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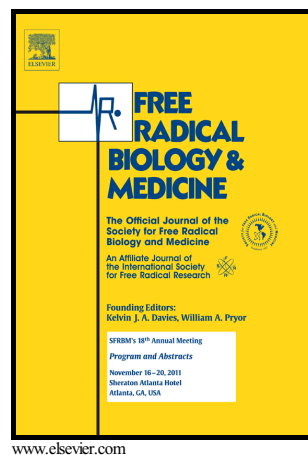
Kathryn Wolhuter, Philip Eaton

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How widespread is stable protein S-nitrosylation as an end-effector of protein regulation?

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Abstract

Over the last 25 years protein S-nitrosylation, also known more correctly as S-nitrosation, has been progressively implicated in virtually every nitric oxide-regulated process within the cardiovascular system. The current, widely-held paradigm is that S-nitrosylation plays an equivalent role as phosphorylation, providing a stable and controllable post-translational modification that directly regulates end-effector target proteins to elicit biological responses. However, this concept largely ignores the intrinsic instability of the nitrosothiol bond, which rapidly reacts with typically abundant thiol-containing molecules to generate more stable disulfide bonds. These protein disulfides, formed via a nitrosothiol intermediate redox state, are rationally anticipated to be the predominant end-effector modification that mediates functional alterations when cells encounter nitrosative stimuli. In this review we present evidence and explain our reasoning for arriving at this conclusion that may be controversial to some researchers in the field.

Introduction

Nitric oxide (NO) is perhaps best known as the endothelium-derived relaxation factor responsible for vasodilation, but it also plays fundamental roles in a diverse array of disparate biological processes [1]. Much of the biology associated with NO production is via it binding and altering the function of heme proteins [2]. For example NO activates soluble guanylyl cyclase in this way, stimulating synthesis of the second messenger cyclic guanosine monophosphate which binds and activates protein kinase G to induce phosphorylation-dependent signaling [3]. NO and a number of related NO chemical variants can also react with cellular molecules to yield nitroso (i.e. R-NO) derivatives [4], which may alter their physiochemical properties and so perhaps also their biological activities. Thiol (R-SH) containing molecules are especially susceptible to such nitrosation reactions, and the formation of these species in proteins is considered to occur widely. Formation of S-nitrosothiols (P-S-N=O), a process commonly referred to as S-nitrosylation, is widely held to be a pervasive post-translational modification that serves as a regulatory mechanism by altering protein function. Indeed, protein S-nitrosylation is often presented as an evolutionary-conserved signalling mechanism that affects most, if not all, classes of proteins. The putative roles for protein S-nitrosothiols within the cardiovascular systems is vast with over 1000 proposed targets in the heart alone [5]. Many reviews ascribe S-nitrosothiols as end-effectors of NO signalling that contribute to homeostasis during health [6-8], with dysregulation of these processes contributing to disease

pathogenesis. For example, hyper- or hypo-S-nitrosylation contributes to a range cardiovascular disease including type 1 and 2 diabetes [9], atherosclerosis [10], cardiac ischemic injury [11], hypertrophy [12] and sepsis [13].

The current consensus is that S-nitrosylation, much like phosphorylation, is a stable post-translational modification that directly mediates signaling by altering the function of end-effector proteins. However, such a widespread role would appear incompatible with the inherent lability of the nitroso bond [14], especially *in vivo* where thiol-containing molecules are abundant and react rapidly with S-nitrosothiols to generate disulfide bonds [15, 16]. These transient S-nitrosothiol intermediates can yield intramolecular or intermolecular protein disulfides, as well as disulfides with small molecular weight thiols such as glutathione (GSH) or cysteine. These disulfide post-translational modifications, which are fully anticipated to occur widely when S-nitrosylation reactions occur in cells, have been known for many decades to regulate protein function [17]. Disulfide formation is also known to play a crucial role in redox signalling within the cardiovascular system, however it is less well studied in the setting of nitrosative stress [18].

Although transient S-nitrosothiols are already acknowledged to be one mechanism by which disulfides form [19, 20], it is important to reconsider and highlight such studies. This is because the vast majority of work relating to protein S-nitrosylation fails to recognise that in many cases, if not most, a disulfide is likely the predominant post-translational modification that mediates NO-dependent alterations in function. This does not mean that protein S-nitrosylation does not occur or play crucial roles in biological systems, but instead that the current vision of many that it is a ubiquitous end-effector of protein function is likely to be significantly incorrect. It is notable that the term nitros**yl**ation chemically refers to NO bound to a transition metal, whereas a more correct chemical term for NO bound to a thiol is S-nitrosation. The inclusion of the '**yl**' may be used to infer that it reflects an enzyme-mediated process, perhaps instead of passive chemistry. S-nitrosylation is not only an inappropriate term that can facilitate confusion, but is perhaps deliberately used in an attempt to conflate and assist with establishing parallels with other post-translation regulatory mechanisms such as phospho**ryl**ation. For this reason, the chemically correct term S-nitrosation is predominantly used in this review. Our aim is to critically evaluate the evidence for S-nitrosation as the stable post-translational and universal end-effector modification that it is so widely perceived or advocated to be.

Stable protein S-nitrosation as a regulatory end-effector mechanism

Strong parallels have been drawn between protein S-nitrosation and other well-established post-translational modifications, especially phosphorylation [21, 22]. Kinases are often co-localised with their substrates, with additional selectivity achieved by consensus motifs in the primary sequence of a target protein that is phosphorylated. Similarly, targets of S-nitrosation can localise with NO synthase (NOS) enzymes [8], and may contain linear amino acid consensus motifs [23], thus mirroring key features of kinase-mediated phosphorylation. Furthermore, denitrosylase enzymes that remove NO from proteins are described to play the corresponding role of phosphatases in phospho-regulated proteins [22]. Protein S-nitrosation as a post-translational regulatory mechanism

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is additionally supported by the fact that it can occur stoichiometrically [24], and that pharmacological inhibition of the enzyme that mediates denitrosation leads to elevated S-nitrosation [25]. Moreover genetic ablation of a target cysteine not only prevents formation of the S-nitroso modification, but also abrogates the functional alteration that otherwise occurs in the wild type protein [26]. It would therefore seem irrefutable that stable protein S-nitrosation is a ubiquitous end-effector regulatory mechanism. However, when the primary evidence for each of the individual facets that support a role for regulation by stable S-nitrosation is considered in depth, we contend that significant discrepancies become apparent. Indeed, as considered below, we suggest that protein S-nitrosation per se is unlikely to directly underlie functional alterations to the extent it is commonly described to.

