not physiological. However, species with NO^+-like reactivity can be formed under milder conditions. For example, NO and nitrogen dioxide (NO_2, another paramagnetic species) can combine to give dinitrogen trioxide (N_2O_3) (**Reaction 4**).

$$NO + NO_2 \rightleftharpoons N_2O_3 \quad (4)$$

This reaction is reversible, suggesting that N_2O_3 can simplistically be described as an adduct of NO and NO_2. However, NO_2 is a reasonable one-electron oxidant, and thus N_2O_3 may possess some $NO^+NO_2^-$ character. In the presence of a nucleophile, a $Nuc\cdots NO^+\cdots NO_2^-$ intermediate would lead to transnitrosation from NO_2^- to the nucleophile, to produce for instance an S-nitrosothiol (**Reaction 5**).

$$N_2O_3 + RS^- \rightarrow RSNO + NO_2^- \quad (5)$$

In this way, N_2O_3 functions as an NO^+ donor through transfer of NO^+ rather than spontaneous release of free NO^+.

Generation of S-nitrosothiols via reaction of thiols with N_2O_3 is a chemically established process. The formation of N_2O_3, as mentioned above, occurs when NO_2 is generated in the presence of NO (**Reaction 4**). One mechanism to produce NO_2 is through reaction of NO with O_2 (autoxidation of NO; **Reaction 6**).

$$4NO + O_2 \rightarrow 2N_2O_3 \quad (6)$$

The paramagnetism of the reactants leads to a low kinetic barrier [high reaction rate constant, k, of $8 \times 10^6 M^{-2}s^{-1}$ (Wink *et al.*, 1993a; Keshive *et al.*, 1996)]. However, the second order dependence on NO (rate = $k[NO]^2[O_2]$) also indicates that significant levels of N_2O_3 are formed only at high concentrations of NO. The high order kinetics of NO autoxidation indicates that the time required for an aerobic solution of NO to decay to half its original concentration varies hugely with concentration (on the order of hours for nanomolar and seconds for micromolar – note that this is *not* a true half life since this is not a first order decay process).

Under normal biological conditions/concentrations autoxidation of NO is generally considered to be too slow to be significant. However, higher levels of NO (and O_2) may concentrate in lipophilic compartments (e.g. membranes) due to the favourable partitioning of these non-polar species from aqueous solution (Liu *et al.*, 1998). Furthermore, inflammation leads to increased levels of NO. Alternatively, NO_2 may be formed by a different route, such as through metal-mediated oxidation of nitrite (Thomas *et al.*, 2008). At present, the biologically relevant mechanisms by which N_2O_3 is formed are not fully established.

However, that N_2O_3-mediated nitrosation chemistry does occur *in vivo* is clear and numerous studies in *in vitro* and *in vivo* systems allude to the relevance of N_2O_3 in mediating NO biology. Shinyashiki *et al.* (2001) provide just one example in which disruption of the actions of the thiol-containing, metal responsive yeast transcription factor ACE1 was observed under conditions consistent with N_2O_3 generation. ACE1 inhibition occurred upon addition of NO under aerobic conditions (**Reactions 6 and 4**), upon addition of nitrite to acidic solution (**Reaction 7**), or when NO and NO_2 were present simultaneously (**Reaction 4**).

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \quad (7)$$

Since all of these conditions are amenable to N_2O_3 formation, it was concluded that N_2O_3 was the species responsible for modifying and inhibiting ACE1 activity.

Oxidation of thiols

S-nitrosothiols may also be formed by one-electron oxidation of the thiol to a thiyl radical followed by direct reaction with NO (**Reactions 8 and 9**).

$$RSH \rightarrow RS\cdot + e^- + H^+ \quad (8)$$

$$RS\cdot + NO \rightarrow RS\text{-}NO \quad (9)$$

Thus oxidation of thiol to thiyl radical followed by addition of NO is termed oxidative nitrosylation. It needs to be stressed that NO *does not* directly react with thiols under *any* biological condition, therefore there is no direct nitrosylation mechanism for RSNO formation via reaction of NO with thiols (*vide infra*). The relevance of this nitrosylation pathway to S-nitrosothiol generation is, of course, dependent on the prevalence of thiyl radicals as well as competing reactions with thiol or O_2. Significantly, many proteins/enzymes are known to possess cysteine thiyls (among other radical species) that are crucial for enzymatic activity (e.g. Stubbe and van der Donk, 1998). Thus, enzymes/proteins with existing thiyl radicals may be expected to be reactive with NO via **Reaction 9**. Oxidation of thiols to thiyl radicals typically is driven by the presence of strong one-electron oxidants such as HOO· and HO·. Two-electron oxidants such as H_2O_2 can instead produce the sulfenic acid, RSOH, which is not susceptible to nitrosylation. Both thiyl radicals and sulfenic acids can undergo further reactions to give other more stable species such as disulfides. Thus, for thiol oxidation to result in S-nitrosothiol formation requires coordination with NO biosynthesis.

