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S-nitrosation/Denitrosation in Cardiovascular Pathologies: Facts and Concepts for the Rational Design of *S*-nitrosothiols

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Abstract: Nitric oxide (•NO) is a physiological mediator of vasorelaxation constitutively synthesized by endothelial nitric oxide synthase. Because •NO has a short half-life, it is stored by proteins through *S*-nitrosation reactions. *S*-nitrosation was recently defined as a post-translational modification of proteins for cellular signalling, as important as glycosylation and phosphorylation. Disulfide forming/isomerizing enzymes like thioredoxin (Trx), protein disulfide isomerase (PDI), which are chaperone proteins, are implicated into transnitrosation reactions, which are the transfer of •NO from one cysteine residue to another one. Furthermore, Trx has been shown to denitrosate *S*-nitrosoproteins depending on its redox status. *S*-nitrosation of Trx on Cys residues apart from active site, under nitrosative or oxidative stresses, enhances its activity, thereby reducing intracellular reactive oxygen species. Trx and PDI have therefore an essential role for cell signalling control which leads, among other actions, to cardio and vasculo-protection. The diminution of either •NO synthesis or bioavailability is implicated into a large number of cardiovascular pathologies associated to hypoxia or vasoconstriction like, endothelial dysfunction, arterial hypertension and atherosclerosis. In order to mimic the physiological storage of •NO as *S*-nitrosothiols, the development of •NO donors should be based on the covalent S-NO bond. The chemical stabilisation of the S-NO bond and protection against enzymatically active proteins such as PDI//Trx are major points for the design of stable compounds. *S*-nitrosothiols entrapment in innovative formulations (films, gels, microparticles, nanoparticles) is an emerging field in order to stabilise and protect them, and to deliver •NO under a sustained release at the targeted site.

Keywords: Thioredoxin, protein disulfide isomerase, nitric oxide, nitrosation, *S*-nitrosothiol, •NO donors, innovative formulation.

INTRODUCTION

The biochemical actions of nitric oxide (•NO) are not easily compatible with its chemical properties. Indeed, from the chemical point of view, •NO is highly reactive with a short half-life and has selective signalling biochemical actions. An intriguing question is "how •NO can exist and move inside the cell without being instantaneously sequestered by other molecules". Possible answer is to consider the implication of peptides and proteins in •NO transport, storage and propagation of the biochemical signalling of •NO. NObonomic is the novel metabonomics approach for the global profiling of •NO metabolism and *in vivo* signalling. It has been defined by Janero *et al.*, as the "global NO metabolic profiling which would encompass the quantitative *in vivo* mapping of NO-related metabolites of either endogenous or exogenous (*i.e.*, extragenomic) origin and how they are affected by disease, drug exposure, or other (patho)physiological influences. The set of NO-related metabolites and any products of their interaction identified under the prevailing conditions of the organism would constitute a NObonome" [1]. Moreover, *S*-nitrosation and nitrosylation of proteins implicated in cellular functional regulation are part of the NO-bonomic. Protein *S*-nitrosation, which is the addition of •NO to a cysteine residue (RSH), forming *S*-nitrosothiols (RSNO), constitutes a post-translational modification of proteins for cellular signalling. This modification is as important as glycosylation and phosphorylation. Nitrosylation is the addition of the radical •NO to the metallic center (mostly iron) of metalloproteins like soluble guanylate cyclase (sGC) and cytochrome P450 regulating their enzymatic activities. •NO is synthesized *in vivo* from L-arginine through catalytic action of three NO synthases (NOS) isoenzymes, including neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS). eNOS is expressed in endothelial cells (EC) as a constitutive enzyme and produces picomolar to nanomolar concentrations of •NO for short periods in response to receptor stimulation (acetylcholine, bradykinin) or shear stress [2,3]. •NO through its vasodilator activity is crucial for the maintenance of vascular homeostasis. Indeed, •NO diffuses from EC to smooth muscle cells where it activates sGC through nitrosylation to produce cyclic guanosine monophosphate (cGMP), activating a signalling pathway inducing vasorelaxation (Fig. (1)).

According to a pharmaceutical point of view, •NO donors are prescribed into cardiovascular diseases. Since •NO is characterised by an extremely short half-life *in vivo* (*ca.* 5 s), it is administered as a prodrug. Nonetheless, classical •NO donors have shown some limitations as a fast release of •NO, a relatively poor distribution to the targeted site (the vascular smooth muscle), an oxidative stress induction, and a tolerance phenomenon generation [4]. A way to solve these problems consists in the development of •NO donors based either on physiological *S*-nitrosothiols (RSNO), such as *S*-nitrosoglutathione (GSNO) and *S*-nitrosoalbumin (AlbNO), or exogenous ones, such as *S*-nitroso-*N*-acetylcysteine (NACNO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP). Endogenous high molecular weight RSNO such as AlbNO, *S*-nitrosohemoglobin and low molecular weight ones (e.g. GSNO), are a physiological reservoir of •NO, thus they can become potential •NO donors for cardiovascular disease treatments. As albumin is the most abundant protein in plasma (35-50 g/L), it is not surprising that the major circulating plasma *S*-nitrosoprotein is AlbNO [5], which has been proposed to act as a reservoir of •NO within the circulation, transporting and releasing •NO inside the vascular beds. A major subset of *S*-nitrosated proteins including circulating AlbNO are in equilibrium through spontaneous transnitrosation reactions with reduced glutathione (GSH), leading to a low molecular weight RSNO, *i.e.* GSNO, which acts as a second messenger to transduce •NO bioactivity [6,7]. Indeed, some studies clearly showed that RSNO denitrosation takes place nonenzymatically by rapid transnitrosation reactions in whole blood between GSNO and albumin or in intact cells between albumin and GSH [8,9]. However, some differences in the denitrosation rate of individual RSNO have been noted, suggesting the existence of additional structural factors [9]. The transfer can also be spontaneous implicating the thiols at the cell membrane [10]. However, the relatively unfavorable kinetics of transnitrosation reactions [11] suggest that enzyme-mediated systems are required for the process to happen in a sufficient fast way to medi-

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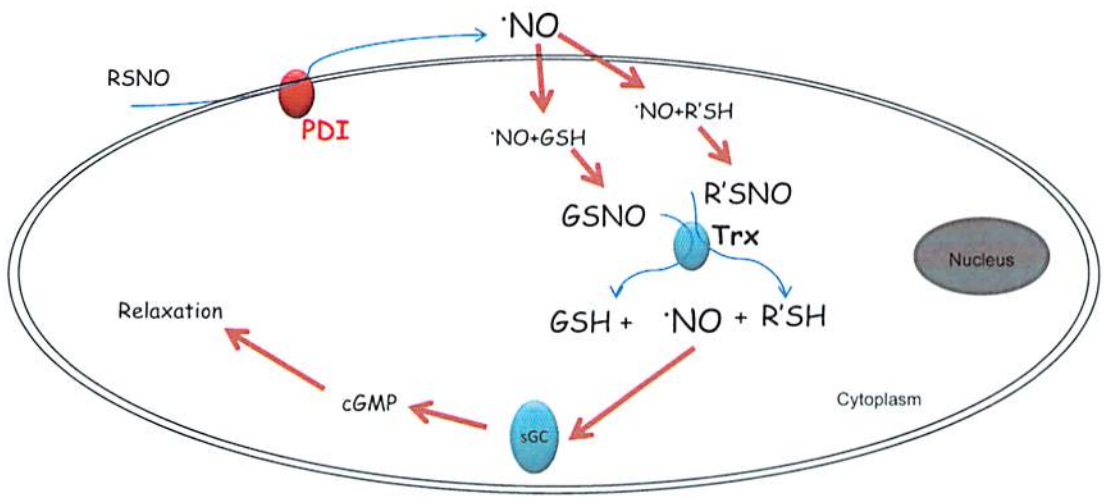


Fig. (1). Transfer and release of •NO by thiol disulfide oxidoreductase enzymes such as protein disulfide isomerase (PDI) and thioredoxin (Trx). Only the PDI at the plasma membrane of the cell is represented here because it is involved in the RSNO influx. Inside smooth muscle cells, •NO induces vasorelaxation through the activation of the soluble guanylate cyclase (sGC) pathway. cGMP: cyclic guanosine monophosphate; GSNO: *S*-nitrosoglutathione, GSH: reduced glutathione; RSNO: *S*-nitrosothiol.

ate signalling events. These systems include disulfide forming/isomerizing enzymes, which are the main enzymes acting to trans/denitrosate RSNO and finally to deliver •NO. The two major enzyme families forming this class are the thioredoxin (Trx) and the protein disulfide isomerase (PDI), both of which have been shown to be involved into transnitrosation and denitrosation processes.