Enzymatic regulation of protein S-nitrosation

A keystone in the conceptual foundations of regulatory S-nitrosation was the identification of so-called 'nitrosylases' and 'denitrosylase' that enzymatically regulate protein nitrosothiol levels by adding or removing the modification respectively. Genetic knock out of neuronal NOS *in vivo* resulted in decreased S-nitrosation of several proteins, illustrating that S-nitrosation is ultimately NOS dependent [27]. NOS enzymes have therefore been described as nitrosylases, playing an analogous role to kinase-mediated phosphorylation, by co-localising with target proteins susceptible to S-nitrosation [28]. NOS-derived reactive nitrogen species can directly transfer to target proteins directly or alternately this can occur indirectly, via a cascade of trans-nitrosation transfer reactions. Direct binding of NOS to a target has been shown experimentally for inducible NOS and cycloxygenase-2 [29]. Indirect trans-nitrosation by NOS via intermediate scaffold and adaptor proteins was summarized by Hess *et al.* [8]. They described how the N-methyl-D-aspartate (NMDA) receptor is S-nitrosated by neuronal NOS [30] via postsynaptic density protein 95 (PSD95), which serves as a scaffold. PSD95 is known to interaction with both the neuronal NOS [31] as well as the NMDA receptor [32], supporting its role as a scaffold. However, whether disrupting PSD95 interactions alters S-nitrosation of NMDA receptor remains unclear. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can also transfer NO from itself to another target protein thiol, therefore acting as a trans-nitrosylase [33]. These examples of localized direct or trans-nitrosation-mediated modification of proteins involving organized multicomponent signaling complex are often assumed to reflect a generic, widespread situation. Whilst there are multitudes of studies describing co-localized, multicomponent signalosomes in the context of phospho-regulation, we note that studies providing primary experimental evidence for protein S-nitrosation to be generically organized in this way are relatively rare.

A significant argument against the existence of regulatory nitrosylases is that S-nitrosothiol formation is principally a chemical reaction between sulfur and various derivatives of NO that occurs rapidly and without enzyme catalysis [34]. As NO is unable to directly oxidise thiols at a meaningful biological rate, S-nitrosothiol formation is dependent on oxidation of NO to a reactive forms in the presence of a one-electron acceptor, such as oxygen [35]. Several mechanisms for attack by NOS-derived reactive nitrogen species have been proposed, which are summarised in **Figure 1** [36].

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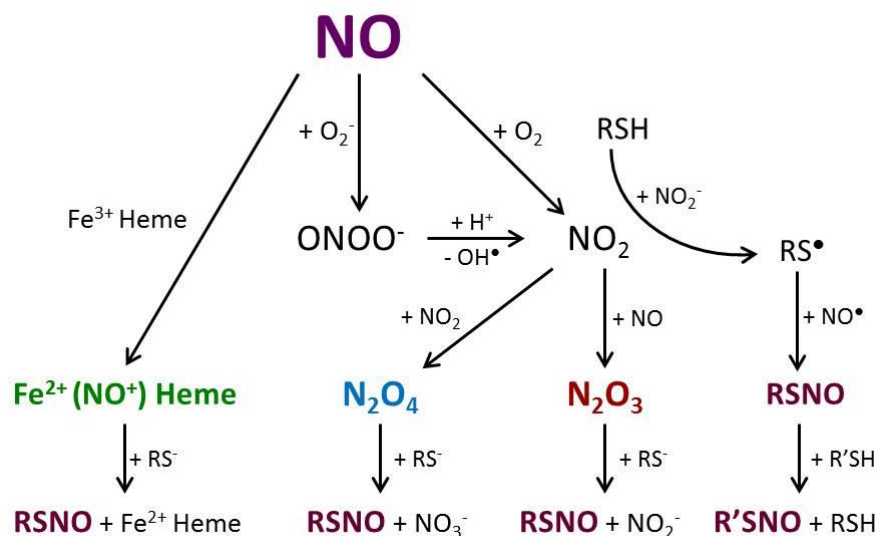
Figure 1. *Proposed mechanisms of S-nitrosation.* Formation of S-nitrosothiols (RSNO) is predicted to occur through multiple distinct pathways. Nitric oxide (NO) can react with transition metals to form an intermediate followed by direct transfer of NO to target thiols. NO will also react with superoxide to form peroxynitrite (ONOO⁻) followed by loss of $^{\bullet}OH$ to form NO_2, although perhaps this is unlikely to occur under physiological conditions. It is perhaps more likely that NO_2 is formed by NO reacting with oxygen. NO_2 then forms nitrosating species, such as N_2O_3 and N_2O_4, that directly S-nitrosate target thiols. Intermediates in the NO/NO_2 pathway can convert a thiol to a thiyl radical ($RS^{\bullet}$) which then directly react with $NO^{\bullet}$ forming RSNO. Finally thiols can be trans-nitrosated whereby one S-nitrosothiol transfers its NO group directly to another thiol.

In the presence of oxygen one prominent theory is S-nitrosation via the formation of N_2O_3 ($^{+}ON...NO_2$) (**eq. 1-2**).

(**eq. 1**) $2NO + O_2 \rightarrow 2\ NO_2$

(**eq. 2**) $NO_2 + NO \rightarrow N_2O_3 = {^{+}ON...NO_2}^{-}$
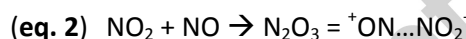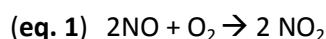
S-nitrosothiols are formed by the electrophilic attack of a deprotonated thiol (PS⁻), known as a thiolate, by NO^{+}. Here formation of N_2O_3 is the rate-limiting step in S-nitrosation as it occurs relatively slowly in aqueous solutions ($6.6 \times 10^6\ M^{-2}.s^{-1}$); although this increases to $8.8 \times 10^7\ M^{-2}.s^{1}$ in the lipid phase or within hydrophobic protein pockets [36]. Kinetic modelling has predicted that physiologically NO concentrations, likely <1 μM, would result in femtomolar concentrations of N_2O_3 [37]. Therefore, when taking diffusion into account, S-nitrosation is likely to primarily occur when a NOS enzyme is proximal to a target protein thiol, especially those in a hydrophobic environment. The efficiency of this third-order reaction is highly dependent on the concentration of oxygen. If oxygen is depleted, such as during hypoxia, S-nitrosation is unlikely to occur via this NO_2/N_2O_3 pathway. At a low oxygen concentration S-nitrosation may instead occur though the recombination of NO with a thiyl radical ($1-3 \times 10^9\ M^{-1}.s^{-1}$) [38]. The amount and complexity of nitrosating species produced *in vivo* is further complicated by the ability of NO to rapidly react with superoxide to form peroxynitrite ($7 \times 10^9\ M^{-1}.s^{-1}$) [39]. Peroxynitrite can protonate and then liberate $^{\bullet}NO_2$ which can combine with NO to generate nitrosating N_2O_3 [40]. However, peroxynitrite can also induce disulfide formation in proteins independently of S-nitrosothiol intermediates [41]. The propensity of NO to

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form peroxynitrite in environments where superoxide or hydrogen peroxide is elevated, such as the mitochondria, adds complexity when considering the extent to which disulfides form via S-nitrosothiols during nitrosative stress.