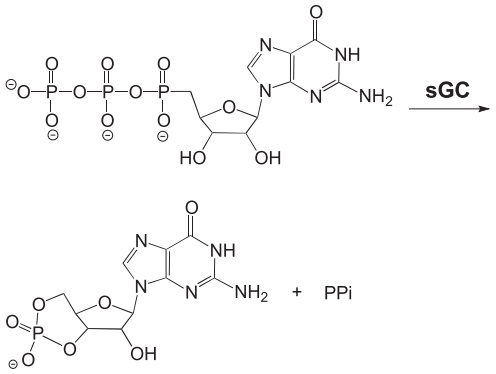
NO_2 is also a strong oxidant that is capable of oxidizing thiols. However, since NO and NO_2 rapidly associate (**Reaction 4**), their simultaneous presence is commonly assumed to produce S-nitrosothiols via the nitrosation pathway. However, there have been several reports suggesting that thiols can effectively compete for the NO_2 generated during autoxidation at low fluxes of NO such that S-nitrosothiol formation under these conditions may not be entirely due to a nitrosation event (Jourd'heuil *et al.*, 2003; Koshiishi *et al.*, 2007). This suggests that there may be more complex reaction mechanisms at low (physiological/pathophysiological) levels of NO. Regardless, it should be noted that the mechanism(s) by which NO-mediates S-nitrosothiol formation requires further investigation.

S-nitrosothiol chemistry

Both nitrosation (e.g. **Reaction 5**) and nitrosylation (**Reaction 9**) can result in production of an RSNO product. Thus, the nomenclature provides a distinction based on the chemical mechanism rather than the nature of the product. In fact, that the same product can arise from both processes is indicative of the nature of S-nitrosothiols. The occurrence of transnitrosation (**Reaction 3**) suggests RS^-NO^+ character. But, S-nitrosothiols are also suggested to function as NO or NO^- donors. Moran *et al.* (2011) have suggested that S-nitrosothiols should be considered as a combination of covalent, zwitterionic and ion pair resonance structures, R-S-N=O, $R\text{-}S^+\text{=}N\text{-}O^-$, RS^-NO^+, with the R-S-N=O form generally being dominant in the resonance hybrid. Whether an S-nitrosothiol undergoes homolytic cleavage to release NO (such as by photolysis) or heterolytic cleavage, transferring either NO^+ or NO^- to a nucleophile or electrophile, respectively, will be highly condition-dependent. This is analogous to the chemistry already described for N_2O_3, which spontaneously dissociates into NO and NO_2 but can also transfer NO^+ to a nucleophile.

Nitrosylation of metal complexes and metalloproteins

Analysis of the interaction of NO with metal centres both in proteins and in model complexes constitutes a very large literature. The reaction of NO with ferrous (Fe^{II}) systems is rapid, and the resulting complexes are highly stable, which are ideal properties for signalling. The ferrous heme protein sGC is commonly referred to as the primary biological target for NO. This enzyme catalyzes the cyclization of GTP to cGMP (**Reaction 10**).



(10)

Although sGC possesses basal activity, it is activated many fold by NO. Elucidation of the full mechanism of this activation process is hampered by the lack of detailed structural data for the large protein. However, it is generally accepted that nitrosylation of the regulatory ferrous heme, producing a ferrous-nitrosyl (Fe^{II}-NO) complex, leads to dissociation of the proximal histidine ligand (Traylor and Sharma, 1992). Although other small molecules such as O_2 and CO also can bind to ferrous hemes, cleavage of the proximal ligand (due to a strong trans directing nature) is unique to NO. In turn, movement of the helix that includes the proximal histidine leads to structural changes that facilitate conversion of GTP to cGMP at a distant catalytic site.

NO reacts with a variety of other Fe^{II} systems including the heme centres of haemoglobin, myoglobin, NO synthase and cytochrome P450 (Khatsenko *et al.*, 1993; Wink *et al.*, 1993b; Stadler *et al.*, 1994) as well as the iron-sulfur cluster of aconitase (Drapier and Bouton, 1996). In contrast to sGC, nitrosylation of most enzymes leads to regulation of function by reducing activity. For example, nitrosylation of the Co(II)(H_2O) form of cobalamin (vitamin B_{12} derivative) results in a diminished ability for this complex to serve as a cofactor for methionine synthase (Brouwer *et al.*, 1996).

Metal complexes can also function as NO scavengers. Haemoglobin, and to a lesser extent myoglobin, in particular is considered to regulate NO levels. In these proteins, NO reacts with the Fe(II)O_2 complex to rapidly produce methaemoglobin (Fe^{III}) and nitrate (**Reaction 11**, shown for haemoglobin, Hb) (Eich *et al.*, 1996).

$$HbFe(II)O_2 + NO \rightarrow HbFe(III) + NO_3^- \quad (11)$$

Nitrate is then excreted [or possibly recycled back to NO (Kapil *et al.*, 2010)], and metHb can be enzymatically reduced back to the O_2-binding ferrous haemoglobin.