Consequently, endogenous RSNO that include •NO-traffic proteins (high molecular weight) or peptides (low molecular weight) can be used as reliable and safe •NO donors [12]. A •NO-traffic protein is a protein with high efficiency of *S*-nitrosation, high stability of the *S*-nitroso group in the blood stream and high efficiency in transnitrosation inside the cells [13]. They protect •NO from degradation (mostly oxidation and radical reactions) and can deliver it to the appropriate site of action (tissues and cells). So, this kind of •NO donors will trigger physiological dynamics to deliver •NO to its cellular target. Considering the lability of the S-NO bond (under exposure to light, metals, reducing agents, enzymes), a rational drug design of •NO donors is required to obtain a prolonged and controlled release of •NO. In this way, the chemical stabilisation of the S-NO bond is the key to increase •NO donors half-life. Indeed, the alpha or beta substitutions on the carbon atom (Cβ - Cα-S-NO), with an electrodonor group and a positive charge respectively, are of great importance to promote RSNO stability [14,15]. Furthermore, RSNO formulation into nanoparticles (NP), making a physical barrier between RSNO and the environment, will help to increase its half-life in the blood stream. Otherwise, physiological RSNO like GSNO are hydrophilic, thus further modifications of RSNO increasing their lipophilicity will ensure a long time •NO delivering formulation.

This review will focus on the process of RSNO denitrosation and transnitrosation *via* disulfide forming/isomerizing enzymes such as Trx and PDI. In addition, we will review the relationship between disulfide forming/isomerizing enzymes and pathology. Finally through the overview of the specificity of the sites and motifs of *S*-nitrosation, we will give some keys to identify potential candidates for new •NO delivery drugs development and their rational design.

TRANSNITROSATION/DENITROSATION

It is widely assumed that the biological effects of RSNO are attributed to the homolytic cleavage of the S-NO bond with release of •NO [16,17]. The rate of •NO release from RSNO is dependent upon many factors, such as pH, partial pressure of O_2, light, metal ion concentration, presence of reducing and chelating agents, excess of thiols and enzymes [18,19]. However, Arnelle *et al.* demonstrated that NO^+ (*via* nitrosyl transfer), •NO, and NO^- (nitroxyl anion) are released from RSNO at physiological pH [18]. Furthermore, the rate of •NO group transfer (transnitrosation) from a RSNO to myoglobin is more rapid than rates of •NO release by a RSNO alone [18]. These results suggest that heterolytic mechanisms of decomposition constitute significant pathways in RSNO metabolism.

S-nitrosothiols can undergo spontaneous [20] or assisted transnitrosation from one thiol to another one. The spontaneous transnitrosation from high to low molecular weight RSNO depends on the pKa of the thiol group. Indeed, transnitrosation occurs as the nitroso-nitrogen atom is attacked by the nucleophilic thiolate anion. So, the reaction rate is dependent on the reactivity of the thiol, which is correlated with the pKa of the sulfhydryl group. Besides, Wang *et al.* showed that transnitrosation rate constant increases in a linear relationship with the pKa of the thiol group [20].

Inside the cell cytoplasm, •NO can react with GSH to form GSNO, an intracellular pool of •NO. Transnitrosation can be catalyzed by disulfide forming/isomerizing enzymes such as Trx and PDI. This class of proteins commonly known as thiol disulfide oxidoreductases catalyzes thiol disulfide exchange *in vivo* reactions [21]. The activity of these enzymes depends on a common motif with one pair of cysteine (Cys) residues arranged in a Cys-X-X-Cys motif (X is any amino acid). This motif is usually integrated in a domain sharing structural homology with Trx. The Cys-X-X-Cys motif has become the hallmark of proteins that are involved in forming or breaking disulfide bonds.

Thioredoxin

The thioredoxin system consists of Trx, NADPH and thioredoxin reductase [22]. It is essential for the regulation of cellular redox state and plays an important role in reducing the disulfide bonds of proteins through an interaction with the redox-active site center of Trx. Human Trx contains five Cys residues, including two, Cys32 and Cys35 in its evolutionary conserved –Trp-Cys-Gly-Pro-Cys-Lys- catalytic center. In their reduced thiol form (rTrx), Cys32 and Cys35 can catalytically reduce specific protein disulfide bonds and can serve as a denitrosase toward specific *S*-nitrosated proteins [23-25], resulting in the formation of a disulfide bond be-

tween Cys 32 and Cys 35 (Fig. (**2**)). There are multiple forms of Trx encoded by different genes. Thioredoxin-1 (Trx-1), the most extensively studied, exists primarily in the cytosol, but is also found in the nucleus and blood plasma [22]. The two other isoforms are thioredoxin-2 (Trx-2) also known as a mitochondrial Trx, and SpTrx, an isoform expressed in spermatozoa [26].

In this review, we will focus on cytosolic and circulating Trx-1, which are the most important location for RSNO (as •NO donor) catabolism. It has been shown that Trx is able to denitrosate low molecular weight peptides (23 to 30 kDa) like GSNO or *S*-nitrosocysteine (CysNO), or high molecular weight proteins like AlbNO, *S*-nitrosometallothionenin and *S*-nitrosocaspases 3 and 8 [23,27,28]. Human Trx-1 is a 12 kDa oxidoreductase enzyme containing a dithiol-disulfide active site. It is a ubiquitous protein induced by oxidative stress that is found in the cytosol and in the mitochondria of cells. Some reports also showed that Trx accumulates in the lipid raft fraction of the plasmic membrane of EC that may be involved in the adherence of leukocytes [29]. *In vitro* experiments showed that Trx is able to reduce nitrosated substrates to thiols in a variety of proteins and peptides like the transcription factor AP-1, caspase-3, metallothionein, and GSNO [27,30,31].

Haendeler *et al.* showed that Trx is constitutively *S*-nitrosated in EC at a single allosteric Cys residue (Cys69), which is located outside of the active site (which contains Cys32 and Cys35) [32]. Furthermore, *S*-nitrosation of Cys69 is the main facilitating determinant of the oxidoreductase capacity in EC. Indeed, Trx can denitrosate and thereby activate auto-*S*-nitrosated NOS, which is consistent with the finding that active Trx helps to maintain cellular SNO content. It has also been shown that auto-*S*-nitrosation of eNOS inhibits its activity [33,34].

Trx also binds and inactivates the apoptosis signal-regulating kinase-1 (ASK1). ASK1 acts as a mitogen-activated protein kinase (MAPK), which activates the Jun N-terminal kinase (JNK) and p38 MAPK pathways and is required for TNFα-induced apoptosis. The analysis of Haendeler *et al.* indicated that *S*-nitrosation of Trx at Cys69 is necessary for its basal anti-apoptotic function in EC [32]. However, it has also been reported that treatment with GSNO can induce *S*-nitrosation of Trx in intact cells and dissociate ASK1 [35], possibly by targeting active-site Cys32 and/or Cys35. Accordingly, whereas basal *S*-nitrosation of Cys69 will facilitate oxidoreductase and anti-apoptotic functions, *S*-nitrosation of active-site Cys (which might be induced by nitrosative stress) might exert an apoptotic effect. This would occur through the release of ASK1 and the inhibition of oxidoreductase function (including ROS scavenging), which would result from active-site thiol modification.

