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### **S-nitrosothiols as selective antithrombotic agents - possible mechanisms.**

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## **S-NITROSO THIOLS AS SELECTIVE ANTITHROMBOTIC AGENTS – POSSIBLE MECHANISMS**

Short running title: S-nitroso thiols as selective antithrombotic agents

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## Summary

S-nitrosothiols have a number of potential clinical applications, among which their use as antithrombotic agents has been emphasised. This is largely because of their well-documented platelet inhibitory effects which show a degree of platelet selectivity, although the mechanism of this remains undefined. Recent progress in understanding how NO-related signalling is delivered into cells from stable S-nitrosothiol compounds has revealed a variety of pathways, in particular denitrosation by enzymes located at the cell surface, and transport of intact S-nitrosocysteine via the amino acid transporter system-L (L-AT). Differences in the role of these pathways in platelets and vascular cells may in part explain the reported platelet-selective action. In addition, emerging evidence that S-nitrosothiols regulate key targets on the exofacial surfaces of cells involved in the thrombotic process (for example, protein disulphide isomerase, integrins and tissue factor) suggests novel antithrombotic actions which may not even require transmembrane delivery of NO.

## Keywords:

S-nitrosothiols, nitric oxide, platelets, coagulation, protein disulphide isomerase, L-AT, nitrosation

## Abbreviations:

csPDI	Cell surface protein disulphide isomerase
cyclic GMP	Guanosine 3':5'-cyclic monophosphate
cysNO	S-nitrosocysteine
DAF-FM	4-amino-5-methylamino-2'7'-difluorofluorescein
GSNO	S-nitrosoglutathione
L-AT	Amino acid transporter system L
PDI	Protein disulphide isomerase
RSNO	S-nitrosothiol
sGC	Soluble guanylate cyclase
tPA	Tissue plasminogen activator

### *Introduction*

Cardiovascular disease is the most common cause of death in developed countries and arterial thrombosis, following rupture of an atherosclerotic plaque, underlies most cases of myocardial infarction and stroke. Thrombus formation involves the rapid accumulation of blood platelets and fibrin into an occlusive mass within the blood vessel. Platelets adhere to collagen and von Willebrand factor exposed in the ruptured plaque, and their activation is upregulated by locally generated thrombin, thromboxane and ADP, with subsequent surface display of integrin adhesion molecules (principally  $\alpha\text{IIb}\beta\text{3}$ ) in their active conformation, allowing fibrinogen binding and platelet aggregation (Mackman, 2008). Thrombin-mediated fibrin formation follows triggering of the blood coagulation cascade by exposure (Steffel, Luscher and Tanner, 2006) or de-encryption (Bach, 2006) of tissue factor, either located within the plaque itself or arriving with the cells accumulated by the thrombus. Following these initiating events, thrombin generation is amplified and propagated by assembly of coagulation enzyme complexes on the surface of activated platelets and other cells (Hoffman and Monroe, 2001) with resulting fibrin deposition.

### *Endothelial dysfunction and loss of nitric oxide bioactivity*

Intravascular platelet activation is evident in ischaemic syndromes affecting both the coronary (Gurbel *et al.* 2004) and cerebral circulations (Badimon and Vilahur, 2007), and interactions between activated platelets and the vessel wall are thought to contribute not only to the final thrombotic events in atherosclerotic disease, but also in the initiation and progression of atheroma (Langer and Gawaz, 2008). Failure to control platelets in such circumstances is due to endothelial dysfunction, in particular the loss of nitric oxide activity (Vanhoutte *et al.* 2009). Common conditions such as diabetes mellitus, hypertension and renal failure are characterised by chronic oxidative stress which increases cardiovascular risk, in part by diminishing the availability of bioactive nitric oxide (NO) and thus permitting platelets to contribute to thrombosis (Freedman and Loscalzo, 2003). These considerations provide a rationale for NO supplementation using donor agents to limit thrombotic risk

and a variety of compounds are available for this purpose (Miller and Megson, 2007). This review will focus on the anti-thrombotic potential of S-nitrosothiols (RSNOs), and particular attention will be paid to the mechanism by which these compounds deliver NO to platelets and other cells of the vascular compartment, since there has been progress in this area which may explain reports of selective anti-platelet action from certain RSNO molecules.