NO also reacts with more oxidized metals. Nitrosylation of ferric (Fe^{III}) complexes is generally considered to be reversible, due to lower binding affinities compared to the corresponding ferrous analogues (see Lim *et al.*, 2005). However, nitrosylation of ferric heme centres can also lead to enzyme inhibition, as observed for catalase (Hoshino *et al.*, 1993; Farias-Eisner *et al.*, 1996). Furthermore, the resulting ferric nitrosyl complex often is written as Fe^{II}-NO^+ rather than Fe^{III}-NO, indicating that significant redistribution of electron density occurs upon binding [**Reaction 12**; also applicable to Mn(III)]. As with N_2O_3 and S-nitrosothiols, ferric nitrosyls can therefore be susceptible to nucleophilic attack (**Reaction 13**).

$$Fe(III) + NO \rightleftharpoons [Fe(III)NO \leftrightarrow Fe(II)NO^+] \quad (12)$$

$$Fe(II)NO^+ + Nuc\text{-} \rightarrow Fe(II) + Nuc\text{-}NO \quad (13)$$

In this case, nitrosylation of the metal in the presence of a nucleophile leads to transnitrosation to the nucleophile and one-electron reduction of the metal centre. A second nitrosylation of the reduced metal centre then produces a stable ferrous nitrosyl complex (**Reaction 14**).

$$Fe(II) + NO \rightarrow Fe(II)NO \quad (14)$$

Together, this multistep process is called reductive nitrosylation (e.g. Gwost and Caulton, 1973; Wayland and Olson, 1973). Wade and Castro demonstrated that ferric nitrosyl complexes could nitrosate biomolecules including N-acetyl cysteine (Wade and Castro, 1990).

Nitrosation of other biological targets

Nitrosation is not limited to thiols, and the study of nitrosation chemistry is more than a century old (reviewed in Williams, 1983). Prior to the discovery that NO is biosynthesized, nitrosation of amines in the gastrointestinal tract became a concern as a potential source of carcinogens (Bartsch *et al.*, 1990) (**Reaction 15**).

$$R_2NH + N_2O_3 \rightarrow R_2NNO + NO_2^- \quad (15)$$

In fact, the detection of N-nitrosamines following activation of the immune system was critical to discovery of endogenous formation of NO (Stuehr and Marletta, 1985; Miwa *et al.*, 1987; Hibbs, 1991). Formation of N-nitrosamines has also been proposed to play a role in carcinogenesis associated with inflammation (Marletta, 1988).

Primary amines (RNH_2) such as found in nucleobases can also be nitrosated by either N_2O_3 or ferric nitrosyl complexes (Castro and Bartnicki, 1994). However, primary N-nitrosamines are unstable to further reaction as they can tautomerize. As shown in Figure 2 for cytosine, the N-nitrosamine can tautomerize and then dehydrate to produce a diazonium ion. This intermediate hydrolyzes to release N_2 to produce an enol. Tautomerization to the keto form produces uracil. Importantly, Caulfield *et al.* (1998) have demonstrated that DNA structure plays an important role in this potentially mutagenic reaction.

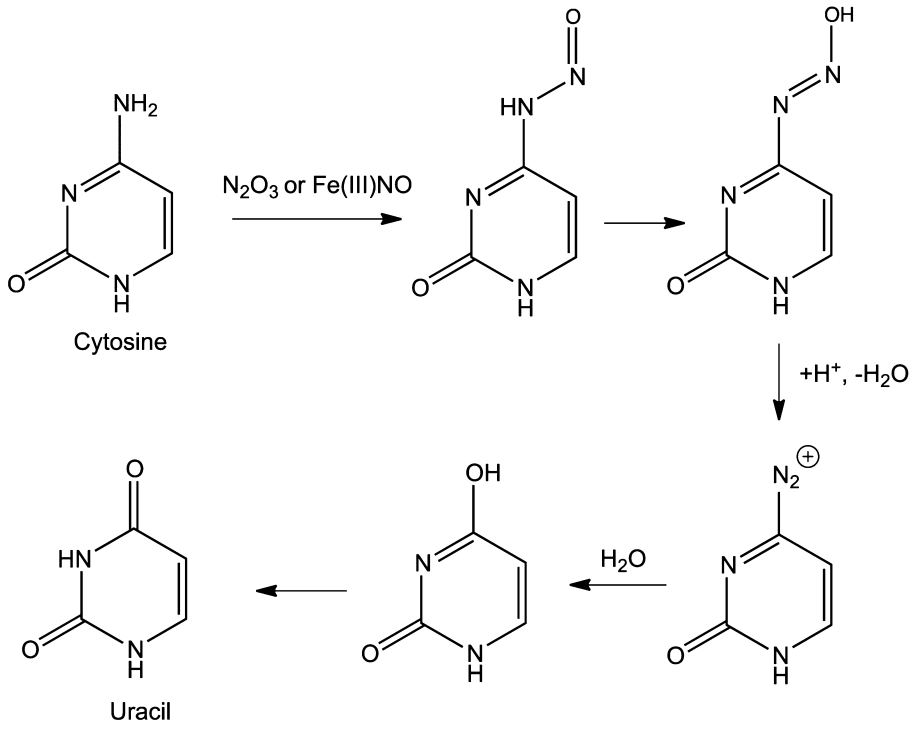


Figure 2
Nitrosation of cytosine leading to the conversion to uracil.

