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Routes for Formation of S-Nitrosothiols in Blood

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Abstract

S-nitrosothiols (RSNO) are involved in post-translational modifications of many proteins analogous to protein phosphorylation. In addition, RSNO have many physiological roles similar to nitric oxide ($\bullet$ NO), which are presumably involving the release of $\bullet$ NO from the RSNO. However, the much longer life span in biological systems for RSNO than $\bullet$ NO suggests a dominant role for RSNO in mediating $\bullet$ NO bioactivity. RSNO are detected in plasma in low nanomolar levels in healthy human subjects. These RSNO are believed to be redirecting the $\bullet$ NO to the vasculature. However, the mechanism for the formation of RSNO in vivo has not been established. We have reviewed the reactions of $\bullet$ NO with oxygen, metalloproteins, and free radicals that can lead to the formation of RSNO and have evaluated the potential for each mechanism to provide a source for RSNO in vivo.

Keywords

S-nitrosothiols; Nitric oxide; Blood; Hemoglobin; S-nitrosation; Metalloproteins

Introduction

S-nitrosothiols (RSNO) are simple organic thio-esters of nitrites or functional groups containing a nitroso group covalently attached to the sulfur atom of a thiol. The life span of nitric oxide ($\bullet$ NO) is <2 ms in blood [1] and <2 s in tissues [2]. It is believed that this short life span is a limitation for the biological activity of $\bullet$ NO. However, when $\bullet$ NO complexes with thiol groups to form RSNO, it increases the life span by protecting the $\bullet$ NO from inactivation by metalloproteins (especially hemoglobin) and free radicals (especially superoxide and lipid peroxyl radical). RSNO can elicit their biological activity by releasing $\bullet$ NO, resulting in the activation of the classical guanylyl cyclase/cGMP-dependent pathway or the cGMP-independent pathway, which includes post-translational modification of proteins [3–7]. RSNO are potent vasodilators [8, 9], strong inhibitors of platelet aggregation [10, 11] and leukocyte binding to the vascular endothelium [12–14]. They are also involved in bronchodilation [15], neuroprotection [16], and exhibit anticancer activity [17]. Recently, considerable interest has been generated in S-nitrosylation of protein cysteine residues, which regulate numerous cellular signaling pathways ranging from G-protein coupled reactions to receptor stimulation and activation of nuclear regulatory proteins [18–20]. The

roles of physiological and nonphysiological levels of RSNO in health and diseases have been reviewed in detail [21, 22].

In spite of varied roles of RSNO in biological systems, the mechanism by which $\bullet\text{NO}$ is converted to a nitrosation agent that reacts with thiols is not yet clearly known [23]. This review is mainly focused on the routes by which RSNO are formed with reference to the blood milieu.

S-Nitrosothiols in Blood

RSNO were first synthesized and characterized by Tasker and Jones in 1909 [24]. In 1973, Needleman et al. originally hypothesized that tissue thiol groups were needed to potentiate the dilation of aortic strips by organic nitrates such as nitroglycerin [25]. In 1980, Ignarro et al. for the first time implicated a role for RSNO in the activation of guanylyl cyclase by nitroso compounds such as nitrosoguanidine, glyceryl trinitrate, and sodium nitroprusside [26, 27]. Subsequently, these investigators reported that the reaction of low molecular weight thiols with nitrogen oxides, such as N_2O_3 generate RSNO with a life span appreciably longer than $\bullet\text{NO}$, which can play a role in $\bullet\text{NO}$ mediated functions [28–30]. In 1992, Stamler et al. reported that the reaction of oxidized derivatives of $\bullet\text{NO}$, such as nitrosonium cation (NO^+) or N_2O_3 , with electron rich SH groups of proteins in vitro generate S-nitrosylated proteins [31, 32]. These S-nitrosylated proteins stabilize the bioactivity of $\bullet\text{NO}$ by increasing the life span of $\bullet\text{NO}$. These important findings have placed research on RSNO in the forefront of $\bullet\text{NO}$ biochemistry and physiology. RSNO have subsequently been detected in almost all intra- and extracellular biological fluids [33].