### *S-nitrosothiols*

S-nitrosothiols are a class of compounds produced by the S-nitrosation of sulphhydryl groups (usually cysteine thiols), and with the general formula R-SNO. They are sometimes referred to as thionitrites in the older chemical literature and their general chemistry and biological properties are documented in several review articles (Al-Sa'doni and Ferro, 2000, Hogg, 2000), as are prospects for their therapeutic use (Richardson and Benjamin, 2002). Their attractiveness as therapeutic agents is increased by the fact that they occur naturally in blood and tissues as endogenous metabolites of NO, suggesting that toxicity associated with their use might be low.

Following the identification of NO as the active component of nitrovasodilator drugs (Murad, 1999) and the molecule responsible for the endogenous activity known as endothelium derived relaxing factor (Palmer, Ferrige and Moncada, 1987), it was suggested that physiological RSNO formation might provide a means to stabilise and extend the activity of NO (Myers *et al.* 1990, Stamler *et al.* 1992a). Despite the fact that NO itself is a poor nitrosating agent, plausible mechanisms exist for RSNO formation within the biological environment (Zhang and Hogg 2005) and these compounds, principally in the form of S-nitrosoalbumin and S-nitrosohaemoglobin, but also as low molecular weight forms (S-nitrosocysteine, s-nitrosocysteinylglycine, S-nitrosogluthione) are found to be naturally occurring in blood and tissues (Giustarini *et al.* 2003). Circulating RSNO concentrations are a matter of dispute, largely through differences in methodology (Stamler, 2004), however most reports put them in the low nanomolar range.

RSNOs are simple and cheap to synthesise in the laboratory, an advantage if they were to be employed as drugs. Among the suggested clinical uses of RSNOs, their potential as anti-thrombotic agents has often been highlighted.

There is a substantial literature recording the platelet-inhibitory action of RSNOs, and a number of mechanisms have been identified. In addition, a smaller number of reports suggest that other components of the haemostatic system (for example coagulation and fibrinolysis) may be influenced by these agents.

#### *Antiplatelet actions of RSNOs*

Very soon after the identification of NO as an endogenous mediator, it was shown that NO inhibited platelet function and that this inhibition coincided with stimulation of soluble guanylyl cyclase (sGC) and intra-platelet cyclic GMP accumulation (Radomski, Palmer and Moncada, 1987). Nitrovasodilator drugs, including various RSNO compounds, had already been shown to suppress platelet aggregation via sGC stimulation (Mellion *et al.* 1983), and further reports documented cyclic GMP-mediated inhibition of platelet adhesion, aggregation, granule secretion and fibrinogen binding by S-nitrosocysteine (cysNO) and S-nitrosoglutathione (GSNO) (Radomski *et al.* 1992, Lieberman, O'Neill and Mendelsohn, 1991, Mendelsohn *et al.* 1990). Accumulation of the second messenger cyclic GMP influences platelet function by activation of protein kinase G, with consequent phosphorylation of numerous intracellular targets and inhibition of processes including calcium mobilisation, integrin  $\alpha$ IIb $\beta$ 3 activation, cytoskeleton rearrangement, granule secretion (Schwarz, Walter and Eigenthaler, 2001), activity of thromboxane receptors (Wang *et al.* 1998) and phosphoinositide 3-kinase (Pigazzi *et al.* 1999). It should be noted that cyclic GMP-induced platelet inhibition can under some circumstances occur independently of NO (Riba *et al.* 2008), however the physiological importance of this mechanism, relative to endothelial NO release, is not yet clear.

Although sGC stimulation probably represents the primary mode of action of NO donor agents, including RSNOs, it nevertheless became evident that platelet control is also exerted by cyclic GMP-independent mechanisms (Gordge, Hothersall and Noronha-Dutra, 1998)(Pawloski, Swaminathan and Stamler, 1998, Sogo *et al.* 2000). A variety of molecular alterations have been proposed to mediate this process, including prevention of thromboxane

synthesis (Tsikas *et al.* 1999), nitration of  $\alpha$ -actinin (Marcondes *et al.* 2006) , inhibition of the platelet P2Y<sub>12</sub> ADP receptor (or, more precisely its cellular signalling partners) (Kokkola *et al.* 2005) and either S-nitrosylation (Walsh *et al.* 2007) or altered phosphorylation (Oberprieler *et al.* 2007) of the important platelet integrin  $\alpha$ IIb $\beta$ 3. There appears to be a requirement for extracellular generation of NO to occur before cyclic GMP-independent inhibition of calcium signalling and platelet aggregation can be brought about by NO donor compounds, including RSNOs (Crane, Rossi and Megson, 2005) .

