Nitric Oxide and Posttranslational Modification of the Vascular Proteome
S-Nitrosation of Reactive Thiols

Diane E. Handy, Joseph Loscalzo

Abstract—Nitric oxide (NO·) is known to exert its effects via guanylyl cyclase and cyclic GMP-dependent pathways and by cyclic GMP-independent pathways, including the posttranslational modification of proteins. Much ongoing research is focused on defining the mechanisms of NO-mediated protein modification, the identity and function of the modified proteins, and the significance of these changes in health and disease. S-nitrosation or thionitrite formation has only been found on a limited number of residues in a subset of proteins in vitro and in vivo studies. Protein S-nitrosation also appears to be reversible. There are several theories about the in vivo S-nitrosating agent, and most suggest a role for oxidation products of NO· in this process. Flux in cellular S-nitrosoprotein pools appears to be regulated by NO· availability and is redox-sensitive. An analysis of S-nitrosation in candidate proteins has clarified the mechanism by which NO· regulates enzymatic and cellular functions. These findings suggest the utility of using proteomic methods to identify unique targets for protein S-nitrosation to understand further the molecular mechanisms of the effects of NO·.

Key Words: endothelial cells • nitric oxide • posttranslational modification • proteomics • S-nitrosation

Nitric oxide (NO·) is produced by many cell types and has many diverse functions. In the cardiovascular system, NO· is a key regulator of vascular tone, where it is known to mediate its effects, in part, by binding to the heme moiety of its effector, soluble guanylyl cyclase, with the subsequent activation of cyclic GMP-dependent signaling. There are many additional cGMP-independent actions of NO· that rely on modifications of biomolecules, including the posttranslational modification of proteins at reactive cysteine residues to form a thionitrite and the nitration of tyrosine at its ortho position.

Much ongoing research is focused on defining the mechanisms of NO-mediated protein modification, the identity of proteins that are modified, and the significance of these changes in health and disease. Research has focused on these modifications for a number of reasons detailed in this review. Importantly, these changes (e.g., S-nitrosation or tyrosine nitration) have each been shown to occur with normal physiological and/or pathophysiological levels of NO·. These modifications have only been found on a limited number of residues in a subset of proteins in vitro and in vivo studies, suggesting that the modifications do not occur randomly and, therefore, may constitute a signaling event, akin to phosphorylation. Several studies have shown that these modifications can alter protein function, and, at least for S-nitrosation, there is evidence to suggest that this process is reversible.

Sources of NO· in the Vasculature
NO· is produced by nitric oxide synthase (NOS) enzymes as a result of the 2-step oxidation of L-arginine to form NO· and L-citrulline. Enzyme activity requires the cofactors NADPH, tetrahydrobiopterin, and flavin for electron transfer. Of the 3 known mammalian NOS isoforms, endothelial (eNOS) and neuronal (nNOS) isoforms are constitutively expressed enzymes that require calcium and calmodulin for their activation. The expression of inducible NOS (iNOS) is regulated by transcriptional activation in response to inflammation and cytokine production. iNOS is a high-flux enzyme highly regulated by substrate (L-arginine) availability. Phosphorylation, cellular localization, protein–protein interactions, and cysteinyl S-nitrosation can also modulate the activity of NOS enzymes.

Under normal conditions, vascular NO· is derived primarily from eNOS in the endothelial cell. iNOS is primarily expressed in activated mononuclear leukocytes, such as those found in atherosclerotic lesions; however, iNOS can also be induced in many cell types, including endothelial and vascular smooth muscle cells, by cytokine stimulation.

In Vivo Formation of S-Nitrosoproteins
There are many theories concerning the endogenous agent capable of S-nitrosation. It is generally believed that higher oxides of nitrogen mediate S-nitrosation; N2O3 is one pro-
Nitrosation of this protein by reaction with NO2 provides supporting evidence for this mechanism, showing that nitrosation of thiols occurs after intermediate oxidation with low concentrations of NO.