Plasma

Albumin is a major plasma protein that contains a single free cysteine at position –34. At physiological pH, the SH group of this cysteine residue exists as a highly reactive thiolate anion ($-\text{S}^-$) that can react with metals and electrophiles. Plasma contains $\sim 600\ \mu\text{M}$ thiol groups most of which are contributed by albumin ($500\ \mu\text{M}$), GSH ($5\text{--}30\ \mu\text{M}$), and cysteine ($15\ \mu\text{M}$) [34]. In principle, the electrophilic attack of these thiols by higher oxides of nitrogen such as N_2O_3 can generate RSNO [23]. Stamler et al. determined the existence of RSNO in μM levels by the photolysis chemiluminescence method in plasma of healthy humans primarily as S-nitrosoalbumin (albSNO), which can serve as a reservoir of $\bullet\text{NO}$ for physiological functions [35, 36]. Other investigators have confirmed the presence of SNO in blood plasma using different methodologies including visible spectrophotometer (Griess reagent), Fluorimetry, HPLC, GC–MS, chemiluminescence (reagents in the purge vessel that cleave RSNO releasing NO) [37–40]. However, the concentrations reported by these investigators were lower than that reported by Stamler and his coworkers. Our measurements of plasma RSNO, which involved chemiluminescence detection, in healthy human subjects are typically in the range of 2–20 nM with the average centered around 7.93 nM [41]. It is now generally accepted that the plasma levels of RSNO are in the low nanomolar (nM) range as against the original claim of $7\ \mu\text{M}$. Needless to say, most of the observed differences can be attributed to different methodologies used to measure RSNO [33, 40].

In support of the existence of RSNO in blood plasma, a significant level of albsNO has been shown to be formed when the whole blood is incubated with donors of NO [42]. In addition, plasma RSNO levels have been reported to decrease when endothelial nitric oxide synthase (eNOS) is inhibited and to increase during •NO inhalation [43], intravenous administration of •NO [44, 45] or treatment of rats with lipopolysaccharide (LPS) [46]. Plasma RSNO equilibrate between high molecular weight protein thiols (albumin) and low molecular weight thiols (GSH and cysteine) by undergoing transnitrosation reactions [47, 48]. Though the concentration of plasma low molecular weight RSNO is less than that of protein RSNO, the low molecular weight RSNO more readily undergo transnitrosation reactions with membrane thiols. In addition, cysteine–NO (Cyst–NO) can cross the cell membranes through amino-acid transporters providing an intracellular source for •NO.

Red Blood Cells

Hemoglobin (Hb) was viewed exclusively as a scavenger of NO until the 1996 paper by Stamler and collaborators demonstrated that Hb conserved the reactivity of •NO by nitrosation of the β -93-cysteine group producing S-nitroso-hemoglobin (SNOHb) [49]. This •NO on the thiol group can be transported and released under hypoxic conditions providing a potential mechanism for hypoxic vasodilation to match the local metabolic demand [50–52]. In support of these observations, the authors have shown that rat arterial and venous blood contains 311 and 32 nM SNOHb, respectively [49]. Human arterial blood was found to contain about 2.5 μ M SNOHb with no detectable SNOHb in venous blood [53]. Both of these studies indicate a significant arterial/venous gradient of SNOHb determined by the photolysis chemiluminescence method. Another study has reported 1.45 μ M SNOHb in human umbilical arterial blood and 2.19 μ M SNOHb in venous blood determined by Saville and Greiss reaction method [54]. Furthermore, Gladwin and coworkers have shown that human arterial and venous blood contains 161 and 142 nM SNOHb, respectively, which corresponds to a much smaller arterial/venous gradient [55]. Subsequently, after improving their methodology to determine RSNO using the tri-iodide chemiluminescence method, the red blood cell SNOHb was shown to be in the range of ~50 nM [56]. The reactions of •NO with Hb and the formation of SNOHb and the potential transfer of the •NO bioactivity from red blood cells to the vasculature has been reviewed in detail [45, 57, 58]. Formation of varying levels of SNOHb has been reported during the reaction of oxyHb, deoxyHb, or methemoglobin (metHb) with physiological or non-physiological concentrations of •NO [59]. The formation of SNOHb has also been observed during the reduction of nitrite to NO by deoxyHb [60–62]. In another study, the generation of N₂O₃, a nitrosation species and subsequent formation of RSNO has been shown during reduction of nitrite by deoxyHb in red blood cells [63]. The physiological relevance of these in vitro studies is not yet known.

Neutrophils and Platelets

RSNO are also detected in neutrophils [64] and platelets [65].

The Source of Nitric Oxide in Blood

Endothelial Cells

The vascular endothelium plays a major role in the regulation of vascular homeostasis by secreting several biologically active substances that control vascular tone, remodeling, platelet aggregation, monocyte adhesion, lipid metabolism, and vascular growth [66]. •NO is one of the predominant vasoactive substances synthesized in endothelial cells from L-arginine by •NO synthase. The •NO synthesized in endothelial cells diffuse randomly to the surrounding cells. It is well established that •NO diffused into smooth muscle cells activate guanylyl cyclase, which, in turn increases cGMP levels resulting in vasodilation. Similarly, •NO can also diffuse into the blood milieu and is therefore amenable for reaction with red blood cell Hb. Even before the discovery that •NO was EDRF, it was well known that oxyHb and deoxyHb scavenge •NO to form nitrate and metHb or iron nitrosylhemoglobin, respectively, with diffusion limited rate constants of $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Eqs. 1–3) (Fig. 1) [67]. Subsequently, it has been shown that the rate for the •NO reaction with Hb engulfed within the red blood cell is at least two orders of magnitude slower ($\sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$) than free Hb, because of the combined effect of the unstirred layer around red blood cells and the diffusion barrier of red blood cell membrane proteins [1, 68]. Though •NO binds strongly to deoxyHb in the presence of oxygen, it slowly oxidizes forming metHb and nitrate.

