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CHAPTER 9

**Low-molecular-weight S-nitrosothiols**

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S-nitrosothiols (thionitrites, RSNO) appear as endogenous reaction products of NO or NO metabolites with sulfhydryl groups. Prime targets are the sulfhydryl groups on cysteine residues found in many different proteins [1,2] or the thiol moieties on endogenous glutathione (GSH) or cysteine (Cys). The nitrosation of the sulfhydryl moiety leads to S-nitrosothiols with high and low molecular weight, respectively. In organisms, the combined pool of S-nitrosothiols varies over the compartments. Table 1 gives an overview of various NO stores in rats, and S-nitroso moieties in organ tissue are 10–100 nM. Much higher levels up to 250 nM are found in erythrocytes, and plasma levels remain below 10 nM. The overwhelming majority (ca 90%) of all S-nitroso groups are anchored to proteins and belong to the class of high-molecular mass [3,4]. The interest in the S-nitrosothiols was motivated by the observation that these compounds form in vivo and may elicit physiological responses that are strongly reminiscent of free NO radicals. Well-documented examples are vasodilatation and the inhibition of platelet aggregation. The formation and functions of S-nitrosated proteins are discussed in Chapter 10, whereas this chapter will be primarily concerned with the S-nitrosothiols of low molecular weight. A number of examples is shown in Fig. 1. Many different forms have been synthesized in vitro and tested for use as NO donor [for example S-nitroso-N-acetyl-pencillamine (SNAP)], but in mammals the most relevant species are S-nitrosocysteine (CysNO) and especially S-nitrosoglutathione (GSNO). The latter is found in all tissues, with basal concentrations in the range of 1–10 nM, but higher concentrations arise in aortic tissue and erythrocytes (cf Table 1). Given that endogenous GSH concentrations are in the mM range, it appears that the degree of S-nitrosation of GSH is only a percent or less. Therefore, S-nitrosation cannot have an impact on the redox status of tissues by shifting the GSH/GSSG ratio. But the basal GSNO levels appear comparable to those of free NO, so that the pool of S-nitrosothiols may be significant for the NO status of the tissue.

The compounds mentioned above are all cysteine based and relevant for mammalian physiology. However, they are definitely not exhaustive for endogenous thiols found in organisms. Other types of cysteine-based thiols like trypanothione (TSH) or mycothiol have been found in certain bacterial strains. A completely independent class of intracellular thiols is based on histidine residues, with very different chemistry. Well-studied examples are ergothioneine...
Table 1. Magnitude of various pools of NO-metabolites in Wistar rats (adapted from [9]). The [GSH]/[GSSG] ratio is a marker for the redox state of the tissue. The rightmost column gives NO-metabolite values from human plasma for Ref. [10].

| NO-metabolite         | Aorta  | Brain  | Heart  | Plasma | Erythrocytes | Human plasma |
|-----------------------|--------|--------|--------|--------|--------------|--------------|
| Nitrite (µM)          | 23 ± 9 | 1.7 ± 0.3 | 0.80 ± 0.08 | 0.29 ± 0.05 | 0.68 ± 0.06 | 0.20 ± 0.02 |
| Nitrate (µM)          | 49 ± 7 | 6.1 ± 1.1 | 5.9 ± 1.7 | 5.7 ± 0.6 | 10.2 ± 1.2 | 14.4 ± 1.7 |
| S-nitroso moieties (nM) | 96 ± 24 | 22 ± 6 | 13 ± 2 | 1.4 ± 0.5 | 246 ± 32 | 7.2 ± 1.1 |
| N-nitroso moieties (nM) | 19 ± 11 | 61 ± 8 | 14 ± 2 | 3.5 ± 0.4 | 95 ± 14 | 32.3 ± 5.0 |
| Nitrosyl-heme (nM)    | Below detection* | 160 ± 30 | 15 ± 1 | Below detection* | 10.8 ± 1.8 | n.d. |
| GSH (mM)              | 0.34 ± 0.08 | 1.28 ± 0.06 | 0.93 ± 0.12 | 0.02† | 0.8 mM‡ (whole blood) | n.d. |
| [GSH]/[GSSG]           | 6.8    | 24     | 14     | 5°     | > 10° (whole blood) | n.d. |

* Detection limit 1 nM.
† [11].
‡ [10].
n.d. = not done.

Fig. 1. Molecular structures of CysNO, GSNO and SNAP.
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ESH and ovothiols [5]. ESH is a low-molecular-weight thiol with established reactivity towards GSNO [6], capacity to scavenge singlet oxygen and hydroxyl radicals, and is found in millimolar concentrations in specific tissues like liver, kidney and erythrocytes [7]. ESH was not reported to form S-nitrosothiols, but it may form disulfides with cysteinic thiols. Although the reactivity towards GSNO is intriguing, the biological function of such histidine-based thiols is still unclear. Therefore, such “unconventional” thiols will not be considered further in this chapter.

Finally, it should be mentioned that S-nitrosation is only one of many pathways via which nitroso moieties can be incorporated into proteins. Organic molecules and peptides may undergo N-, O-, C- as well as S-nitrosation, depending on the structural motif to which the NO moiety is attached. Mixed nitrosations are of course possible also. An interesting example is albumin which may be N-nitrosated on one of its two tryptophan residues as well as S-nitrosated on the Cys34 residue [8]. Significantly, the N-nitrosated tryptophan residue also elicited vasodilatory response from precontracted aortic rings of rabbits [8]. Since the nitrosation was carried out under extreme non-physiological conditions (acidification of nitrite), it remains unclear whether this N-nitrosation of tryptophans has significance for in vivo conditions.

In contrast, the physiological significance of S-nitrosation has been proven beyond doubt. Many examples will be given at the end of this chapter. Under biological conditions, S-nitrosation is facile, fast and affects a wide range of proteins in vitro and in vivo. Judging from the citation numbers in scientific literature, the nitrosation of sulfhydryl groups has highest relevance for physiology so far. Therefore, this chapter will be primarily concerned with S-nitrosocompounds.

SPECTROSCOPIC PROPERTIES

In general, RSNO have red, pink or green color due to strong absorption of visible light. As an example, Fig. 2 shows the UV–VIS spectrum of pink GSNO with two prominent absorption bands from the S–NO bond. The UV peak at 336 nM ($\varepsilon_{336} = 778 \text{ (Mcm)}^{-1}$) is attributed to a $\pi \rightarrow \pi^*$ transition, and the secondary band at 545 nM ($\varepsilon_{545} = 34 \text{ (Mcm)}^{-1}$) to a $nN \rightarrow \pi^*$ transition [12,13]. The intensity of these absorption bands is quite sensitive to the type of thiol. In albumin, S-nitrosation of the Cys34 residue leads to a slightly higher extinction of $\varepsilon_{335} = 870 \text{ (Mcm)}^{-1}$ [14]. Multiple S-nitrosation of different residues leads to correspondingly higher extinctions. Multiple nitrosated albumin (poly-SNO-albumin) can reach a molar extinction of $\varepsilon_{335} = 3870 \text{ (Mcm)}^{-1}$[15]. In the infrared region, the RSNO moiety has additional characteristic absorptions from the N–O stretch vibration (1480–1530 cm$^{-1}$) and the C–S stretch vibration (600–730 cm$^{-1}$).