Mechanisms of protein denitrosation

Several enzyme systems are thought to specifically catalyse the denitrosation of S-nitrosothiols. This includes the thioredoxin (Trx) system, comprising Trx and Trx reductase (TrxR) [22, 42]. Denitrosation and subsequent activation of caspase-3 was shown by Benhar *et al.* to be regulated by the Trx-TrxR system *in vivo* [43]. The ability of Trx to remove S-nitrosothiols is dependent on its active site Cys-X-X-Cys motif, which accepts the NO from the target by a trans-nitrosat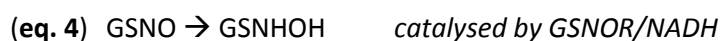ion reaction. Glutaredoxin (Grx) and GSH reductase (GR) may also remove protein S-nitrosothiols in a similar thiol-dependent manner [44]. Thus, at face value it seems clear that the cell is equipped with several denitrosylase enzymes. However, this somewhat simplistic view needs further consideration. Each of these enzymes is thiol-dependent and given S-nitrosothiols react rapidly with thiols, one wonders if any protein with a reactive thiolate could serve this denitrosation role. Indeed, even less reactive protonated thiols such as in GSH, as discussed below, react rapidly to catalyze protein denitrosation. When we also consider that a disulfide is the product of this passive chemical reaction of a nitrosothiol with a resolving thiol, we then have to consider the extent to which the abundant Trx, Grx or GR enzymes are likely to selectively denitrosylate proteins in cells. Is it possible that that these enzymes simply play their well-established roles as disulfide reductase enzymes after an S-nitrosothiol transitions to the disulfide state [45]? Pharmacological inhibition of Trx with auranofin leads to the accumulation of S-nitrosated proteins, supposedly providing evidence of this enzyme as a denitrosylase [43]. However, blockade of this well-established, classical disulfide reductase activity is anticipated to generate the same result by limiting the resolution of S-nitrosothiols via disulfide formation. Similarly, recombinant Trx was used to reduce cellular proteins induced to the S-nitrosated state by treatment with S-nitrosocysteine [46], which were then alkylated and identified by mass spectrometry – to supposedly identify targets of S-nitrosation. A potential caveat is that that S-nitrosocysteine also induces widespread protein disulfides [47], which Trx disulfide reductase is fully capable of reducing and so many disulfide-modified proteins are likely to have been misidentified as S-nitrosated. To be clear, we fully anticipate Trx to be capable of reducing S-nitrosated proteins, as thiol-containing molecules will generically do this, but whether it does so directly in cells, or whether they first transition to a disulfide is difficult to establish (**Figure 2**). Discriminating between protein S-nitrosation and disulfide formation, as discussed more below, can be problematic and the possibility that the latter may form is often not considered.

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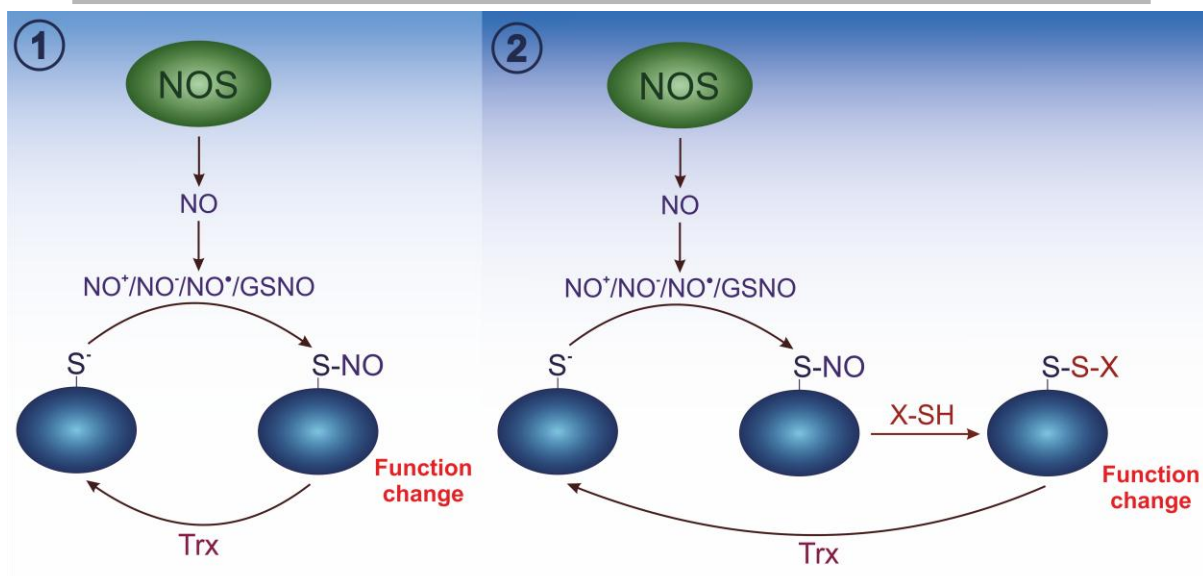
Figure 2. *Are the end-effectors in NO-dependent signal transduction S-nitrosothiols or disulfide bonds?* Nitric oxide synthase (NOS)-derived nitric oxide (NO) forms reactive nitrogen species including $NO^+/NO^-/NO^{\bullet}/S$-nitrosogultathione (GSNO) which may in turn react with protein thiolates (S^-) to generate protein S-nitrosothiols (S-NO). S-nitrosothiols are widely thought to directly alter the function of a protein, serving as the end-effector modification in NO-mediated signalling pathways ①. However, the nitroso bond is inherently labile and has the propensity to rapidly react with free thiols (X-SH) resulting in disulfide bond (S-S-X) formation ②. Disulfide bonds are also known to change the function of proteins. Due to the high concentration of free thiols as well as the inherent lability of S-nitrosothiols, it is likely that the majority of S-nitrosothiols act as transient intermediates in the formation of regulatory disulfide bonds. Both nitro-oxidative modifications are reduced by thioredoxin (Trx) making it difficult to discriminate which modification was in place.

Cells also contain an S-nitrosoglutathione (GSNO) reductase (GSNOR) system comprising of GSH, GSNOR and GR [13, 48]. GSNOR is thought to be the key enzyme in the regulation of plant S-nitrosothiols, with some evidence that it plays the same role in mammals. Much of the evidence comes from GSNOR$^{-/-}$ mice where protein S-nitrosation in the heart and lung increased during disease. It should be noted however, at base line their global protein S-nitrosative state was indistinguishable between genotypes, apart from elevated S-nitrosated haemoglobin in the transgenic [49]. GSNOR does not directly catalyse the removal of NO from protein thiols but rather reduces the trans-nitrosation 'NO pool' by removing GSNO. GSNO is converted to GSNHOH using NADH as a cofactor. GSNHOH can either react with GSH to produce glutathione disulfide (GSSG) and hydroxylamine (NH_2OH) (**eq. 3-6**), which is rapidly reduced by thiolate anions to ammonia [50], or rearranges and then spontaneously hydrolyses to produce GSO_2H and ammonia [51]. GSSG is then reduced back to GSH by GR using NADPH as a cofactor.