•NO is a non-polar gaseous molecule which is 6–9 times more soluble in hydrophobic solvents than in aqueous media [69]. Therefore, it was thought that •NO that diffused into blood milieu will readily pass through the hydrophobic membrane into red blood cells where it is immediately scavenged by oxyHb or deoxyHb and converted to nitrate (a biologically inert molecule), that is excreted by the kidneys.

Diffusion model studies actually predicted that this very efficient scavenging of •NO by red blood cell Hb limits its diffusion into smooth muscle cells [1, 70, 71]. Hence, the blood has been viewed as a sink for most of the •NO synthesized in endothelial cells, and any •NO that diffused into the blood can no longer act as a signaling molecule. However, it is well established that •NO synthesized by eNOS is involved in vasodilation and the regulation of blood pressure, which is based on the observation of decreases in vasodilation when eNOS is inhibited by L-NAME. The logical question then would be if most of the endothelial •NO is scavenged by Hb, how does •NO cause vasodilation? This dilemma has been the basis for

studies investigating the conversion of $\bullet\text{NO}$ into forms that are resistant to scavenging by Hb and which can release $\bullet\text{NO}$ in the vasculature. In order to unravel this dilemma, studies have been carried out to further understand the chemistry and physiology of $\bullet\text{NO}$ that is released into the circulation.

Blood Cells

Red Blood Cells—The presence of eNOS and inducible nitric oxide synthase (iNOS) proteins have been reported in red blood cells [72, 73]. These enzymes had been shown to be in an inactive form thought to be remnants of the developing erythroblast or reticulocyte [74]. However, recent and more detailed studies have demonstrated that human red blood cells contain catalytically active NOS that release $\bullet\text{NO}$ species, which are involved in the maintenance of red blood cell deformability and platelet function [75, 76]. Whether such $\bullet\text{NO}$ is involved in vasodilation needs to be further investigated.

Platelets— $\bullet\text{NO}$ has been shown to be synthesized in platelets by eNOS and iNOS [77, 78]. Usually, iNOS is in an inactive form when platelets are in the resting stage, and is only activated when the platelets are stimulated [77]. Thus, on activation platelets can be a significant source of $\bullet\text{NO}$.

Neutrophils— $\bullet\text{NO}$ is involved in immunity by acting as a toxic agent to infecting organisms. The presence of NOS enzymes in neutrophils has been reported [62, 79, 80]. There are, however, some reports that neutrophils do not have NOS proteins [81]. Neutrophils make up about 50–60% of the leukocytes in the circulation, and if they have NOS proteins would contribute a significant amount of the $\bullet\text{NO}$ in the lumen, especially during infection and/or inflammation [79].

Eosinophils—Eosinophils play a major role in allergic diseases. An elevated level of exhaled NO has been reported in patients of allergic asthma. This elevated level of NO is correlated with the presence of NOS isoforms in eosinophils [82].

Monocytes/Macrophages—Several studies have shown that monocytes/macrophages contain functional iNOS protein. Monocytes/macrophages produce low levels of $\bullet\text{NO}$ under normal healthy conditions. However, $\bullet\text{NO}$ is produced at higher levels on the expression of iNOS during infection and inflammatory conditions [83]. Macrophages can become a significant source of $\bullet\text{NO}$ under such conditions.

Reduction of Nitrite to $\bullet\text{NO}$ by deoxyHb—Human plasma contains nitrite in the range of 50–250 nM [84]. Recent studies have demonstrated that under hypoxic conditions this nitrite has vasodilatory activity associated with the reduction to $\bullet\text{NO}$ by red blood cell deoxyHb (Eq. 4) [85–87]. Therefore, this $\bullet\text{NO}$ also serves as a source of $\bullet\text{NO}$ in blood. The mechanism of this $\bullet\text{NO}$ bioactivity and its potential transfer to the vasculature is under intense investigation [88, 89].

