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The chemical biology of dinitrogen trioxide

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A B S T R A C T

Dinitrogen trioxide (N_2O_3) mediates low-molecular weight and protein S- and N-nitrosation, with recent reports suggesting a role in the formation of nitrating intermediates as well as in nitrite-dependent hypoxic vasodilatation. However, the reactivity of N_2O_3 in biological systems results in an extremely short half-life that renders this molecule essentially undetectable by currently available technologies. As a result, evidence for *in vivo* N_2O_3 formation derives from the detection of nitrosated products as well as from *in vitro* kinetic determinations, isotopic labeling studies, and spectroscopic analyses. This review will discuss mechanisms of N_2O_3 formation, reactivity and decomposition, as well as address the role of sub-cellular localization as a key determinant of its actions. Finally, evidence will be discussed supporting different roles for N_2O_3 as a biologically relevant signaling molecule.

1. Dinitrogen trioxide reactivity

Dinitrogen trioxide exists in an equilibrium with nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2$), where $k_1 = 8.1 \times 10^4\ s^{-1}$, and $k_{-1} = 1.1 \times 10^9\ M^{-1}s^{-1}$ [1] (Reaction 1).

$$N_2O_3 \rightleftarrows {}^{\bullet}NO_2 + {}^{\bullet}NO \quad \text{(R. 1)}$$

Thus, concentrations of 1 μM of $^{\bullet}NO_2$ and $^{\bullet}NO$ each, result in the formation of just 13.6 nM N_2O_3 at equilibrium, indicating that this reaction system favors N_2O_3 dissociation. Notably, N_2O_3 dissociation increases with temperature and decreasing solvent polarity, and is particularly efficient in the gas phase where the produced radicals are not constrained by a solvent cage [2]. Nitrogen dioxide is a strong one-electron oxidant, capable of reacting with thiols with rate constants of 1.9×10^7 and $4.8 \times 10^7\ M^{-1}s^{-1}$ for glutathione (GSH) and cysteine (Cys), respectively [3]. Based on these rates and considering a concentration of approximately 5 mM GSH in the cytosol, Ford et al. calculated that the rate of N_2O_3 formation from $^{\bullet}NO$ and $^{\bullet}NO_2$ is 100 times slower than the rate of $^{\bullet}NO_2$ consumption by thiols, while results from Madej et al. suggest an even larger difference in favor of thiol oxidation [3,4]. As a result, it is likely that the dissociation of N_2O_3 will be an essentially irreversible process under most physiological conditions. In addition, N_2O_3 is also hydrolyzed upon reacting with water to generate nitrite (NO_2^-), with an observed first order rate constant of $k_2 = 530\ s^{-1}$ and a $t_{1/2} = 1.3$ ms (Reaction 2) [1]. Furthermore, bicarbonate and phosphate accelerate N_2O_3 hydrolysis [5,6].

$$N_2O_3 + H_2O \rightarrow 2\,NO_2^- + 2\,H^+ \quad \text{(R. 2)}$$

From a biological perspective, perhaps the best-characterized reaction of N_2O_3 is its ability to react with nucleophilic thiols and amines to generate the corresponding nitrosated products with rate constants $k_3 > 6 \times 10^7\ M^{-1}s^{-1}$ [6,7]. (Reaction 3).

$$N_2O_3 + RS^- \rightarrow RSNO + NO_2^- \quad \text{(R. 3)}$$

Although controversial [8,9], thiol S-nitrosation has been associated with not only the regulation of individual protein function but also with conserved homeostatic processes, and with having a pivotal role in many diseases when dysregulated [10–12]. Many of the challenges associated with stringently defining physiological roles for protein S-nitrosation as a bonafide signaling process stem from the use of differential labeling techniques for the identification of labile S-nitrosated proteins in biological matrices. In particular, the biotin switch method and other SNO-capture techniques rely on the use of thiol alkylating agents to block non-nitrosated cysteine residues, followed by selective reduction of S-nitrosothiols to generate the corresponding free thiols that are then labeled with thiol-reactive reagents to enable detection [13–15]. Notably, these approaches are susceptible to artifacts due to incomplete blocking or non-specific oxidized thiol reduction [8,16,17]. However, the more recent development of phosphine probes capable of specifically reacting with SNO moieties while preserving both the S and N atoms in the resulting disulfide-iminophosphorane product has allowed the direct quantification of endogenous S-nitrosoglutathione in resting and activated macrophages, as well as in cancer cell lines [18]. Furthermore, the use of biotin-tagged phosphine-based SNOTRAP reagents has confirmed the presence of an endogenous S-nitrosated proteome, and has suggested a potential role for its dysregulation in the

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progression of neurodegenerative diseases [19–21].