(**eq. 3**) RSNO + GSH $\rightarrow$ GSNO + PSH

(**eq. 4**) GSNO $\rightarrow$ GSNHOH   *catalysed by GSNOR/NADH*

(**eq. 5**) GSNHOH + GSH $\rightarrow$ GSSG + HN$_2$OH

(**eq. 6**) GSSG $\rightarrow$ GSH   *catalysed by GR/NADPH*
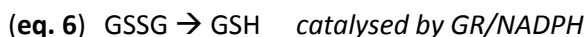
However, S-nitrosothiols also react with GSH non-enzymatically to generate S-glutathiolated protein (RSSG) in a reaction that also generates nitroxyl (HNO). Formation of HNO adds additional complexity, as it also reacts with GSH (6×10^6 M^{-1} s$^-$ [52]) to produce the same products without the need for catalysis by GSNOR (**eq. 7-9**).

(**eq. 7**) RSNO + GSH $\rightarrow$ RSSG + HNO

(**eq. 8**) GSH + HNO $\rightarrow$ GSNHOH

(**eq. 9**) GSNHOH + GSH $\rightarrow$ GSSG + HN$_2$OH

S-glutathiolated proteins can then be reduced by another GSH molecule, resulting in a free protein thiol and GSSG [53]. This process highlights how, when viewed from an alternate perspective, S-nitrosation is fundamentally regulated by non-enzymatic chemistry. Above we highlighted that the reputed protein denitrosylase enzymes Trx, Grx or GR are perhaps simply abundant proteins known to resolve disulfides, but because of their reactive thiols they are capable of resolving S-nitrosothiols. Similarly GSNOR also has well-established aldehyde and formaldehyde dehydrogenase activities, which again are dependent on catalytic thiols [54]. That perturbations in a major thiol-containing proteins impacts on GSNO levels and perhaps also protein S-nitrosation, may not be definitive evidence that it formally serves as an endogenous cellular denitrosylase.

A consensus motif for selective and targeted protein S-nitrosation

An important aspect of a post-translational regulatory control system is selectivity in terms of targeting proteins for modification. In the case of kinase-dependent phosphorylation this is in part mediated by linear consensus motifs within the substrate. Perhaps based on an inclination to draw or anticipate parallels between protein S-nitrosation and phosphorylation, it was rational to assess whether there was also a linear consensus motif that provides selectivity in target S-nitrosation. Indeed, a consensus motif for S-nitrosation was proposed in 1994 when it was found that many protein S-nitrosothiols have basic or acidic amino acid preceding the cysteine followed by an acidic amino acid [55]. From this a consensus motif consisting of a core of three residues (K/R/H/D/E)–C–(D/E) was proposed to predict S-nitrosation in hydrophobic protein domains [23]. The computational prediction software GPS-SNO (http://sno.biocuckoo.org/) was used to analyze 467 experimentally verified S-nitrosothiol sites in 302 proteins, to develop algorithms that computationally 'predict' S-nitrosation from a primary sequence alone. Experimentally these algorism were found to be 75% accurate at predicting sites of S-nitrosation [56]. However, these motifs have been criticised due to the high concentrations of NO donor used in the founding experiments to map S-nitrosation sites [57, 58], and so provide a platform for the prediction algorithm. Supra-physiological concentrations of NO may have resulted in the identification of motifs that lack biological significance. As considered below all molecules (including reactive NO species) will progressively interact with more targets, i.e. become less selective, as they are used at higher concentrations – binding and modifying proteins it would not at lower, physiological levels.

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Instead of a simple linear motif, the residues surrounding a thiol in its tertiary structure are perhaps more likely to control its reactivity with S-nitrosating species. The impact of the residues surrounding a thiol was studied using GAPDH as a model. GAPDH Cys-149 was shown to be activated by an adjacent histidine, which deprotonates it to render it a stronger nucleophile, enabling cleavage of the S-N bond within GSNO resulting in either S-glutathiolation or S-nitrosation [19]. Pérez-Mato *et al.* described the crystal structure of methionine adenosyl transferase and found Arg-357 and Asp-363 are in proximity to the active site Cys-131. These residues facilitate S-nitrosation by acting as partial electron acceptors for NO, enhancing its electrophilicity to increase its reactivity with the target thiol [59]. These observations indicate that S-nitrosation is significantly determined by protein topology, perhaps more so than a linear consensus motif, by influencing the chemical reactivity between a specific derivative of NO and the target thiol. Furthermore, topology may also determine whether target S-nitrosation occurs via trans-nitrosation by a protein or non-protein S-nitrosothiol, or directly by NO-derived species. Finally, even if there is some form of linear or 3D motif for S-nitrosation, it does not mean the resulting S-nitrosothiol is the end effector that alters function. The formation of an S-nitrosothiol may target a protein to undergo further redox modification, including disulfide formation, as part of a redox signalling cycle.

Considerations on the stoichiometry of protein S-nitrosation

For a post-translational alteration to impact on the function of a protein and so modulate cellular signalling, the modification should occur to a significant proportion of the protein pool, i.e. occur with significant stoichiometry. It is difficult to reconcile a low stoichiometry with a regulatory role for a modification as most of the protein would remain unaffected, unless the post-translational modification induced a new activity which could provide a useful means of regulation. If S-nitrosation was to serve as a simple activity switch, it becomes more difficult to conceive it as an efficient control mechanism without significant target occupancy. The stoichiometry of modification occupancy is especially important when considering the potential for regulatory protein S-nitrosation, as its inherent chemical lability at least conceptually limits it in this role. As discussed above, S-nitrosothiols readily transition to disulfide species by reaction with thiols, which are abundantly present in proteins, as well as many small 'antioxidant' biomolecules such as cysteine, lipoic acid or GSH. GSH alone is typically present at millimolar concentrations therefore, unless a S-nitrosothiol is shielded, it is likely to rapidly react to form an S-glutathiolated protein, a modification that widely regulates protein function [35].

Though the stoichiometry of S-nitrosation is rarely investigated there are a few studies that address this. Perhaps the most frequently cited example is Mannick *et al.*, who found ~85% of mitochondrial capase-3 is basally S-nitrosated at its Cys-136 active site [24]. They suggested this prevents inappropriate caspase auto-activation, but failed to consider that this may be disulfide mediated. Indeed, S-glutathiolation of caspase-3 at Cys-136 also induces inhibition [60]. Complex I was shown to be stoichiometrically S-nitrosated *in vitro* by GSNO, with 7 mol of S-nitrosothiols per mol of complex I. But unexpectedly this only corresponded to a 20% reduction in activity, casting doubt on the functionality of this modification [61]. Soluble guanylyl cyclase was inhibited by S-nitrosation which occurred with a stoichiometry of 2:1 mol S-nitrosothiol per mol protein [62]. This inhibition was readily reversed in the presence of GSH, perhaps indicating that in the reducing environment of the cell the S-nitrosated state would not accumulate unless GSH was also depleted. Finally, Wang *et*

al. used mass spectrometry to compare the relative stoichiometry of S-nitrosation and disulfides formation on several cysteines within glutathione-S-transferase Pi after exposure to S-nitrosocysteine. S-nitrosation was accompanied by disulfide formation, but is was notable that disulfides formed at low concentrations of S-nitrosocysteine (5 µM), preceding measurable increases in S-nitrosation [47]. Only after excess S-nitrosocysteine (>400 µM) was utilised did the proportion of S-nitrosated protein surpass that of disulfide. This is consistent with S-nitrosothiols rapidly transitioning to disulfides, with stable S-nitrosothiols only accumulating when supra-physiologic S-nitrosating variants of NO are present. Such conditions likely compromise the many cysteine-dependent cellular reducing enzymes via S-nitrosation-mediated inactivation of their catalytic thiols.

