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S-Nitrosothiols and the S-Nitrosoproteome of the Cardiovascular System

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Abstract

Significance: Since their discovery in the early 1990's, S-nitrosylated proteins have been increasingly recognized as important determinants of many biochemical processes. Specifically, S-nitrosothiols in the cardiovascular system exert many actions, including promoting vasodilation, inhibiting platelet aggregation, and regulating Ca^{2+} channel function that influences myocyte contractility and electrophysiologic stability. **Recent Advances:** Contemporary developments in liquid chromatography–mass spectrometry methods, the development of biotin- and His-tag switch assays, and the availability of cyanide dye-labeling for S-nitrosothiol detection *in vitro* have increased significantly the identification of a number of cardiovascular protein targets of S-nitrosylation *in vivo*. **Critical Issues:** Recent analyses using modern S-nitrosothiol detection techniques have revealed the mechanistic significance of S-nitrosylation to the pathophysiology of numerous cardiovascular diseases, including essential hypertension, pulmonary hypertension, ischemic heart disease, stroke, and congestive heart failure, among others. **Future Directions:** Despite enhanced insight into S-nitrosothiol biochemistry, translating these advances into beneficial pharmacotherapies for patients with cardiovascular diseases remains a primary as-yet unmet goal for investigators within the field. *Antioxid. Redox Signal.* 18, 270–287.

Introduction

NITRIC OXIDE ($\text{NO}^\bullet$) is a ubiquitous molecule that participates in a versatile range of key biological functions. As a small gaseous, lipophilic nitrogen oxide, the biochemistry of $\text{NO}^\bullet$ affords diffusion-limited, rapid penetration of the mammalian cell membrane, whereupon it may interact with various target molecules to induce a wide range of biological effects. In blood vessels, for example, protein kinase G (PKG)-dependent vascular smooth muscle cell relaxation and inhibition of platelet aggregation are induced by $\text{NO}^\bullet$ *via* activation of soluble guanylyl cyclase (sGC), which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), the key cofactor for PKG. Although abnormal $\text{NO}^\bullet$ -cGMP signaling is well established as a pathobiological mechanism in the development of many human cardiovascular diseases, cGMP-independent post-translational modification(s) of proteins by $\text{NO}^\bullet$ has emerged as an important process involved in the regulation of cardiovascular function.

It has long been recognized that a significant discrepancy exists between the half-life of pure $\text{NO}^\bullet$ in an aqueous solution and that observed for $\text{NO}^\bullet$ *in vivo* (0.1 *vs.* 6 s) (49). This observation raised speculation regarding the formation of $\text{NO}^\bullet$ -harboring molecular adducts, proposed conceptually as a mechanism to protect $\text{NO}^\bullet$ from inactivation, thereby

accounting for differences in the half-life. Owing to the reactivity of organosulfurs and the abundance of reduced thiols in plasma, S-nitrosothiols evolved as a potential candidate pool of $\text{NO}^\bullet$ adducts. In support of this hypothesis was the seminal observation that under certain experimental conditions S-nitrosylation of the nucleophilic sulfur can occur (128). Moreover, such S-nitrosothiols were found to be functional, capable of promoting vasodilation and inhibiting platelet aggregation (70, 128).

Over the ensuing two decades, advances in mass spectrometry and the development of biotin- and His-tag switch assays have overcome many early procedural and biochemical limitations preventing the ready detection of post-translationally modified S-nitrosylated proteins. This cumulative work has expanded substantially the catalogue of proteins (into the hundreds) known to undergo S-nitrosylation *in vitro* and/or *in vivo*, as well as emphasized the mechanistic importance of protein denitrosylation, trans-S-nitrosylation, and S-thiolation in modulating the biological effects of S-nitrosoproteins (59, 144).