Cysteines to yield S-nitrosothiols.19,24 Studies suggest a role for peroxynitrite in the oxidation of cysteines to yield S-nitrosothiols.19,24 This reaction would require some denitrosation/transnitrosation pathways.

To form NOX, NOX reacts with O2 to form the NOX intermediate (2 NOX + O2 → 2 NOX2) followed by the reaction of NOX with NOX to form NOX2, the nitrosating agent.18,19 Formation of these nitrogen oxides depend on NOX-concentration, and, thus, these reactions are predicted to occur more readily in hydrophobic compartments of cells and proteins because of the relative hydrophobicity of NOX and O2. NO2 is also transferred into the intracellular space via the action of protein disulfide isomerase at the cell surface. The accumulation of NOX and O2 in the cellular membrane may promote the formation of N2O3, a nitrosating agent. RSNO may enter the cell through other as of yet undetermined mechanisms. Various enzyme systems, such as formaldehyde dehydrogenase (FD)34 and cellular glutathione peroxidase (GPX-1), may play a role in regulating some denitrosation/transnitrosation pathways.

Commonly Used Methods to Detect S-Nitrosoproteins

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<tr>
<th>Method*</th>
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<tr>
<td>Photolysis chemiluminescence</td>
<td>Gas phase measurement of released NO</td>
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<td>Cu(+) / Cysteine chemiluminescence</td>
<td>Spectrophotometric measurement of released NO</td>
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<td>Tri-iodide chemiluminescence</td>
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<td>Saville-Greiss†</td>
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<td>S-nitrosothiol antibodies</td>
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†These methods are often used after immunoprecipitations to study levels of S-nitrosation in candidate proteins.

Many questions remain regarding the formation of S-nitrosoproteins in vivo. Experimental evidence suggests that only certain cysteinyl side chains on certain proteins are targeted; however, the biochemical basis for this selectivity is unclear. It has been hypothesized that the microenvironment of the site or the pKa of the cysteinyl group may influence its reactivity and the stability of the S-nitrosated product.36 Others have suggested that S-nitrosation occurs in target proteins that are in proximity to an NOX generator.37 Myeloperoxidase may catalyze the formation of NOX from NOX and H2O2 at the endothelial surface, and may thereby also contribute to S-NO formation in vivo.38,39 A global picture of S-nitrosoproteins and their subcellular localization is necessary to answer these and other questions on this important topic.

Detection of S-Nitrosoproteins

The labile properties of the S-NO bond have hindered general identification of S-nitrosated proteins and the specific cysteinyl residues that are modified. The S-nitroso bond is subject to decomposition by light,40 transition metals,41 excess thiols, and antioxidants, including ascorbate.42 Another confounder is sample contamination with nitrogen oxides, which can be measured as NOX by some methods or can cause nitrosation of thiols during sample preparation. The Table lists some of the commonly used methods for S-nitrosoprotein detection.
Photolysis-chemiluminescence is a sensitive method used to identify S-nitrosoproteins in biological samples. This method relies on the light-activated cleavage of the S-N bond, followed by detection of NO\textsuperscript{-} gas by chemiluminescence in the presence of ozone.\textsuperscript{5} Alternatively, S-nitrosoproteins can be detected by ozone-based chemiluminescence after Cu(+) / cysteine\textsuperscript{43} or acidic tri-iodide\textsuperscript{44} release of NO\textsuperscript{-}. These assays require highly specialized equipment and are sensitive to nitrite contamination. In addition, NO\textsuperscript{-} may be released from iron–nitrosyl complexes and/or O-nitroso and N-nitroso bonds.\textsuperscript{54–57} For accurate measurement of the S-nitrosothiol pool, free thiols must be blocked during sample purification to prevent postisolation S-nitrosation, and acidified sulfanilamide can be used to eliminate nitrite contamination. To distinguish the S-nitrosothiol pool, sequential assays with and without mercuric chloride pretreatment are performed because only the S-NO bond is susceptible to mercuric chloride cleavage. These highly sensitive chemiluminescence assays have mostly been used to quantify S-nitroso content in complex biological samples (eg, cell or tissue extracts).