Further considerations of the stability of protein S-nitrosothiols

For a post-translational modification to participate in signalling it must occur in biologically-relevant time scales. The reaction of NO with iron present in heme centers is a rapid, second-order reaction with a rate in the order of 10^7 $M^{-1}.s^{-1}$ [63, 64]. It is postulated that under aerobic conditions the most likely mechanism of protein S-nitrosation is via formation of N_2O_3 which is third-order with a rate of 6.6×10^6 $M^{-2}.s^{-1}$ [36]. For this slower, oxygen-dependent reaction to compete with higher-order reactions for biological significance the products must be very stable. The bond energy of an S-N bond has been found to vary from 22 to 32 kcal/mol, meaning the lifetime of the bond could theoretically span from seconds to years [8]. However, the stability of S-nitrosothiols is unclear as published half-lives vary dramatically and are clearly condition-dependent [65-68]. Non-protein S-nitrosothiols studied *in vitro* in the presence of metal chelators are relatively stable with a half-life of S-nitrosocysteine reported at 11 hours [69], although this is decreased to less than 2 minutes when metal ions are present [70], or to less than 20 seconds in the case of cysteine ethyl ester cysteine [16]. The rate of S-nitrosothiols decomposition via transnitrosation is second-order, although rates vary depending on the chemical properties of the thiols involved [66]. Transnitrosation between S-nitroso-N-acetylpenicillamine and GSH was observed to be 5.4 $M^{-1}.s^{-1}$ whereas transfer of NO from GSNO to cysteine was 675.8 $M^{-1}.s^{-1}$ [71]. Decomposition of non-protein S-nitrosothiols in the presence of free thiols is also predicted to generate disulfides, as discussed in more detail below, although the rate of disulfide formation varies greatly. For example, rapid disulfide formation in dihydrolipoic acid though an S-nitrosothiol intermediate occurred too rapidly for the S-nitrosothiol intermediate to be detected by standard techniques [16], whereas the rate of disulfide formation is GSSG generated by GSH reacting with GSNO is predicted to be relatively slow *in vitro* with a rate of 8.3×10^{-3} $M^{-1}.s^{-1}$ [72]. How these *in vitro* kinetic experiments on low-molecular-weight S-nitrosothiols relate to the stability of protein S-nitrosothiol, which is more relevant to this review, is less clear. Given that proteins are well established targets of S-nitrosation, the rate at which they individually react with thiols they encounter is perhaps more important, although less studied. Paige *et al.* exposed cell lysates to GSNO and used the biotin-switch method to identify ten proteins resistant to reduction by GSH, and thus considered them stably S-nitrosated under basal conditions [73]. However, as they examined over one hundred proteins and only found ten to be stable over a half hour period, another interpretation of this study is that the majority of protein S-nitrosothiols are not stable in the presence of GSH.

The lability of S-nitrosothiols may mean they are in most cases too unstable to provide a widespread means of post-translational regulation. Alternatively, it is perhaps conceivable that rapid S-

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nitrosation and denitrosation reactions allow regulation on a shorter time scale than other more stable post-translational modifications. For example, the co-localisation of ion channels with NOS enables NO synthesis to parallel calcium concentrations. Ion channels regulate cardiac excitability and contractility with millisecond timescales, raising the possibility that thiol-reactive NO species modulate heart function via protein S-nitrosation on a beat-to beat basis [74]. Recently we reported that disulfide formation in myocardial protein kinase G Iα rapidly regulates diastolic relaxation [75], consistent with this oxidative modification also being capable of regulation with a millisecond timescale. Whilst both these are possibilities are interesting, the half-life of these modifications has yet to be determined.

Reversible protein S-nitrosothiol formation is thought to contribute to cardioprotection afforded by ischemic preconditioning [76]. One concept is that S-nitrosylation prevents over-oxidation of important cysteinyl residues during ischemia and reperfusion. This protective S-nitrosylation comes at the expense of inhibiting these thiol-dependent enzymes, but this is reversible and eventually the activity can recover, whereas over-oxidation causes permanent inactivation [6]. But to reiterate the considerations above, S-nitrosylation is anticipated to yield disulfides, which have been observed in isolated hearts after nitrosative stress [77]. Of course protein disulfide formation would also offer cardioprotection from cysteine over-oxidation reactions, and indeed preconditioning also induces widespread disulfide bond formation [78], perhaps via S-nitrosothiol intermediates.

Is NO-dependent signal transduction mediated by stable S-nitrosothiols or disulfide bonds?

Whether S-nitrosothiols or disulfides formed though S-nitrosothiol intermediates are the predominant functional end-effectors in the majority of NO-mediated signalling pathways has yet to be determined. Many proteins that have long been known to be regulated by disulfide formation are now being 'rediscovered' as targets for S-nitrosation, as discussed below. There are potential advantages to disulfide formation over S-nitrosothiols in terms of mediating post-translational regulation. For example, disulfides are more stable with a bond energy of 60 kcal/mol and do not undergo spontaneous degradation [79]. As a result functional changes to the protein are anticipated to be more long-lived, potentially enhancing their ability to control cell signalling by providing a more stably robust regulatory mechanism [80].

S-thiolation has been shown to be directly regulated by NO levels *in vivo*, with mice overexpressing cardiac-specific inducible NOS showing elevated protein S-glutathiolation [81]. This is likely explained by the elevated NO inducing S-nitrosated protein, which as explained above, then react with thiols, especially with abundant GSH, to form disulfides. Additionally, acetylcholine increased cellular protein S-glutathiolation which was attenuated by inhibition of endothelial NOS [81]. As well as directly regulating protein function, S-thiolation is also thought to protect proteins from oxidative stress, possibly by abrogating cysteine hyperoxidation to the sulfinic or sulfonic acid states [82, 83]. Mechanistic studies suggest that during nitrosative stress protein S-glutathiolation is kinetically more likely via a protein S-nitrosothiol intermediate than an exchange reaction of the protein thiol with GSSG [84]. Despite protein S-thiolation having been recognised early on as a cellular response to nitrosative stress [85], the rates at specific protein S-nitrosothiols react with small thiols to promote S-thiolation have yet to be comprehensively determined.