Reaction of NO with radical species

Unpaired electrons can impart high reactivity to many radical species in that they can be electron poor and, therefore, oxidants. The resistance of NO to dimerization is highly unusual for a radical, but is critical to its function as a signalling agent. NO does react rapidly with other radicals, as shown in **Reactions 4 and 9**. In these reactions, two radicals combine to form a diamagnetic species (formally via nitrosylation). Since O_2 has two unpaired electrons, two NO molecules react with it (**Reaction 6**). Another important radical to consider with NO is superoxide (O_2^-). Although reduction of O_2 is unfavourable, interaction of O_2 with strong reductants can result in production of O_2^-. In particular, O_2^- can be produced by the respiratory chain necessitating a high concentration of superoxide dismutase in mitochondria. However, immune cells have evolved to utilize O_2^- in the control of invading microorganisms. Since the immune system also produces NO, the interaction of NO and O_2^- has received substantial attention both as a scavenger of NO and in the formation $ONOO^-$ (**Reaction 16**).

$$NO + O_2^- \rightarrow ONOO^- \quad (16)$$

Peroxynitrite itself is not a radical species (i.e. it does not have any unpaired electrons). However, proton dependent decomposition of $ONOO^-$ has the potential to generate potent odd electron oxidants NO_2/HO· as fleeting intermediates contained in a solvent cage in a reaction pathway that gives predominantly nitrate (NO_3^-) (**Reaction 17**) (e.g. Gunaydin and Houk, 2008).

$$ONOO^- + H^+ \rightarrow ONOOH\ (pKa\ 6.8) \rightarrow [NO_2/HO\cdot] \rightarrow NO_3^- + H^+ \quad (17)$$

The biochemical fate of $ONOO^-$ is highly dependent on its environment. Under normal biological conditions, reaction of $ONOO^-$ with CO_2 is likely the major reaction giving nitrosoperoxycarbonate (**Reaction 18**). Further decomposition of nitrosoperoxycarbonate (via possible homolytic cleavage of the O-O bond, analogous to **Reaction 17** above), generates potent oxidants, $NO_2/CO_3^{\cdot-}$, as fleeting intermediates on a reaction path that eventually forms nitrocarbonate (which will further decompose to give NO_3^-) (**Reaction 19**).

$$ONOO^- + CO_2 \rightarrow ONOO\text{-}C(O)O^- \quad (18)$$

$$ONOO\text{-}C(O)O^- \rightarrow [NO_2/CO_3^{\cdot-}] \rightarrow O_2NOC(O)O^- \quad (19)$$

Thus peroxynitrite is not itself a nitrosating agent. However, the intermediates in H^+- or CO_2-mediated decomposition can produce powerful oxidants (NO_2, HO· or $CO_3^{\cdot-}$). Yet, in excess NO, these oxidants can be converted to a variety of species including the nitrosating agent N_2O_3 (**Reactions 20, 21, 22**). This balance between oxidative chemistry and nitrosative chemistry may provide an important interface between these two chemical conditions.

$$OH\cdot + NO \rightarrow NO_2^- + H^+ \quad (20)$$

$$NO_2 + NO \rightarrow N_2O_3 \quad (21)$$

$$CO_3^{\cdot-} + NO \rightarrow CO_2 + NO_2^- \quad (22)$$

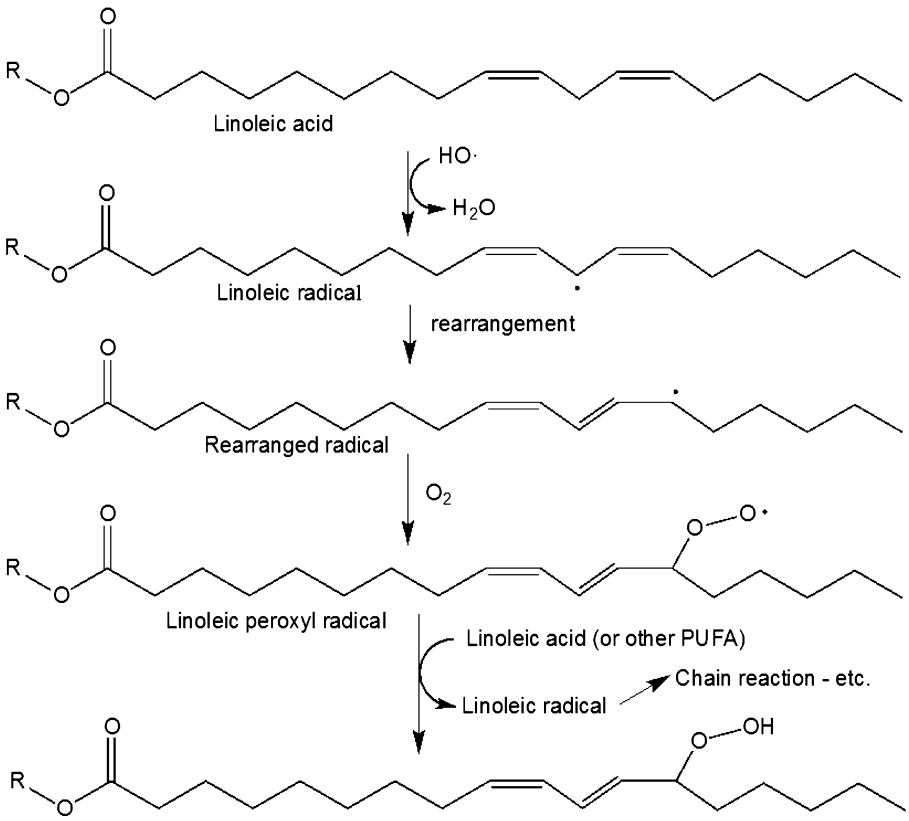


Figure 3
Lipid peroxidation mechanism.