Human Trx contains five Cys (Cys32, Cys35, Cys62, Cys69, and Cys73) that can be readily *S*-nitrosated both *in vitro* and *in vivo* [32,35,36,37]. There are inconsistent results pertaining to the specificity of GSNO-dependent nitrosation of Trx. Nitrosation has been reported on all three of the non-active-site Cys, Cys62 [38,39], Cys69 [32,40,41] and Cys73 [37,40,42,43,44]. More recently, Barglow *et al.* showed that the differences in Cys selectivity for nitrosation between the reduced (rTrx) and oxidized (oTrx) forms of Trx may represent a redox-dependent "switch" for signal transduction *via* cascading transnitrosation reactions (Fig. (**3**)) [45]. Because Cys73 and Cys62 are on opposite faces of the protein, it is possible that oTrx-SNO and rTrx-SNO have different binding partners and thus different downstream transnitrosation targets such as caspase-3 under oxidative stress. This would provide an alternate mechanism of signal transduction during periods of nitrosative or oxidative stress.

From all of these considerations on nitrosation of Cys belonging to the active site or the three other Cys residues, arose ideas on the distinction between transnitrosation and denitrosation activities of Trx. Indeed, it is known that oTrx can be nitrosated on Cys73 and can still act as a transnitrosating agent [37,44]. When Cys32 and Cys35 are reduced, Trx can act as a denitrosylase or as a reductase. Clear evidences have been provided that Trx can denitrosate some proteins and transnitrosate other ones. Indeed, Benhar *et al.* demonstrated that Trx is unable to denitrosate the key antioxidant enzyme peroxiredoxin 1 [24] whereas Wu *et al.* found that peroxiredoxin 1 is transnitrosated by SNO-Trx [44]. Furthermore, a recent study investigated the distinction between Trx transnitrosation and denitrosation activities and target proteins [25]. For example, both α-tubulin and GAPDH are sensitive to both SNO-Trx transnitrosation and rTrx denitrosation, while peroxiredoxin 1, peptidyl-propyl cis-trans isomerase A and α-enolase are only SNO-Trx transnitrosating targets and not Trx denitrosating targets [25].

Thioredoxin is a key oxidoreductase enzyme that has firstly been shown to be implicated in the denitrosation of *S*-nitrosoproteins. Multiple studies presently cited, have clearly demonstrated that Trx can undergo post-translational modifications through *S*-nitrosation of two Cys not included in its active site. These post-translational modifications lead to the transnitrosating activity of Trx. However, Trx is not the only oxidoreductase enzyme present in eukaryotic cells. PDI must have also its own role in •NO transfer.

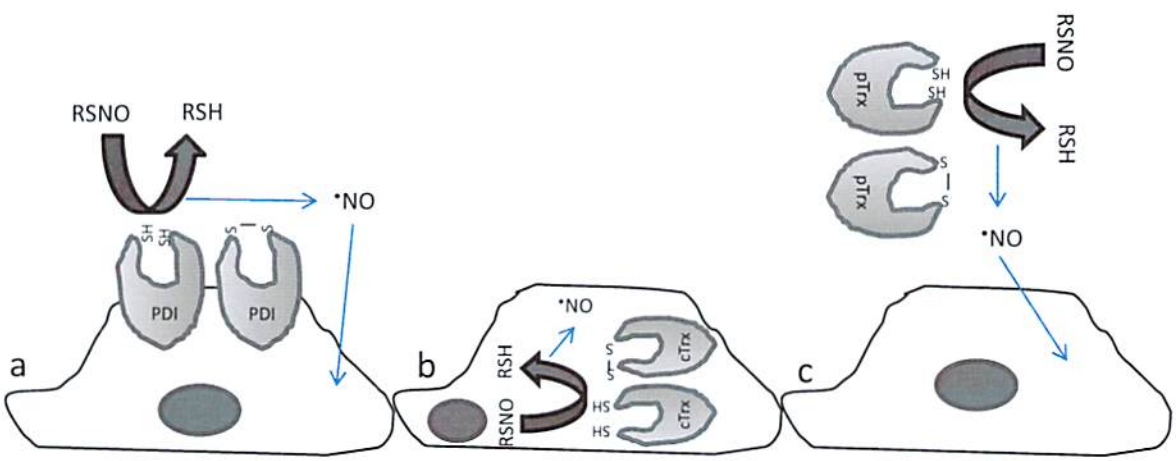


Fig. (2). The different locations of thiol disulfide oxidoreductase enzymes such as protein disulfide isomerase (PDI) and thioredoxin (Trx) implicated into the transfer of •NO from the blood circulation to the cytoplasm of cells. The S-NO bond is catabolized by the reduced Cys of thiol disulfide oxidoreductase enzymes releasing •NO and oxidizing the enzyme into its disulfide form. a) Protein disulfide isomerase (PDI); b) Cytoplasmic thioredoxin (cTrx); c) Plasmatic Trx (pTrx). RSNO: *S*-nitrosothiol; RSH: thiol.

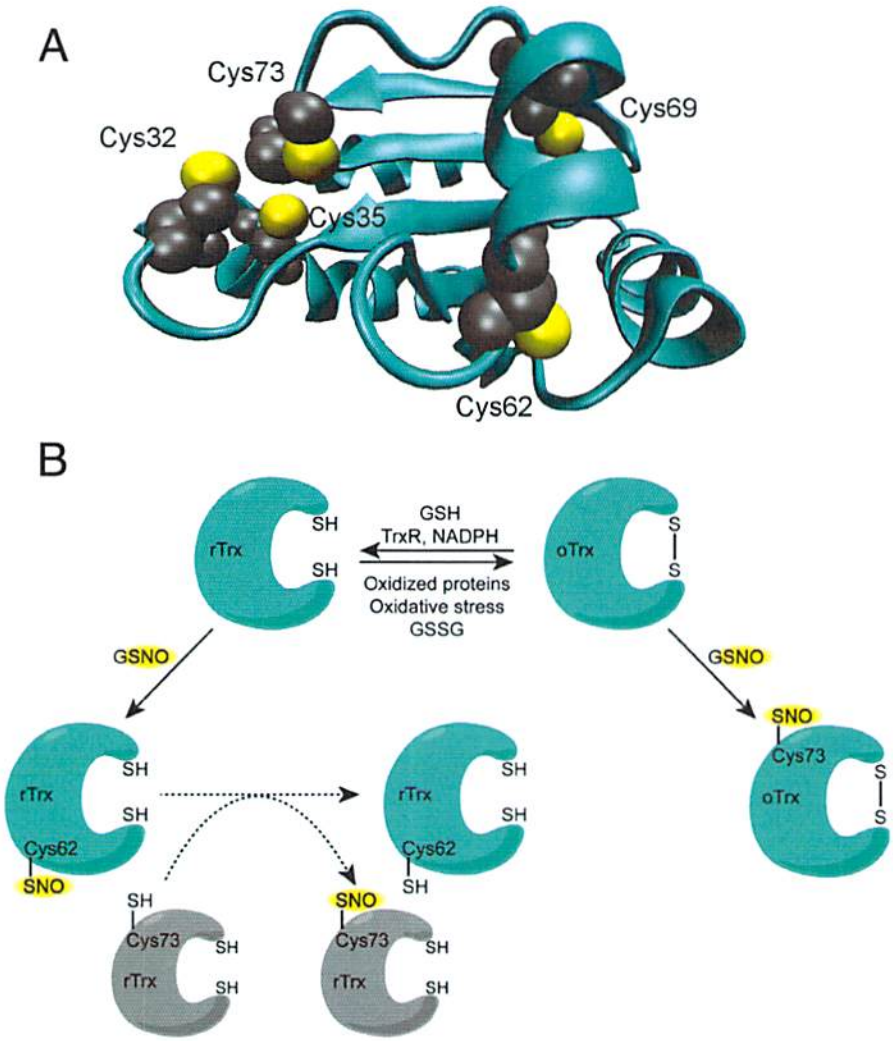


Fig. (3). Model of nitrosation selectivity for oxidized and reduced thioredoxin (oTrx and rTrx). (**A**) rTrx structure (based on PDB ID code 1ERT) showing the location of the 5 Cys. (**B**) Proposed model for interconversion and nitrosation of rTrx and oTrx. From [45]; copyright authorized by PNAS.