From a structural perspective, N_2O_3 is in equilibrium between at least three different conformations: asymmetric (*asym*N_2O_3), symmetric (*sym*N_2O_3) and *trans-cis*-N_2O_3, with theoretical and experimental measurements suggesting similar isomeric stabilities [22–25]. Reactivity modelling indicates that all three isomers are susceptible to nucleophilic attack by thiols or amines, resulting in the transfer of a nitroso moiety and the production of nitrous acid (Scheme 1, adapted from Ref. [26]); with calculations showing that *sym*N_2O_3 and *trans-cis*-N_2O_3 are the more effective nitrosating agents [2,26,27]. Interestingly, while N_2O_3 has mostly been associated with nitrosative chemistry, the fact that this molecule can dissociate into $^{\bullet}NO$ and $^{\bullet}NO_2$, suggests a potential role in promoting nitration reactions. In this regard, and while possibly less efficient at promoting nitration than other $^{\bullet}NO_2$-generating systems such as peroxynitrite- or myeloperoxidase-dependent reactions, a role for N_2O_3 in the nitration of conjugated diene-containing fatty acids has been demonstrated [28–31]. In these experiments, Vitturi et al. showed that while nitrosonium tetrafluoroborate ($NOBF_4$) is unable to promote the nitration of conjugated linoleic acid (CLA) by itself, addition of nitrite (NO_2^-) results in the formation of NO_2-CLA. These results, in combination with studies assessing the isotopic distribution of nitrosation and nitration products obtained from the reaction between $^{\bullet}NO$, O_2 and $^{15}[N]^{18}[O]_2$-labeled NO_2^-, strongly suggested that NO_2^- reacts with N_2O_3 resulting in the formation of a symmetrical isomer of this molecule [31]. This was the first demonstration of the formation of *sym*N_2O_3 under physiologically relevant conditions *in vitro* and *in vivo*, as experimental proof for the existence of alternative N_2O_3 conformations had been limited to studies in inert low-temperature liquid matrices [22,25,32].

Despite substantial evidence of its ability to participate in nitrosation reactions, N_2O_3 formation is not the only pathway that can lead to nitrosative chemistry *in vivo*. In principle, any molecule capable of oxidizing a thiol or an amine by one electron in the presence of $^{\bullet}NO$ has the potential to promote nitrosation. Potential oxidants include $^{\bullet}NO_2$ and the carbonate radical anion ($CO_3^{\bullet-}$), with the resulting thiyl or aminyl radicals reacting with $^{\bullet}NO$ with rate constants approaching the diffusion limit to generate the corresponding nitrosated products (Reactions 4–5) [4,7,33,34].

$$^{\bullet}NO_2 + RS^- \rightarrow RS^{\bullet} + NO_2^- \quad \text{(R. 4)}$$

$$RS^{\bullet} + {}^{\bullet}NO \rightarrow RSNO \quad \text{(R. 5)}$$

Besides free radical intermediates in solution, alternative mechanisms involving metal center-assisted nitrosation have been proposed. For instance, a reaction between glutathione (GSH) and cytochrome *c* in the presence of $^{\bullet}NO$ has been proposed as a significant source of intracellular S-nitrosothiols. In this pathway, GSH binds to hexa-coordinated ferric (Fe^{III}) cytochrome *c* facilitating a reaction in which $^{\bullet}NO$ reduces the heme to the ferrous state (Fe^{II}) and simultaneously generates S-nitrosoglutathione (GSNO, Reactions 6, 7) [35,36]. A similar reaction is observed with ceruloplasmin, except that in this case it is the copper atoms that mediate GSNO formation from GSH and $^{\bullet}NO$ [37]. Subsequent transnitrosation reactions between GSNO and other nucleophilic thiolates enables downstream protein S-nitrosation [38].

$$Fe^{III}Cyt\ c + GSH \rightleftarrows Fe^{III}Cyt\ c\text{–}GSH \quad \text{(R. 6)}$$

$$Fe^{III}Cyt\ c\text{–}GSH + {}^{\bullet}NO \rightarrow Fe^{II}Cyt\ c + GSNO \quad \text{(R. 7)}$$