#### *Other effects of RSNOs on the haemostasis process*

Haemostasis involves the interaction of a variety of components, including platelets, vascular cells and proteins of the coagulation and fibrinolytic systems, however relatively little work has been published on the direct influence of nitric oxide on coagulation and fibrinolysis. Clot formation via thrombin-induced polymerisation of fibrin and its subsequent crosslinking by factor XIII is accelerated by nitrating, but not non-nitrating oxidants, suggesting that nitrosative stress may bring about a pro-thrombotic state (Vadseth *et al.* 2004) , although paradoxically others have reported inhibition of fibrin polymerisation by peroxynitrite (a recognised nitrating agent) (Lupidi *et al.* 1999). Conversely, exposure of fibrinogen to the RSNO compound GSNO suppresses fibrin polymerisation (i.e an anti-thrombotic effect) through what appears to be an allosteric interaction separate from covalent modification of the fibrinogen molecule (Geer *et al.* 2008, Akhter *et al.* 2002). RSNOs may further oppose thrombosis by inhibiting the action of transglutaminase enzymes, including coagulation factor XIII (Lai *et al.* 2001, Catani *et al.* 1998) . The major vascular initiator of fibrinolysis is tissue plasminogen activator (tPA), and S-nitrosylation of tPA, as might be brought about via transnitrosation from RSNO molecules, confers antiplatelet properties on tPA without altering its fibrinolytic action (Stamler *et al.* 1992b). A further intriguing, and possibly crucial aspect of coagulation control by RSNOs involves their involvement in switching tissue factor (the main physiological trigger for coagulation) into a coagulation inactive form (Ahamed *et al.* 2006). This fits into an emerging paradigm in which tissue factor activity

is regulated by a variety of post-translational modifications (Egorina, Sovershaev and Osterud, 2008), in particular redox modification of an allosteric disulphide bond (Chen *et al.* 2006). It should be noted, however, that the concept of redox regulation of tissue factor is contested (Pendurthi *et al.* 2007).

Thus, via a variety of pathways beyond simple inhibition of platelet function, RSNO compounds show potential for anti-thrombotic action.

#### *Anti-thrombotic action of RSNOs - in vivo studies*

Both low molecular weight and protein forms of RSNO suppress platelet activation in animal models (Radomski *et al.* 1992, Keaney *et al.* 1993), and the anti-platelet action of GSNO coincided with improved tissue survival in a rat model of ischaemia/reperfusion injury (Kuo *et al.* 2004). Novel anti-thrombotic RSNO molecules with chemical modifications conferring selectivity for areas of vascular injury (Miller *et al.* 2003) or for platelets (Vilahur *et al.* 2004) have been developed, and shown to be effective in rabbit and porcine models.

In human patients, GSNO limits platelet activation in severe preeclampsia (Lees *et al.* 1996). In addition, a number of small clinical trials have documented a significant anti-thrombotic and/or anti-embolic effect of GSNO administration, following surgical procedures such as coronary artery bypass grafting (Salas *et al.* 1998), carotid endarterectomy (Molloy *et al.* 1998) and carotid angioplasty (Kaposzta *et al.* 2002). An interesting property of GSNO identified during human in vivo studies was that it showed a degree of platelet selectivity, in that platelet inhibition could be demonstrated with doses of GSNO that failed to produce significant vasodilation (de Belder *et al.* 1994). The mechanism of this platelet selective behaviour was not fully defined, but the authors speculated that it might relate to different abilities of platelets and other vascular cell types to mediate enzymatic release of NO from GSNO.