Possible Routes for Formation of S-Nitrosothiols in Blood

$\bullet\text{NO}$ reacts with thiols very slowly under anaerobic conditions to form $\text{RS}\bullet\text{NOH}$, but does not generate RSNO [90, 91]. However, one electron oxidation of $\bullet\text{NO}$ forms NO^+ which reacts with nucleophiles such as thiols and amines to produce RSNO and nitrosamines, respectively [92]. In order to generate NO^+ ion another molecule has to accept the electron from $\bullet\text{NO}$. Oxygen, metals, metal compounds, and metalloproteins can accept this electron following the reaction with NO . RSNO can also be produced by the reaction of $\bullet\text{NO}$ with thiyl radicals and superoxide anion in the presence of thiol groups (see below). The potential mechanisms for producing RSNO by the reaction of these molecules with $\bullet\text{NO}$ in the presence of thiol groups are discussed.

Oxygen-Dependent Autoxidation of NO

It is well established that the reaction of $\bullet\text{NO}$ with thiols in the presence of oxygen generate RSNO in vitro [93, 94]. The reaction of $\bullet\text{NO}$ with oxygen forms nitrogen dioxide ($\bullet\text{NO}_2$), which immediately reacts with another molecule of $\bullet\text{NO}$ to form dinitrogen trioxide (N_2O_3), with the same +3 nitrogen oxidation state as the NO^+ ion and which has reaction characteristics similar to the NO^+ ion. The N_2O_3 is either rapidly hydrolyzed to nitrite or reacts with thiols to form RSNO (Eqs. 5–8) (Fig. 1).

In addition, $\bullet\text{NO}_2$ can oxidize thiols to form thiyl radicals, which can directly react with another $\bullet\text{NO}$ molecule to form RSNO (Eqs. 9, 10) [93, 95, 96].

The relevance of these reactions involving $\cdot\text{NO}$ and oxygen for the formation of RSNO in the circulation has, however, been challenged. The rate for the reaction of $\cdot\text{NO}$ with oxygen to produce N_2O_3 is second order in $\cdot\text{NO}$ concentration, and first order in O_2 concentration with an overall third order rate constant of $6.3 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$ [93, 97]. The rate limiting step in this reaction is the initial reaction of $\cdot\text{NO}$ with O_2 [94]. The rate of this reaction is largely dependent on the de nova concentration of $\cdot\text{NO}$ and oxygen. At the steady-state physiological levels of 100 nM $\cdot\text{NO}$ and 100 μM oxygen in blood, the half-life of $\cdot\text{NO}$ is predicted to be >30 min. With the reported life span of $\cdot\text{NO}$ in blood of <2 ms [1] and in tissues of <2 s, it is unlikely that $\cdot\text{NO}$ reacts with oxygen in vivo to form the nitrosating agent (N_2O_3) or thiyl radicals. Therefore, it does not seem probable that RSNO are formed by oxygen-dependent autoxidation of NO that diffuses from endothelial cells into the circulation.

In spite of these kinetic constraints, reports indicate the formation of RSNO in plasma following the incubation of $\cdot\text{NO}$ donors with whole blood [42], intravenous infusion of $\cdot\text{NO}$ in human subjects [44], inhalation of NO [43, 98], and rats treated with LPS [46]. These results suggest that there are mechanisms, other than oxygen-dependent $\cdot\text{NO}$ autoxidation, involved in the generation of RSNO in blood. A recent report [99] suggests that $\cdot\text{NO}$ directly reacts with thiols to produce a radical intermediate ($\text{RS}\cdot\text{NOH}$), which reduces oxygen to superoxide to form RSNO. These results could not be reproduced by others when the reaction was carried out under anaerobic conditions by incubating GSH with $\cdot\text{NO}$ in the presence of NAD^+ (as an electron acceptor) [96]. In addition, the rate of reaction of $\cdot\text{NO}$ with thiols ($10 \text{ M}^{-1} \text{ s}^{-1}$) [96] is nearly six orders of magnitude slower than the reaction of $\cdot\text{NO}$ with erythrocytic Hb ($\sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$) [1, 68]. Hence, the possibility of a direct reaction of $\cdot\text{NO}$ with thiol groups seems to be very unlikely in the blood.

It has been reported that the hydrophobic interior domain of proteins [100] such as albumin [101], and other membrane proteins [18, 102–104] can catalyze S-nitrosation. This observation is based on the increase in local concentration of $\cdot\text{NO}$ and O_2 in the hydrophobic region of membranes and proteins due to their lipophilic nature that can result in acceleration of the autoxidation of $\cdot\text{NO}$, resulting in the formation of nitrosating species. The location and stabilization of these nitrosating species at specific sites in proteins would nitrosate the thiols to produce RSNO. These reports are further supported by the finding that albSNO accounts for the majority of the RSNO found in plasma. However, a recent report contradicts the contention that thiols located in the deep hydrophobic core of membranes more likely to undergo nitrosation in the presence of oxygen and $\cdot\text{NO}$ [96, 105]. This recent data and the short life span of $\cdot\text{NO}$ in blood indicates that it is very unlikely that RSNO are generated by accelerated $\cdot\text{NO}$ autoxidation in membranes or the hydrophobic domain of proteins present in the blood.