The biochemical consequences of $ONOO^-$ formation and degradation will be discussed further later.

In addition to small radicals such as NO_2 or O_2^-, NO can react with carbon-centred radicals (R·) to give the corresponding C-nitroso compound (**Reaction 23**).

$$R\cdot + NO \rightarrow R\text{-}NO \quad (23)$$

Most alkyl nitroso compounds are unstable with respect to tautomerization to the corresponding oxime (**Reaction 24**).

$$R_2HC\text{-}NO \rightarrow R_2C = NOH \quad (24)$$

Such reactivity (i.e. tautomerization to an oxime) is not available to tertiary or phenyl nitroso compounds.

In aerobic systems, carbon-centred radicals can be converted into oxy- and peroxy-radicals. This can be particularly deleterious since these radicals can initiate and/or propagate chain reactions. That is, a single radical initiating event can lead to the destruction or modification of numerous molecules via propagation by chain-carrying and O_2-derived reactive intermediates. One of the most important examples of this is lipid peroxidation. Production of a one-electron oxidant (e.g. HO·, NO_2, HOO·, etc.) in a membrane lipid environment can lead to the generation of a carbon-centred radical via one-electron oxidation of an unsaturated fatty acid (typically by hydrogen-atom abstraction or addition to an unsaturation). In the presence of polyunsaturated fatty acids (PUFA), membranes are more susceptible to lipid peroxidation due to the fact that PUFAs are more easily oxidized compared to saturated or monounsaturated fatty acids. In an aerobic environment, the fatty acid radical will react with O_2, but only one of the two unpaired electrons of O_2 will be paired by this process. The resulting alkyl peroxyl radical (LOO·) is a reasonable one-electron oxidant that can propagate the chain reaction by oxidizing another fatty acid. This process is schematically depicted in Figure 3 using linoleic acid as an example.

Lipid peroxidation can be initiated during the inflammatory process and can ultimately lead to cell death by disrupting cell membranes (e.g. Higdon *et al.*, 2012). There are multiple radical intermediates in the lipid peroxidation process (HO·, lipid radical, lipid peroxyl radical), all of which can react with NO (e.g. **Reaction 25**).

$$LOO\cdot + NO \rightarrow LOONO \quad (25)$$

This quenching by NO of the radical nature of intermediates involved in the initiation or propagation of lipid peroxidation leads to termination of the process (e.g. Padmaja and Huie, 1993; Rubbo *et al.*, 1995; Wink *et al.*, 1995). This protective function of NO has been observed in both endothelial (Struck *et al.*, 1995) and macrophage cells (Hogg *et al.*, 1995). Clearly, the utility of NO in this regard will be highly dependent on having NO generated in proximity to these radical events and having it at high enough concentrations. Importantly, NO will partition favourably into lipid environments (Liu *et al.*, 1998).

The build up of cholesterol in arteries produces atherosclerotic plaques, which are the cause of most coronary artery disease and strokes. Such plaques are initiated by accumulation of oxidized lipoproteins produced by the inflammatory response. Donors of NO have been found to prevent the macrophage-dependent oxidation of low-density lipoproteins by termination of lipid peroxidation (Hogg *et al.*, 1993). This study indicates that NO may provide endogenous protection against plaque development. In fact, NO has been shown to be a more potent inhibitor of lipid peroxidation than the antioxidant α-tocopherol (vitamin E) (O'Donnell *et al.*, 1997).

NO has also been designated as a pro-oxidant, capable of initiating radical chain chemistry. Such effects are not likely due to NO itself since it is such a poor oxidant (one-electron reduction of NO is highly unfavourable). Rather, NO_2 and other strong oxidants, derived from the reaction of NO for instance with O_2 and O_2^-, are likely responsible. Again, association of NO with such oxidants will lead to radical quenching and thus will limit lipid oxidation.