Unlike cytochrome *c* and ceruloplasmin, thiol nitrosation by dinitrosyl iron complexes (DNIC) is independent from existing metal-containing prosthetic groups. In this case, nitrosothiol formation requires the generation of nitrosyl complexes with the labile iron pool, a weakly coordinated form of endogenous intracellular iron that is accessible to exogenous chelators [39–41]. According to one proposed mechanism of DNIC-dependent nitrosation, the coordination of two $^{\bullet}NO$ molecules to a ferrous iron atom results in a redistribution of electrons leading to the production of bound NO^- and NO^+ equivalents, which upon reaction with a third $^{\bullet}NO$ molecule and a proton, generates a $Fe^+(NO^+)_2$ complex and nitroxyl (HNO). Reaction of this $Fe^+(NO^+)_2$ complex with a nucleophilic thiol results in S-nitrosation (Scheme 2) [42,43]. Alternatively, another mechanism posits that S-nitrosation occurs as a consequence of the formation of DNIC with low molecular weight thiol ligands. These authors suggest that DNIC are formed first via a mononitrosyl $Fe^{II}(NO)(RS)_2$ intermediate that undergoes autoreduction to generate $Fe^{I}(NO)(RS)$ and a thiyl radical, which in the presence of excess $^{\bullet}NO$, results in the formation of an S-nitrosothiol together with the stable $[Fe^{I}(NO)_2(RS)_2]^-$ DNIC product (Scheme 3) [44–46].

The co-existence of several proposed mechanisms for biological S-nitrosation is in part a reflection of the challenges associated with dissecting reaction pathways that involve transient intermediates and that lead to the formation of relatively labile products detected by artifact-prone methods [47–50]. Furthermore, S-nitrosation reactions do not occur in isolation, and are often accompanied by conditions that are conducive to nitrative and oxidative processes. In this regard, kinetic simulations of complex systems suggest that oxidation reactions often predominate over both nitration and nitrosation under inflammatory conditions [33]. Nevertheless, the relative yields of these pathways is determined by the mechanism of formation of the precursor reactive species, their sub-cellular compartmentalization, the presence of competing substrates, and by changes in tissue acidity.

2. Nitric oxide autoxidation

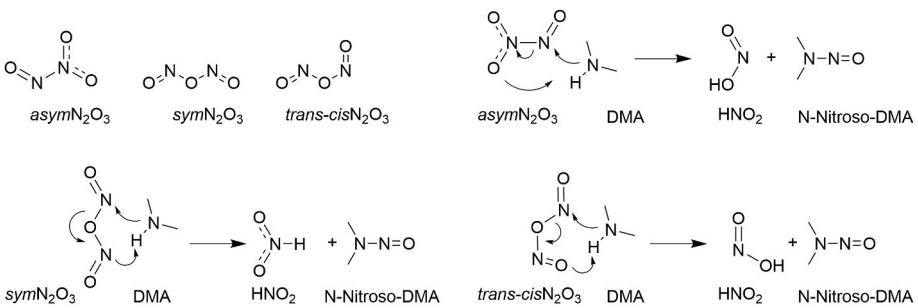
The contributions of N_2O_3 as a nitrosating agent have often been studied in the context of the reaction between $^{\bullet}NO$ and oxygen (O_2), a process also known as "$^{\bullet}NO$ autoxidation" (Reactions 8–10) [1,51,52].

$$^{\bullet}NO + O_2 \rightleftarrows ONOO^{\bullet} + {}^{\bullet}NO \rightleftarrows N_2O_4 \quad \text{(R. 8)}$$

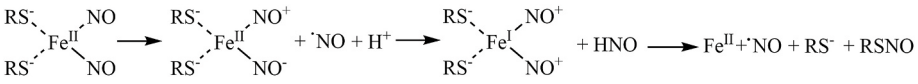
$$N_2O_4 \rightleftarrows {}^{\bullet}NO_2 + {}^{\bullet}NO_2 \quad \text{(R. 9)}$$

$$^{\bullet}NO_2 + {}^{\bullet}NO \rightleftarrows N_2O_3 \quad \text{(R. 10)}$$

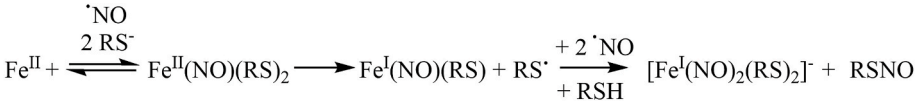
Although this reaction system generates N_2O_3, its yield of formation is dictated by the relative concentrations of $^{\bullet}NO$ and other substrates



Scheme 1. Structural isomers and nitrosating reactivity of dinitrogen trioxide.