Thiyl Radicals

Plasma contains about 600 μM thiols, including low and high molecular weight thiols. Thiyl radicals are formed in biological systems due to the detoxification of reactive oxygen, nitrogen species and reactive chemical metabolites by thiols (Eq. 11) [106]. The reaction

between these thiyl radicals and $\bullet\text{NO}$ has been suggested as one of the potential mechanism for the formation of RSNO (Eq. 12) [93, 107, 108].

The rate constants for the reaction of thiyl radical with $\bullet\text{NO}$ was originally reported to be $1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [107, 109]. In a later study, a rate constant nearly two orders of magnitude lower of $2.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [110] was reported. Subsequently, Madej et al. reevaluated this reaction and reported a rate constant of $2.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ which is close to the originally reported value [111]. This high rate is typical for radical–radical reactions and is appreciably higher than the rates for the quenching of thiyl radical by other reductants such as GSH, ascorbate, and urate in blood [107, 112]. In order to react $\bullet\text{NO}$ with these thiyl radicals in blood, thiyl radical has to compete with erythrocytic Hb. The reported rate constant for the reaction of $\bullet\text{NO}$ with thiyl radicals is four orders of magnitude faster than that of the reported rate constant for the reaction of $\bullet\text{NO}$ with erythrocytic Hb [1, 68]. Thus, the rate of reaction of $\bullet\text{NO}$ with thiyl radicals if present in blood is about four orders of magnitude faster than the rate of reaction of $\bullet\text{NO}$ with erythrocytic Hb. This may, therefore, be one of the potential route for the formation of RSNO in plasma in spite of the very high level of Hb.

Peroxynitrite

Superoxide generated by leukocytes, macrophages, and endothelial cells are released into the vascular lumen in addition to $\bullet\text{NO}$. Peroxynitrite anion (PN) is formed by the rapid radical–radical reaction of $\bullet\text{NO}$ with superoxide (Eq. 13) [113]. The rate constant for the reaction of $\bullet\text{NO}$ with superoxide ($1.6 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) [114] is nearly four to five orders of magnitude higher than that for the reaction of NO with erythrocytic Hb [1, 68].

Hence, there exists a possibility for the reaction of $\bullet\text{NO}$ with superoxide in the lumen before being scavenged by erythrocytic Hb. Consistent with this prediction low levels of plasma 3-nitrotyrosine, which is a marker for PN has been detected in healthy humans. Furthermore, an increase in plasma 3-nitrotyrosine levels and RSNO has been reported under inflammatory or infectious conditions, where superoxide and $\bullet\text{NO}$ production is increased relative to their normal level [115–118].

Peroxynitrite is a strong oxidant and is considered to be a toxic molecule [119]. However, several in vitro and in vivo studies have shown that PN displays some beneficial effects like vasodilation, inhibition of platelet aggregation and protection against ischemia reperfusion injury [117, 120]. These beneficial effects are thought to be mediated by the formation of RSNO following the reaction of PN with thiols (Eq. 14) [113, 117, 121–125].

The formation of RSNO in conjunction with PN raises the possibility that PN might contribute to the formation of RSNO in blood milieu [115, 116]. However, some other studies have shown that PN does not generate RSNO in vivo or in vitro [126–128]. Additional studies are required to get a better understanding of PN-mediated RSNO formation in blood.

Metals, Metal Compounds, and Metalloproteins

*NO is known to readily form complexes with metal centers including metalloproteins [67]. Most of the biological functions of *NO are governed by its interaction with heme proteins such as guanylyl cyclase. As already discussed *NO has to undergo oxidation by losing an electron to form NO^+ to be able to react with thiols. Oxidized metals, metal compounds, or and metalloproteins are good candidates for accepting the electron from NO to form NO^+ . One problem with this mechanism is that the life time of NO^+ is 3×10^{-10} s in aqueous solutions. Hence, NO^+ that is generated cannot diffuse to other sites to react with thiols. Thus, the formation of RSNO requires that thiols are located in the immediate vicinity where NO^+ is released. Therefore, researchers have started looking into the involvement of metals, metal compounds, and metalloproteins, where NO^+ is not immediately released or where thiols are located in close proximity to the NO that is being oxidized to a NO^+ for the formation of RSNO.

Iron and Copper Ions

*NO readily forms complexes with transition metals like Fe(III) and Cu(II) which can be reduced by *NO resulting in the reduction of the metal ion and the oxidation of the *NO to NO^+ . This NO^+ immediately hydrolyzes to nitrite before reacting with thiols. However, it has been shown that Cu(II) that complexes with thiol groups of peptides, bovine serum albumin, and Hb catalyzes S-nitrosation by oxidizing *NO or the thiolate anion [129, 130]. Metal ions are, however, mostly sequestered by proteins in the blood and may not be available for the formation of RSNO. Thus, free metal ions may not significantly contribute to RSNO formation in blood.