Protein Disulfide Isomerase

PDI was the first thiol disulfide oxidoreductase identified about 40 years ago [46]. Seventeen human PDI-family members have now been identified in the endoplasmic reticulum (ER), with a wide range of domain architectures and active-site chemistries [47]. PDI is a versatile enzyme. Indeed, depending on the redox environment and the characteristics of the protein substrates, PDI can catalyse the formation, the reduction or the isomerization of disulfide bonds. Although large levels of PDI are found in the ER, it can also be secreted from cells and associates electrostatically with cell surface, for instance in endothelial, smooth muscle cells and platelets [48-50]. This enzyme family shows little specificity among its protein substrates and it acts on a wide variety of proteins ranging from small, single-domain proteins to large oligomers with multiple domains.

An additional novel activity of PDI is its ability to denitrosate RSNO. PDI is a candidate for specific reactivity toward $^{\bullet}NO$ on the cell surface (Fig. (**2**)). Indeed, some studies have shown evidence for the involvement of PDI expressed on the cell plasma membrane in the RSNO influx (*e.g.* $^{\bullet}NO$ donors based on RSNO) [10,49,51,52]. Furthermore, the active site of PDI includes two Cys residues, one of which has a low pK_a (4.5) [53], allowing the resulting thiolate anion available for reaction with the nitrosonium ion (NO^+) or facilitating the nucleophilic attack of the S-NO bond. Finally, PDI is involved in thiol–disulfide exchange reactions, which are mechanistically similar to RSH-RSNO exchange reactions.

PDI has been shown to denitrosate GSNO [54] and it is postulated that the $^{\bullet}NO$ released through this reaction combines with dioxygen in the hydrophobic environment of either the cell membrane or the PDI protein itself, to form N_2O_3. In that way, PDI becomes a $^{\bullet}NO$ carrier *via* N_2O_3 mediated auto-*S*-nitrosation of its thiol active sites.

PDI and Trx are two disulfide forming/isomerizing enzymes involved in the transfer of $^{\bullet}NO$ from a protein to another one. However, this transfer has to satisfy nitrosation specificities in term of Cys environment.

SPECIFICITY OF *S*-NITROSATION, SNO SITES AND MOTIFS

The identification of SNO proteins was initially an analytical challenge. In fact, the first techniques available to detect SNO proteins were limited to mercury (Hg^{2+}) reaction coupled with photolysis/chemiluminescence or spectrocolorimetry. However, these techniques were unable to identify neither the SNO proteins nor the

modified Cys within the proteins. The development of the biotin switch technique by Jaffrey *et al.* made the identification of SNO-proteins possible [55]. Furthermore, the biotin switch technique enables the identification of the modified Cys within the protein when coupled to mass spectrometry.

Among the multiple Cys residues in a protein, only specific ones are modified and are responsible for altering protein functions [56-58].

It has been proposed that nitrosable Cys residues are flanked within 6 Å by basic (Lys, Arg, His) and acidic (Asp, Glu) residues. These residues could regulate *S*-nitrosation and denitrosation by altering thiol nucleophilicity [59]. Recently, Greco *et al.* identified specific proteins and motifs of *S*-nitrosation in human vascular smooth muscle cells [60]. In smooth muscle cells incubated with Cys-NO, a •NO donor, up to 18 *S*-nitrosocysteine-containing peptides belonging to 16 proteins such as cytoskeletal proteins (myosin heavy chain 9, vinculin), chaperones, and calcium binding proteins were identified. Sequence alignment of these 18 *S*-nitrosocysteine-containing peptides revealed a higher occurrence (50% and 40%) of acidic residues (Asp and Glu, respectively) at positions -3 and -4 relative to the Cys residue. The highest occurrence of basic (Lys, Arg, His) residues was 30% at position 2. There was an occurrence of 10% for acidic residues at position 2 and no occurrence for basic residues at positions 3 and 4. Although primary sequences analyses are useful to determine structural features for specific posttranslational modifications, they do not reveal motifs that result from three dimensional protein structures. *In silico* analyses based on 55 crystalized proteins that have been shown to be nitrosated by •NO donors were recently performed by Marino *et al.* [61]. They tested first parameters well-known to play a role in post-translational Cys modifications such as pK_a, and the residue exposure. They found that the pK_a values of the Cys residues targeted for *S*-nitrosation are higher (9.1) than those of redox and non-redox catalytic Cys (5.5). Furthermore, 65% of the Cys-NO sites were exposed. *S*-nitrosation seems to be favorized by hydrophobic regions around the target Cys increasing thiol nucleophilicity [62] and concentrating nitrosating agents like albumin in the plasma [63]. Indeed, Rafikova *et al.* showed that in the presence of a physiological concentration of •NO, albumin becomes saturated with •NO and accelerates the formation of low-molecular-weight RSNO, *in vitro* and *in vivo*. This mechanism is due to micellar catalysis of •NO oxidation in the hydrophobic core of albumin. The hydrophobic region within the modified Cys seems also to be of great importance since Sun *et al.*, showed that the ryanodine receptor was nitrosated on only one Cys (cys3635) out of about 50 [62] and located in a hydrophobic pocket.

RELATIONSHIP BETWEEN THIOREDOXIN AND NITRIC OXIDE DONORS IN CARDIOVASCULAR DISEASES

Cardiovascular diseases like hypertension, atherosclerosis, thrombosis and cerebrovascular diseases are often associated with oxidative stress. Under oxidative stress (for example exposure of cells to sublethal amounts of hydrogen peroxide) [64], Trx-1 is released from cells, and it appears as a good marker of oxidative stress in plasma [65]: 15-30 ng/mL for healthy volunteers, rising to 40-140 ng/mL in patients suffering from diseases related to oxidative stress [66]. Trx-1 has a protective role in different cardiovascular diseases [67]. Indeed, data indicated that serum Trx-1 levels were found to be significantly increased in patients with acute coronary syndromes and dilated cardiomyopathy [68].

As mentioned in the section concerning Trx, this enzyme has been shown to be *S*-nitrosated at Cys-69 in EC [32]. Interestingly, Tao *et al.* documented that the intraperitoneal administration of *S*-nitrosated human Trx in a mouse myocardial ischemia model after 30 min of ischemia and 10 min prior to reperfusion, potentiated the cardioprotective effects of human Trx [36]. These results suggested a possible association between Trx-1 secretion and cardiovascular diseases. Literature data on interactions between RSNO and Trx, as stated in the section concerning Trx, have focused on GSNO, which is the most abundant low molecular weight RSNO inside the cells [27]. In contrast, data on denitrosation of CysNO, *S*-nitrosohomocysteine (HCysSNO), and SNAP by Trx are rather limited. The Vmax values for CysSNO, HCysSNO, and SNAP were 1.2, 0.3, and 0.1 nmol of substrate (nmol of Trx)$^{-1}$ min^{-1}, respectively [23].

Reduced •NO bioavailability, due to either endothelial dysfunction or eNOS deficiency, seems to be involved into the onset and the development of pathologies cited above [69]. Due to its short half-life (few seconds), •NO is not administrated *per se* (as a gas) to induce prolonged effect but as •NO donor such as sodium nitroprusside or organic nitrates. The indications, which are validated worldwide by the Health Regulatory Authorities, concern angina pectoris, chronic cardiac failure and hypertension in emergency cases. Although, a wide panel of beneficial pharmacological properties is attributed to this radical [70] and a reduced level of •NO has been implicated into the onset and the progression of various diseases, the therapeutical indications of •NO donors still remain limited. Moreover, RSNO cerebrovascular beneficial effects have already been described. One of the RSNO, i.e. GSNO, was studied in rat under various diseases such as subarachnoid hemorrhage [71], traumatic brain injury [72], and cerebral ischemia [73]. The reported beneficial effects were as follows: increase of cerebral blood flow and improvement of blood perfusion as a consequence of capillaries relaxation; decrease of inflammatory environment; protection *versus* traumatic penumbra in the specific case of the traumatic brain injury; improvement of the overall structure of the tissue with the conservation of the blood brain barrier and the reduction of the post-accident neurological deficit. From a mechanistic point of view, •NO provided by GSNO was deemed to suppress iNOS induction but also to enhance at the same time eNOS expression, thus explaining the therapeutic effect of the molecule [74].