S-nitrosylation in the cardiovascular system

Specific to cardiovascular (patho)physiology, protein S-nitrosylation appears to play a role in virtually every aspect

of cardiac function, including the regulation of inflammation, apoptosis, and angiogenesis that contribute to atherogenesis (79, 130); nitric oxide synthase (NOS) activity that modulates blood vessel tone (31); trans- and intramyocyte Na^{2+} , Ca^{2+} , and K^{+} flux that regulates myocardial contractility and electrophysiologic signaling (13); cardiovascular responses to hypoxia (129); mitochondrial bioenergetics in vascular tissue (22); and platelet function, among others (19, 34, 127, 134) (Table 1). Thus, a central focus of current research in the field is the relationship between S-nitrosylation and the pathogenesis of ischemic heart disease, ischemic preconditioning, pulmonary hypertension, and cardiac arrhythmias, such as atrial fibrillation and cardiomyopathy-associated ventricular fibrillation. Protein S-

nitrosylation is also implicated as a key mechanism of action for many widely used cardiovascular pharmacotherapies, including HMG-CoA reductase inhibitors (statins), and, possibly, combination isosorbide dinitrate/hydralazine (4, 28).

This review of the S-nitrosoproteome aims to discuss this multifaceted biochemistry of the thiol proteome through the prism of cardiovascular medicine. Here, we will provide a contemporary perspective on the biochemistry of S-nitrosothiols, discuss current techniques in use for detecting S-nitrosylated proteins *in vitro* and *in vivo*, present novel proteins known to undergo S-nitrosylation and the associated downstream biological effects of this post-translation modification, and consider potential therapeutic strategies that

TABLE 1. KEY S-NITROSYLATION TARGETS IMPORTANT IN CARDIOVASCULAR (PATHO)PHYSIOLOGY

<i>S-nitrosothiol target</i>	<i>S-nitrosylation site</i>	<i>Functional effect(s)</i>	<i>Pathophysiological relevance</i>
Glutathione	Cys2	Vasodilation Platelet inhibition ↓ ICAM-1 ↓ MMP-9	Atherosclerosis Stroke
Human serum albumin	Cys34	$\text{NO}^{\bullet}$ storage form and carrier	Sickle cell anemia
eNOS	Cys101 (within zinc-tetrathiolate)	Vasodilation Platelet inhibition	Essential hypertension Pre-eclampsia Atherosclerosis Arterial injury (<i>i.e.</i> , following balloon angioplasty)
sGC	Cys122	Tonic suppression of eNOS	S-nitrosylation as a regulatory mechanism by which NO inhibits eNOS activity
Hemoglobin	Cys93	Inhibition of sGC activity (Hypoxic) vasodilation	Tolerance to nitrate therapy Sickle cell anemia Pulmonary hypertension Essential hypertension Ischemic stroke Subarachnoid hemorrhage
Angiotensinogen	Cys18 Cys138	Inhibits renin binding	Essential hypertension Pre-eclampsia
Hcy	Site not yet determined	Decreases Hcy-induced oxidant stress and apoptosis	Atherosclerosis
Mitochondrial complex I	34 candidate thiols	Decreases mitochondria-derived oxidant stress	Protective against ischemia reperfusion injury
p47 ^{phox} (NADPH oxidase)	C378 (predicted)	Decreases NADPH oxidase-derived oxidant stress	Protective against ischemia reperfusion injury
Cyclooxygenase-2	13 candidate thiols	Increased synthesis of prostaglandin I ₂	Protective against ischemia reperfusion injury
L-type Ca^{2+} channel	Site not yet determined	Modulates intracellular Ca^{2+} in ventricular myocytes	↓ in CHF → ventricular fibrillation
RyR2	89 candidate thiols	Modulates sarcoplasmic reticulum Ca^{2+} flux	Promotes normal myocardial contractility and electromechanical function in CHF
I _{Ks} channel	Cys445	Shortens myocyte action potential	Modulates chronotropic responses to autonomic stimulation.
N-ethylmaleimide-sensitive factor	Cys91 and Cys264	Inhibition of Weibel-Palade body exocytosis	Protective against vascular inflammation and thrombosis
MMP-9	Cys99	Promotes neuronal apoptosis and neuronal tissue injury	Ischemic stroke

CHF, congestive heart failure; Cys, cysteine; eNOS, endothelial nitric oxide synthase; Hcy, homocysteine; ICAM, intercellular adhesion molecule; MMP, matrix metalloproteinases; NADPH, nicotinamide adenine dinucleotide phosphate; $\text{NO}^{\bullet}$, nitric oxide; RyR, ryanodine receptor; sGC, soluble guanylyl cyclase.