Dinitrosyl Iron Complexes

These complexes are generated by various cells and tissues on reaction of *NO with thiol rich proteins or low molecular weight thiols (cysteine, GSH) in the presence of iron or as a result of a direct reaction with iron–sulfur proteins [131, 132]. Dinitrosyl iron complexes (DNIC) can act as *NO donors and have several physiological functions similar to *NO [131]. The general formula is shown in Scheme 1. Unlike free metal ions, NO^+ formed from DNIC does not immediately hydrolyze in aqueous solutions. The transfer of this NO^+ to low molecular weight thiols (GSH and cysteine) and proteins has been observed with the formation of RSNO [133, 134]. In fact, generation of DNIC (low and high molecular weight) were detected by EPR, in endothelial cells incubated with *NO donors [135], in rat aortic rings [136, 137], or macrophages [138] treated with LPS, and in various mammalian

cells [139]. The activation of guanylyl cyclase or dilation of vascular rings potentiated when DNIC was incubated with cysteine supports the hypothesis that DNIC can transfer $\bullet\text{NO}$ to cysteine. The low molecular weight RSNO (cysteine-NO), formed by catalysis of DNIC in endothelial cells, macrophages and leukocytes, can diffuse into blood where it can transfer its $\bullet\text{NO}$ (transnitrosation) to high molecular weight proteins like albumin to form protein RSNO [140–142]. It is also possible that RSNO can be formed in plasma as a result of the reaction of DNIC with thiols, although this remains to be demonstrated.

Ceruloplasmin

Ceruloplasmin (CP), is a major multicopper containing enzyme, and has been shown to catalyze the formation of *S*-nitrosoglutathione (GSNO) and albSNO in the presence of GSH or albumin and $\bullet\text{NO}$ [129, 143]. Cu(II) of CP being an oxidized metal can accept the electron from NO to form NO^+ , and this can react with GSH to form GSNO (Eqs. 15–17).

The in vivo formation of RSNO by CP is supported by Inoue et al. who observed the formation GSNO in CP media released from HepG2 hepatic carcinoma cells [143]. Furthermore, the role for CP in producing RSNO in blood is supported by the finding that the level of RSNO in plasma correlated with the levels of CP in hypercholesterolemic subjects [144]. However, Shiva et al. were unable to observe CP catalyzed SNO formation in whole blood in the presence of $\bullet\text{NO}$ donors [145]. They interpreted their results by suggesting that most of the NO^+ formed from the reduction of Cu(II) is hydrated to nitrite (Eq. 16). These findings are consistent with the very short half-life of NO^+ (<1 ns) ruling out the diffusion of NO^+ into media to be able to react with GSH. Although $\bullet\text{NO}$ can react with CP before being taken up by red blood cells, it is very unlikely that CP can catalyze the formation of RSNO in blood, unless low molecular weight thiols are coordinated to the metal centers of CP.

Hemoglobin

The fundamental view that red blood cell scavenging of $\bullet\text{NO}$ results in the formation of inactive nitrate and Hb(II)NO has been altered following the reports of Stamler and his colleagues. The interactions of $\bullet\text{NO}$ with red blood cell Hb has been proposed to preserve the bioactivity of $\bullet\text{NO}$ by forming SNOHb [49, 52]. As discussed above, NO neither reacts with β -93 cysteine directly nor with oxygen to form the nitrosating agent (N_2O_3) required to form SNOHb in red blood cells. It has, however, been proposed that at the very low

physiological levels of $\bullet\text{NO}$ ($<1\ \mu\text{M}$) preferentially binds to the few vacant uncoordinated hemes ($<1\%$) in predominantly oxygenated red blood cells in a cooperative manner without reacting with oxyhememes [146]. This $\bullet\text{NO}$ remains bound to the iron as the red cell is deoxygenated and is proposed to be transferred intramolecularly to the β -93-cysteine residue forming SNOHb when the Hb quaternary conformation changes from the T to R quaternary conformation on oxygenation in the lungs. This $\bullet\text{NO}$ transfer processes requires the removal of an electron from $\bullet\text{NO}$ to facilitate the formation of NO^+ and its subsequent reaction with β -93 cysteine. The acceptor for this electron and the preferential binding of $\bullet\text{NO}$ to vacant hemes in the presence of an excess of oxyhememes has not been convincingly explained. Although this mechanism is supported by some workers, many investigators contradict the reported mechanism for SNOHb formation as well as the allosteric release of $\bullet\text{NO}$ under hypoxic conditions [147–153].