Alternatively, the Saville reaction is used to release the nitrosonium ion (NO\textsuperscript{+}). This mercury-mediated decomposition of S-NO is followed by a colorimetric or fluorimetric method to measure nitrogen oxide products. Commonly, the Griess reaction is used to produce a nitrogen oxide-dependent colored azo complex or, alternatively, 2,3-diaminonaphthalene (DAN) is used to form a highly fluorescent product to measure indirectly NO\textsuperscript{-} derived from S-nitrosated thiols (reviewed in\textsuperscript{48}). Of these methods, the DAN assay is the more sensitive method to measure S-nitrosated thiols.\textsuperscript{49} The Saville-Griess or DAN reactions have been used to quantify levels of S-nitrosation in complex biological samples but have also been used in combination with targeted protein immunoprecipitation to quantify levels of protein-specific S-nitrosation in candidate proteins. Importantly, because these methods will measure contaminating nitrite as well as nitrite formed by S-NO cleavage, measurements with and without mercuric chloride treatment are necessary to assess nitrite levels; as in all of these methods, care must be taken to limit S-NO degradation during sample processing.

Other promising methods for the detection of S-nitrosoproteins include antibody reagents developed to S-nitrosocysteine by using S-nitrosoalbumin as an immunogen.\textsuperscript{50,51} Some of these antibodies are commercially available, but their specificity has been questioned. Several publications have used these reagents to characterize in vitro S-nitrosated proteins,\textsuperscript{52} or S-nitrosated proteins in situ in vascular tissue and/or endothelial cells.\textsuperscript{50,51,53} Although these antibodies have been shown to react with mercuric chloride-sensitive epitopes during immunohistochemical analysis of cells grown in culture or aortic rings,\textsuperscript{50,51,52} additional studies are necessary to prove the utility of these reagents in identifying unequivocally specific S-nitrosated proteins.

A different approach was used in a recent study that labeled available thiol pools with a biotin compound in NO-treated and NO-untreated samples.\textsuperscript{54} Although this method detects changes in available thiols that can be caused by any thiol oxidation (and not solely S-nitrosothiol formation), it may prove useful as a means to identify proteins with highly reactive thiols.

The recently developed biotin switch method by Jaffrey et al\textsuperscript{55,56} has been used by us with modification\textsuperscript{57} and by others to characterize global S-nitrosoprotein changes in endothelial cells.\textsuperscript{57,58} This method has also been used in numerous studies to follow the specific S-nitrosation of candidate proteins that were first isolated by immunoprecipitation. This elegant method relies on the differing susceptibility of the S-nitroso bond for cleavage compared with other thiol linkages. There are 3 major steps to the procedure: blocking free thiols; cleaving the S-N bond with ascorbic acid; and then blocking the resulting, newly formed thiols with a sulfhydryl-specific biotinylating reagent. The biotin moiety provides a convenient epitope for isolation or detection with antibodies to biotin or streptavidin reagents. Successful application of this method requires complete blocking of free thiols in the first step. Removal of this initial blocking agent is also crucial to avoid blocking ascorbate-sensitive sulfhydryl groups that are generated in the subsequent steps. As shown in the original report,\textsuperscript{59} coupling of the biotin switch method with proteomic methods, such as 2-dimensional electrophoresis, followed by in-gel tryptic digestion of protein spots, and subsequent mass spectrometry, has allowed for the identification of S-nitrosated proteins. Because of the limitations of mass spectrometry and the lability of the thiol-biotin linkages, this method has not permitted the identification of the actual modified cysteinyl residues in biological samples.

We recently developed a modification of the biotin switch method to follow the formation and localization of S-nitrosoproteins in situ in endothelial cells.\textsuperscript{57} In our studies, we found it necessary to use 10-fold higher concentration of alkylator, ie, 200 mmol/L methyl methanethiosulfonate (MMTS),\textsuperscript{57} than in the original published method\textsuperscript{55} to block free thiols. We also used a higher concentration of ascorbate to cleave the S-N bonds. Because of the highly reactive and specific nature of MMTS compounds for thiols, after reducing S-nitroso bonds, we used a Texas red derivative of MMTS to produce fluorescently tagged proteins that can be detected in situ. A biotinylated form of this compound was used for S-nitrosoprotein isolation and proteomic identification.