#### *Delivery of NO signalling by RSNO compounds*

The tissue effects of RSNOs (cyclic GMP generation, vasodilation, platelet aggregation inhibition) are not shown by the non-nitrosated parent thiol compounds (Mathews and Kerr, 1993), implying that RSNOs must act via

transmission of NO-related signals. Nevertheless it was recognised in the early 1990s that the rate of spontaneous NO release from different RSNOs failed to correlate with their corresponding potencies in bioassay systems, so therefore spontaneous NO liberation could not explain the biological actions of RSNOs (Mathews and Kerr, 1993, Kowaluk and Fung, 1990) . This lack of correlation probably reflects the fact that NO release from RSNOs into solution almost always results from catalysis by copper (I) ions, whereas *in vivo* other pathways are involved. The mode of intracellular delivery of NO from RSNOs is more complex and RSNOs cannot be viewed as simple “NO donors”.

#### *Cellular metabolism of RSNOs*

Cellular metabolism is one possible means of NO delivery and a number of studies have documented NO release from RSNOs mediated by intact platelets and other cell types (Simon *et al.* 1993a, Gordge *et al.* 1998, Liu *et al.* 2001, Zeng, Spencer and Hogg, 2001, Cornwell *et al.* 2003, Shah *et al.* 2003). In addition, RSNOs are substrates for a variety of enzymes including glutathione peroxidase (Freedman *et al.* 1995), a copper (I)-dependent enzyme (Gordge *et al.* 1996),  $\gamma$ -glutamyl transferase (Hogg *et al.* 1997), thioredoxin reductase (Nikitovic and Holmgren, 1996), superoxide dismutase (Jourdeheuil *et al.* 1999), protein disulphide isomerase (Sliskovic, Raturi and Mutus, 2005), cytoplasmic metalloprotein (Mani *et al.* 2006) and GSNO reductase (glutathione-dependent formaldehyde reductase, or alcohol dehydrogenase 3) (Liu *et al.* 2001, Liu *et al.* 2004, ). This latter enzyme appears to play a crucial role in regulating nitrosative stress via adjustment of intracellular levels of S-nitrosylated proteins (Staab *et al.* 2009, Foster *et al.* 2009), however there is no direct evidence yet for a role in the transfer of NO signalling from extracellular RSNOs.

#### ***Cell surface protein disulphide isomerase promotes NO delivery across the plasma membrane***

Of the enzymes mentioned above, protein disulphide isomerase (PDI) has perhaps the best credentials as a mediator of RSNO signalling. PDI was

originally characterised as a resident of the endoplasmic reticulum, assisting in the correct folding of nascent proteins (Gruber *et al.* 2006). In recent years PDI has been documented at locations outside the ER, including the cell surface, cytosol and nucleus (Turano *et al.* 2002). Cell surface isomerases, including PDI (csPDI), have attracted particular research interest through their involvement in infectious disease (Conant and Stephens, 2007), HIV entry into CD4 positive lymphocytes (Barbouche *et al.* 2003), platelet aggregation (Burgess *et al.* 2000, Lahav *et al.* 2003, Jordan *et al.* 2005, Robinson *et al.* 2006, Manickam *et al.* 2008), and control of tissue factor activity (Chen *et al.* 2006, Versteeg and Ruf, 2007). Studies in mice have confirmed an *in vivo* role for csPDI in both fibrin generation and platelet thrombus formation (Cho *et al.* 2008), thus csPDI has a direct bearing on haemostatic regulation.

A further line of research has shown that csPDI plays an important role in NO signalling, specifically the transfer of NO from extracellular membrane-impermeant RSNOs across the plasma membrane of target cells (Zai *et al.* 1999, Ramachandran *et al.* 2001, Bell, Shah and Gordge, 2007)). The best-developed model described so far postulates that csPDI denitrosates RSNO molecules in the vicinity of the plasma membrane, releasing NO which then enters the membrane by virtue of its lipophilicity and combines there with oxygen to produce the nitrosating agent  $N_2O_3$ . When this, in turn, nitrosates target molecules on the cytoplasmic side of the plasma membrane the goal of NO internalisation is achieved (Ramachandran *et al.* 2001). Our own experimental studies have confirmed the role of csPDI in delivery into platelets of NO-related signalling from RSNOs, however we also found that active csPDI was necessary for signal delivery from donors of nitroxyl ( $NO^-$ ) and of NO (Bell, Shah and Gordge, 2007). csPDI-mediated denitrosation should not be required for entry of NO, and further work is therefore needed to reconcile these results. Redox mechanisms involving the vicinal thiols of the csPDI active site underlie the process of RSNO denitrosation (Sliskovic, Raturi and Mutus, 2005). The published scheme shows plausibly how a single enzyme turnover brings about NO release, but the mechanism of active site thiol regeneration, required to continue RSNO signalling, is not yet defined. Several studies have documented thiol oxidation within csPDI and loss of enzyme activity as a result of the interaction with RSNO (Zai *et al.* 1999, Root