A GSH/GSNOR Denitrosylation: **B** Trx/TrxR Denitrosylation:

FIG. 2. Two proposed mechanisms for denitrosylation. (A) The glutathione/S-nitroso-glutathione reductase (GSH/GSNOR) system requires nicotinamide adenine dinucleotide (NADH), reduced glutathione (GSH), and S-nitrosogluthathione (GSNO) for selective denitrosylation of GSNO. Trans-S-nitrosylation from RSNO to GSH forms GSNO. In the presence of NADH, GSNO is converted to glutathione N-hydroxysulfenamide (GSNHOH) by GSNOR. (B) Thioredoxin (Trx) reduces RSNO to induce denitrosylation in a reaction that generates NO[•] or HNO₂ and the oxidized form of thioredoxin (TrxSS). Reduction of TrxSS to recycle Trx is catalyzed by thioredoxin reductase (TrxR) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). Se, selenium.

in which Cys63 is ultimately oxidized to form sulfenic acid (R-SOH) and sulfinic acid (R-SO₂H) (6).

The stability of S-nitrosothiols is also affected by trans-S-nitrosylation and S-thiolation (60). In trans-S-nitrosylation, the nucleophilic thiolate of R'SH attacks the nitrogen of S-nitrosothiols to form R'SNO and RSH, whereas in S-thiolation, the nucleophilic thiolate interacts with the sulfur of an S-nitrosothiol to form a mixed disulfide and NO⁻ (Fig. 3). Importantly, nitrogen is more electrophilic than sulfur. Thus, trans-S-nitrosylation, which involves the attack on the nitrogen of an S-nitrosothiol by a nucleophilic thiolate, is predicted to be more favorable than S-thiolation, in which the thiolate attacks a sulfur. Indeed, in *in vitro* studies, a higher concentration of S-nitrosothiols is required in the S-thiolation reaction to yield mixed disulfides (76), whereas trans-S-nitrosylation is a reversible second order reaction with rate

Transnitrosylation **S-Thiolation**

FIG. 3. Trans-S-nitrosylation and S-thiolation reactions. In trans-S-nitrosylation, the nucleophilic thiolate (R'S⁻) of R'SH attacks the nitrogen of an S-nitrosothiol to form R'SNO and RSH, whereas in S-thiolation, the nucleophilic thiolate interacts with the sulfur of an S-nitrosothiol to form a mixed disulfide (RSSR') and NO⁻.

constants of 1–100 M⁻¹·s⁻¹ and equilibrium constants close to 1 (61). Trans-S-nitrosylation, therefore, occurs under normal physiological conditions (106, 116), while S-thiolation likely prevails under conditions of prolonged nitrosative stress.

Protein disulfide isomerase (PDI), which is present on the cell membrane surface and catalyzes thio-disulfide exchange reactions, is believed to facilitate exogenous NO[•] entry into cells, possibly *via* trans-S-nitrosylation reactions. The potential for PDI-mediated trans-S-nitrosylation has been demonstrated previously using a NO[•]-dependent oxymyoglobin oxidation assay (147). In the presence of GSNO, PDI caused a 15-fold increase in the NO[•]-dependent conversion of oxymyoglobin to metmyoglobin compared to GSNO alone. These data suggest that PDI catalyzes the trans-S-nitrosylation of NO[•] from GSNO to oxymyoglobin to promote the formation of metmyoglobin through the following two reactions: Fe²⁺·O₂ + NO[•] → Mb-Fe²⁺·O₂NO → Mb-Fe³⁺ + NO₃⁻ (26). Moreover, the molecular inhibition of cell surface PDI is associated with decreased GSNO-stimulated activation of (intracellular) sGC in human erythroleukemic cells, indicating a functional importance of PDI in facilitating the transfer of bioavailable NO[•] from extracellular S-nitrosothiols to the intracellular compartment. Importantly, data derived from human vascular endothelial cells and lung hamster fibroblasts indicate that the K_m for denitrosylation of bovine serum albumin (BSA)-NO by PDI is 12–30 μM, or ~17% of the predicted V_{max} (109); thus, the contribution of PDI to denitrosylation under physiological conditions is unclear. Furthermore, the role of PDI in maintaining endogenous NO[•] levels *in vivo* is less well characterized, owing, in part, to a lack of mammalian PDI-knockout models (56).