Direct confirmation of S-nitrosoproteins by mass spectrometry has only been possible on purified proteins subjected to in vitro modification with NO donors.\textsuperscript{59,60} Although these methods can detect the NO adduct in modified proteins or peptides, these procedures cannot necessarily identify the cysteinyl residue that is modified by nitrosation because of the lability of the S-NO bond: the S-N bond cleaves at lower energies than more stable peptide bonds. To date, targeted mutagenesis is the only method available to confirm unequivocally the role of specific cysteine residues in S-nitrosation reactions.

**Regulation of S-Nitrosoprotein Formation in Endothelial Cells**

From the earliest studies of protein thiol modification by nitrogen oxides by our group, it was apparent that S-nitrosoproteins were of biological importance. Plasma pro-
teins that were modified in vitro were shown to inhibit platelet activation and to promote vasodilation in vivo, thus suggesting a role for S-nitrosoproteins in the transport and storage of NO. Importantly, the identification of circulating plasma S-nitrosalbumin and other S-nitrosoprotein species under normal physiological conditions supported the notion that susceptible thiols are stably nitrosated in vivo. Because these initial studies, many biological effects of NO have been correlated with S-nitrosation of target proteins, including receptors, enzymes, and transcription factors.

It is now widely accepted that S-nitrosoproteins exist in all tissues. Several recent studies have examined the global formation and proteome-wide identification of S-nitrosoproteins in endothelial cells. These studies provide some information regarding the formation and diversity of S-nitrosoproteins in these cells.

The precise relationship of S-nitrosoprotein formation to NOS activity is currently unclear. One study in human umbilical vein endothelial cells found that S-nitrosoprotein pools decreased in response to 10 to 18 hours of incubation with pro-inflammatory and proatherogenic stimuli; however, inhibition of basal NOS activity with L-arginine (L-NMMA) had no effect on detectable S-nitrosoprotein formation until 48 hours of treatment with the NOS inhibitor. This finding is in contrast to whole animal studies that reported NOS inhibitors decreased detectable S-nitrosoprotein formation, indicating that cellular S-nitrosoprotein pools are dynamic and sensitive to changes in NO concentrations and flux. Other studies in umbilical vein and arterial endothelial cells have found that NOS inhibitors decreased basal or stimulated S-nitrosoprotein pools. Global S-nitrosoprotein formation can also be augmented by stimulation of NOS activity by shear stress or calcium ionophores. Global S-nitrosoprotein formation is redox-sensitive.

In our recent study, we also acquired evidence for a central role of mitochondria in protein S-nitrosation: S-nitrosoproteins localized to the mitochondria or perimitochondrial space, and inhibition of mitochondrial respiration or use of rho(0) cells that have greatly diminished numbers of functional mitochondria substantially decreased S-nitrosoprotein formation. The mitochondrial role in S-nitrosoprotein formation may be caused, in part, by its role as a source of superoxide that may promote peroxynitrite generation (Figure 2). Two recent studies showed that chronic stimulation of cells with NO targets the S-nitrosation of mitochondrial proteins involved in respiration, including cytochrome c oxidase, a protein previously reported to be modified by NO.

Proteomic Identification of Endothelial S-Nitrosoproteins

From the use of the biotin switch method, we and others have identified a number of S-nitrosated proteins from the proteome of endothelial cells treated with NO donors. These proteins were not targeted for study a priori but were isolated from 2-dimensional gels and identified by peptide fingerprints and/or peptide sequencing using mass spectrometry. Many of these proteins have known reactive thiols that are involved in protein–protein interactions or enzymatic functions and, therefore, fit one of the logical criteria for endogenous targets of S-nitrosation. This “subproteome” of S-nitrosated targets overlaps with that of S-thiolated proteins, as S-thiolation may also occur at reactive cysteines. S-thiolation involves the formation of a disulfide between a cysteine residue in a protein and a low-molecular-weight thiol, such as cysteine or glutathione. S-glutathiolation, in particular, has been studied as a protein modification that is inducible by increased oxidative stress and is capable of modifying protein function. S-glutathiolation is also considered a protective event that may prevent irreversible oxidation of reactive cysteine residues. Some studies show that S-NO formation may precede S-thiolation; however, S-nitrosation and S-thiolation can occur at different cysteine
residues. The exact relationship among various forms of cysteine modification is still unclear and will require precise definition of each of these subproteomes.