Lipid peroxidation can also be initiated indirectly by oxidation of metal centres. In biological systems, Fe^{II} and Fe^{III} are the most common oxidation states. However, more oxidized species can form transiently, for example, due to reaction of H_2O_2 (**Reaction 26**).

$$Fe(II) + H_2O_2 \rightarrow Fe(IV) = O + H_2O \text{ (or } Fe(III) + HO\cdot + HO^-) \quad (26)$$

Such highly reactive species [i.e. Fe(IV)=O or HO·, **Reaction 26**] can lead to cellular damage if uncontrolled (Puppo and Halliwell, 1988). NO can inhibit this chemistry by reducing these oxidized centres to a normal valence state (**Reaction 27**) (Kanner *et al.*, 1991; Wink *et al.*, 1994), providing a protective mechanism against peroxide-mediated cytotoxicity (Wink *et al.*, 1993c).

$$Fe(IV) = O + NO \rightarrow Fe(III) + NO_2^- \quad (27)$$

Oxygen-based radicals are also produced during normal metabolism, most notably the tyrosyl radical. Both ribonucleotide reductase and cyclooxygenase/prostaglandin H-synthase contain a catalytically important tyrosyl radical. Reaction with NO results in enzyme inhibition (for a review, see Guittet *et al.*, 1999). The resulting suppression of DNA or prostaglandin synthesis is a possible deleterious function of NO. However, the mammalian immune system utilizes this inhibitory effect to combat pathogens.

As discussed immediately above, the anti- and pro-oxidative properties of NO are highly dependent on the concentration of NO as well as the biochemical environment. Thus, it is often difficult to predict the effects NO will have on a system without an intimate understanding of these issues.

Nitration via NO-derived species

In a formal sense, the term nitration is analogous to that of nitrosation, referring specifically to chemical reactions involving the addition of a nitronium ion (NO_2^+) to a nucleophilic group. However, nitration has been used extensively, particularly by organic chemists, as a general term to denote formation of a nitro compound without implying any specific mechanism of X-NO_2 formation (X representing an atom to which an NO_2 group has been added). As with NO^+, the highly electrophilic NO_2^+ is only generated under highly acidic conditions (e.g. nitric acid in sulfuric acid). Such conditions have been used extensively to nitrate organic compounds but are not relevant to biological conditions. In contrast to nitrosation, a physiologically relevant NO_2^+ transfer agent has not been established. Thus, the likely and primary mechanism for nitration in cellular systems involves association of NO_2 to existing radicals (comparable to nitrosylation). However, a unique term has not been defined for this process.

As already described, NO_2 can be formed from the autoxidation of NO (**Reaction 6**) and from the reaction of NO with O_2^- (**Reaction 16**). In the first case, as discussed earlier, the second order dependence of NO on autoxidation to give NO_2 limits its prevalence in the aqueous environment of cells but to a lesser extent in lipophilic regions. Also, NO_2 generated from NO autoxidation will be kept at low levels due to further reaction with NO leading to N_2O_3 formation (thus not allowing accumulation NO_2) (**Reaction 4**). Significantly, NO_2 can also be formed in the absence of NO from oxidation of NO_2^- by H_2O_2 in the presence of metals (e.g. Burner *et al.*, 2000; Monzani *et al.*, 2004) (**Reaction 28**).

$$2NO_2^- + H_2O_2 + 2H^+ \rightarrow 2NO_2 + 2H_2O \quad (28)$$

Again, the metal centres function as peroxidases as shown here for Fe^{III} systems. In the first step, H_2O_2 leads to production of a more oxidized species (compound I; **Reaction 29**; please note that the metal oxidation states are shown primarily as a means of electron accounting and may not accurately represent the actual oxidizing species).

$$Fe(III) + H_2O_2 \rightarrow Fe(V) = O + H_2O \quad (29)$$

This oxidized species can participate in two sequential one-electron oxidations of NO_2^-, ultimately regenerating the Fe^{III} starting complex (**Reactions 30 and 31**).

$$Fe(V) = O + NO_2^- \rightarrow Fe(IV) = O + NO_2 \quad (30)$$

$$Fe(IV) = O + NO_2^- + 2H^+ \rightarrow Fe(III) + NO_2 + H_2O \quad (31)$$

There has been significant speculation that biological nitration primarily follows this pathway. Nitrite is an end-product of NO biosynthesis, and thus can lead to NO_2 formation by **Reactions 30 and 31**. Importantly, the possible temporal separation of NO biosynthesis from metal-mediated NO_2^- oxidation reduces the likelihood of reaction of NO_2 with NO, which would lead to nitrosation rather than nitration (*vide supra*). Nitrite can also be ingested in the diet, which completely eliminates the dependence on NO biosynthesis.

In biological systems, typically an oxygen, carbon or other main group element is nitrated. It is well established that nitrated adducts, including those of tyrosine, tryptophan, fatty acids and guanosine, are prevalent biological species under conditions of NO-generation (e.g. Akuta *et al.*, 2006; Souza *et al.*, 2008; Nuriel *et al.*, 2011; Schopfer *et al.*, 2011) (Figure 4). Unlike many of the chemical processes involving nitrogen oxides, as described above, nitration is currently considered to be a biologically irreversible modification. That is, biologically mediated conversion of a nitrated species back to the original molecule is not reported to happen (although nitro group reduction has been reported).