Protein sulfenic acids (P-SOH) are well-established redox state-intermediates that lead to formation of disulfide bonds in proteins after exposure to oxidants such as hydrogen peroxide and peroxynitrite [86, 87]. The sulfenic acid reacts with a second thiol that it comes in contact with to liberate water and form the disulfide. Studies by Arnelle and Stamler found that the rate of intramolecular disulfide formation is associated with hydroxylamine formation and *in vitro* when DTT was added to GSNO or S-nitrosocysteine hydroxylamine was detected. Additionally, 1,3-dithiols have a faster rate of formation than 1,4-dithiols, which indicates that the spatial distances between thiols critically influences the rate of S-nitrosothiol-induced disulfide formation. They postulated that S-nitrosation of a neighbouring thiol via trans-nitrosation can lead to the formation of nitroxyl anion by oxidation of thiol to disulfide [16]. S-nitrosation of Cys-296 in Akt1/protein kinase Bα was observed to accelerate its interaction with Cys-310 to form Cys-296/Cys-310 intramolecular disulfide [88]. These data provide evidence that S-nitrosothiols not only act as an intermediate in the formation of disulfides but actively accelerated their formation.

Proteomic screens have reputedly identified thousands of proteins in the heart that are susceptible to S-nitrosation [89-91], however the corresponding functional effects of S-nitrosation have only been determines for a handful of these targets. Many proteins that are reported to be regulated by S-nitrosothiol formation in the cardiovascular system have also been shown, most often previously, to be regulated by disulfides, often at the same cysteine residue. For example, S-nitrosation and disulfide formation has been shown to induce the same functional change in hypoxia-inducible factor 1α-subunit [92, 93], caspase-3 [60, 94], Na^+/K^+ ATPase [95, 96] and actin [84, 97]. A summary of proteins that form S-nitrosothiols as well as disulfides is provided in **Table 1**, some of which are discussed in more detail below. For proteins that form both S-nitrosothiols and disulfide bonds after nitrosative stress it is important to determine which modification is stoichiometrically dominant and therefore likely underpins the functional change.

Table 1. Selected examples of cardiovascular proteins that are regulated by both S-nitrosation and disulfide bond formation.

Protein	Nitroso site	Effect of S-nitrosothiol	Detection method and tissue	Ref.	PSSG PSSP PSSH	Disulfide site	Effect of disulfide	Detection method and tissue	Ref.
actin	374	Impaired polymerization and NO donor	Cross-linking reactions and activity assay in vascular smooth muscle	[98]	PSSG	10, 374	Impaired polymerization	MALDI-TOF/MS of aorta, western blotting of ischemic heart	[84] [99]
aldose reductase	298	Activation – denitrosation "switched off" enzyme	ESI⁺–MS on purified protein	[100]	PSSG	298	Inhibition of activity	MALDI-TOF/MS of IR heart	[101]

ATP synthase α-subunit	294	Inhibition of activity and reduces ATP consumption during IR	MALDI-TOF/MS of Langendorff-perfused heart	[76]	PSSG **PSSP**	163, **294**	Disrupt nucleotide binding and inhibition of activity	MALDI-TOF/MS of aorta	[84]
caspase 3	163	Inhibits activity	Biotin-switch assay of cardiomyocytes	[94]	PSSG	163, 184, 220	Protects protein from cleavage and cell death	Western blotting in HUVECs	[60]
complex I	137**?**	Inhibits activity, and attenuates ROS generation during IR	Activity assay and biotin-switch in mitochondria isolated from heart	[102]	PSSG	206	Regulates superoxide production	MS/MS of purified complex I from heart tissue	[103]
endothelial nitric oxide synthase	99, 101	Inhibition of activity	Activity assay and biotin-switch in BAECs, MALDI-MS of purified protein	[104] [105]	PSSG	689, 908	Switch to superoxide production	EPR spin trapping and MS/MS in EC and aorta	[106]
glyceraldehyde phosphate dehydrogenase	150	Mitochondrial trans-nitrosylase (In neurones: nuclear translocation of the complex)	MS/MS of Langendorff-perfused heart (MALDI-TOF/MS of cerebral granule neurons)	[33] [107]	PSSG *PSSH*	150	PSSG: Inhibition of enzyme activity PSSH: activation of enzyme activity	PSSG: HPLC analysis of Langendorff-perfused heart PSSH: MS/MS on HEK-293	[108] [109]
hypoxia-inducible factor 1α-subunit	533	Protein stabilised	Biotin-switch in melanoma cells, MI in GSNOR⁻/⁻ mice	[92]	PSSG	533	Protein stabilised	MS/MS in ischemic muscle	[93]
myosin	697**?** 707**?**	Reduction in ATPase activity	SNO-RAC and biotin-switch assay in myotubes and muscle	[110]	PSSG	400, 695, 947	Conformational change and altered ATPase activity	MALDI-TOF/MS of myocardium	[111]
Na⁺/K⁺ ATPase	46	Inhibition of pump at β1 subunit	Biotin-switch assay of myocardium	[95]	PSSG	46	Inhibition of pump at β1 subunit	Pump activity assay in cardiac myocytes	[96]
RAS p21 protein activator 1	118	Chemical process of PSNO formation results in enhanced guanine nucleotide	MS and NMR on purified protein	[112]	PSSG	118	Increased activity and activation of downstream pathways	Western blotting of BAECs, MALDI-TOF/MS of cardiac myocytes	[113] [114]

		exchange						
ryanodine receptor type 1	235, 315, 811, 906, 1040, 1303, 3635	Enhances Ca^{2+} activation, increases probability of channel open	MALDI-TOF/MS of skeletal myotubes	[115] [116]	PSSG **PSSP**	**36**, 235, 315, 811, 906, 1591, **2326**, **2363**, 3193, **3635**	Decreases Mg^{2+} inhibition, promotes channel opening	MALDI-TOF/MS of skeletal myotubes	[115] [116]
sarco/endoplasmic reticulum Ca2+-ATPase	344, 349, 471, 498, 525, 674, 3635	No change in Ca^{2+} uptake activity	HPLC-MS of skeletal muscle	[117] [40]	PSSG	674	Increased SR Ca^{2+} uptake	MALDI-TOF/MS of aorta	[118]

Glyceraldehyde-3-phosphate dehydrogenase

Several studies have shown that S-nitrosation of GAPDH at Cys-150 modulates its function. S-nitrosation of GAPDH in neurones is associated with its translocation to the nucleus and initiation of apoptosis by stabilising the E3 ubiquitin ligase Siah1 [107]. More recently GAPDH has also been described as a cardiac trans-nitrosylase in the heart, transferring NO between proteins [33]. However GAPDH is also regulated by disulfide formation at the very same Cys-150 site, for example undergoing both S-glutathiolation and S-nitrosation in response to GSNO [19]. Each of these modifications inhibited the dehydrogenase activity of GAPDH *in vitro*, but when only S-nitrosation was induced activity returned within 30 minutes - whereas inhibition by S-glutathiolation did not [108]. This shows that GAPDH S-nitrosation can occur but is unstable and the inhibition it mediates is quickly reversed, and this occurs independently of denitrosylase enzymes. Moreover, it demonstrates the increased stability and functionality of disulfides. GAPDH is also activated by persulfidation of Cys-150 [109], highlighting that different oxidative modifications on the same cysteine may have the same or differing functional effects.