In 2 separate reports, peroxyredoxin, actin, and GAPDH were identified as S-nitrosoproteins. The function of peroxyredoxin relies on an active cysteine during the decomposition of peroxide. Actin is known to contain reactive cysteine residues; actin modification by alkylation and S-nitrosation at reactive thiol has been previously correlated with alterations in actin function. S-nitrosation of GAPDH has also been shown to inhibit enzyme activity in multiple cells, including endothelial cells.

Using this proteomic approach, other proteins were identified that could potentially play a significant role in NO-mediated signaling, including ubiquitin-conjugating enzyme (Ubch7) and 14 to 3–3 isoforms. Ubch7 S-nitrosation may be important because NO is known to inhibit proteasomal degradation, and a catalytic cysteine (cys86) in Ubch7 is required for this function. 14 to 3–3 isoforms are known to play a role in protein signal transduction. However, the exact role of a reactive cysteine in these processes is unclear.

The total list of S-nitrosoproteins that have been identified to date is extensive. Most of these proteins have been identified in targeted studies involving in vitro S-nitrosation of purified candidate proteins or by analysis of NO release from immunoprecipitated proteins. The latter method may allow for detection of S-nitroso-adducts in proteins that are S-nitrosated at low levels, compared with the proteomic methods; however, it requires pre-selection of candidate proteins. Importantly, one cannot exclude post hoc (ie, after cell lysis) formation of S-nitrosoproteins identified by this conventional methodology. The application of streamlined proteomic methods, such as the use of in-line liquid 2-dimensional chromatographic separation of proteins or peptides, may improve recovery of less abundant proteins, reduce artifacts during protein isolation, and increase the ability to identify novel S-nitrosoproteins using the biotin switch method.

The effects of S-Nitrosation on Cellular Function

Several studies have identified S-nitrosoproteins by using the targeted antibody approach. Many of these candidate proteins were chosen because they play a role in processes regulated by NO. For example, in endothelial cells, NO is known to inhibit apoptosis; thus, caspases that control the apoptotic cascade were studied. Caspase-3, a proapoptotic caspase, was shown to be S-nitrosated at the active site, cys163, as well as at other cysteinyl residues by in vitro studies. In endothelial cells, S-nitrosation at the cys163 was functionally related to NO-mediated inhibition of the caspase signaling cascade. In other cell types, NO-induced S-nitrosation of caspases was also found to inhibit cytokine-induced programmed cell death. These data confirm the importance of NO-mediated signaling in regulating cell death and provide a mechanism to explain this effect.

Interesting findings are also emerging concerning the NO-mediated regulation of NOS activity itself. Exposure to NO was shown to inhibit the activity of NOS in endothelial cells in a manner that was reversed by thioredoxin/thioredoxin reductase in in vitro assays. More recent studies found that both eNOS and iNOS are susceptible to NO-induced thiol modification, suggesting a possible feedback mechanism to control NOS activity. NO-mediated inhibition can occur by endogenous stimulation of NO-production, or by the use of exogenous NO generators. The susceptible cysteinyl residues are part of the zinc-tetra-thiolate cluster, which functions to stabilize NOS dimers. S-nitrosation at these cysteines was shown to interfere with NOS dimerization in vitro and in cell culture, and to decrease enzyme activity. In endothelial cells, the S-nitrosation of eNOS showed a dynamic pattern that was inversely related to eNOS activity in resting cells, during states of eNOS inactivity, eNOS was S-nitrosated; activation of NOS activity corresponded with a loss of S-nitrosation, which returned to the basal S-nitroso-state within 15-minutes after insulin activation and within 60 minutes after VEGF activation.