Scheme 2. Proposed mechanism for thiol nitrosation via FeI(RS)2(NO+)2 formation.



Scheme 3. Proposed mechanism for thiol nitrosation via FeII(RS)2(NO) autoreduction.

capable of reacting with $^{\bullet}NO_2$ (Reaction 4). In the case of nitrosation reactions, and assuming that $^{\bullet}NO$ concentrations are not limiting, some substrates will exhibit preferential reactions towards N_2O_3 and others will follow the radicalar pathway illustrated in Reactions 4–5 [7]. Under experimental conditions, this differential reactivity can be elucidated by assessing the dependence between the yields of the S- or N-nitrosated product and the concentration of $^{\bullet}NO$. Thus, if a substrate is exclusively nitrosated via the radicalar pathway, the yields will be expected to decrease at higher $^{\bullet}NO$ concentrations, as excess $^{\bullet}NO$ would divert $^{\bullet}NO_2$ from substrate oxidation to generate N_2O_3. In contrast, if nitrosation is exclusively dependent on N_2O_3 formation, then the yield of the reaction should be independent of $^{\bullet}NO$ concentration. Following this approach, Goldstein and Czapski concluded that while cysteine, GSH, dithiothreitol and penicillamine are preferentially S-nitrosated via the radicalar pathway (Reactions 4, 5); N-acetyl-penicillamine, morpholine and captopril are preferentially nitrosated by N_2O_3 [7].

Regardless of the nitrosation mechanism, the rate limiting step for the formation of $^{\bullet}NO_2$ and N_2O_3 is Reaction 8. This reaction exhibits first order dependence on O_2 concentration and second order dependence on $^{\bullet}NO$, resulting in global third-order kinetics with $k = 2.9 \times 10^6\ M^{-2}s^{-1}$ (Equation (1)) [1].

$$\frac{-d[NO]}{dt} = 4k[NO]^2[O_2] \quad \text{(Eq. 1)}$$

Using this information, Lancaster calculated the half-life of $^{\bullet}NO$ under constant physiological concentrations of O_2 to be between 42 s and 5.8 days for initial $^{\bullet}NO$ concentrations of 1 μM and 5 nM, respectively [48]. These results not only indicated that $^{\bullet}NO$ is stable under physiological O_2 levels, but also that the formation of N_2O_3 and $^{\bullet}NO_2$ via $^{\bullet}NO$ autoxidation is too slow to be relevant *in vivo*. However, it was subsequently found that this reaction is accelerated between 30 and 300 times in the presence of lipid membranes, and more modestly by proteins [53–55]. This effect has been observed in a variety of biological samples and biomimetic ensembles, including hepatocyte membranes, purified mitochondria, phospholipids, low-density lipoproteins (LDL), proteins, detergent micelles, and perfluorocarbon emulsions [53–57]. This acceleration was termed "lens effect", and it occurs because both $^{\bullet}NO$ and O_2 are slightly hydrophobic molecules and are more soluble in the hydrophobic regions of lipid membranes and proteins than in the aqueous surrounding [58]. As mentioned, the rate of $^{\bullet}NO$ autoxidation has a second-order dependence on $^{\bullet}NO$ concentration, and a first-order dependence on O_2 concentration (Eq. (1).) [1,59]. Therefore, an increase in the local concentration of $^{\bullet}NO$ and O_2 in hydrophobic regions will result in significant acceleration of the reaction [53]. Chemical effects altering the rate constant could also be involved in accelerating the reaction in hydrophobic phases, but this possibility has been dismissed after careful consideration [58,60].

The concentration of molecules such as $^{\bullet}NO$ and O_2 in lipid hydrophobic regions relative to water are conveniently expressed as the partition coefficient K_P. The K_P for O_2 and $^{\bullet}NO$ in egg yolk phosphatidylcholine (EYPC) membranes is approximately 3, meaning that both O_2 and $^{\bullet}NO$ are 3 times more concentrated in the lipid membrane than in the aqueous surrounding. If we include these K_P values in Eq. (1), a theoretical acceleration factor equal to $3 \times 3 \times 3 = 27$ can be obtained. Using an $^{\bullet}NO$-selective electrode, it was observed that adding EYPC liposomes resulted in a 28-fold increase in the rate of $^{\bullet}NO$ autoxidation, consistent with the calculations derived from the K_P values [54]. Of note, the accelerated formation of $^{\bullet}NO_2$ could similarly be observed [54]. Nitrogen dioxide is also more soluble in organic than in aqueous solvents, and by combining experimental data with quantum calculations, a K_P of 1.5 was estimated between lipid membranes and water [60,61]. Molecular dynamics also support the hydrophobicity of $^{\bullet}NO_2$ [62,63]. Therefore, $^{\bullet}NO_2$ reaction with $^{\bullet}NO$ to make N_2O_3 will also be promoted by hydrophobic phases. In this regard, the rate of thiol nitrosation is increased by membranes and LDL, supporting a role of hydrophobic phases in biological nitrosation [54].