It is important to note that mitochondria represent the primary subcellular location of S-nitrosoprotein formation in eukaryotic cells. Mitochondria are a key site of reactive oxygen species (ROS) generation owing to the oxygen-rich environment of the intramitochondrial space and the electron transport chain, which plays host to the thermodynamically favored reaction in which one electron is transferred from molecular oxygen to form superoxide (•O₂⁻) (98). In turn, the interaction of •O₂⁻ with exogenous or endogenous NO[•] forms the nitrosating agent(s) peroxynitrite (ONOO⁻) and/or N₂O₃. Pharmacological inhibition of the electron transport chain or removal of mitochondria results in a significant decrease in S-nitrosoprotein formation, such as has been observed in endothelial cells (145).

Detecting S-nitrosothiols: Contemporary Proteomic and Molecular Strategies

Several factors hamper efficient detection of biological S-nitrosothiols. First, although stable under physiologic conditions (pH 7.4, 37°C), S-nitrosothiols tend to degrade in the presence of microenvironmental perturbations to pH and temperature, and in the presence of light or transition metal ions (120). Secondly, even in S-nitrosothiol-rich tissues, the concentration of S-nitrosothiols relative to the total protein thiol content is low (86). Even basal levels of S-nitroso serum albumin (SNO-SA), a relatively abundant S-nitrosothiol species in human plasma, is often below assay detection limits; when detectable, concentrations of 30–40 nM have been reported (81, 110). This observation does not, however, indicate that

absolute S-nitrosoprotein levels are necessarily negligible within a given biological compartment, since rapid degradation and/or increased reactivity of S-nitrosoproteins could produce a negative bias in detection results. Thirdly, radiolabeling of a nitroso group, which would enhance detection efficiency, is not chemically possible (60).

To overcome these obstacles, early S-nitrosothiol identification methods relied on prototype-generation [^{15}N] nuclear magnetic spectroscopy or mass spectrometry, and were limited to analyzing only purified thiol-containing proteins, such as BSA, cathepsin B, and tissue plasminogen activator (128). However, contemporary proteomic- and molecular-based strategies afford the investigation of S-nitrosylation of lower abundance proteins and under a wider range of empiric conditions, including *in vivo*.

Liquid chromatography–mass spectrometry

In-tandem, liquid chromatography–mass spectrometry (LC-MS) offers the site-specific identification of the S-nitroso bond within peptide fragments. However, sample preparation requirements and laser energies necessary for performing conventional LC-MS, such as matrix-assisted laser desorption (MALDI)/ionization reflection time-of-flight (ReTOF) mass spectrometry or electrospray ionization (ESI), often induce S-nitrosothiol degradation that results in poor detection yields (85). At the expense of limited peptide coverage rates, however, ESI LC-MS experimental methods may be optimized for detecting S-nitrosylated proteins as a +29 Da ion (+NO $^{\bullet}$ and –H) (64, 96). Overall, despite increasingly sophisticated LC-MS methods (such as quadrupole time-of-flight MS) and

software analysis programs, coupling LC-MS with an upstream strategy designed to stabilize S-NO groups before LC-MS remains a more effective strategy for S-nitrosothiols detection compared with LC-MS alone, particularly *in vivo*. In fact, direct detection by LC-MS of S-nitrosoproteins *in vivo* was only recently reported from experiments conducted in phytochelatin isolated from *Arabidopsis thaliana* cells (30, 136). Proteins from these flowering plant-derived cells are typically cysteine-rich, and, thus, are optimal for analyzing post-translational modifications of cysteinyl thiols by LC-MS. The application of LC-MS alone for the direct detection of protein S-nitrosylation in mammalian cells *in vivo*, however, has not yet been reported.