The therapeutic use of RSNO is relevant based on their unique capacity to deliver •NO and beneficial effects, particularly in the cerebrovascular system. At this time, only GSNO and NACNO have been involved in several clinical trials (Table 1) demonstrating therapeutic interest. Nevertheless, GSNO is quite unstable in the bloodstream (50 min) [75], one of the reasons being its specific breakdown occurring through catalytic reaction with γ-glutamyltranspeptidase (GGT) [76]. GGT is a central enzyme involved in the degradation of GSH through its specificity for the γ-Glu amino acid [77]. Furthermore, the structure of RSNO has to be modulated to obtain a greater stabilisation and/or protection *versus* environmental attacks through catalytic action of Trx and PDI, to increase the duration of •NO delivery in the bloodstream. Thus the potential applications of RSNO require appropriate chemical modification and/or formulation.

RATIONAL DESIGN OF *S*-NITROSOTHIOLS FOR A SUSTAINED RELEASE

Due to the lability of •NO and its limited diffusion from 40 to 200 μm [92], this drug cannot be administered *per se* to reach constant systemic effects, thus it has to be carried by a prodrug. Various kinds of carriers have been designed for this purpose; some of them were based on molecular concepts. Indeed, to delay the interaction with environmental factors as well as proteins such as disulfide forming/isomerizing enzymes, the moiety wearing –NO has to confer a high stability to the final molecule. Moreover, macromolecular as well as macroassembly approaches and lastly drug delivery systems were designed in order to increase either the grafting of –NO moieties *per* molecule or the time of drug release (ensuring a good protection of –NO) or both. As it will be later explained, this can guarantee a good protection of the sensitive –NO part. Besides, the biological effect of the drug is also strongly related to the delivered concentration therefore, its sustained release at the targeted site remains a crucial challenge.

Table 1. Main characteristics of clinical trials based on administration of *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetylcysteine (NACNO) reported in the literature. In all these studies, a research ethics committee was consulted

Main aim of the study Targeted application	Main conclusion	Number of included patients	References
Reduction of cerebral embolization			
Carotid angioplasty carotid endarterectomy and/or recurrent stroke and/or systemic embolization	Highly significant reductions in the number of Doppler cerebral embolic signals in GSNO groups	42	[78]
	GSNO resulted in a rapid reduction in the frequency of cerebral embolic signals of 95% at 0 to 3 h and 100% at 6 h	16	[79]
		20	[80]
	Significant reduction of cerebral embolic signals in the GSNO group	24	[81]
Inhibition of platelet activation and/or vasodilatation and/or effect on blood flow			
Percutaneous transluminal coronary angioplasty	Significant inhibition of the increase in platelet surface expression of P-selectin and glycoprotein IIb/IIIa without altering blood pressure	13	[82]
Anti-thrombotic effect	GSNO is a relevant drug for anti-thrombotic effect	10	[83]
Coronary artery saphenous vein grafts	GSNO significantly inhibited platelets activation	24	[84]
Severe Preeclampsia	Reduction of maternal mean arterial pressure, platelet activation and uterine artery resistance without further compromising foetal Doppler indices.	10	[85]
Systemic vasodilatation	Intravenous application of GSNO has similar hemodynamic effect as compared to inhaled $^{\bullet}NO$	10	[86]
Dermal wound dressings and local vasodilation in ischemic tissues	Release of GSNO from films induced local vasodilatation relevant for dermal wound dressings or for promoting local vasodilation in ischemic tissues	7	[87]
Wound healing	Increase of blood flow parallel with $^{\bullet}NO$ delivery after application of GSNO or NACNO hydrogel skin application	4	[88]
Female sexual disorder	Increase of clitoral blood flow	40	[89]
Replenishment of GSNO pool			
Effect on left ventricular function	Positive effects on left ventricular function	12	[90]
Effect on cystic fibrosis	Modest improvement of general oxygenation	20	[91]

Molecular $^{\bullet}NO$ Carriers: *S*-nitrosothiols

The $^{\bullet}NO$ donors class can be divided into organic nitrates (R-O-NO_2), for instance glyceryl trinitrate (GTN), nitrites (R-O-NO) and *X*-nitroso- derivatives (R-X-NO). Moreover, research is currently focusing on hybrid or dual molecules able to deliver $^{\bullet}NO$ associated with various drugs (for example, in the non-steroidal anti-inflammatory drug class, both aspirin modified as an organic nitrate derivative, and diclofenac bearing an *S*-nitroso-moiety were studied; the antihypertensive drug captopril was modified on its

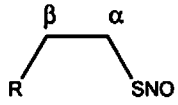
RSH group with the grafting of NO) [93,94]. The strategy developed in these cases will not be detailed here.

Among the *X*-nitroso-compounds, -NO can be linked either to metal ligands (named nitrosyl derivative such as sodium nitroprusside) or to carbon, nitrogen and sulfur atoms. As compared to classical •NO donors, RSNO induce neither oxidative stress nor tolerance phenomenon [95]. Moreover, some RSNO are physiological *i.e.* CysNO, GSNO, and AlbNO and they are considered as an important means of •NO storage in the organism [96]. Due to RSNO unique properties that can be associated to a minimal toxicity, they are nowadays extensively studied. Different clinical trials were also led with GSNO and NACNO as already mentioned (Table 1). Therefore, in this review, only the RSNO family will be described, taking into account the main parameters to stabilise the sulfur-nitrogen bond against environmental attacks (for the other •NO donors, see [97]).

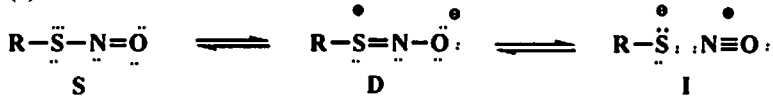
S-nitrosothiols are sensitive to the environment conditions, which may be explained by the weak sulfur-nitrogen bond characterised by a homolytic dissociation energy value of 126 kJ/mol. As a consequence, degradation occurs following a treatment by external factors such as light, temperature and internal factors including cationic ions (Cu^{2+} *via* Cu^{+}), other sulfur containing compounds such as the gaseous messenger H_2S, ascorbate, and proteins like disulfide forming/isomerizing enzymes. These stress conditions are frequently used to study the stability of newly synthesized products [98]. In order to confer a great stability to the sulfur-nitrogen bond, four main parameters have to be highlighted. Three of them are deeply linked to the three resonance structures described for RSNO (Fig. (4)) [99].

The dominant form S (70 to 80 %) is the usual structure characterised by a single bond between sulfur and nitrogen. In the D form (15 to 25 %) the S-N bond is strengthened since a double bond is

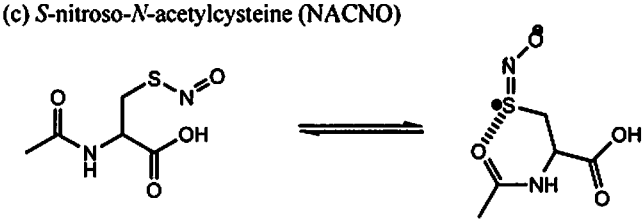
(a) General formula of *S*-nitrosothiol (RSNO)



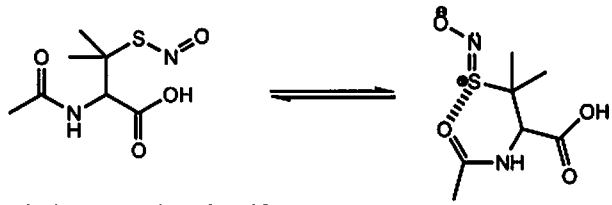
(b) Resonance structures



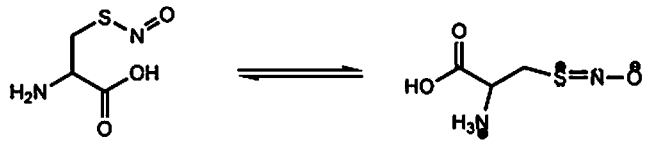
(c) *S*-nitroso-*N*-acetylcysteine (NACNO)



(d) *S*-nitroso-*N*-acetylpenicillamine (SNAP)



(e) *S*-nitrosocysteine (CysNO)



(f) *S*-nitrosoglutathione (GSNO)

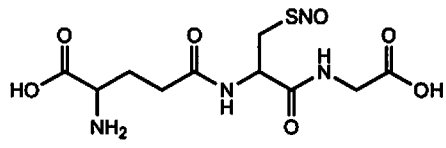


Fig. (4). Structures of low molecular weight *S*-nitrosothiols.

created between these atoms. In contrast, in the ionic form **I** (5 to 10 %) the S-N bond is weak. As a result, each modification that favors the **D** structure will increase the stability and decrease the homolytic cleavage probability. This stable form can be generated by *(i)* the well-controlled synthesis of *S*-nitrosocompounds, *(ii)* the chemical substitution of carbon atoms located either at the alpha position of -SNO group or at its beta position. The last parameter that may induce a better stability *versus* metallic cation attack relies upon the increase of the molecule lipophilicity or its steric hindrance.