Aldose reductase

Another example of the divergent function of redox modification on protein function is aldose reductase. Analysis of purified aldose reductase by mass spectrometry indicated S-nitrosation of Cys-298 activated the protein [100]. However, in the heart, ischemia and reperfusion resulted in S-glutathiolation of aldose reductase and this was inhibitory [101]. S-glutathiolation, which was determined to have occurred though the S-nitrosothiol intermediate, operated as an 'off' switch that limits NO-dependent nitrosative activation.

RAS p21 protein activator 1

S-nitrosation of Cys-118 was originally thought to enhance guanine nucleotide exchange on Ras [119]. Recently a combination of MS and NMR techniques were used to show that stable S-

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nitrosation of Ras does not alter its structure, but instead changes its interaction with its down-stream signalling partner Raf or, perhaps more importantly, alters its guanine nucleotide exchange rate. However, the formation of a thiol radical intermediate during S-nitrosation interfered with guanine nucleotide substrate binding which mediated enhanced guanine nucleotide dissociation [112]. Although S-nitrosation of Ras occurs, this is followed by a rapid exchange reaction and Cys-118 S-glutathiolation [120], which is associated with increased activation of downstream partners.

Ryanodine receptor type 1

Large proteins such as ryanodine receptor type 1 (RyR1) that contain multiple cysteines susceptible to modifications add significant complexity, with the theoretical possibility that different combinations of S-oxidation or S-nitrosation may create subtle differences in function. Several reviews have attempted to 'pull apart' the complex oxidative regulation of RyR1. Out of 101 cysteines RyR1 contains at least 12 known to be either S-nitrosated, S-glutathiolated or involved in the formation of inter- or intramolecular disulfides [121]. Though all forms of oxidative modification were associated with channel opening, S-nitrosation is thought to enhance Ca^{2+} activation, whereas S-glutathiolation decreased the inhibitory effect of Mg^{2+} without altering Ca^{2+} activation [116]. To date Cys-3635 remains the only cysteine residue shown to be functionally relevant in the redox sensing properties of the channel. Cys-3635 was shown to modulate calmodulin-dependent NO modulation of channel activity, as HEK293 cells expressing C3635A RyR1 were resistant to activity modulation by NO [122]. However, prior to this, experiments in sarcoplasmic reticulum membranes from rabbit showed Cys-3635 formed a disulfide bond with a cysteine in a neighbouring subunit upon calmodulin binding. The formation of this inter-subunit disulfide bond resulted in structural changes to the tetrameric from of RyR1 and was thought to protect RyR1 from further oxidation [123].

Sarco/endoplasmic reticulum Ca2+-ATPase

NO stimulates the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) to relax muscles by decreasing the intracellular Ca^{2+} concentration. There are 7 proposed sites of S-nitrosation within SERCA [40]. Selective inhibition of neuronal NOS in rat hearts resulted in a significant decrease in the contractile response to isoproterenol, with a corresponding 50% decrease in S-nitrosation of SERCA [124]. This highlighted how basal cardiac contractility is dependent on the rapid and reversible S-nitrosation of SERCA and other Ca^{2+}-handling proteins. A decrease in cardiac NO resulting from decreased expression and activity of eNOS and increased superoxide dismutase activity in rat hearts lead to a reduction in S-nitrosation of SERCA. This was associated with increased left ventricular end-diastolic pressure and impaired myocardial relaxation [117]. However, although these studies suggested peroxynitrite was responsible for the S-nitrosation of SERCA they did not measure disulfide formation [41]. Prior to these S-nitrosation-focused studies, peroxynitrite was shown to directly increase SERCA-dependent Ca^{2+} uptake activity due to a disulfide, namely S-glutathiolation of Cys-674 [118]. During atherosclerosis Cys-674 was oxidised to a sulfonic acid, preventing S-glutathiolation and limiting SERCA activity, illustrating the functional importance of disulfide in the control of SERCA activity.

Endothelial NOS

In 2004 Ravi *et al.* attributed a loss of endothelial NOS enzymatic function to S-nitrosation of Cys-99 [125]. Further to this, in 2011 it was revealed that Cys-99 is located in a zinc-thiolate cluster that is essential to the heme binding site [126]. Treatment with peroxynitrite resulted in loss of zinc from the zinc-thiolate cluster and formation of disulfide bonds between the monomers. This resulted in decreased synthesis of NO and potentiated superoxide production [127]. Additional regulation of endothelial NOS activity by disulfides was found when mass spectrometry analysis of aortas, which indicated that S-glutathiolation of Cys-689 and Cys-908 uncoupled endothelial NOS so that it generated superoxide instead of NO [106]. Thus NOS, like so many other enzymes reported to be S-nitrosated, are also observed to form disulfides.

Potential pitfalls and considerations when studying S-nitrosation

Whilst the evidence suggests that many of the signalling events attributed to protein S-nitrosation are instead functionally mediated protein by disulfide formation, this likely and rational conclusion is mostly ignored or remains elusive to many in the NO community. A significant reason for this may relate to fashion, together with methodological developments such as the biotin-switch protocol which made the study of protein S-nitrosation amenable without the need for complex equipment or extensive user training. Furthermore, the importance of NO in the maintenance of health and disease is appreciated by a broad audience, and so it might appear simply implicit that the stable post-translational modification of proteins by this molecule would be important. Whilst the words "nitrosylation" and "nitrosation" appear in PubMed in 1980, the study of protein disulfides started at least half a century earlier. Thus disulfides may lack contemporary novelty, which combined with less amenable methods for their study and a lack of a direct connection to the biology of NO, may explain why they have garnered less attention in recent years. These considerations, together with a broad failure to understand the intrinsic reactivity of S-nitrosothiols with another thiol and thus their readiness to transition to a disulfide, have probably contributed to disulfide formation though an S-nitrosothiol-intermediate being significantly overlooked.

Use of *NO donors in the study of protein S-nitrosation*

Although protein S-nitrosothiols generated in cells and tissues through the action of endogenously arising S-nitrosation reactions can be detected [128], the majority of studies still use exogenous NO donors. In is quite typical for NO donors to be used at concentrations of 100-500 μM, including in studies published in prominent journals [43, 129, 130]. This adds significant complexity, especially in the context as to whether a protein is endogenously regulated by S-nitrosation.

Any drug administered becomes increasingly less selective for the targets it modifies as it is progressively used at higher concentrations. Thus we should recognise that many of the proteins that become S-nitrosated when NO donors are added, or indeed NOS enzymes are overexpressed, are unlikely to be modified at endogenous levels. Further complexity arises from the variable cell permeability and chemistry of different NO donors, which means the accumulation of intracellular NO is likely to be variable in terms of its concentration, as well as the precise molecular species formed, each of which differ in their S-nitrosative capability. High levels of NO donor will not only

limit selectively, but also have the potential to compromise the cellular reducing systems thus stabilising S-nitrosothiols, allowing them to accumulate at a higher concentration. S-nitrosocysteine is known to deplete GSH levels in a concentration-dependent manner in multiple cell types [131, 132]. Severe depletion of the reduced thiol pool was shown to generate non-physiological levels of intracellular S-nitrosothiols. This is compelling evidence that indicates that many stable S-nitrosated proteins reported in the literature may be a result of NO donors depleting reducing thiols, leading to the artefactual modification of proteins that would not occur in the absence of exogenous S-nitrosating agents.