SYNTHESIS AND DETECTION IN VITRO AND IN VIVO

The easiest synthetic pathway for S-nitrosothiols is via the equilibrium with nitrous acid, obtained by acidification of a solution of nitrite [16,17].

$$\text{RSH} + \text{HNO}_2 \rightleftharpoons \text{RSNO} + \text{H}_2\text{O}$$ (1)
A typical stock of CysNO is obtained by adding equimolar amounts (ca 50 mM) of CySH and sodium nitrite to 0.1 M HCl. The reaction is rather fast and requires less than a minute to complete. The kinetics of CysNO formation at various pH can be seen in Fig. 3. The reaction products always appear as a mixture of CysNO and disulfides, with the relative balance being strongly dependent on pH (cf Table 2). At physiological pH the disulfide is dominant, but at pH ~ 1 the CysNO yield is nearly 100%. Near pH = 1, the conversion of Cys to CysNO is completed within a minute at 37°C, at room temperature within a few minutes.

As S-nitrosothiols are prone to photolysis and thermolysis, the reaction should proceed in a dark and cool location. The formation of CysNO and GSNO is indicated by its intense
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Table 2 Yields of CysNO and cystine obtained in a reaction mixture of 0.04 M CysH with 0.04 M NaN O2 in aqueous buffers at 37°C. The yields show that all cysteine is incorporated in either S-nitrosothiol or the cystine disulfide. Data adapted from Ref. [18]

| pH | CysNO% | Cystine% |
|----|--------|----------|
| 0.5 | 100 | 0 |
| 1.5 | 92.1 | 7.9 |
| 2.0 | 90.2 | 9.8 |
| 2.5 | 85.8 | 14.2 |
| 3.5 | 75.1 | 24.9 |
| 4.65 | 35.1 | 64.9 |

The pink color. The formation of small gas bubbles with time indicates the escape of a certain quantity of NO gas, and the final yield should be verified spectrophotometrically just prior to use. Stock solutions are best freshly prepared and should not be stored in liquid form for more than a few hours. Storage up to a week is possible in frozen state, but longer term storage is possible only for refrigerated crystalline GSNO or CysNO (pink powders).

It should be noted that the equimolar mixture of thiol and nitrite always leaves some residual deprotonated thiol in the solution. This holds for synthesis of any kind of RSNO. The presence of a certain quantity of RS− in assays with RSNO is highly significant because of its capacity to reduce spurious copper ions in the solution

\[ \text{Cu}^{2+} + \text{RS}^- \rightarrow \text{Cu}^+ + \text{RS}^* \]  

This reaction leads to thyl radicals and a small quantity of monovalent copper Cu+. The latter is an effective catalyst for RSNO decomposition (vide infra) and the pathway of spurious copper often introduces artefacts. The problem can be avoided by inhibiting the redoxactivity of the copper ions by chelators like EDTA or neocuproine.

Although the reaction Eq. (1) has been known for a long time, its molecular mechanism is still not fully understood. Recent reports [18] showed that the major nitrosating species at low pH < 3.5 is nitrosonium NO+ and the reaction is fast. At higher pH the release of nitrosonium is blocked, and N2O3 acts as the main nitrosating species in the reaction

\[ \text{GSH} + \text{N}_2\text{O}_3 \rightarrow \text{GSNO} + \text{NO}_2^- + \text{H}^+ \]

This nitrosation by N2O3 is very fast with a second order rate of \( k = 6.6 \times 10^7 \text{ (Ms)}^{-1} \) [19]. The rate of formation of N2O3 is much lower (cf Chapter 1) and acts as the rate limiting step.

Interestingly, the NO radical itself does not show significant reactivity towards the sulphhydryl group in anaerobic conditions [20–22]. Phrased otherwise, NO is not a S-nitrosating compound by itself in the absence of oxygen. Instead, the reaction of NO with thiols leads to formation of disulfides RS–SR [23–25]

\[ \text{RSH} + \text{NO} \rightarrow \text{RS}^- \text{N}^- \text{OH} \]

\[ 2\text{RS}^- \text{N}^- \text{OH} \rightarrow \text{RS}^- \text{SR} + \text{HONNOH} \rightarrow \text{RS}^- \text{SR} + \text{N}_2\text{O} + \text{H}_2\text{O} \]

\[ \text{RS}^- \text{N}^- \text{OH} + \text{NO} \rightarrow \text{RSOH} + \text{N}_2\text{O} \]

\[ \text{RSOH} + \text{RSH} \rightarrow \text{RS}^- \text{SR} + \text{H}_2\text{O} \]  

It should be noted that this sequence of reactions is pH dependent since the thiolate anion shows far higher reactivity than the protonated thiol. For [NO]<([GSH], the sequence (3)
was reported [25] to have an apparent second order reaction rate of 0.080 ± 0.008 (Ms)$^{-1}$ (37°C, pH 7.4). Table 1 shows that physiological [GSH] $\sim$ 1 mM. At this concentration, reaction (3) would give NO radicals a lifetime of ca $10^4$ s. Therefore, it is unlikely that the anoxic reaction (3) cause significant loss of NO in vivo.

In this context it is important to note that acid dissociation constant pK$_a$ of thiols shows a large variation, depending on the functionalization of the cysteine residues. The thiolate moiety of free cysteine has pK$_a$ = 8.3. In glutathione, the constant for the cysteine residue has been raised to pK$_a$ = 8.7. The constant is drastically lowered for the cysteine residues of many proteins, like protein tyrosine phosphatase (PTP1) with pK$_a$ $\sim$ 5.4 or protein disulfide isomerase with pK$_a$ $\sim$ 3.5. This large variation in dissociation constant implies a large variation in the balance between deprotonated/protonated forms of the sulphydryl group, with concomitant influence on reaction rates and yields of S-nitrosation.

So how are S-nitrosothiols formed in vivo? A modest release of RSNO from catalytic action of ceruloplasmin and NOS itself has been reported [26], but potent enzymatic pathways for S-nitrosation seem not to have been identified so far [2]. It suggests that non-enzymatic pathways dominate in vivo. The transformation of RSH to RSNO requires the appearance of S-nitrosating intermediates like nitrosonium NO$^+$, NO$_2$ or N$_2$O$_3$. The reaction pathways for the formation of these species from free NO were discussed in Chapter 1. Although the reactions are well studied in vitro, there remains considerable uncertainty as to the dominant mechanism for S-nitrosation of sulphydryls in vivo [22,27]. Via what pathway do S-nitrosocompounds appear in live tissue? This question cannot be addressed without noting that S-nitrosothiols forms only one class of nitrosocompounds that is closely linked to other pools via slow chemical equilibria.