The acyl chain region of lipid membranes is often considered as comparable to hydrocarbon solvents such as decane or hexadecane. However, although they are similar in polarity, the hydrocarbon chains in lipid bilayers are oriented parallel to each other and restricted in motion, resulting in general in a lower solubility of solutes because of an exclusion effect. To exemplify this, the K_P of O_2 in decane relative to water at 25 °C is 8.7, whereas the K_P of O_2 in dilauroyl PC membranes relative to water is 3.2 [61]. Furthermore, lipid composition can alter the order in the bilayer and this has effects on K_P. For instance, PC composed of saturated acyl chains such as dimyristoyl PC and dipalmitoyl PC undergo phase transitions from an ordered gel phase to a disordered fluid phase at 24 and 43 °C, respectively. The K_P of O_2 between these membranes and water in the gel phase is approximately 1, but in the fluid phase K_P is above 3 [64]. Another factor that can modify the solubility of hydrophobic molecules is cholesterol, mostly by ordering membranes and reducing free volume [65,66]. Therefore, it is expected that more compact and less fluid membranes, such as those rich in sphingomyelin and cholesterol will also show lower K_P for O_2 and $^{\bullet}NO$.

A provocative study suggested extremely high acceleration of $^{\bullet}NO$ autoxidation by proteins, but these observations could not be reproduced and the acceleration effect was later shown to be only modest [55,67,68]. In proteins, hydrophilic amino acid residues are usually distributed on the protein surface and exposed to the solvent, whereas hydrophobic amino acid residues form the protein core. In theory, this hydrophobic core could be a favorable site for $^{\bullet}NO$ and O_2 partition and accelerated autoxidation, but proteins have an average density of 1.37 g/mL and are densely packed, thus resembling molecular crystals and providing limited room to accommodate other molecules. Nevertheless, most proteins are dynamic structures, as illustrated by the ability of different exogenously added molecules to quench the fluorescence of internal tryptophan residues [69]. Furthermore, some proteins such as serum albumin can accommodate hydrophobic ligands such as fatty acids in interior sites that are not evident in the crystal structure [70]. Thus, protein dynamics would allow for $^{\bullet}NO$ and O_2 to accommodate in the hydrophobic core and accelerate their reaction. It was found that the degree of $^{\bullet}NO$ autoxidation correlated with protein size and even more with their compressibility, indicating the importance of protein dynamics and cavities within the hydrophobic core of proteins [55]. Denaturing albumin, which leads to higher exposure of hydrophobic

surface and lower compressibility, resulted in a decrease in •NO autoxidation, indicating that the hydrophobic core rather than a hydrophobic surface is necessary to accelerate •NO autoxidation [55]. An overall 1.38-fold acceleration of •NO autoxidation was calculated for human albumin, but under normal vascular conditions, the very fast reaction between •NO and intraerythrocytic hemoglobin will outcompete this process [55].

The accelerated rate of •NO autoxidation and the downstream generation of N_2O_3 suggest that nitrosation reactions should be favored in close proximity to membranes. In addition, while the half-life of N_2O_3 in the cytosol is limited by hydrolysis (Reaction 2), the hydrophobic environment of the membrane protects N_2O_3 and should increase its chances of reacting with targets. To test this concept, peptides incorporating thiols at different depths within a lipid bilayer were designed, and it was found that nitrosation yields decreased as thiols were located deeper into the membrane [71]. This paradoxical result is explained by the lower ionization of thiols in non-polar environments. Thiolates rather than thiols are the main substrates for nitrosation reactions, and the low polarity of the membrane interior results in decreased thiolate availability.