*et al.* 2004, Shah *et al.* 2007). Redox regeneration of csPDI may derive from both internal sources, via trans-membrane oxidoreductases such as NAD(P)H oxidase, and/or from reducing equivalents present in blood plasma. The relative importance of these various systems, and the effects of oxidative / nitrosative stress on csPDI-mediated processes, need to be known for a full understanding of csPDI pathophysiology.

### ***Some RSNO molecules are delivered intact via membrane transporters***

An alternative means of RSNO-mediated signalling is by cellular uptake of an intact RSNO molecule via a membrane transporter. Evidence has emerged from a number of different laboratories showing that the low molecular weight RSNO compounds S-nitroso-L-cysteine (CysNO) and S-nitrosohomocysteine act as substrates for the widely-distributed amino acid transporter system-L (L-AT). This mechanism for transmembrane transport of cysNO could explain why stereoselective haemodynamic effects are seen following administration of L-cysNO and D-cysNO to rats (Davisson *et al.* 1996), and experimental studies carried out *in vitro* have confirmed its presence in a number of cell types including erythrocytes, (Sandmann, Schwedhelm and Tsikas, 2005), endothelial cells (Broniowska, Zhang and Hogg, 2006), vascular smooth muscle cells (Li and Whorton, 2007, Riego *et al.* 2009), epithelial cells (Granillo *et al.* 2008), and various transformed cell lines (Zhang *et al.* 2004, Li *et al.* 2005) although to date there has been no direct demonstration of cysNO uptake via L-AT in platelets. In these published studies other forms of RSNOs, including GSNO, S-nitroso-cysteinyl-glycine, S-nitroso-N-acetyl-penicillamine and S-nitrosoalbumin, failed to be transported via L-AT, nor could they mediate NO-related signalling in target cells unless extracellular cysteine was supplied. In the presence of extracellular cysteine, cysNO is formed from the inert RSNO by a process of transnitrosation, with subsequent uptake on the L-AT system and intracellular signal transmission. If only cystine is available, a cystine-cysteine shuttle mediated by the  $X_c^-$  aminoacid transport system can import cystine and subsequently release cysteine into the surrounding medium following intracellular reduction, thus providing substrate for transnitrosation and L-AT mediated uptake of cysNO (Li and Whorton, 2005, Zhang and Hogg, 2004). This mechanism has been shown to be relevant for a

wide range of signalling events and also for the accumulation of intracellular RSNOs. Experiments have generally been performed using relatively high RSNO concentrations (20  $\mu$ M upwards) and endpoints measured after RSNO exposure for at least 15 minutes. An interesting feature to emerge from these studies is that in general, cellular effects mediated by cysNO / L-AT are insensitive to the presence in the extracellular medium of NO scavengers, such as oxyhaemoglobin, thus excluding NO release from the mechanism (Zhang and Hogg, 2004, Zhu *et al.* 2008). An exception is when cysNO-mediated stimulation of sGC is considered – this process is inhibited by oxyhaemoglobin, but only because intracellular reduction of cysNO to NO is required before a cyclic GMP response can occur (Riego *et al.* 2009).

***Different modes of RSNO delivery may explain their selective antithrombotic action***

RSNOs are potent platelet inhibitors (see above) but it is not yet clear that their antiplatelet actions require prior conversion of RSNO to cysNO and transport into the platelet via the L-AT system. *In vitro* aggregation of washed platelet suspensions is inhibited by a range of RSNO molecules without need for addition to the surrounding medium of cysteine or cystine (Radomski *et al.* 1992, Gordge, Hothersall and Noronha-Dutra, 1998, Mathews and Kerr, 1993, Simon *et al.* 1993b), and unlike L-AT mediated actions, platelet inhibition (both cyclic GMP-dependent and –independent) is inhibited by haemoglobin (Radomski *et al.* 1992, Crane, Rossi and Megson, 2005, Megson *et al.* 2000), implying that release of free NO must occur as part of the process. For protein RSNOs, such as S-nitrosoalbumin, to inhibit platelet aggregation, there does appear to be a requirement for prior transnitrosation to a low molecular weight thiol for the anti-platelet action to be realised, however this function can be fulfilled as efficiently by glutathione or cysteinyl-glycine as by cysteine (Crane *et al.* 2002). Studies using washed platelets have not included measurement of cysteine concentrations in the surrounding medium, and it is therefore possible that cysteine released by the platelets themselves mediates NO signal transfer via L-AT, however this seems unlikely since the anti-platelet action of GSNO, for example, is evident at lower concentrations (10  $\mu$ M or