In-vitro experiments indicate that aerobic mixtures of NO/GSH or GSNO/GSH release a certain quantity of highly reactive oxygen species (ROS). The release of free ROS was manifested from significant strand breaking in DNA and protection against strand breaking by scavengers like catalase and superoxide dismutase (SOD) [28]. The reaction mechanisms still remain controversial. A mechanism for S-nitrosation involving the reduction of O$_2$ to superoxide O$_2^-$ was proposed and confirmed in vitro [29]:

$$RSH + NO \rightarrow RS-N-OH$$

$$RS-N-OH + O_2 \rightarrow RS-NO + O_2^- + H^+ \quad (4)$$

$$O_2^- + NO \rightarrow ONOO^-$$

This mechanism does not involve trace metal ions. However, such trace metal ions are known to accelerate both formation and decomposition of S-nitrosothiols in vitro [30] and in vivo, possibly by catalyzing the formation of nitrosating species like NO$^+$, NO$_2$ or N$_2$O$_3$ from NO in the presence of oxygen. This makes it unlikely that a mechanism like Eq. (4) be the dominant pathway for formations of S-nitrosothiols in vivo. Certain enzymes like ceruloplasmin and NOS itself were seen to catalyze the formation of S-nitrosothiols [26] but yields remain low. Alternatively, dinitrosyl iron complexes (DNICs) are known to participate in a complex reaction equilibrium between thiols, NO and S-nitrosothiols. This equilibrium is discussed in detail in Chapter 11 of this book. In-vitro experiments have confirmed that the presence of iron induce formation of RSNO from NO via intermediate DNIC even in anoxic conditions [31]. Intriguingly, the formation of small quantities of DNIC from endogenous
iron has been detected in many biological systems after exposure to exogenous NO or after 
stimulus of endogenous NO production. Many examples were reported from cell cultures 
to plant and animal tissues. However, decisive proof of the physiological relevance of this 
DNIC pathway is still lacking.

From the above it is clear that thiols participate in many reaction pathways with endoge-
nous reactants like oxygen or the various nitrogen oxides. The relative importance of these 
pathways is determined by the corresponding reaction rates. A useful selection of reaction 
rates for glutathione has been collected in Ref. [32].

Detection and quantification of \( S \)-nitrosothiols poses a considerable challenge in biological 
systems. Special techniques for \( S \)-nitrosated proteins have been recently reviewed in Volume 
396 of *Methods in Enzymology*. A widely used technique is homolytic cleavage of the \( S \–NO 
bond by copper/iodide, and detection of the gaseous NO via chemiluminescence with an ozone 
analyzer. Alternatively, the NO may be detected electrochemically with an NO electrode, 
or by spin-trapping with electron paramagnetic resonance (cf Chapter 18). Selectivity for 
high or low molecular mass may be obtained via filtering with ultrafiltration membranes [4] 
or high performance liquid chromatography (HPLC). Photolysis-chemiluminescence spec-
troscopy [33] is a sensitive method to analyze mixtures of \( S \)-nitrosothiols. It consists of an 
instrumental cascade comprising a HPLC pump, photolysis chamber and a chemilumines-
cence spectrometer or ozone analyzer. The method works by homolytic cleavage of the \( S \–NO 
bond by irradiation in the photolysis chamber, followed by detection and quantification of 
the escaping gaseous NO with the ozone analyzer.

Alternative methods are based on fluorescence detection of decomposition products of 
the \( S \)-nitrosothiol. In the Saville reaction [34], mercuric chloride is used to catalyze 
the release of free NO and subsequent trapping with 2,7-dichlorofluorescein (DCF) or 
2,3-diaminonaphthalene (DAN). The fluorescence detection is highly sensitive, but the 
specificity of the detection is compromised by artefacts and impostors for true NO [35].

To conclude this section, it should be remarked that the reaction of \( S \)-nitrosation in a 
complex biological system might proceed significantly different from that in a homogenous 
aqueous phase like a buffered solution. The coexistence of hydrophobic and hydrophilic 
compartments in cells, tissues and even individual proteins may modify the reactions as 
known in water. The main reason is the tendency of neutral species like NO, \( O_2 \) and \( N_2O_3 \) to 
accumulate in hydrophobic compartments like the membrane fraction [26,36] or hydropho-
bic pockets of proteins. This effect could promote the \( S \)-nitrosation of the \( \beta_{33} \) cysteine of 
hemoglobin which is located in a hydrophobic region of the protein [32]. In addition, it has 
been recognized that the presence of nearby amino acids affects the rate of \( S \)-nitrosylation 
by modifying the polarity and effective acidity in proximity of the cysteine residue [2]. It has 
been shown that the presence of a lipid compartment accelerates the oxidation of NO by \( O_2 \) 
by orders of magnitude [36].

**STABILITY OF GSNO IN VITRO**

Stability of RSNO is compromised by thermal decomposition, photolysis and the catalytic 
decomposition by trace metal ions like iron and copper. Although the various RSNO share the 
common C–S–N≡O motif, they show a big variation in intrinsic stability when in solution.
The reason for this variation is not well understood. Given this instability, aqueous solutions of RSNO tend to release a certain quantity of gaseous NO and accumulate typical decomposition products like thiols, disulfides and reaction products of thyl radicals. It was recently discovered that, in presence of oxygen, a certain quantity of disulfide \( S \)-oxides (GS(O)SG) and disulfide \( S \)-dioxides (GS(O\(_2\))SG) \([37,38]\) is formed as well. These metabolites of GSH are effective agents for \( S \)-glutathiolation of sulfhydryl groups. They can inactivate glycer-aldehyde 3-phosphate and alcohol dehydrogenases, and release zinc from metallothionin and zinc finger proteins. Such mixtures of decomposition products result from a combination of pathways operating simultaneously. In practical applications usually more than just a single pathway contributes significantly. The basic decomposition reactions will be discussed below.

In solution, RSNO undergo slow thermal decomposition into a disulfide and free NO according to

\[
2\text{RSNO} \rightleftharpoons \text{RS}^- \text{SR}^- + 2\text{NO} \quad (5)
\]

The rate of thermal decomposition depends on the concentration and type of thiol \([39,13]\). At starting concentrations of 50 mM, the thermal decomposition of GSNO and CysNO exceeds 10% after ca 6 and 1 h, respectively. At low concentrations in absence of UV or blue light, the stability of RSNO is quite high. In presence of the metal chelator DTPA, even CysNO achieves a half life of 11 h \([40]\). Therefore, Eq. (5) is not significant unless catalyzed by trace metal ions (see below).