Biotin-switch assay

The biotin-switch assay exploits the selective reactivity of biotin with reduced cysteinyl thiols as part of a three-step proteomic strategy to detect S-nitrosylated proteins (Fig. 4). Jaffrey *et al.* originally used this technique in murine brain tissue to demonstrate S-nitrosylation of numerous proteins involved in cell metabolism, structure, and signaling (64). To accomplish this goal, an experimental system was developed in which protein samples were incubated with methyl-methanethiosulfonate (MMTS) to bind free thiols selectively, but not S-nitrosylated thiols or pre-existing disulfide bonds. With free thiols blocked by MMTS, protein samples are then exposed to a reducing agent that converts S-nitrosothiols to thiols. In the final step, reduced S-nitrosylated thiols are labeled with N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP) and recovered following sample elution. The presence

FIG. 4. Biotin-switch method for the detection of S-nitrosothiols. Free cysteines (–SH) are blocked (–SX) following exposure of protein samples to methyl-methanethiosulfonate (MMTS). Excess MMTS is removed by running the sample through a microspin column or by protein precipitation with acetone. The sample is then treated with ascorbate and copper sulfate. Ascorbate reduces $\text{Cu}^{2+} \rightarrow \text{Cu}^{3+}$, which cleaves S-NO bonds, and previously S-nitrosylated (now free) cysteines are labeled with N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP). Biotin-labeled proteins are subjected to Western immunoblotting, target bands are cut from the gel, and then trypsin-digested before liquid chromatography–mass spectrometry (LC-MS) analysis. Alternatively, streptavidin agarose may be used to segregate biotinylated proteins before LC-MS. Adapted from Kettenhofen *et al.* (71).

of biotinylated cysteinyl thiols, which represent *S*-nitrosylated proteins, may then be assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (using anti-biotin antibodies or streptavidin), LC-MS performed on in-gel trypsin digested target bands, or LC-MS on bands or spots excised from two-dimensional (2D) gel electrophoresis. Numerous iterations of this procedure have since been described, which aim to enhance detection efficiency and range of coverage by utilizing various alternative compounds (144). Substitution of biotin with a His-tagged peptide, for example, may provide a detection advantage as some have suggested that biotin (but not the His-tag) is often lost during sample elution (17, 136).

A key limitation of the conventional biotin-switch assay is poor detection of proteins that undergo *S*-nitrosylation *in vivo*. Moreover, this method does not provide information regarding biological, stereoisomeric, or other biochemical factors that regulate the formation of target *S*-nitrosothiols. Methods to develop a model for predicting protein- and cysteine-specific targets of *S*-nitrosylation have relied on alternative methods. Doulias *et al.* exploited the reactivity of phenylmercury compounds with *S*-nitrosocysteine to induce the formation of a stable thiol–mercury bond, and then used an organomercury resin and a phenylmercury–polyethyleneglycol–biotin compound to capture *S*-nitrosylated proteins and peptides. Through a complex bioinformatic analysis of *S*-nitrosoproteins isolated from murine livers, they demonstrated that by increasing the accessibility of cysteine to NO[•], factors that increase the probability of *S*-nitrosylation are the adjacency of cysteinyl thiols to a charged amino acid (within 6-Å distance) in the tertiary structure of the protein, and cysteinyl thiol positioning within protein α -helices (25).