less) and within a more rapid timeframe (< 2 minutes) than effects reported for L-AT-mediated signalling. Furthermore, if the inhibitory action of GSNO depended upon cysNO / L-AT then it might be expected to be more potent in platelet rich plasma (where plasma cysteine/cystine is available) than in washed platelet suspensions, whereas in fact the reverse is true (Radomski *et al.* 1992). In a recent publication addressing the possible modes of intra-platelet transport of NO, neither cyclic GMP accumulation nor DAF-FM fluorescence in response to GSNO was significantly inhibited by the L-AT inhibitors BCH or L-leucine (Bell, Shah and Gordge, 2007). Therefore, despite a wealth of evidence for the importance of the cysNO / L-AT system in endothelial and smooth muscle cells of the vascular wall, platelets appear to respond to RSNOs in a different way (figure 1). If this anomalous behaviour of platelets, compared with other vascular cells, can be confirmed then a possible explanation for the reported platelet-selectivity of GSNO is suggested (de Belder *et al.* 1994), since, at low concentrations, this molecule may have access to a direct anti-thrombotic action on platelets that is not available to endothelial or smooth muscle cells.

The susceptibility of GSNO's anti-platelet action to NO scavenging by haemoglobin suggests instead that it undergoes platelet-mediated metabolism, either by a (so far uncharacterised) copper-dependent surface enzyme (Gordge *et al.* 1995, Gordge *et al.* 1996), or by csPDI which is known to be present on platelets (Essex, Chen and Swiatkowska, 1995) and capable of releasing NO (Root, Sliskovic and Mutus, 2004). If differences exist in csPDI expression between platelets and vascular cells, then it might be possible to exploit this to provide selective anti-thrombotic action. The inhibition of platelet csPDI which results from interaction with RSNO molecules (Shah *et al.* 2007) is potentially a major antithrombotic mechanism, since there is abundant evidence that active csPDI is required for platelets to function efficiently during haemostasis (Essex, 2004).

Another possible reason why RSNOs might mediate selective anti-thrombotic effects is that a number of functionally important nitrosation targets exist on the *external* surface of platelets. These include csPDI itself but also the adhesion molecules glycoprotein 1b (Burgess *et al.* 2000) and integrin  $\alpha$ IIb $\beta$ 3

(Walsh *et al.* 2007, Yan and Smith, 2000). Allosteric disulphides on tissue factor and other haemostatically active extracellular proteins (Chen and Hogg, 2006) present further possible targets for alteration by plasma RSNOs. Thus although RSNO-mediated signalling to the vessel wall via intracellular thiol modification requires processing via the cysNO / L-AT system, the ability of GSNO (and other RSNOs) to modify important exofacial targets on platelet and other cells may confer selective anti-thrombotic action (figure 2).

### *Conclusion*

Possible mechanisms for selective anti-thrombotic action of RSNOs may be summarised as:

1. Differences between platelets and cells of the vascular wall in expression of RSNO metabolising enzymes, such as csPDI, and / or dependence on csPDI for signal transmission.
2. Differences between platelets and cells of the vascular wall in expression of the L-AT system, or dependence on cysNO / L-AT as a mode of NO delivery.
3. A heightened role in platelets, compared with cells of the vascular wall, for modification of target proteins on the *external* surface of the plasma membrane, thus allowing at least partial inhibitory effects to be achieved without need for intracellular delivery of NO.
4. RSNO-mediated regulation of blood coagulation, in particular tissue factor exposed at sites of vessel injury or on circulating monocytes or platelet microparticles. This might be either a direct effect or secondary to csPDI modification.