Illumination with UV or blue light causes homolytic cleavage of the \( S \)–\( N \) bond. The probability for photolysis is highest at wavelengths near the absorption maximum of RSNO at 336 nM. The quantum yield for photolysis is quite high and exceeds that of photodissociation of the nitrosyl ligand from heme. Fig. 4. shows the photolysis of GSNO by successive sets

![Fig. 4. Photolysis of GSNO by pulsed UV irradiation at 355 nM at room temperature in oxygenated PBS buffer. The successive curves differ by 10 pulses of 50 mJ/pulse. The inset shows the simultaneous photolytic release of free NO radicals as measured with an NO electrode. (From Ref. [41].)](image_url)
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of 10 laserpulses (355 nM, 50 mJ/pulse). The photolytic decomposition of S–NO bonds has raised interest in the application of S-nitrosated complexes for photodynamic therapy of cancers [41]. It should be mentioned that S-nitrosothiols are not the only endogenous species showing such photolytic release of NO: Alongside endogenous S-nitrosothiols, also nitrite was shown [42] to contribute significantly to the light-induced release of NO from vascular tissues of rats.

Reduced trace metal ions like Fe$^{2+}$ and Cu$^{+}$ are efficient catalysts for the decomposition of RSNO [43,44]. Fig. 5 shows the effect of copper and iron on the kinetics of CysNO. The decomposition of GSNO is slower, but qualitatively similar.

The reaction mechanism [46] is given by the following sequence

\[
\begin{align*}
RSNO + Cu^{+} &\rightarrow RS^{-} + NO + Cu^{2+} \\
Cu^{2+} + RS^{-} &\rightarrow Cu^{+} + RS^{*} \\
2RS^{*} &\rightarrow RS – SR
\end{align*}
\]

The reaction releases disulfides and free NO. Fe$^{2+}$ also can catalyze a similar sequence.

The above reactions might leave the erroneous impression that iron and copper act on S-nitrosothiols in similar ways. In reality the iron-catalyzed decomposition of RSNO is complicated by a competing reaction mechanism involving iron complexes carrying two nitrosyl ligands. In principle, copper also may form (di)nitrosyl species like EPR silent
$\text{Cu}^+ (\text{NO}_2)$ or paramagnetic $\text{Cu}^+(\text{NO})$ or $\text{Cu}^{2+}(\text{NO})_2$. However, these species have so far only been reported as adsorbants in dry anoxic porous solids [47] but not in aqueous solutions. Therefore, in solutions, iron alone can interact with $\text{S}$-nitrosothiols to form such DNICs. In the presence of iron, a pool of DNIC will form at the expense of $\text{S}$-nitrosothiols [43,45]. These reactions were well studied in vitro and are described in Chapter 11.

Transnitrosation to other types of thiols may have significant effect on the lifetime of $\text{S}$-nitrosothiols. The basic transnitrosation reaction involves the transfer of a nitrosonium NO$^+$ moiety

$$R_1 - \text{S} - \text{NO} + R_2 - \text{S} \leftrightarrow R_1 - \text{S}^+ + NO - R_2$$

(7)

Here $R_1 - \text{S}^-$, $R_2 - \text{S}^-$ can be various deprotonated thiols, from small cysteine anions to sulphydryl groups on macromolecular proteins. The rates for this reversible transfer vary considerably with the type of thiol, pH and temperature. Transnitrosation rates for cysteine and glutathione were reported [14] at around 80 (Ms)$^{-1}$ (37°C, pH 7.4). Lower rates of 3–9 (Ms)$^{-1}$ apply for transnitrosation from $\text{S}$-nitrosoalbumin to cysteine and glutathione. The transfer rates increase significantly when pH is raised and a larger fraction of thiols is deprotonated. It was shown that 50 $\mu$M CysNO and $\text{S}$-nitrosoalbumin establish their equilibrium by transnitrosation on a timescale of several minutes in vitro (cf Fig. 6) [48]. Transnitrosation from GSNO is slower due to a combination of smaller transfer rate and orders of magnitude lower concentration of GSNO (Table 1). Therefore, the characteristic timescale for equilibration with GSNO should be many minutes. In human plasma, albumin was confirmed to be the dominant target for transnitrosation from CysNO and GSNO [49], with metal chelators having only insignificant effect on the extent and rate of $\text{S}$-nitrosation of the albumin target. In whole blood, the reaction balance is shifted considerably because Hb may be $\text{S}$-nitrosated or nitrosylated at the heme. In addition, oxyHb is an efficient scavenger.
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of free NO (but not for S-nitrosothiols, see below). In whole blood, S-transnitrosation from GSNO to S-nitrosohemoglobin was reported [49] to remain below a few percent.

The previous in-vitro results should not leave the impression that S-transnitrosation be an exclusively passive process with timescales largely determined by the chemical characteristics of the exchanging thiols. In biological systems, the crossing of cellular membranes will act as a significant barrier for S-nitrosation and affect the rate of exchange between the intra- and extracellular compartments. This effect has been shown in vivo: Protein disulfide isomerase (PDI) is a protein in cellular membranes that normally catalyzes thiol-disulfide exchange. The activity of the PDI protein was found to enhance the intracellular pool of S-nitrosothiols in cultured human erythroleukemia cells [50]. The mechanism of membrane crossing of S-nitrosothiols will be discussed later in this chapter.

The process of transnitrosation from low to high molecular weight has been demonstrated in vivo in rabbits [51]. A collection of transnitrosation rates between various thiols has been collected in Ref. [14]. Analogous results were obtained with transnitrosation from S-nitrosoglutathionyl-sepharose beads [52] to free thiols in solution. The transnitrosation to cysteine and glutathione was rapid and accelerated by an order of magnitude when pH was raised from 5 to 9. In contrast, transnitrosation from the beads towards bovine serum albumin was negligible.

It should be noted that reaction (7) proceeds in the presence of a competing pathway which leads to the formation of disulfide bonds and the release of some free NO. It has recently been shown [53] that the sulfhydryl groups of certain proteins can also be modified by nucleophilic attack of the protein thiolate on the sulfur of GSNO rather than on the NO\(^+\) moiety. This reaction pathway amounts to S-glutathiolation of the sulfhydryl moiety. Several proteins underwent a combination of S-nitrosation and S-glutathiolation when exposed to GSNO. In contrast, bovine serum albumin, actin and alcohol dehydrogenase were only S-nitrosated by fresh GSNO. S-glutathiolation of intracellular proteins has been demonstrated in vivo: upon incubation with exogenous CysNO, NIH-3T3 fibroblasts underwent combined S-nitrosation and S-glutathiolation of the cysteine residues of H-ras protein [54]. High capacity of S-glutathiolation was attributed [37] to glutathione disulfide S-oxide (GS(O)SG) which appears as one of the decomposition products of GSNO itself.