S-nitrosohemoglobin in the presence or absence of GSH, fresh human red blood cells, and SNOHb incorporated red blood cells have been shown to have vasodilatory properties by bioassays using vascular rings or organ perfusion methods [49, 53, 154, 155]. Increased coronary blood flow was also observed in vivo when SNOHb enriched red blood cells, but not SNOHb depleted cells were infused into dogs [156]. One salient aspect is that $\bullet\text{NO}$ that complexes with the sulfur residue of proteins in red blood cells can transfer to the vasculature in the presence of a large excess of Hb. RSNO present in red blood cells can, therefore, potentially act as donors of $\bullet\text{NO}$ to the vasculature, although the mechanism for the transfer of $\bullet\text{NO}$ from SNOHb to the red cell membrane thiols and then to the vasculature is yet to be elucidated.

Methemoglobin

Oxyhemoglobin continuously undergoes autooxidation to form metHb. This metHb is reduced back to ferrous Hb by cytochrome *b5* reductase. During this transition, a steady-state level of $<0.5\%$ metHb still exists in red blood cells. $\bullet\text{NO}$ reacts with Fe(III)Hb , albeit much slower than with Fe(II)Hb , forming an Hb(III)NO complex which has Hb(II)-NO^+ character. This complex is finally converted to Hb(II)NO in a process called reductive nitrosylation, which frequently results in the formation of SNOHb (Eqs. 18–21) [59–63, 107, 157, 158].

MetHb is the only protein known to generate S-nitrosylated protein on reaction with $\bullet\text{NO}$ under anaerobic conditions. The mechanism for the formation of this SNOHb is not clearly known. The NO^+ on the heme can either transfer to β -93 cysteine through the globin to form SNOHb or react with water to form nitrite. X-ray crystallography of Hb shows that the distance between the heme-Fe and β -93-cysteine group is about 13 Å. Since the life span of NO^+ is $<3 \times 10^{-10}$ s, it is very unlikely that NO^+ can be transferred over this distance to react with cysteine. It is possible that the life span of NO^+ is longer in the hydrophobic domain of the heme pocket promoting the reaction with cysteine to form SNOHb. Other possibilities suggested by Luchsinger et al. involve the oxygenation of the Hb(II)NO formed by reductive nitrosylation that trigger the transfer of heme bound NO to the thiol (see above) [61]. Rifkind et al. have suggested that delocalization of an electron from the β -93 cysteine to NO^+ forms a transient thiyl radical which binds with another NO to form SNOHb [60, 151]. Herold and Rock suggest that either NO^+ reacts with H_2O to form H_2NO_2^+ or HNO_2 , which react with β -93 cysteine, or NO^+ transfers intramolecularly to the cysteine to form SNOHb [59, 107].

Ford et al. reported that nitrite, a common contaminant in solutions of $\bullet\text{NO}$, catalyzes the reductive nitrosylation of ferriheme models of metHb by $\bullet\text{NO}$ [157]. They propose that nitrite accelerates this reaction by reacting with NO^+ to form N_2O_3 , which nitrosylate the β -93 cysteine to form SNOHb (Eqs. 22, 23).

Kim-Shapiro and his colleagues have proposed a mechanism in which metHb forms a complex with nitrite [159], which has the characteristic of a ferrous-nitrogen dioxide ($\text{Hb(III)}-\bullet\text{NO}_2$) complex (Eq. 24) [63]. $\bullet\text{NO}$ rapidly reacts with this complex to form N_2O_3 (Eq. 25). The occurrence of this reaction has been further confirmed by Roche and Friedman using sol-gel encapsulated metHb to identify the reaction intermediates by slowing the reaction rates [160]. This N_2O_3 can diffuse out of red blood cells to react with plasma proteins forming RSNO (Eq. 26). The physiological relevance of this mechanism is uncertain since $\bullet\text{NO}$ has to compete with the mM levels of Hb to react with nM levels of $\text{Hb(III)}-\bullet\text{NO}_2$ in red blood cells.

Although the role of metHb in intact RBCs is questionable, small amount of hemolysis takes place continuously in circulation releasing Hb into the plasma compartment. This Hb is rapidly oxidized to metHb, which can complex with nitrite or $\bullet\text{NO}$ to generate SNOHb that in turn can transnitrosate to other plasma thiols.

We recently observed the formation of SNOHb when iron nitrosylHb (Hb(II)NO) without any nitrite contamination is oxidized by excess potassium ferricyanide under anaerobic and aerobic conditions [161]. We interpret these results in terms of the oxidation of Hb(II)NO to Hb(III)NO by potassium ferricyanide and resultant formation of NO^+ in the hydrophobic heme pocket (Eq. 18), which then reacts with β -93 cysteine to form SNOHb (Eq. 20).