The 'lens effect' suggests that the formation of •NO-derived oxidizing and nitrosating species will occur mainly within lipid membranes. Considering that membranes account for 3 % of the cellular volume and that •NO autoxidation occurs 30 times faster in this compartment, it was estimated that 50 % of all the •NO autoxidation will occur within cellular membranes [58]. As a result, biomolecules in close proximity to membranes will be exposed to higher fluxes of oxidizing and nitrosating species. However, as these species are diffusible, the yields of oxidative and nitrosative reactions will be significantly affected by individual substrate reactivity.

3. Gastric N_2O_3 formation

Nitrate and nitrite are central components of the human diet and are particularly abundant in green leafy vegetables, red beetroot, celery, fennel, and leeks; as well as in cured and uncured meats [72,73]. Dietary nitrate has a high bioavailability with close to 100 % of any given dose recovered in plasma over 24 h following ingestion [74,75]. Importantly, nitrate reabsorption by salivary glands results in its active uptake from the circulation and secretion into the saliva, where bacterial components of the oral flora reduce it to nitrite [76–78]. In this regard, the concentration of nitrite in saliva increases from a basal value of approximately 2 mg/mL to over 70 mg/mL following consumption of 400 mg nitrate, the equivalent to 200 g of spinach [79]. In the stomach, parietal cells secrete between 1 and 2 L of hydrochloric acid daily, resulting in a strong acidic environment in the gastric lumen (pH $\leq$ 3) [80]. Under these conditions, dietary nitrite is in equilibrium with nitrous acid (HNO_2, pKa ~3.2), with the protonated form 15- to 150-fold more abundant than the anion. Notably, nitrous acid undergoes disproportionation with $k = 13.4\ M^{-1}s^{-1}$ leading to the formation of N_2O_3 and thus •NO and $^{\bullet}NO_2$ (Reactions 11–12) [81–83].

$$NO_2^- + H^+ \rightleftarrows HNO_2 \quad \text{(R. 11)}$$

$$HNO_2 + HNO_2 \rightleftarrows N_2O_3 + H_2O \quad \text{(R. 12)}$$

The formation of nitrosating intermediates in the stomach has been studied extensively due to its potential to generate carcinogenic nitrosamines, nitrosamides and related compounds, although the pathological relevance of this pathway is a subject of debate [84,85]. From a mechanistic perspective, both radical and N_2O_3-mediated nitrosation pathways are likely to occur in the stomach, but these are modulated by the presence of other dietary components [86–88]. An interesting feature of the gastric compartment is that unlike other organs, it comprises both aqueous and lipid phases as well as a gas phase containing approximately 60 mmHg O_2 [89,90]. As discussed previously, •NO and O_2 tend to partition preferentially into lipidic compartments where N_2O_3 and N_2O_4 are protected from hydrolysis. This observation has important implications for reactivity, as indicated by assessing the effect of ascorbate on the yields of nitrosamine formation in the presence or absence of lipid phases. In this regard, Combet et al. found that while ascorbate potently inhibits nitrosamine formation from nitrite acidification in a monophasic solution, the addition of 10 % lipid to the reaction system results in ascorbate significantly increasing nitrosation yields [91]. Furthermore, the same physicochemical properties that dictate the preferential partition of •NO, O_2 and N_2O_3 into hydrophobic layers, also determine an even more favorable partition into the gas phase. As a result, nitrous acid disproportionation and N_2O_3 dissociation are main contributors to gastric •NO formation as evidenced by its detection in the exhaled breath [92]. Additional mechanisms contributing to intragastric •NO formation include univalent reduction of nitrite by dietary ascorbate and polyphenols [93,94]. The formation of •NO and $^{\bullet}NO_2$ in the stomach has important roles in gastric physiology, including regulating tissue blood flow and mucosal thickness, as well as preventing the proliferation of pathogenic microorganisms such as *Helicobacter pylori*, *Escherichia coli* and *Candida albicans* [95–97]. Importantly, a recent study of over 80,000 hospitalized patients found that disruption of gastric N_2O_3 formation secondary to elimination of the oral microbiome with mouthwash is associated with a small but significant increase in death rates, particularly in those patients at the lower risk of mortality [98]. Although indirect, this evidence suggests an important contribution of gastric N_2O_3 formation to •NO homeostasis and cyto-protective signaling [99]. Finally, gastric $^{\bullet}NO_2$ generation secondary to N_2O_3 homolysis is also conducive to nitration reactions as originally appreciated by the groups of Joao Laranjinha and Marco d'Ischia [88,100,101]. In particular, the nitration of dietary derived CLA is thought to be the main route for the endogenous formation of NO_2-CLA, a potent electrophilic fatty acid capable of promoting antioxidant and anti-inflammatory signaling in the gastrointestinal tract and beyond [102–104]. Importantly, the formation of NO_2-CLA has been proposed as an anti-hypertensive mechanism associated with the consumption of a Mediterranean diet, as well as a potentially protective factor in the context of the cardiac arrest survival and recovery [105–107].