The lifetime of hours for GSNO may be shortened to minutes by addition of other thiols, in particular SNAP and cysteine [55–57]. The effect may be inhibited by the thiol-blocking compound N-ethylmaleimide [58]. Two different mechanisms have been identified for this phenomenon: transnitrosation and formation of disulfides. In the first case, the GSNO is depleted by the transnitrosation to the shorter lived CysNO. In the same spirit, transnitrosation to more stable species may stabilize the pool of S-nitrosothiols up to a certain degree. The second mechanism is mediated by the reductive nature of the thiol anions and involves the formation of a disulfide bridge under release of a nitroxyl anion [24,59]:

\[
RS\text{-NO} + GS^- \rightarrow RS^- + SG + NO^-
\]

(8)

If RS represents glutathione, the reaction releases GS–SG disulfide at a rather slow rate with a second order rate constant of \(k = 8.3 \times 10^{-3} (\text{Ms})^{-1}\) [24]. Given that tissues contain GSH below mM concentrations (Table 1), this decay channel of GSNO appears insignificant in vivo. If on the other hand RS represents a sulfhydryl group on a protein, the reaction (8) amounts to S-glutathiolation of the protein. It has been confirmed that the coincubation with a
mixture of GSNO and glutathione causes S-glutathiolation of the sulphydryl groups on a wide range of different proteins. [53,60]. More detailed studies [37] have shown that intermediate oxides like GS(O)SG or GS(O2)SG are better S-glutathiolating agents than GSNO itself (see below). Therefore, the process of S-glutathiolation may in fact be more complex than suggested by Eq. (8). S-glutathiolation maybe reversed by dithiothreitol [60].

Release of NO radicals from RSNO can be caused by reductants, exogenous as well as endogenous. Ascorbate was found to have a pronounced effect on the lifetime of RSNO [39]. Without chelation of spurious copper, small quantities of ascorbate reduced Cu2+ to Cu+ and initialized the reaction Eq. (6) with formation of disulfides and the release of free NO. After chelation of spurious copper, the true reduction of RSNO became apparent with the release of free NO and thiols instead of disulfides. The effective rate constant accounts for the reduction pathway plus a thermal decomposition rate $k_T$

$$k_e = k[A] + k_T$$

At pH = 7.4, the value of $k$ was 0.25, 0.015 and 0.032 (Ms)$^{-1}$ for CysNO, GSNO and SNAP, respectively. Careful observations showed that the ascorbate monoanion HA$^-$ and the dianion $A^{2-}$ have different rates of reduction. Therefore, the rate $k$ was highly dependent on pH and increased over four orders of magnitude when the pH was changed from 3.6 to 11 [39].

It might seem plausible to expect that the stability of nitrosothiols be inversely related to its potency as vascular effector. More concretely, one might expect that the more rapidly decaying nitrosothiols elicit stronger physiological responses for relative short times. When tested for the responses of vasorelaxation and platelet aggregation, no such correlation was found [61]. This result confirms that S-nitrosothiols are potent physiological effectors in their own respect. In particular, they can act without having to release free NO.

**STABILITY OF RSNO IN VIVO**

In biological systems, additional pathways exist for removal of RSNO. The consumption of S-nitrosothiolates (S-denitrosation) by endogenous enzymes has not been often reported in the literature. Mammalian physiology does not seem to require a dedicated system to remove excess quantities of RSNO. A modest capacity for S-denitrosation has been recently reported for the protein disulfide isomerase enzyme [62] and for anaerobic xanthine oxidase [63]. Cu–Zn superoxide dismutase was capable to denitrosate low concentrations of GSNO in vitro, but the significance of this finding is questionable since the reaction was inhibited by in-vivo levels of GSH (Table 1). Thioredoxin reductase (TR) [64] and glutathione peroxidase [65] will denitrosate GSNO to GSH under release of free NO. Finally, the γ-glutamyl transferase enzyme was implicated in the stereoselective effect of l-CysNO on posthypoxic ventilation of mice [66]. The preceding examples do not consume large quantities of LMW nitrosothiols and are not expected to have significance for GSNO levels in vivo.

In contrast, S-nitrosoglutathione reductase (GSNOR) was found to have an impact on GSNO metabolism *in vivo*. In the literature, this enzyme is also referred to as alcohol dehydrogenase-3 (ADH3) or as GSH-dependent formaldehyde dehydrogenase. It can consume large quantities of GSNO [4,67–70]. GSNOR activity is found in many different
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tissues and found highest in liver [71]. The physiological significance of the GSNOR path-
way was impressively demonstrated by experiments with GSNOR knockout mutant mice [4],
which showed greatly enhanced levels of RSNO and became hypotensive under anesthesia.
Human asthmatics show the combination of enhanced GSNOR levels and depressed GSNO
in the bronchial fluids. It suggests that chemically incorporated NO stores are depleted
in asthmatic patients. Since the remainder of NO is exhaled, the unbalance between NO
and S-nitroso compounds may be the reason for the observed increase of exhaled NO in
asthma [70,72]. Recent studies on the decomposition of low-molecular-weight S-nitrosothiols
in tissue homogenates of rats have provided more evidence that enzymatic pathways con-
tribute significantly to the process [73]. The above publications leave no doubt that significant
enzymatic pathways for S-denitrosation of LMW nitrosothiols operate in mammals. Their
role in the regulation of endogenous pool of S-nitrosothiols remains the subject of intense
research for the moment being.

In addition to this enzymatic pathway, GSNO may be depleted by superoxide according
to the third order reaction [74]

\[
\frac{d[GSNO]}{dt} = -k[GSNO]^2[O_2^-]
\] (10)

The third order reaction rate \textit{in vitro} was reported as \( k = 6 \times 10^8 \text{ M}^{-2} \text{s}^{-1} \). With GSNO
concentrations in tissue below ca 50 nM (cf Table 1) and superoxide levels below micromolar
range, the loss of GSNO via this pathway is insignificant in comparison to loss by catalytic
action of spurious metal ions. This observation supports the hypothesis that the transient
formation of RSNO may help to increase the effective lifetime of NO in biological systems
by protection against the reaction with superoxide.

The hydroxyl radical \( \text{OH}^+ \) is a very reactive oxygen species that can be generated in
solutions when hydrogen peroxide undergoes a Fenton reaction with spurious ferrous iron.
Using pulse radiolysis for generation of the hydroxyl radicals at pH = 7, the main reaction
products were found to be disulfides at nitrite. The reaction was second order with diffusion
limited reaction rates as usually found for hydroxyl. The reaction rates for CysNO, GSNO
and ACysNO were 2.27, 1.46 and 1.94 \( \times 10^{10} \text{ (Ms)}^{-1} \), respectively [75].