GSNO is often presented as a 'physiologically relevant' NO donor that is anticipated to be naturally abundant in cells and is therefore commonly used. Although functional changes in proteins exposed to GSNO are typically attributed to S-nitrosation, generally the stoichiometry of this modification will not be determined and the ability of this agent to readily induce S-glutathiolation is wholly ignored. To control for possible S-glutathiolation, treatment with GSH may be considered suitable. However, GSH treatment is an inappropriate control because alone it will not react itself with thiols to generate disulfides [133]. However, if a thiol has been S-nitrosated by GSNO, the freed GSH may then attack the labile and thiol-reactive S-nitroso bond to generate an S-glutathiolated protein. This has been observed experimentally, whereby GSNO promoted S-glutathiolation of actin and S-nitrosation was not detected [84]. This further illustrates how S-nitrosating chemical reactions can readily generate protein disulfides.

Some methodological considerations

A major limitation of studying oxidative modification has been the lack of reliable methods that can be routinely applied. Although attempts have been made to make anti-S-nitrosothiol antibodies [134] this has been largely unsuccessful probably due to the labile nature of the nitroso bond. Certainly they are not widely used, which may question their utility. With the advent of the ascorbate-dependent biotin-switch in 2001, pioneered by Jaffrey and Snyder, detection and purification of S-nitrosated proteins became available to a broader community [135]. Whilst many redox modifications have a cyclic turnover flux, the ascorbate-dependent biotin-switch only takes a snap shot of these complex events. The ascorbate-dependent biotin-switch is typically presented as selective for S-nitrosothiols, which even if it is, there are many other redox modifications such as disulfides that can form during nitrosative stress. Combining an S-nitrosating agent with a method where only S-nitrosothiols are monitored helps focus attention on S-nitrosation of the protein. Perhaps this approach creates a myopic view and draws attention away from the important role of S-nitrosation-dependent disulfide formation. Selective detection of S-nitrosothiols by the ascorbate-dependent biotin-switch is further complicated by the frequent addition of copper. Copper I is thought to directly reduce the nitroso bond, resulting in copper II which is then reductively regenerated by ascorbate [136]. Whilst the addition of copper I dramatically increases the sensitivity of the method, what other oxidative modifications may be reduced is not well defined.

There have been methodological advances that have increased the sensitivity of S-nitrosation assays. Resin-assisted cysteinyl peptide enrichment is a method of quantitative reactivity profiling [137]. This method attempts to sort 'functionally relevant' thiols from random oxidation events and to date has identified 281 sites of S-nitrosation in 145 proteins. A limitation is the assumption that if a site is

reactive the modification will be of functional significance. Three 'functional' S-nitrosation sites were detected on GAPDH, however one of these, namely Cys-245, is hidden within a β-sheet and two (Cys-150 and Cys-154) are involved in disulfide bond formation [138]. Whilst this method may be valuable for determining which thiols are likely to undergo oxidative modification, it does not provide evidence that S-nitrosothiols are the end-effectors that underlie the associated signalling.

Combining tandem mass tags (TMT) with a thiol reactive group has allowed concurrent identification and multiplexed quantitation of post-translational modifications by mass spectrometry. Therefore, changes in S-nitrosation and disulfides can be quantified within the same experiment and even the same sample. CysTMT utilises a dithiopyridine reactive group to label thiols and successfully revealed 171 proteins which were both S-nitrosated as well as S-glutathiolated, although this only relates to a 10% overlap in pools of proteins that were either S-nitrosated or S-glutathiolated. Furthermore, endothelial NOS knockout mice showed decreased S-nitrosothiol site occupancy with no significant difference in S-glutathiolation [139]. These data seemingly indicate functionally distinct protein networks are modulated by the two modifications. IodoTMT tags have an advantage over CysTMT as they irreversibly label thiols and allow samples to undergo routine reduction and alkylation before analysis. Interestingly results from parallel labelling by CysTMT and iodoTMT showed that there is only a 25% overlap in S-nitrosothiols detected [140]. This highlights the incomplete labelling by both these tags and that they label different populations of proteins. CysTMT labelled S-nitrosothiols with a higher aliphatic index than those surrounded by positively charged amino acids. This therefore calls into question how reliable the CysTMT data set mentioned above is along with the conclusion that there was only 10% overlap of S-nitrosated and S-glutathiolated proteins.

Limitations with the use of cysteine mutant proteins

Molecular genetic support for S-nitrosothiols as the functional end-effectors in NO signalling often comes through mutagenesis of target cysteines. Cysteine mutagenesis typically results in loss of a functional response to a nitrosative intervention [130, 141, 142], with samples derived from transgenic NOS knockout mice also showing attenuated protein S-nitrosation signals. [143]. That a protein activity is altered after S-nitrosative intervention and this redox regulation is lost after mutation of a cysteine is often considered as definitive proof that the protein is directly regulated by S-nitrosation, even if the stoichiometry of the modification was not determined. Such conclusions ignore the fact that S-nitrosothiols transition to disulfides. It is reasonably obvious that mutation of a target thiol to prevent S-nitrosation will also prevent a disulfide, the modification that likely mediates the functional regulation, at the same site. The same argument can be made for NOS knockout mice; if S-nitrosylating species are decreased in a model system; this is also fully anticipated to limit the formation of disulfides as explained in detail above. Such oversights are perhaps in part due to S-nitrosation being in vogue, as well as the complexities of selectively measuring global disulfide bond formation.

Conclusions and perspectives

Although S-nitrosation is widely considered as a stable, enzymatically-regulated post-translational modification that directly regulates the function of proteins, this paradigm may be significantly

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incorrect. Evidence presented here highlights the flaws in the assertion that protein S-nitrosation is a ubiquitous end-effector of protein function. Although protein S-nitrosation is considered to have the key features of a post-translational regulatory system, as outlined above, this evidence primarily emanates from relatively few studies focussing on select proteins. It is perhaps unsafe to conclude that stable S-nitrosation is a widespread regulator of protein function, especially with the content of this review in mind. Whilst it is clear that a multitude of proteins are susceptible to S-nitrosation, especially in the presence of high concentrations of exogenous S-nitrosating NO donors, how commonly this modification occurs with significant stoichiometry to directly and widely regulate the function remains unclear and probably is over-stated in the literature. We contend that in most cases S-nitrosothiols probably primarily serves as a transient, redox state intermediates leading to disulfide formation (**Figure 2**). This does not mean that stable, regulatory protein S-nitrosation does not occur, but rather that this is likely the exception and probably occurs when the target thiol is shielded from the abundant thiol reducing molecules found within cells.

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Highlights

Protein S-nitrosation is widely held to play a similar role as phosphorylation

However, S-nitrosated proteins react rapidly with abundant thiols to form disulfides

Protein S-nitrosation mainly serves as a redox-state intermediate leading to disulfide

Disulfides may be the predominant end-effector modification during nitrosative stress

Stable, regulatory protein S-nitrosation may occur - but perhaps relatively rarely