Often cited limitations to the biotin switch method include the nonspecific effects of ascorbate, which may reduce disulfide bonds to enable the false-positive identification of *S*-nitrosation sites (36, 38). Murray *et al.* have recently reported on the use of the cysteine mass tag (cysTMT) reagent, which offers several technical advantages over the use of conventional biotin for the specific identification of *S*-nitrosylation sites. First, cysTMT provides a permanent mass tag that enhances LC-MS analysis sensitivity. Second, cysTMT allows for the ability to fragment up to six reporter ions, which, in turn, allows for multiplex quantitative mass spectrometry analysis. Third, the cysTMT switch assay utilizes a low pH elution from the TMT affinity resin for reduction, rather than ascorbate, to protect mass tag binding to the modified cysteine(s) (100). Their methods identified 220 *S*-NO-modified cysteines on 179 proteins isolated from pulmonary artery endothelial cells. Relative quantification of the sensitivity of cysteines to *S*-nitrosylation was calculated according to the slope generated by the reporter ion intensity for each cysteine across a range of GSNO (as an NO donor) concentrations.

Two-dimensional difference gel electrophoresis

The cyanide dye-labeling (CyDye)-switch assay utilizes CyDye for in-gel fluorescence scanning, enabling direct comparative proteomic analysis of *S*-nitrosoproteins between two or more samples (71). Modeled closely after the biotin-switch assay principle, the CyDye-switch method incorporates difference gel electrophoresis (DIGE) technology to visualize differences in protein *S*-nitrosylation profiles between

control and treated (*i.e.*, *S*-nitrosylated) samples (71). With this protocol, following the thiol blocking and reduction steps (as outlined in the biotin-switch method), free thiols in both control and experimental samples are labeled with a succinimidyl ester of the cyanine dyes, Cy3 (green) or Cy5 (red) (Fig. 5A). In the control condition, cysteinyl thiols unblocked by MMTS (*i.e.*, cysteinyl thiols previously involved in a disulfide bond, and now reduced) will fluoresce green on a 2D gel. In turn, cysteinyl thiols unblocked by MMTS in the experimental condition (*i.e.*, *S*-nitrosylated cysteinyl thiols or reduced disulfide bonds) fluoresce red (Fig. 5B). Samples are then run simultaneously on a 2D gel, and images acquired using a dual laser scanning device or xenon arc-based instrument equipped with different excitation/emission filters to generate two separate images, one in red and one in green (108). Band differences present after gel overlay analysis indicate modified proteins and, thus, represent targets for LC-MS analysis. It is important to note that Cy labeling induces a subtle shift in the molecular mass of the target protein, which is responsible for an elevated false identification rate of excised spots on LC-MS analysis (108, 135). Thus, poststain treatment with the SYPRO Ruby dye is often necessary to enhance the accuracy of target band excision before LC-MS analysis (71, 108, 135, 150).

Resin-assisted capture of *S*-nitrosoproteins

Detection of *S*-nitrosylated proteins through a resin-assisted capture method is among the latest modifications of the biotin-switch assay. In this methodology, following the free thiol blocking and reducing steps discussed in the biotin-switch assay, nascent thiols are covalently trapped with a thiol-specific resin-bound 2- or 4-pyridyl functionality (37). Samples are then eluted with a reductant, separated electrophoretically by SDS-PAGE, and visualized using Western immunoblotting or a specialized stain. Resin-trapped thiols may also undergo trypsin cleavage before isotopic labeling, which improves LC-MS detection rates of target proteins. In Langendorff-perfused hearts, Kohr *et al.* used this methodology to demonstrate the *S*-nitrosylation of 31 proteins in response to ischemic preconditioning (77).

Antibody-based methods of detecting *S*-nitrosoproteins

The use of an antibody against conjugated *S*-NO has been reported for immunoblotting, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry analyses *in situ* (21). The use of anti-*S*-NO antibodies, however, requires *a priori* verification of antibody specificity to the *S*-nitrosylated form of the target protein, and, in general, is unable to provide complete identification of all cysteines involved in *S*-nitrosylation of a particular protein (48). Moreover, it has been suggested that the stereometric effects of the *S*-NO bond on protein structural conformation may in and of itself impede recognition of the target (*i.e.*, *S*-nitrosylated) protein by anti-*S*-NO antibodies, thereby limiting further the sensitivity of immunoblotting in these experiments.