Other accessory proteins that modulate NOS activity are functionally inactivated by S-nitrosation. Thiol S-nitrosation of Hsp90 apparently reduces ATPase activity of this protein and, in vitro, reduces the ability of Hsp90 to potentiate eNOS function. Similarly, argininosuccinate synthetase (AS) is inactivated by S-nitrosation at cys132. AS is involved in the regeneration of L-arginine from the NOS product L-citrulline. Under basal conditions, S-nitroso-AS was not detected, but the formation of S-nitroso-AS was stimulated after lipopolysaccharide stimulation of vascular smooth muscle cells and in tissues after in vivo lipopolysaccharide induction of iNOS. AS production of L-arginine may be especially important for maintaining the high flux activity of iNOS. Taken together, these data suggest that NO-mediated inhibition of susceptible thiol may reduce NOS activity directly, and S-nitrosation of accessory proteins may serve to regulate NOS function indirectly. Nitrosation may also provide a negative feedback mechanism for the transcriptional stimulation of the eNOS gene accompanying shear stress. Shear stress causes an NFkB-dependent increase in eNOS transcription, followed by an eNOS-dependent accumulation of S-nitrosated p50. S-nitrosation of the NFkB subunit correlates with a decrease in nuclear localization of NFkB-dependent, thereby decreasing transcription.

Another important target for S-nitrosation is thioredoxin. Thioredoxin is a small protein that mediates the actions of many redox-sensitive proteins directly or indirectly by reducing these proteins or accessory proteins by mechanisms involving a Cys-Gly-Pro-Cys active core. Overall there are 5 cysteinyl residues in thioredoxin. Cys69, one of the cysteinyl residues outside the active core, is susceptible to S-nitrosation. S-nitrosation of thioredoxin has been associated with activation of thioredoxin and reduction of intracelular reactive oxygen species in endothelial cells. Thioredoxin is known to interact with many proteins by disulfide interactions. In kidney cells, S-nitrosation of thioredoxin reduced the association between thioredoxin and apoptosis signal regulated kinase 1, and promoted an apoptotic program in these cells. These consequences of S-nitrosation may be cell type-specific; however, it is likely that the status of
thiol-modification may affect many of thioredoxin’s protein–protein interactions.

Conclusions and Future Avenues
Many biological effects of NO are mediated through cGMP-independent pathways via posttranslation modification of proteins by S-nitrosation of target proteins, including receptors, enzymes, and transcription factors. Importantly, there is evidence to suggest that S-nitrosation can occur under physiological conditions, and that it is a reversible process that may modulate enzyme activities. Many studies have defined the particular cysteinyl residues involved in S-nitrosation and the role of these changes in cellular function by analysis of candidate proteins. These studies, including those cited in this review, have provided important insights into the role of S-nitroso-modification in regulating some of the cellular effects of NO; however, there is much that is unknown about NO-mediated signaling. In proteomics, no a priori selection of targets is made; instead, proteins are selected by other criteria. The development of the biotin switch method has allowed for the substitution of a thiol-selective biotin moiety in place of thiol-specific nitroso-adducts, providing an indirect method to select for S-nitrosoproteins that can be identified through proteomic methods using mass spectrometry. In practice, this method has allowed for the identification of a modest number of proteins, some of which have susceptible cysteinyl residues that could be a target for S-nitrosation. Currently, sensitivity is one issue that limits the identification of S-nitrosoproteins by available proteomic means. Application of new techniques, such as liquid 2-dimensional chromatography performed in line with mass spectrometry, should allow for identification of additional S-nitrosoproteins in endothelial cells and in vascular tissue samples. These data will help in further understanding the biological effects of NO in health and disease.

Acknowledgments
This work was supported by NIH grants HL59876, HL55993, HL61828, HV28178, and HL81587. The authors thank Stephanie Tribuna for assistance with the preparation of this manuscript.

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Arterioscler Thromb Vasc Biol. published online March 16, 2006;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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