Finally, the reaction with oxy-heme proteins, in particular oxy-hemoglobin (oxyHb) and
oxy-myoglobin (oxyMb) should be considered. Oxy-hemoglobin is known as the major sink
for free NO radicals in the vascular system. The reaction products are methemoglobin and
nitrate

\[
\text{oxyHb} + \text{NO} \rightarrow \text{metHb} + \text{NO}_3^-
\]
\[
\text{oxyMb} + \text{NO} \rightarrow \text{metMb} + \text{NO}_3^-
\] (11)

The reaction is very fast with second order reaction rates of \( 8.9 \times 10^7 \text{ (Ms)}^{-1} \) for oxyHb
and \( 4.4 \times 10^7 \text{ (Vs)}^{-1} \) for oxyMb (pH = 7, 20°C) [76]. Therefore, one might expect a
significant oxidation of S-nitrosothiols by oxyHb as well. However, \textit{in-vitro} experiments
show that the NO moiety of S-nitrosothiols is well protected against oxidation to nitrate
by oxy-heme. Exposure of a small quantity of CysNO to oxyHb leads to S-nitrosation of
the Cysβ93 residue of the protein but not to the formation of nitrate [77,78]. In fact, the
rates and extent of transnitrosation from CysNO were very similar for oxyHb and metHb.
This transnitrosation reaction to oxyHb was completely inhibited by the addition of the metal chelators neocuproine and DTPA [78]. Therefore, the transnitrosation of oxyHb seems to require the presence of catalytic traces of copper, either in free form or in the form of Cu–Zn dismutase.

Clearly, reaction (11) is highly significant for free NO radicals but does not apply to S-nitrosothiols. Phrased otherwise, a small quantity of low-molecular-weight S-nitrosothiols can survive for a very long time in the presence of oxy-heme. This property is a very significant distinction with free NO. It explains why significant quantities of S-nitrosothiols can coexist with oxygenated erythrocytes and blood (Table 1), in clear contrast to free NO. Oxidation to nitrate becomes feasible only if GSNO is supplied in excess over Cysβ residues [78]. This situation is very unlikely to happen in vivo.

Under anaerobic conditions, GSNO can be reduced by deoxyhemoglobin in a slow and irreversible reaction [79]. In presence of excess deoxyHb, the half life of GSNO is about an hour with concomitant release of equimolar quantities of GSH and nitrosylated ferric hemoglobin. The reaction rate was dependent on the conformer state (R or T) of the Hb tetramer. Interestingly, transnitrosation from GSNO to the Cysβ residue of Hb did not occur in this assay. As with oxyHb, the reaction pathway with deoxyHb seems not relevant for the lifetime of GSNO in vivo.

Recapitulating these various reactions, we recall that in vivo RSNO concentrations remain below ca 100 nM (Table 1). At such low levels, the thermolytic and photolytic pathways are negligible in comparison with the effect of trace metal ions, superoxide and the chemical equilibria with other stores of NO. Significantly, S-nitrosothiols are essentially stable against oxidation by oxyHb, whereas free NO is rapidly oxidized to nitrate.

TRANSPORT OF LMW S-NITROSOThIOLS

Long-distance transport of S-nitrosothiols is possible via the blood flow along the vascular tree, but the kinetics of transport on a cellular scale in tissues is still being investigated. The transport of S-nitrosothiols across cell membranes was particularly controversial [50,80,81]. Experimental evidence suggests that molecules like GSNO or GSH cannot cross cellular membranes, neither by passive diffusion nor by an active transport mechanism. The carboxyl groups of glutathione are only weakly acid, so that GSNO and GSH should be a dynamic mixture of neutral, anionic and dianionic forms. But even the neutrals are not capable of crossing into the intracellular compartment. Studies [82] on NIH/3T3 fibroblasts showed that exposure to extracellular GSNO or SNAP failed to increase intracellular S-nitrosation. Low levels of extracellular CysNO (<0.1 mM) enhanced intracellular cysteine levels but failed to raise S-nitrosation. Significant S-nitrosation was only observed at high levels (1 mM) of extracellular CysNO. S-nitrosation of the intracellular compartment took place on a timescale of about 10 min [82]. NMR studies [83] confirmed that no significant exchange of GSH takes place over the membrane of human erythrocytes. Moreover, the extracellular glutathione affected neither the intracellular GSH/GSSG ratio nor the glucose metabolism of the erythrocytes. This indicates that extracellular GSH did not provoke intracellular release of thiols from cleavage of disulfide bonds in the interior compartment. Taken together, this study showed that intracellular GSH cannot be raised by suppletion of extracellular GSH.
The cellular transport of small $S$-nitrosothiols was extensively studied by Hogg et al. [84,85]. They showed that extracellular GSNO and SNAP did not significantly increase the pool of intracellular $S$-nitrosothiols in RAW 264.7 macrophages. However, the coincubation of GSNO with $L$-cysteine or its disulfide ($L$-cystine) greatly enhanced the intracellular $S$-nitrosation. The $S$-nitroso moieties were predominantly located on the cysteine residues of proteins. Significant intracellular $S$-nitrosation was also observed after incubation with only extracellular $L$-CysNO. The data suggested that $L$-CysNO was transported intact across the membrane, and initiated subsequent transnitrosation towards the intracellular proteins. The effect of $L$-cysteine on extracellular GSNO was plausibly explained by the following sequence of events: First, transnitrosation from GSNO to $L$-CysNO. Second, active transport of $L$-CysNO across the membrane into the interior compartment. Third, transnitrosation from $L$-CysNO to the sulfhydryl groups of the proteins. The transport of $L$-CysNO was stereospecific for the $L$-isomer and could be blocked by inhibitors of the cellular transport system of amino acids. The uptake of $L$-CysNO was specifically attributed to the amino acid transporter system $L$ ($L$-AT) [81,85].

The amino acid transporter system of the cellular membrane is not the only factor determining entry of $S$-nitrosothiols into the interior compartment and the timescale of intracellular transnitrosation. The cell membrane itself is rich in thiol-containing proteins, and each one of these is a potential carrier for cellular entry of NO. Protein disulfide isomerase (PDI) is a membrane enzyme that normally catalyzes thiol–disulfide exchange reaction. This protein was found to raise the level of $S$-nitrosothiols inside human erythroleukemia cells [50]. Additional pathways for the entry of $S$-nitrosothiols will undoubtedly be recognized in the future.

**BIOLOGICAL ACTIONS OF LMW NITROSOTHIOLS**

A large body of data attests to the effect of low-molecular-weight RSNOs on various physiological and biochemical processes. We can roughly classify these effects in three categories. In the first, RSNO operate as donors of free NO and the NO subsequently acts as the true effector, just as would NO released by NO donors or by NOS enzymes. In the second, RSNO acts as a transnitrosator of other sulfhydryls as found on proteins, and the effects result from the $S$-nitrosation of the group. Effects in this category can also be evoked by exposure to other nitrosating agents like N$_2$O$_3$. In the third category, RSNO itself acts as an effector in its own right, for example as activator of guanylate cyclase enzymes (GC). We also include S-glutathiolation into this third category.