4. Vascular N_2O_3 formation

In 1998, Lancaster published a provocative manuscript in which mathematical modelling of the half-life of •NO in the vascular compartment suggested that hemoglobin at a concentration approximately 10 % of that found in blood would be expected to scavenge over 90 % of all •NO produced by endothelial cells [108]. This conclusion was based on the fact that •NO reacts with both oxyhemoglobin and deoxyhemoglobin with rate constants between 10^7-$10^8\ M^{-1}s^{-1}$ (Reactions 13–14) [109,110], and thus questioned the ability of *free* •NO to function as a vasodilator *in vivo* [111].

$$^{\bullet}NO + HbFe^{II}O_2 \rightarrow HbFe^{III} + NO_3^- \quad \text{(R. 13)}$$

$$^{\bullet}NO + HbFe^{II} \rightarrow HbFe^{II}NO \quad \text{(R. 14)}$$

Notably, it was later found that •NO scavenging by intraerythrocytic hemoglobin at 50 % hematocrit is 50–150 times slower than by the same concentration of cell-free hemoglobin, with earlier microvessel bioassay determinations suggesting this difference to be approximately three orders of magnitude [112,113]. Different mechanisms have been proposed to explain these observations, including the presence of a cell-free zone adjacent to the vascular endothelium, the existence of a •NO-depleted unstirred layer around the extracellular side of the erythrocyte membrane, and controversially, the potential existence of an intrinsic membrane barrier to •NO diffusion in the erythrocyte [114]. However, these observations also led to hypotheses proposing the existence of stabilized forms of •NO that could escape scavenging by

hemoglobin and could become activated along the arterial to venous gradient [115,116]. One such mechanism proposed that nitrite is reduced to •NO by deoxyhemoglobin, with allosterically controlled reduction rates that are maximal at hemoglobin fractional saturations approaching 50 % (Reaction 15) [117,118].

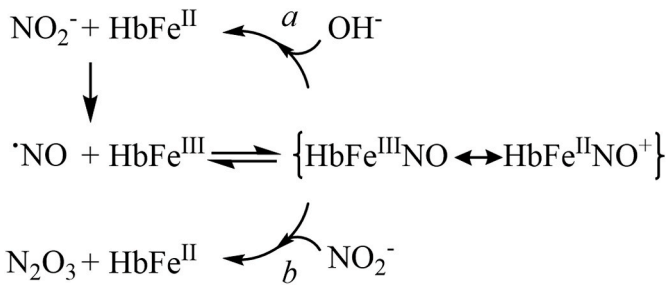
$$NO_2^- + HbFe^{II} + H^+ \rightarrow {}^\bullet NO + HbFe^{III} + OH^- \quad \text{(R. 15)}$$

This reaction elegantly links •NO generation from nitrite to the lower hemoglobin oxygen fractional saturations typically found in resistance arterioles, and to hypoxic vasodilation responses in general [119,120]. In this regard, nitrite addition to deoxygenated erythrocytes leads to the induction of extracellular •NO-dependent responses such as the inhibition of platelet aggregation, inhibition of mitochondrial respiration and vasorelaxation [116,117,121–123]. However, a main challenge to the mechanism proposed in Reaction 15 is that nitrite reduction occurs intracellularly in the presence of a large excess of oxygenated and deoxygenated hemoglobin (20 mM heme, corresponding to four hemes per hemoglobin tetramer), thus suggesting that any •NO generated would be immediately consumed (Reactions 13–14) [108,124]. These arguments, together with previous work on reductive heme nitrosylation by Ford et al. (Scheme 4a), suggested the possibility that the diffusible product of nitrite reduction by deoxyhemoglobin might not be •NO but rather N_2O_3 [125,126]. It was hypothesized that this mechanism would limit •NO scavenging by hemoglobin through the generation of diffusible N_2O_3 that can then homolyze to produce •NO in the extracellular compartment (Reaction 1). Notably, this proposal is in line with observations that nitrite supplementation is often associated with increased intraerythrocytic S-nitrosothiol formation *in vitro* and *in vivo* [116,126–128]. While several related mechanisms have been proposed for the hemoglobin-catalyzed generation of N_2O_3 from nitrite, the formation of a nitrosyl-methemoglobin complex that can then react with nitrite in the distal heme pocket through either an outer or inner sphere reaction appears to be the more favored possibility (Scheme 4b) [129–132].