There are few experimental data directly comparing these different mechanisms between platelets and cells of the vascular wall, so it is difficult to grade the mechanisms in order of importance. Nevertheless, the evidence currently available suggests a scenario in which denitrosating enzymes on platelets permit low concentrations of RSNO to mediate anti-thrombotic NO signalling, whereas higher concentrations of RSNO are required for vasodilatory signalling via the cysNO / L-AT mechanism. A further impeding factor may be the need for RSNO to cross the endothelial monolayer to gain

entry into vascular smooth muscle, both of which steps involve cysNO / L-AT. At present these ideas remain speculative, however they do suggest lines of enquiry that might help define and realise the anti-thrombotic potential of RSNO compounds.

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#### Statement of conflicts of interest

None

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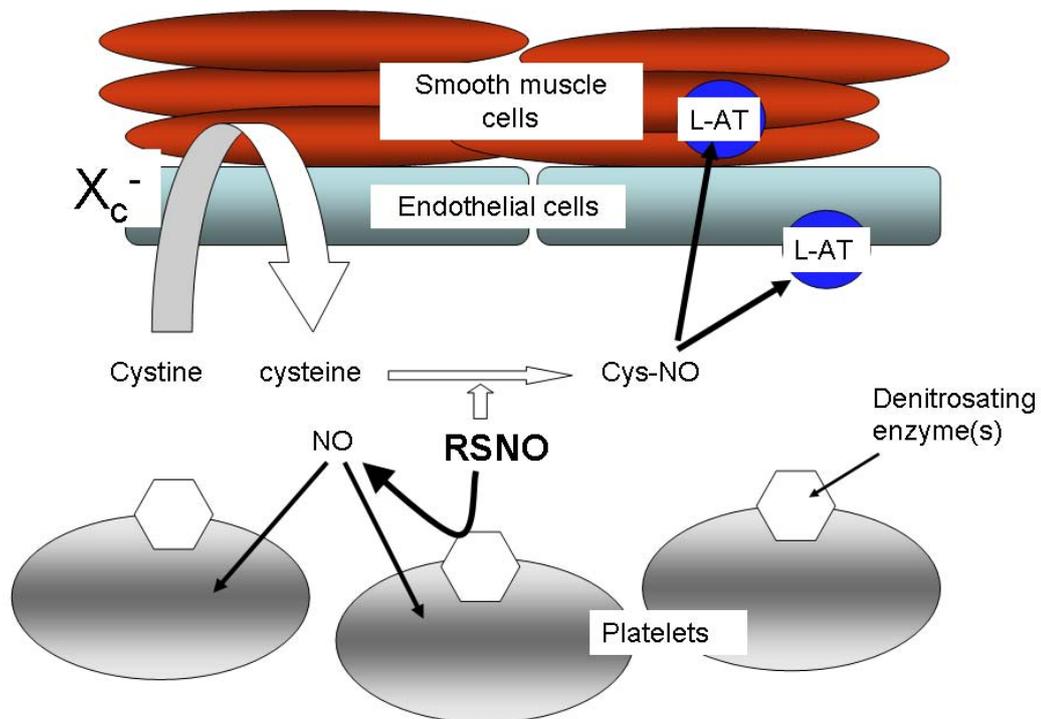
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## Figure Legends

### Figure 1

*Different modes of NO delivery from RSNOs.* Cells of the vascular wall import NO principally via uptake of cysNO on the L-AT amino acid transporter system, following an extracellular process of transnitrosation from RSNO to cysteine. A cystine-cysteine shuttle mediated by the  $X_c^-$  transporter may act as a supply of extracellular reduced cysteine. In contrast NO delivery into platelets relies on the activity of cell surface denitrosating enzymes, such as csPDI. This scheme indicates the main routes of NO uptake, but does not exclude the possibility that alternative or additional routes are available for each cell type.



**Figure 2.**

*Cell surface targets for the antithrombotic action of RSNOs.* Inhibition of the thrombotic process may be mediated without the need for intracellular NO entry, by inactivation of platelet surface adhesion molecules and/or of tissue factor exposed on the surface of the damaged vascular wall, on activated monocytes or circulating microparticles. These modifications occur indirectly via RSNO-induced inhibition of csPDI, and also possibly via direct transnitrosation of the target molecule.