A summary of the two physico-chemical as well as vasorelaxant properties of molecules that will be exposed below is proposed in Fig. (**5**).

Measuring vasorelaxation of isolated and preconstricted rat aortic ring [110,111] is now recognized as the most frequently used *ex vivo* model to test efficiency of •NO donors. The high standardisation level of the assay gives rise to less and less variations between research teams, facilitating data comparison. Another *in vitro* cellular model used to evaluate efficiency of •NO donors consists in the measurement of cGMP produced by an enzymatic reaction catalysed by sGC; however, most of cultured cell lines derived from muscular cells express low levels and/or inactive sGC to set the assay in a reliable way [112].

Several chemical procedures have been usually described to synthesize RSNO. In aqueous medium, nitrosation of a thiol is usually provoked using sodium nitrite in acidic medium, by mixing $^{\bullet}NO/O_2$ gas or transnitrosation with another RSNO. Nevertheless, in non-stoechiometric conditions between nitrating reagent and thiol, *S*-nitroderivatives, $RSNO_2$, can be produced [113,114]. Such products show the same UV-visible spectrum; as a consequence, another methodology have to be operated (*e.g.* HPLC [113,114]) to facilitate their identification. But this reaction is described not to favor the stability of the product because the **D** structure formation does not tend to occur. Indeed, Balazy *et al.* estimated the half-life of $GSNO_2$ (generated with a five-fold excess of nitrating agent) at *ca.* 30 min in the absence of other destabilising stimuli [113]. The same result was observed in our laboratory not only on the cited product but also after the formation of *S*-nitro-*N*-acetylcysteine $NACNO_2$ (generated by the same excess of nitrite reagent) and was also characterised by a half-life of less than 30 min. Therefore, the operating conditions to obtain RSNO have to be well-defined.

The two carbon atoms located at the alpha and beta positions of the –SNO group (Fig. (**4**)) are of major importance to design stable RSNO. Firstly, regarding the alpha position, the –SNO stabilisation was reported to be increased with the substitution degree of this carbon atom [14,15]. Nevertheless, the theory was not in accordance with all experimentations, since NACNO (rate of •NO release = 0.025 $nmol.mL^{-1}.min^{-1}$ in phosphate buffer) was described as more stable than its tertiary substituted derivative, SNAP (0.046 $nmol.mL^{-1}.min^{-1}$ in phosphate buffer pH 7.4) (Fig. (**4**) and Fig. (**5**)) [108]. Recent computational work on these molecules (undergoing

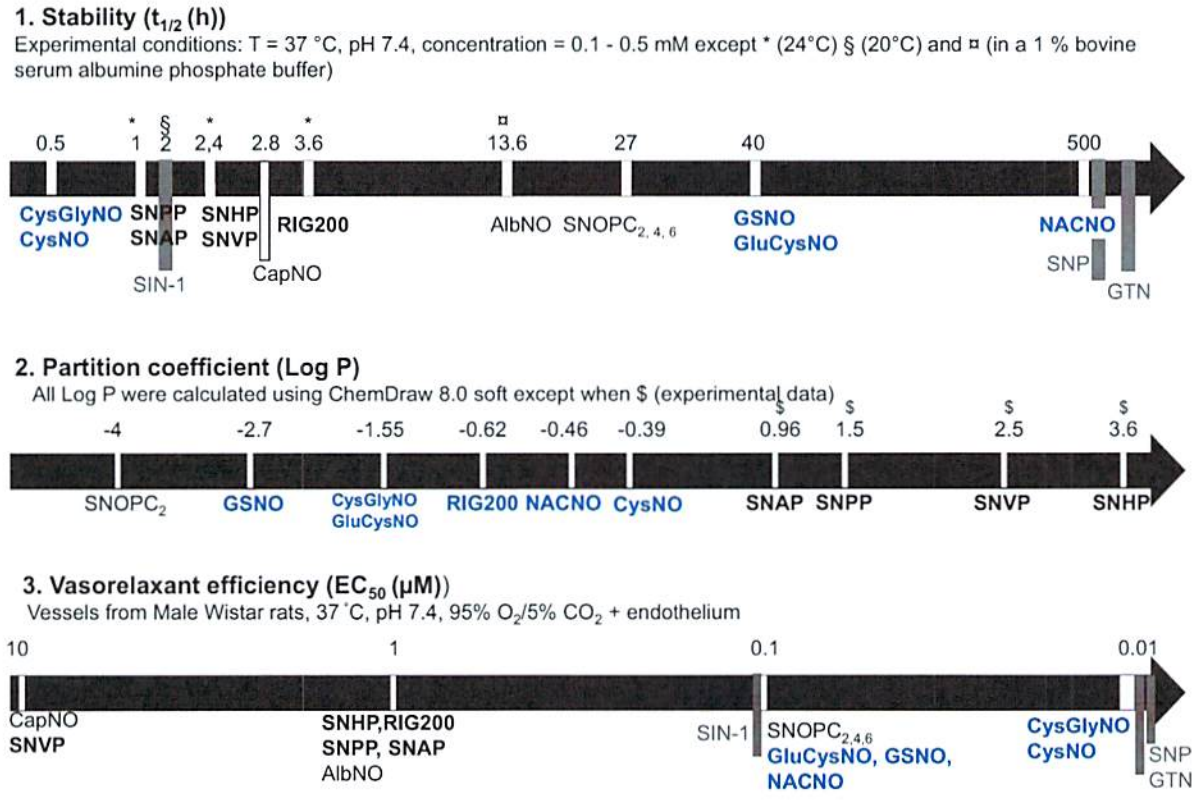


Fig. (5). Classification of main physico-chemical and physiological properties of various •NO donors according to literature data [100-109]: *in vitro* stability, partition coefficient (log P) and vasorelaxant efficiency (corresponding to the half maximal effective concentration EC_{50} obtained in vasorelaxation measurement of isolated and preconstricted rat aortic ring). AlbNO: *S*-nitrosoalbumin, CapNO: *S*-nitrosocaptopril, CysNO: *S*-nitrosocysteine, CysGlyNO: *S*-nitrosocysteinylglycine, GluCysNO: *S*-nitroso- γ-glutamylcysteine, GSNO: *S*-nitrosoglutathione, GTN: Glyceryl trinitrate, NACNO: *S*-nitroso-*N*-acetylcysteine, RIG200: N-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-O-acetyl-β-*D*-glucopyranose, SIN-1: Linsidomine, SNAP: *S*-nitroso-*N*-acetylpenicillamine, SNHP: *S*-nitroso-*N*-heptanoyl-*D,L*-penicillamine, $SNOPC_2$: *S*-nitrosophytochelatin-2, $SNOPC_4$: *S*-nitrosophytochelatin-4 , $SNOPC_6$: *S*-nitrosophytochelatin-6, SNP: Sodium nitroprusside, SNPP: *S*-nitroso-*N*-propanoyl-*D,L*-penicillamine, SNVP: *S*-nitroso-*N*-valeryl-*D,L*-penicillamine.

hydrolysis in acidic medium) [114] proved that the sulfur protonation of SNAP is favored by the presence of the two methyl groups and as a consequence, the equilibrium of the resonance is modified towards the I (51 %) and S (49 %) forms which may ease the nucleophilic attack of -NO moiety [115].