S-Nitrosylation in Cardiovascular (Patho)physiology

Glutathione

Glutathione is a low-molecular-weight tripeptide that contains a cysteine bound to the carboxyl group of the

GSNO may also interact with deoxyhemoglobin to form glutathione, NO[•], and ferric hemoglobin ($k = 0.01$ to $0.03 \text{ M}^{-1} \cdot \text{s}^{-1}$) (123) in a reaction that occurs more readily in high oxygen tension. Hemoglobin is rapidly S-nitrosylated by nitric oxide that is generated from this reaction (123). The inverse relationship between pO₂ and the propensity for GSNO-derived NO[•] to S-nitrosylate hemoglobin to form S-nitrosylated hemoglobin (SNO-Hb) supports a role for oxygen tension as a mitigating factor in the bioactivity of NO[•] (see *Hemoglobin* section, below) (47, 148). In the presence of reactive thiols, GSNO is metabolized in a rapid and cell-independent manner (47, 148). Additionally, GSNO may be irreversibly converted to GSNHOH (glutathione N-hydroxysulphenamide) by the alcohol dehydrogenase enzyme GSNOR (10). Indeed, GSNOR antagonism to enhance bioavailable GSNO appears to hold pharmacotherapeutic potential. Sanghani *et al.* recently reported three novel compounds that selectively inhibit GSNOR, resulting in an increase in GSNO in macrophages, decreased NF- κ B activation, and increased cGMP formation (114). The benefits of direct GSNO administration on vascular responses to vascular injury have also been tested. In a murine *in vivo* model of traumatic brain injury, infusion of GSNO decreased intercellular adhesion molecule-1 and matrix metalloproteinase (MMP)-9 levels and expression of inducible NOS (iNOS) in macrophages (72). Similar beneficial effects of GSNO therapy have been reported in a rat model of ischemic stroke (73), and in decreasing recurrent carotid events in patients with atherosclerotic carotid disease (67).

Human serum albumin

Human serum albumin (HSA) is a single polypeptide consisting of 585 amino acids, including 35 cysteines and 17 disulfide bridges (104). In human plasma, S-nitroso-serum albumin (SNO-SA) accounts for an impressive 82% of all S-nitrosothiols; thus, it follows that the single free cysteine (Cys34) (pK_a = ~5) is observed to be highly reactive with nitrogen oxide species (126). The trans-S-nitrosylation kinetics of SNO-SA are slow relative to other S-nitrosothiols. For example, under physiologic conditions (pH 7.4, 37°C), the second order rate constant (k_2) for S-nitroso-thiol exchange reactions between GSNO and N-acetyl-penicillamine is $0.9 \text{ M}^{-1} \cdot \text{s}^{-1}$, whereas that for SNO-SA is $9.1 \text{ M}^{-1} \cdot \text{s}^{-1}$. Together with the high abundance of SNO-SA in plasma, these kinetic data implicate albumin as a leading candidate for the major long-term NO[•] storage adduct under physiologic conditions (95).

The functional effects of SNO-SA were originally described in denuded rabbit aortic strips in which a concentration-dependent decrease in vascular tone was observed as was inhibition of platelet aggregation; at a SNO-SA concentration of $10 \mu\text{M}$, an ~80% decrease in both measures could be elicited (126, 128). SNO-SA is protective against ischemia/reperfusion injury in the skeletal muscle and myocardial tissue. In a rabbit model of hindlimb ischemia/reperfusion injury, SNO-SA administration 30 min before reperfusion or ischemia resulted in sustained increases in NO[•] levels (assessed continuously by porphyrinic microsensor in the wall of the femoral artery), and decreased vasoconstriction and interstitial edema following reperfusion (51, 52).

The protective effects of SNO-SA-mediated increases in bioavailable NO[•] has been investigated in other cardiovas-

cular diseases, including sickle cell anemia, hypertension, and vascular injury following transluminal coronary arterial stent deployment (24, 68, 90). Conversely, impaired NO[•] release from SNO-SA may be an under-recognized pathophysiological mechanism in certain vascular diseases. For example, in pre-eclampsia, a hypertensive condition of pregnancy, decreased circulating levels of ascorbate, a key modulator of NO[•] release from S-nitrosothiols, are associated with increased SNO-SA levels, but decreased SNO-SA denitrosylation and NO[•]-dependent vasodilation (137). This observation notwithstanding, there are insufficient data derived from relevant animal models or patients with pre-eclampsia to characterize the mechanistic contribution of ascorbate deficiency to the hypertensive response in this disease.