**First category effects**

The first category contains the majority of effects mediated by the guanylate cyclase-dependent pathway in tissues and cellular assays [86,87]. The RSNO can be of low molecular weight like GSNO or high molecular weight like $S$-nitrosohemoglobin. It is now widely accepted that the release of free NO from cleavage of the $S$–NO bond in cells and tissues is dominated by catalytic action of copper [88]. Recent studies [86] report that GSNO and...
S-nitrosated oxy-hemoglobin is vasodilating without release of free NO (cf Fig. 7). The sensitivity of aortic tissue to GSNO is very high, and of the same order of magnitude as the sensitivity to the physiological stimulant acetyl choline (Ach) (Fig. 8). The vasodilation of aortic rings is considerably reduced when superoxide levels in the organ bath are artificially raised by coincubation with pyrogallol [89], a generator of superoxide radicals. Seen as NO donors various RSNOs can produce both beneficial and adverse effects on cells and tissues. This state of affairs reminds us of the ongoing controversy surrounding the benefits/disadvantages of upregulation of NOS enzymes. Many detailed studies in vitro and in vivo suggest that raising levels of free NO be beneficial in vascular ischemia, but harmful in brain ischemia. Accordingly, many therapeutic treatments of ischemia involve NO donors for vasculature, and NOS inhibitors for brain. Experiments confirm many similarities between the effect on ischemia of LMW S-nitrosothiols and true NO donors. The similarities include relaxation of blood vessels or bronchial smooth muscle, inhibition platelet aggregation, etc. Just as with true NO donors, high doses of RSNO can be harmful and activate the apoptotic chain [90–92]. The mechanism is still being investigated. A significant contribution is attributed to the oxidation of excess NO to the highly noxious peroxinitrite. The latter induces so-called nitrosative stress [93].

In many cases, moderate doses of S-nitrosothiols are seen to provide protection against oxidative stress: Just like true NO donors, GSNO stimulated the recycling of ascorbate by human erythrocytes [94]. The mechanism was not fully understood but did not significantly depend on the presence of free transition metal ions in solution.

It is interesting to note that the iron status of cells is an important parameter in the response of cultured cells to exogenous RSNO. NO is implicated in the activation of the apoptotic chain. Details of this mechanism are discussed in Chapter 12. Interestingly, the apoptotic action of NO depends on the presence of iron. Cellular apoptosis could also be abrogated in a range of cultured human tumor cell lines by the addition of exogenous iron to the medium [91].

Fig. 7. Vasorelaxation by GSNO and S-nitrosated hemoglobin in precontracted aortic rings from rats. The relaxation was stimulated by 100 µM GSH, followed by either 50 nM GSNO (A); SNOoxyHb (B); SNOmetHb (C). At a later timepoint 10 µM oxyHb was added to scavenge free NO. Experiments in Krebs buffer at 37°C at high oxygen tension (95%O₂ + 5%CO₂). (From Ref. [86].)
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Fig. 8. Dose response curves for relaxation of precontracted rat aortic rings by acetylcholine (left panel) and GSNO (right panel). The presence of 0.3 mM pyrogallol (open triangles) cancels the stimulus from Ach, and reduces the sensitivity to GSNO by an order of magnitude. Experiments in Krebs buffer at 37°C at high oxygen tension (95%O₂ + 5%CO₂). (From Ref. [89].)

RAW264.7 macrophages contain a comparatively low amount of non-heme iron. These macrophages proved quite susceptible to the pro-apoptotic action of RSNOs. Preincubation with exogenous iron reinforced their resistance to apoptosis and EPR spectroscopy revealed the formation of intracellular DNIC with thiol-containing ligands [90]. These DNIC were evidently assembled from RSNO and non-heme iron. In contrast, hepatocytes contain fairly large amounts of endogenous iron and show much higher resistance to the pro-apoptotic effect of NO [90]. The cellular reaction pathways for DNIC are considered in detail in Chapter 11. Intracellular formation of DNIC was also observed after addition of ferrous iron to cultured leukemic cells incubated with S-nitrosocysteine (Vanin, A.F., unpublished data). The incubation of cells with Cys-DNIC or GS-DNIC did not affect the cell viability.

The mechanism of apoptotic protection was investigated in cellular assays [90]. It was shown that apoptosis was suppressed by inhibition of caspase 3 by S-nitrosation of this protein. The S-nitrosation could be reversed by the addition of dithiothreitol. It demonstrated that extracellular DNIC enhanced S-nitrosation of the intracellular proteins. Details of the mechanism remain unclear since transport of intact DNIC across cellular membranes remains unproven at this moment.

We just noted that both RSNO and DNIC can act as NO donors in their own respect. Usually, activation of a physiological response requires higher doses of GSNO than DNIC [90,95]. As donors, they are clearly distinguished by their response to alterations in the pool of free iron and copper in the solutions. Addition of a metal chelator like bathophenanthroline disulfonate (BPDS) will remove the iron from DNIC and induce instantaneous release of the nitrosyl ligands into the solution. The effect on GSNO is exactly opposite in that its lifetime is greatly extended by chelation of the free metal ions. But the pools of RSNO and
DNIC are always coupled in a dynamic equilibrium by the presence of free iron ([96] and Chapter 11). It should be noted that tissues always contain a certain quantity of loosely bound iron (experimental evidence for this iron pool in tissues is reviewed in Chapter 2).

Second category effects

Besides acting as NO donors, RSNOs can modulate physiological and biochemical processes by transnitrosation. Certain effects were found to be stereoselective and are specific for the L-isomer of S-nitrosocysteine. Stereoselectivity was observed for neuronal stimulation in brain of conscious rats [97] and in transnitrosation of the intracellular compartment [84,85]. Mounting evidence suggests that these effects are manifestations of a cascade of events involving the exchange of S-nitrosation between exogenous or endogenous low-molecular RSNOs and sulfhydryl moieties on intracellular proteins. [84,98]. This chemical modification is reversible and, in principle, potentially useful for regulation of the enzymatic activity inside cells.

Effects of S-nitrosation on enzymatic activity have now been confirmed for dozens of enzymes in vitro as well as in vivo. The involvement of S-nitrosation is usually established beyond doubt by being reversible under treatment with the denitrosating agent dithiothreitol. S-nitrosation can inhibit glutathione reductase [99], seven members of the caspase family [100], including the inducer of apoptosis, caspase 3 [100,101], creatine kinase [102], glutathione-S-transferase [103], adenosyltransferase [104], transcription factor Yin Yang [105], cathepsin K [106], glyceraldehyde-3-phosphate dehydrogenase [107], alcohol dehydrogenase [108], c-Jun N-terminal kinase 2 [109], HIF-1α protein [110], protein tyrosine phosphatase [111], inactivate aconitase [112], activate the ryanodine receptor for calcium release [113], inhibit adenyl cyclase enzyme in rat thymocytes [81,114], etc. Also S-nitrosation of crucial thiol groups by exogenous or endogenous low-molecular RSNOs can inhibit the ligand-binding ability of glucocorticoid receptor [115], disrupt the mitochondrial electron-transfer chain [116], induce cytostasis or cytotoxicity [117], inhibit NF-κB binding to DNA [118,119], inhibit papain [111] and ornithine decarboxilase [120], inhibit Complex I from the mitochondrial respiratory chain [121], regulate the redox state and anti-apoptotic function of thioredoxin [122]. This list is exemplary only and by no means exhaustive. As a rule, these inhibitions are reversible, and enzymatic activity may be restored by S-denitrosation, for example by adding excess glutathione or dithiothreitol. It should also be noted that enzymes may interact with nitrosospecies in more than just one way. A well-known example is Hb which undergoes nitrosylation of the heme and also S-nitrosation of its Cys93 residue. Another is Complex I of the respiratory chain which undergoes nitrosylation of the catalytic Cu-B/heme-a3 site as well as multiple S-nitrosation of cysteine residues [94].