Secondly, as far as the beta position is concerned, the role of this carbon atom as a stabiliser was highlighted when comparing *S*-nitrosocysteine (CysNO) and NACNO (half-lives of 0.54 h and > 500 h in phosphate buffer, respectively [107]) (Fig. (4) and Fig. (5)). The substitution by a quaternary ammonium functional group, as seen in CysNO, dramatically decreases the stability of the S-NO bond. On the contrary, the presence of a hydroxyl or an acetamido group such as in the second molecule has the opposite effect. Indeed, in the case of CysNO, the positively charged ammonium group leads to an electrostatic repulsion with the sulfur of the S-NO bond. On the opposite, the acetamido substituent of NACNO has a stabilizing effect by the formation of a 6-membered cycle between the carbonyl function of the acetamido and the sulfur of S-NO bond which lacks electrons (Fig. (4)). As a conclusion, the proportion of the D resonance form is highly increased in the case of NACNO and decreased for CysNO [14]. This also gives an explanation to the high stability of GSNO (Fig. (4) and Fig. (5)) *versus* its active metabolite *S*-nitrosocysteinylglycine (CysGlyNO) (half-lives of 41 h and 0.5 h in phosphate buffer respectively [107]) after GGT activity or *S*-nitroso- γ -glutamylcysteine (GluCysNO) (half-life of 42 h in phosphate buffer [107]).

Lastly, other chemical modifications of RSNO were directed in order to increase the drug lipophilicity or steric hindrance to improve the drug stability *versus* metallic ion attack. Two main strategies were undertaken, both being performed on SNAP as the core. On the one hand, a chemical modification was performed with the grafting of a tetra-*O*-acetylglucosamine moiety on the carboxylic function to obtain *N*-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-O-acetyl-β-*D*-glucopyranose (RIG200) to modify steric hindrance [104]. On the other hand, a carbon chain was added to the amido group of the molecule: with either a C1 to C4 *via* a grafted glycine upstream [116] or directly a C3 (*S*-nitroso-*N*-propionylpenicillamine, SNPP), a C5 (*S*-nitroso-*N*-valerylpenicillamine, SNVP) to C7 (*S*-nitroso-*N*-heptanoylpenicillamine, SNHP) [106,117]. The last cited modification led to the establishment of a correlation between measured partition coefficient (log P) and the carbon chain length, from log P = 1.5 for the C3 to 3.5 for C7 *versus* nearly 1 for SNAP, which is much higher than GSNO ($_{calculated}$log P = -2.7) or NACNO ($_{calculated}$log P = -0.46) (Fig. (5)). In each study, the new derivatives were more stable than the mother molecule *versus* catalytic decomposition by copper ions. Although, the efficient concentration 50 (EC_{50}) was not improved (Fig. (5)), a sustained release of •NO was demonstrated (> 1 h for SNHP compound [106] and > 4 h for the tetra-*O*-acetylglucosamine derivative, RIG200 [104] in presence of vessels). A linear correlation was observed between the lipophilic character of the compound (C3 to C7 RSNO) and the sustained vasorelaxation [106]. As a result, the mechanism of sustained vasodilation was hypothesised to be correlated to retention of the lipophilic RSNO derivatives into the lipid rich domains of the sub-endothelial layers of arteries. Indeed, the effect was obvious into endothelium-denuded vessels. The authors concluded to a selectivity of the compounds towards injured vessels [106].

According to these results, the physico-chemical data of *in vitro* stability and partition coefficient and the vasorelaxant efficiency are difficult to perfectly correlate, and therefore from Fig. (5), no general rule can be formulated. As a consequence, the design of the ideal RSNO appears as a real challenge. Although SNAP and its derivatives showed a delayed degradation through metallic ion attack but a vasorelaxant efficiency lower than GSNO, the sustained release of •NO appears interesting from a therapeutical point of view. Nevertheless, the development of *N*-acetylpenicillamine derivatives must include some immunological investigations since penicillamine is known to induce autoimmunity [118]. Moreover, the molecules based on a Cys core present a better vasorelaxant effect and for some of them a satisfying stability (GSNO and NACNO) nonetheless, a sustained delivery of •NO has never been described from these derivatives to the best of our knowledge.

Macromolecular Concepts and Macroassemblies for •NO Transport

The design of macromolecular •NO donors is based not only on polypeptides, or proteins, or polymeric structures but also on mixed structures.

Taking the previous chemical data into account and because GluCysNO has a greater stability (half-life of 42 h in phosphate buffer), *S*-nitroso derivatives of GSH were synthesized [107]. In this way, phytochelatins, (γ-glutamylcysteinyl)$_n$-glycine (n = 2 to 6 for $SNOPC_{2,\ 4,\ 6}$), were nitrosated and characterised [98,109]. The modified-glutathione derivatives showed a •NO release with a half-life of *ca.* 27 h under *in vitro* conditions. Their efficiency to induce a vasorelaxant effect was in relationship with the number of grafted –NO moiety on the molecule with EC_{50} of 0.14, 0.08 and 0.06 μM for $SNOPC_{2,\ 4,\ 6}$, respectively (Fig. (5)). These oligopeptides did not manage to produce a sustained release of the drug and the authors concluded to combine *S*-nitrosophytochelatins to drug delivery systems.

Among *S*-nitrosoproteins, the most studied ones remain AlbNO and its derivatives. As previously mentioned, AlbNO is the main nitrosated protein encountered in the blood [5]. Several procedures are described to obtain the modified protein. Different approaches are usually envisaged: either the simple nitrosation of albumin introducing one –NO moiety on the protein (involving the Cys34) [104,111], or more complex chemical modifications that aim at inserting more –NO groups on albumin *via* S-NO as well as N-NO bond formation (using an excess of nitrating agent) [119] or with the grafting of chemical groups to anchor more thiols into the protein structure that will then be nitrosated (i.e. Poly-SNO-BSA) [120,121]. Regarding the pharmacokinetic parameters, the half-lives of the nitrosated proteins were improved by the binding of a polyethylene glycol (PEG) group: 147 h for PEG- AlbNO *versus* 13.6 h for AlbNO in a buffer containing 1 % bovine serum [120] as compared to 5.5 h in human blood under *in vitro* conditions [104]. The chemical modification of albumin with the introduction of more than one –NO group leads to the highest content of –NO (up to 10 mol –NO/mol albumin [120]). Nevertheless, it also deeply modifies the physico-chemistry of albumin [119], its interaction with cells [121] and finally may induce some immunological properties conferred by the nitrosated as well as the denitrosated macromolecule [122].

Dendrimers which are tree-like polymers were used as a strategy to increase the loading of -NO onto a macromolecule. Thus, generation-4 polyamidoamine dendrimers were modified with RSNO, synthesized and characterised [123,124]. Sixty four units of either SNAP or NACNO were grafted onto the dendrimer skeleton. The *in vitro* half-lives in presence of copper (0.2 mM) of the as-synthesized polymers were evaluated at 2.3 min and 106 min for the derivatives of SNAP and NACNO in phosphate buffer, respectively [123]. Using the same concentrations, the dendrimer derivatives of SNAP showed a more pronounced efficiency to inhibit platelet aggregation compared to the unmodified RSNO. As a result, the authors concluded that these dendrimers appeared as a relevant strategy in ischemia-reperfusion injury.

A polypeptide/polymeric mixed macroassembly was also designed in order to control •NO release. Either GSNO or SNOPC was covalently linked to a polymer (poly(vinyl methyl ether-*co*-maleic anhydride)) and then interpolymer complexes were created with a poly(vinyl pyrrolidone) thanks to hydrogen bonds between both polymers [125]. As concerns GSNO, a 50 % grafting yield was

reached. Moreover, a controlled release of •NO was effective until day 10 under *in vitro* conditions for both RSNO. The complexes were applied to wound healing with interesting positive results.

Thus, the macromolecular structures cited here improved the loading of –NO and some of them induced a sustained release.

Innovative Drug Delivery Systems Applied to *S*-nitrosothiols

Various concepts were designed in order to enhance the RSNO stability towards the environment and also to ensure a sustained release of the drug. Studies describing RSNO delivery systems are summarised in (Table 2) and they are based on approaches related to particles, matrix or films or both.