Hemin

Hemin and heme are prosthetic groups in a large number of cellular hemeproteins that carry out diverse functions. They both contain four pyrrole rings and an iron atom. Hemin is the oxidized form, whereas heme is the reduced form of iron protoporphyrin. Hemin is found on red cell membranes at a concentration of 0.23 pmol/mg membrane protein. Hemin is also found in plasma (<10 μM) mainly bound to albumin and hemopexin. Most of this hemin comes from the dissociation of the hemin moiety of metHb generated due to autooxidation of oxyHb in red blood cells or lysed red blood cells. In 1987, Shviro and Shaklai reported that under anaerobic conditions GSH scavenges free hemin by complexing with the iron under anaerobic conditions [162]. Ferric iron in hemin has a high electron affinity compared to GSH. Hence, the charge can transfer from GSH to iron forming a charge transfer complex (a weak electron resonance complex) (Eq. 27). We observed that this GS–hemin complex readily reacts with NO resulting in the robust formation of GSNO and reduced heme under both anaerobic and hypoxic (2% O_2) conditions (Eq. 28) [163].

Reactions of $\bullet\text{NO}$ with the albumin–hemin complex also generate low levels of albsNO and heme. The reaction of $\bullet\text{NO}$ with GS–hemin complex in the presence RBCs also resulted in the formation of low levels of GSNO (5%) suggesting that a small part of $\bullet\text{NO}$ reacts with GS–hemin before being scavenged by red blood cells (unpublished results). The reaction of $\bullet\text{NO}$ with hemin–protein complexes may contribute to the formation of RSNO in blood.

Conclusions

Our analysis of the routes for the formation of RSNO implies that in vivo there are most probably multiple pathways involved in the formation of the physiological important RSNO. The possible pathways for the formation of RSNO in the vascular lumen are summarized in Fig. 1. Under conditions of inflammation the simultaneous generation of appreciable concentrations of both NO and superoxide and the rapid formation of PN will likely increase the level of RSNO. The radical–radical reaction between thiyl radicals and NO is very rapid. However, the formation of RSNO by this mechanism is expected to be limited to conditions where one electron oxidation of thiols produce thiyl radicals at the same time that $\cdot\text{NO}$ is released.

The dominant pathways for the formation of RSNO, however, involve metal ion interactions which can oxidize $\cdot\text{NO}$ to nitrosating agent that can be either the NO^+ cation or (particularly in the presence of nitrite) N_2O_3 . The extremely short life span for the NO^+ cation and the relatively short life span for N_2O_3 require that the thiol to be nitrosylated is in close proximity. These metal ion reactions can involve either proteins or low molecular weight compounds. While there are many metalloproteins that can bind $\cdot\text{NO}$ and oxidize $\cdot\text{NO}$, the formation of an S-nitrosylated protein is limited to situations with a thiol in the active site.

The role of Hb has been extensively studied because of the extremely high concentration of Hb that is known to react with $\cdot\text{NO}$. Although a thiol is not located in the heme pocket there is an active thiol group in the β -chain $\sim 13 \text{ \AA}$ away from the heme iron. S-nitrosylation resulting from the reactions of $\cdot\text{NO}$ with ferrous Hb, which are not accepted by many investigators, have been postulated where S-nitrosylation can take place by coupling an oxidative reaction to the S-nitrosation reaction. However, it has been established that the reaction of $\cdot\text{NO}$ with the Fe(III)Hb does result in S-nitrosation of the β -93 cysteine.

The two low molecular weight thiol reactions involve DNIC and hemin compounds found in blood and tissues. In both cases, the short life span of one electron oxidation species of NO^+ is bypassed (Scheme 1, Eq. 28). For DNIC, this is accomplished by the formation of a stable complex with $\cdot\text{NO}$ that releases the NO^+ ion in the presence of a thiol. For hemin, this is accomplished by a charge transfer stable complex with thiols that results in the formation of RSNO when these complexes interact with $\cdot\text{NO}$.

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Abbreviations

Hb	Hemoglobin
RSNO	S-nitrosothols
$\cdot\text{NO}$	Nitric oxide
CP	Ceruloplasmin

PN	Peroxynitrite
DNIC	Dinitrosyl iron complexes
NO⁺	Nitrosonium cation
LPS	Lipopolysaccharide
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
Cyst–NO	Cysteine–NO

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Fig. 1.

The possible pathways for generation of S-nitrosothiols in the vascular lumen based on in vitro experiments: endothelium is a major source of $\bullet\text{NO}$ in the blood and most of the $\bullet\text{NO}$ is consumed by red blood cells. A small amount of $\bullet\text{NO}$ may react with indicated molecules to form either directly RSNO or intermediary products. These intermediates in turn react with thiol groups to form RSNO

Scheme 1.