Mathematical models and experiments performed in glassy matrices and in solution/sol-gel suggest that this reaction scheme is feasible, and that it can extend the half-life of •NO in the circulation [130,132–135]. However, whether the physiological levels of nitrite in the erythrocyte are sufficient to sustain •NO formation at functionally relevant concentrations remains to be established. Finally, a new mechanism that is independent of N_2O_3 formation has been recently proposed suggesting that nitrite reduction by deoxyhemoglobin leads to the formation of a diffusible nitrosyl-ferroheme species in the erythrocyte membrane that can transfer to circulating albumin and eventually promote cGMP-dependent signaling in the smooth muscle [136].

5. Conclusion

Although impossible to detect directly by current methods, the physiological formation of N_2O_3 is a possible phenomenon, particularly in lipid compartments and in the acidic conditions of the stomach (Fig. 1). These locational preferences are defined either by the favorable partition of precursor species into nonpolar environments that exclude competing thiolates and water, or by conditions that simultaneously decrease thiolate availability by protonation and promote N_2O_3 generation from relatively abundant dietary nitrite via nitrous acid disproportionation. Regardless of its mechanism of formation, N_2O_3 is expected to exist at very low steady-state levels even in the absence of substrates, as this species is not only susceptible to hydrolysis but it also quickly dissociates into •NO and $^\bullet NO_2$. Despite its short lifetime, the consequences of N_2O_3 reactivity are highly pervasive and include the generation of stable carcinogenic nitrosamines, the modulation of cysteine-dependent signaling pathways via S-nitrosation, the production of local and systemically distributed bioactive nitrated fatty acids, and potentially the regulation of vascular physiology in health and disease



Scheme 4. Reductive nitrosylation (a) and nitrite dehydration (b) pathways of hemoglobin.

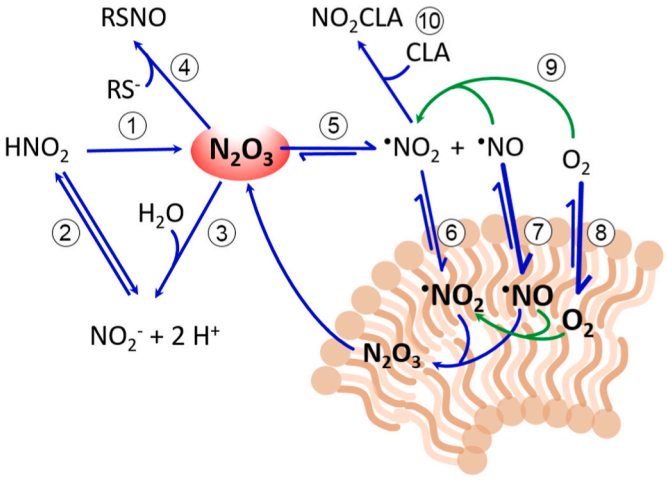


Fig. 1. Summary of the main reactions leading to N_2O_3 formation and consumption under physiological conditions. 1: $k_1 = 13.4\ M^{-1}s^{-1}$ [81]; 2: pKa = 3.2 [138]; 3: $k_3 = 530\ s^{-1}$ [1]; 4: $k_4 > 6 \times 10^7\ M^{-1}s^{-1}$ [7]; 5: $k_5 = 8.1 \times 10^4\ s^{-1}$ [1], $k_{-5} = 1.1 \times 10^9\ M^{-1}s^{-1}$ [1]; 6: $K_{P6} = 1.5$ [61]; 7: $K_{P7} = 3.6$ [54]; 8: $K_{P8} = 3.2$ [54]; 9: $k_9 = 2.9 \times 10^6\ M^{-2}s^{-1}$ [1]; 10: CLA nitration follows a complex mechanism. For a detailed discussion see Refs. [102,139].

[82,85,103,104,137]. Therefore, understanding the factors that determine the formation, reactivity and decomposition of N_2O_3 is essential for the elucidation of key signaling mechanisms that contribute to both homeostatic and pathological states.

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CRediT authorship contribution statement

Matías N. Möller: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Darío A. Vitturi:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Dario A. Vitturi reports financial support was provided by National Institutes of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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