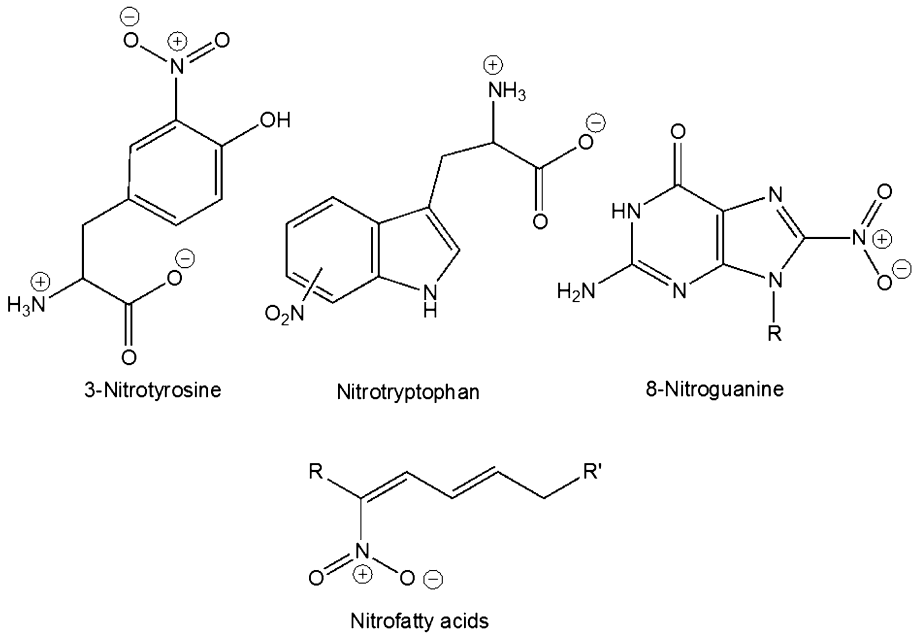


Figure 4
Examples of nitrated biological species.

The term 'nitrative stress' is often used to indicate conditions associated with nitration events on, for example, proteins or nucleotides leading to pathologies (e.g. Zaki *et al.*, 2005). It is important to note the distinction between the terms 'nitrosative stress' and 'nitrative stress'. As discussed earlier, nitrosative stress results from the nitrosation of biological molecules (e.g. protein cysteines) while nitrative stress is associated with nitration of biological molecules (e.g. protein tyrosines). 'Nitrative stress' has been characterized primarily via the detection of nitrated proteins. For example, nitration of tyrosines (3-nitrotyrosine formation) detected by antibodies is often used to characterize/indicate a nitrative stress. However, several important questions regarding this phenomenon remain. For example, is nitration merely a dosimeter of a chemical/biochemical condition? Are there any significant functional consequences of nitration? To date, answers to these questions are not well established. A further complication is that nitration of tyrosine may result from oxidative or nitrosative conditions (*vide infra*) indicating that nitration does not represent or result from a unique biochemical condition.

One mechanism of 3-nitrotyrosine formation from interaction with $ONOO^-$ and derived species has been determined to involve first oxidation of tyrosine by the carbonate radical anion ($CO_3^{\cdot-}$) generated from the nitrosoperoxycarbonate intermediate (*vide supra*), and then reaction of the tyrosyl radical with NO_2 (**Reactions 17, 18, 19, 32, 33**) (e.g. Gunaydin and Houk, 2009).

$$CO_3^{\cdot-} + \text{Tyr-OH} \rightarrow HCO_3^- + \text{Tyr-O}\cdot \quad (32)$$

$$\text{Tyr-O}\cdot + NO_2 \rightarrow \text{3-}NO_2\text{-Tyr} \quad (33)$$

It should be noted that the yield of 3-nitrotyrosine by this chemistry is highly dependent on the relative concentrations and fluxes of NO and O_2^- (the precursors to $ONOO^-$) with the highest yield occurring at equal fluxes. Moreover, even under ideal conditions a less than 40% yield is realized under purely chemical conditions (Goldstein *et al.*, 2000).

Since 3-nitrotyrosine is formed by the reaction of the tyrosyl radical with NO_2 (**Reaction 33**), any oxidant capable of removing an electron from tyrosine in the presence of NO_2 can lead to 3-nitrotyrosine production. Importantly, NO_2 itself is capable of oxidizing tyrosine to Tyr-O·, and thus any system that generates NO_2 can lead to the formation of 3-nitrotyrosine.

The reaction of the tyrosyl radical with NO is also facile. Interestingly, the product 3-nitrosotyrosine has been suggested to further oxidize to give 3-nitrotyrosine via an intermediate iminoxyl radical (Gunther *et al.*, 2002). In this pathway, an existing tyrosyl radical reacts with NO to give a 'weak complex' that tautomerizes to 3-nitrosotyrosine. Subsequent tautomerization to an oxime can be followed by one-electron oxidation generating an iminoxyl radical intermediate that is further oxidized to give an electrophilic species capable of reacting with water to give 3-nitrotyrosine (Figure 5).