S-nitrosothiol delivery systems manage to protect the molecule and to control the release of •NO. A complete proof-of-concept with an increase of the vascular flow was provided [133]. •NO delivery systems (including RSNO delivery systems) have already been extensively reviewed [92,134,135]. Nevertheless, some remarks have to be taken into consideration for further developments:

- The safety and biocompatibility of the formulations have to be fully demonstrated. This includes:
  - the stability of the drug delivery system tested in physiological conditions (temperature, ionic strength, pH) in order not to release some impurities;
  - a total absence of toxicity: since most of the delivery systems are characterised by a release half-life in the range of hours even days to develop a chronic treatment (for example 7 days of release half-life in *in vitro* conditions *i.e.* phosphate buffer [136]);
  - no activation of the immune system with respect of antigen presenting cell phenotype after administration of the formulations, not to induce an inflammatory phenomenon [137].
- Concerning the particle design with a vascular target aim, following points have to be considered:

Table 2. *S*-nitrosothiol Delivery Systems Formulated as Particles, Matrix or Film, or Both

Drug delivery system	S-nitrosothiol	Physicochemical characterisation	Main conclusions	References
Particle-based systems				
Particles prepared by ionic gelation with alginate/chitosan	GSNO	Diameter > 300 nm	Decrease of GSNO decomposition rate when entrapped into NP especially in the first hours.	[126]
Liposomes	NACNO associated with a photosensitizer	Liposomes (made of dipalmitoylphosphatidylcholine) filtered through a 0.45 µm membrane	Photodynamic therapy is possible using such particles.	[127]
Mercaptosilane-based silica	Nitrosation of the mercaptomoieties after NP preparation	Diameters tunable from 240 to 720 nm -SH content from 0.5 to 20 % wt/wt and 55 to 100 % nitrosation efficiency	•NO release from the NP exceeded 48 h.	[128]
Particles entrapped into matrices				
S-nitrosothiol-derivatized fumed silica polymer filler particles	CysNO, SNAP, NACNO	Diameters from 7 to 10 nm Total -NO loading from 21 to 138 nmol/mg NP	SNAP derived NP showed the highest –NO loading. Polymeric films containing the NP were prepared and released •NO upon photoexcitation	[129]
Liposomes entrapped into a porous silica matrix	SNAP	Liposomes (made of dimyristoyl-*sn*-glycero-3-phosphocholine, cholesterol and dihexadecyl hydrogen sulphate) diameters of 150 nm Loading equivalent to 35 nM into the final material	Release of •NO is controlled by photoexcitation with a rate comparable to a biological active one.	[130]
Matrix and films				
Polyethylene glycol matrix	NACNO	-	Stability enhanced into the PEG matrix. Storage possible for 10 weeks at -20°C	[131]
Polymeric films	GSNO	20.4 wt % into a film made of polyvinyl alcohol and polyvinyl pyrrolidone	Release of •NO and GSNO from the films with a half-life of 35.5 min.	[132]
Polymeric films	GSNO	5 to 30 % of GSNO with a loading efficiency of 30 to 70 %	Increase of vaginal blood flow during 210 min with the loaded films compared to 20 min for unformulated GSNO	[133]

- to improve the blood circulation half-life, addition of PEG onto the particle to ensure its stealthness from the mononuclear phagocytic system may be required [138];
- the size of particle is one major point since particles with a diameter/dimension in the range 100 to 200 nm were shown to be able to accumulate into the inner layers of the arteries [139].

Development of a drug delivery system dedicated to the transport and protection of a •NO donor is one way to avoid the interaction with degrading factors such as proteins. It was already mentioned that an increase of Trx concentration in the blood can be associated to diseases linked with oxidative stress such as cardiovascular pathologies. In these cases, in which a •NO donor can be a relevant therapeutic strategy, a particulate system that on the one hand limits Trx •NO release and on the other hand allows a controlled delivery at the targeted site should be interesting. Moreover, it is noteworthy that drug delivery systems can be internalized by cells. Regarding particles, two major kinds of cells have to be distinguished: antigen presenting cells which are professional phagocytes (since they are the first line of defence and have to screen the entire organism to fight the non-self) and the others for which the phagocytic activity is not the main one. Three main pathways of internalisation are nowadays well described: phagocytosis, endocytosis (including clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin and caveolae independent endocytosis and macropinocytosis) [140] and the fusion with cell membrane. The last one is observed in the case of some liposomes or some vesicles [141], and it allows the release of the particle content directly into the cytosol, whereas the other processes may confine the particle inside an organelle (for example endolysosomes). Therefore, using the appropriate drug delivery system, the interaction with proteins can be limited (for example circulating Trx and membrane PDI) to deliver •NO and/or to nitrosate protein inside the cell. In summary, the assembly of a stable RSNO and a carrier appears the best way to deliver •NO under a controlled pathway.

GENERAL CONCLUSION

The present review points out the emerging role of RSNO as a new therapeutic class fighting vascular dysfunctions and permitting to overcome drawbacks of actual •NO donors. This is demonstrated by:

(i) the actual intense investigation on RSNO interaction with proteins leading to *S*-nitrosation, a translational pathway of main importance in cell signalling leading to physiological action such as vasorelaxation,

(ii) and also by many clinical trials based on administration of GSNO, a physiological RSNO.

As GSNO bioavailability has to be improved, it is necessary to define which parameters should be taken in consideration in order to increase the half-life of new designed RSNO. Structural properties of the molecular skeleton bearing the pharmacophore group SNO as well as the molecule lipophilicity appear interesting to improve their stability and to facilitate their (nano)formulation, respectively, even if no exact relationship between these physicochemical properties and *in vivo* efficiency has been established at this time. Another real perspective in RSNO design is also to consider their interaction with enzymatically active proteins such as PDI//Trx presently reviewed.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

AlbNO	=	*S*-nitrosoalbumin
ASK1	=	Apoptosis-regulating kinase-1
CapNO	=	*S*-nitrosocaptopril
cGMP	=	Cyclic guanosine monophosphate
Cys	=	Cysteine
CysGlyNO	=	*S*-nitrosocysteinylglycine
CysNO	=	*S*-nitrosocysteine
EC	=	Endothelial cells
ER	=	Endoplasmic reticulum
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
sGC	=	Soluble guanylate cyclase
GGT	=	γ-glutamyltranspeptidase
GluCysNO	=	*S*-nitroso- γ -glutamylcysteine
GSH	=	Reduced glutathione
GSNO	=	*S*-nitrosoglutathione
GTN	=	Glyceryl trinitrate
HcysNO	=	*S*-nitrosohomocysteine
JNK	=	Jun N-terminal kinase
Lys	=	Lysine
MAPK	=	Mitogen-activated protein kinase
NP	=	Nanoparticles
NACNO	=	*S*-nitroso-*N*-acetylcysteine
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NOS	=	Nitric oxide synthases
eNOS	=	Endothelial nitric oxide synthase
iNOS	=	Inducible nitric oxide synthase
nNOS	=	Neuronal nitric oxide synthase
NP	=	Nanoparticles
PDI	=	Protein disulfide isomerase
PEG	=	Polyethyleneglycol
Pro	=	Proline
RIG200	=	*N*-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranose
RSH	=	Thiol
RSNO	=	*S*-nitrosothiol
SIN-1	=	Linsidomine
SNAP	=	*S*-nitroso-*N*-acetylpenicillamine
SNHP	=	*S*-nitroso-*N*-heptanoyl-*D,L*-penicillamine
$SNOPC_2$	=	*S*-nitrosophytochelatin-2
$SNOPC_4$	=	*S*-nitrosophytochelatin-4
$SNOPC_6$	=	*S*-nitrosophytochelatin-6
SNP	=	Sodium nitroprusside
SNPP	=	*S*-nitroso-*N*-propanoyl-*D,L*-penicillamine
SNVP	=	*S*-nitroso-*N*-valeryl-*D,L*-penicillamine
TNFα	=	Tumor necrosis factor alpha
Trp	=	Tryptophan

Trx	=	Thioredoxin
oTrx	=	Oxidized thioredoxin
rTrx	=	Reduced thioredoxin

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