Before concluding the effects in the second category, we recall that DNIC plays a very important role in the process of protein S-nitrosation by LMW RSNOs. As was mentioned earlier, the probability of transnitrosation from low-molecular RSNOs to protein thiol groups is low and takes many minutes to establish equilibrium (Fig. 6). Spurious iron and copper help significantly, but the formation of DNIC really accelerates transnitrosation. It was shown by Boese et al. [123] that Cys-DNIC is much more effective for S-nitrosation of serum albumin
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than CysNO itself. It is conceivable that loosely bound iron and DNIC rather than spurious copper determine the rate and extent of S-nitrosation of the proteins in cultured cells and tissues.

Third category effects

Here we list a number of physiological responses via mechanisms other than S-nitrosation. More precisely, processes for which S-nitrosation does not play a role or in which S-nitrosation has not been recognized yet. S-nitrosothiols provide neuroprotection [124], protect against amyloid β-peptide neurotoxicity [125], induce bacteriostatic effect of α₁-protease inhibitor [126], interrupt the replication of the coronavirus [127], inactivate HIV-1 protease [128], inhibit dystrophin proteolysis by Coxsackieviral protease 2A [129], promote expression of the S-lipoxygenase (5-LO) in several human bronchial cells types [130] and stimulate noradrenalin release in rat brain [81]. Nitroprusside and SNAP activate a complex extracellular signal-regulated kinase (ERK) pathway via an as yet unknown mechanism [131]. S-nitrosothiols are often found to influence the life cycle of viruses, for example human immunodeficiency [132], Herpes Simplex [2] and Epstein-Barr [133]. In many cases the mechanism has not been fully understood, and it is speculated that S-nitrosation of thiols might have significant effect on virion maturation [2,127]. Upon incubation with S-nitrosothiols, the cysteine residues of proteins often show a certain degree of S-glutathiolation. The S-glutathiolation pathway is often operating simultaneously with S-nitrosation. The process of S-glutathiolation requires the presence of oxygen and seems to be dominated by intermediate oxides like GS(O)SG or GS(O₂)SG [37]. S-glutathiolation of intracellular proteins has been demonstrated in vivo: upon incubation with exogenous CysNO, NIH-3T3 fibroblasts underwent combined S-nitrosation and S-glutathiolation of the cysteine residues of H-ras protein [54]. S-glutathiolation may be reversed by dithiothreitol which is an effective reductor for disulfide bonds [60]. Again, the above list is not exhaustive.

This growing body of evidence attests to the signaling role of various RSNOs. These agents are clearly involved in the control of enzyme activity and in various intracellular messenger pathways.

THERAPEUTIC USES OF LMW NITROSOTHIOLS

Hitherto, therapeutic application of LMW nitrosothiols was limited because they elicit a rapid and strong vasodilatory response which exceeds the margins of safety for humans. In short, they are too effective as vasodilators. Alternative donors like organic nitrates or nitroglycerine are considered safer for clinical applications as they provide better control over the vasodilation. These alternative NO donors are described in detail in Chapter 17 of this book. The risk of excessive hypotension makes that clinical studies of the effect of GSNO in humans are very few. A clinical study into the effects of infusion of GSNO into the bloodstream of pregnant women with severe preeclampsia [134] has shown promising improvement in maternal blood pressure (Fig. 9) and uterine arterial resistance (Table 3).
RSNO has been implicated in a number of respiratory diseases. Inflammatory state as in pneumonia gives higher RSNO levels in the lungs. Asthmatic patients exhale high levels of NO but have depressed RSNO levels in the airways. The modulation of RSNO status for clinical therapy is only now being considered [66,70,135].

Injection of modest doses of GSNO has also been found to promote wound healing in rats. In this case, repeated intraperitoneal injection of GSNO was found to accelerate healing of skin lesions by promoting deposition of new collagen in the affected areas [136] (Fig. 10).

External application of RSNO avoids the risk of exceedingly strong vasodilation and life threatening loss of blood pressure. Very promising results have been recently reported for external application of hydrogels containing GSNO to promote skin repair [137].

Table 3 Effect of venal infusion of GSNO on blood circulation parameters of pregnant women with severe preeclampsia. PI and RI are pulsatility and resistance index, respectively. P-selectin expression is a marker for activation of blood platelets and has a value 1.1 ± 0.2% in healthy pregnancies. Adapted from Ref. [134]

| Parameter                  | Before infusion | During infusion |
|----------------------------|-----------------|-----------------|
| Mean arterial pressure (mm Hg) | 125 ± 5         | 104 ± 4         |
| Pulse rate (beats/min)      | 74 ± 6          | 90 ± 4          |
| Mean uterine artery RI      | 0.76 ± 0.03     | 0.70 ± 0.03     |
| Umbilical artery PI         | 1.9 ± 0.2       | 1.6 ± 0.2       |
| Fetal thoracic aorta PI     | 2.5 ± 0.2       | 2.3 ± 0.2       |
| P-selectin expression (%)   | 3.0 ± 0.5       | 1.2 ± 0.2       |
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Fig. 10. Hydroxyproline content of scar tissue at two time points after dorsal incision in rats. Hydroxyproline is the main component of collagen. After incision GSNO and GSH were injected intraperitoneally at 24 h intervals with dose of 0.3 mg/kg. The treatment with GSNO significantly enhances the collagen deposition in the affected tissue. (From Ref. [137].)

The beneficial action was attributed to a combination of factors. First, the sterilizing action of NO radicals released from the GSNO. Second, the vasodilation and ensuing improvement of blood supply to the affected tissues.

The older literature on potential therapeutic applications of S-nitrosothiols was reviewed in Refs. [138–140].

Summarizing the above results, it appears surprising that such a powerful vasodilator has found relatively few practical therapeutic applications. As mentioned before, this state of affairs is primarily caused by the practical problems regarding the need to keep complete control over the dosage to avoid life threatening loss of systemic blood pressure. S-nitrosothiols, whether they be of low or high molecular weight, clearly elicit strong physiological responses. Some of these responses are acute (e.g. vasodilation) and some induced on a longer timescale (like apoptosis). But strong that they are and since the S-nitrosothiols are endogenously formed, these compounds deserve constant attention and consideration by researchers in the field of NO.

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