Conclusion and summary of terms

The biological chemistry of NO is dominated by its ability to coordinate to metals and to react with other radical species. The subsequent chemistry that is initiated by the formation of metal nitrosyls and NO-radical adducts is important to the ultimate fate and chemical biology of NO. Importantly, much of the lexicon of nitrogen oxide chemistry was established prior to the relatively recent explosion of biological interest/

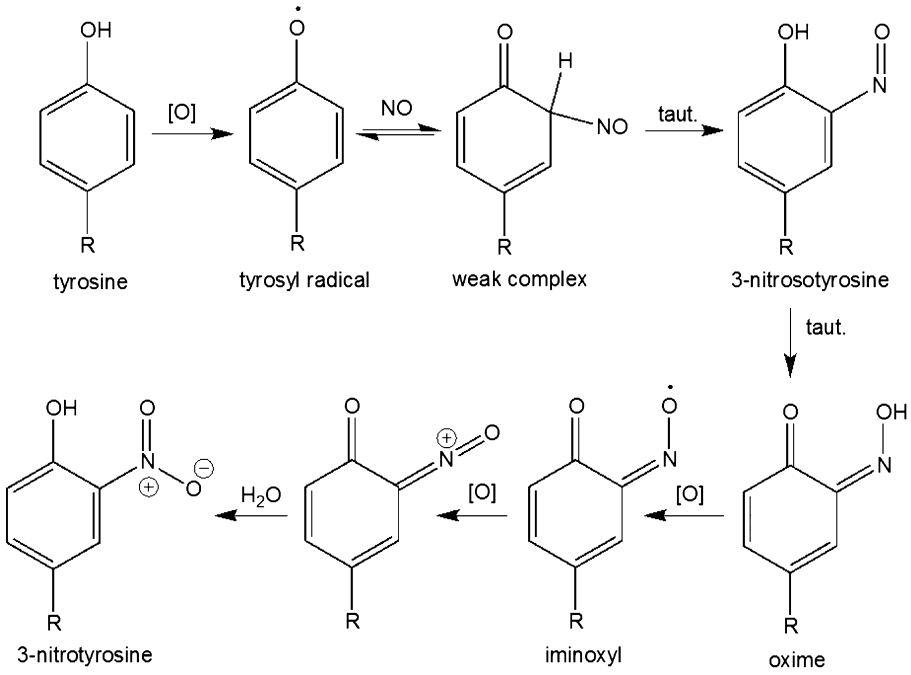


Figure 5

Possible mechanism of 3-nitrotyrosine formation from tyrosyl radical and NO.

application. Unfortunately, a lack of adherence to the established chemical terminology can lead to problems or misunderstandings regarding the intimate chemical mechanisms and processes being described. Herein described are some of the biologically relevant chemistry of NO (and derived species) along with what we believe to be the proper terminology.

To be sure, not all biological/biochemical terms are as chemically rigorous or descriptive as would be preferred. For example (and particularly relevant to this discussion and mentioned above) is the term 'phosphorylation'. In biochemistry textbooks, the term phosphorylation is used to describe the transfer of a phosphate from the atom of one biomolecule to another (typically from one oxygen atom to another). 'Phosphoryl' groups are derivatives of phosphoric acid (H_3PO_4). Therefore, 'phosphorylation', as used in biochemistry, is a term that describes the products of the reaction but not the mechanism. However, it needs to be noted that biochemical phosphorylation occurs via attack of a nucleophilic atom of a biomolecule at an electrophilic phosphorous atom of the phosporyl donor (to our knowledge, there are no other known reaction pathways) thus precluding a need for numerous mechanistically distinct terms.

Since formation of an X-NO bond can occur by two distinct mechanisms, (described above), it is preferable to use a mechanistically defined term when possible. The question then arises as to the proper nomenclature when the mechanism is neither known nor implied. Both nitrosation and nitrosylation are often used in the literature to denote the general (mechanistically ill-defined) formation of X-NO compounds. However, given that both terms inherently describe a mechanism of formation, neither term is appropriate in cases where the mechanism is unknown or ambiguous. Rather, a more generic description such as thiol modification or S-nitrosothiol production is recommended (in the case of thiol targets). Currently, there is the tendency for many to view 'nitrosation' as a chemical term that specifically denotes NO^+-mediated modification of a nucleophilic species (such as a thiol) and 'nitrosylation' as a biological term that denotes the mere formation of an X-NO species (X = S, N, etc.) without regard to mechanism. However, it needs to be stressed that nitrosylation is also a chemical term with mechanistic implications (*vide supra*) and use of nitrosylation to merely draw analogy to, for example, phosphorylation seems unwarranted at this time.

Below is a summary of the terms discussed in this review.

- Nitrosation: refers to the addition of a nitrosonium ion (NO^+) to a nucleophilic centre (e.g. a thiol or amine) either directly or by transfer from an NO^+ donor (e.g. N_2O_3 or $Fe^{II}NO^+$).
- Transnitrosation: the transfer of NO^+ from one nucleophilic centre to another.
- Nitrosative stress: a biological stress caused by nitrosation of biological molecules.
- Nitrosylation: refers to the direct formation of a nitrosyl species (X-NO; X = metal centre or radical species) via direct reaction with NO.
- Transnitrosylation: technically, this term would refer to the transfer of NO from one molecule to another. This process will likely involve NO dissociation from one system before binding to another. Use of transnitrosylation to indicate transfer of NO^+ is incorrect and should be discontinued.

- Nitration: any process that leads to the generation of an X-NO_2 species (nitro group formation). This term is not mechanistically specific.
- Nitrative stress: refers to a biological stress associated with nitration of biological molecules.

Conflict of interest

There are no potential conflicts of interest.

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