Endothelial NOS

Endothelial NOS (eNOS) is a homodimeric enzyme and the primary source of endogenously derived NO[•] in vascular tissue. Activation of eNOS releases suppression of electron transfer between the N-terminal heme-binding (*i.e.*, oxygenase) and C-terminal reductase domains to catalyze the 5-electron oxidation of the guanidino nitrogen of L-arginine that generates NO[•] (2) [for a complete review of eNOS, see (27)]. It is believed that S-nitrosylation of eNOS at Cys101, a zinc-tetrathiolate cysteine, modulates the tonic suppression of eNOS catalysis, whereas activation of eNOS results in rapid denitrosylation (31). S-Nitrosylation of eNOS may occur secondary to eNOS-derived NO[•] or *via* pharmacologic NO[•] donors, but requires translocation of eNOS to the cell membrane *via* acylation-dependent post-translational modification(s) of eNOS (32). For example, acylation-deficient eNOS, which is expressed exclusively in the cytosol, exhibits levels of S-nitrosylation that are significantly lower than wild-type forms of the enzyme (31, 32).

The mechanism by which S-nitrosylation inhibits enzyme activity appears to be as a consequence of eNOS uncoupling. Compared to control, treatment of aortic endothelial cells with NO[•] to induce eNOS S-nitrosylation (as detected by optical absorption spectroscopy) decreases eNOS dimer expression and activity levels, while increasing eNOS monomer levels. Site-directed mutation of Cys99 (within the zinc tetrathiolate cluster), however, induces a relative increase in monomer expression levels compared to dimer expression levels following sample treatment with NO[•], supporting the postulate that the tetrathiolate cluster is involved in S-nitrosylation-dependent regulation of eNOS structure and function (112). The subcellular effects of eNOS-derived NO[•] on S-nitrosylation of other proteins have been evaluated. In COS cells transiently transfected with a nuclear localized eNOS fused with a monomeric red fluorescent protein, eNOS stimulation enhanced the fluorescence signal in the Golgi and the cell nucleus (63). In the Golgi, S-nitrosylation of N-ethylmaleimide-sensitive factor (NSF) is associated with decreased cellular trafficking of proteins, including the von Willebrand factor (93).

Soluble guanylyl cyclase

sGC is a heterodimeric enzyme that catalyzes the conversion of cytosolic GTP to cGMP, a critical secondary messenger necessary for normal PKG-dependent vascular smooth muscle relaxation. Nitric oxide derived from eNOS, iNOS, or

pharmacologic NO[•] donors (*e.g.*, nitroglycerin, isosorbide dinitrate, isosorbide mononitrate, *etc.*) interacts with the sGC prosthetic heme group located near His105 on the β_1 -subunit to stimulate sGC activation ~20-fold above basal levels. Recently, Fernhoff *et al.* proposed that sGC activation by NO[•] occurs in an hierarchical manner (35). In this model, sGC activation by NO[•]-heme binding (1-NO) is enhanced further by the binding of NO[•] to regulatory thiol(s) (xsNO) in sGC. Their work using recombinant rat sGC *in vitro* demonstrated that the V_{max} for GTP turnover was 14.7 *versus* 64.1 *versus* 3610 nmol·min⁻¹·mg⁻¹ for the basal, 1-NO, and xsNO states, respectively, indicating that the S-NO form is the most active form of sGC. The regulatory function of S-NO binding on sGC activation was confirmed by experiments in which sGC was exposed to MMTS, which modifies thiols through the formation of methyl disulfide. These investigators observed that without affecting sGC activity under basal conditions, MMTS inhibited significantly NO[•]-stimulated sGC activation.

Prolonged use of NO[•] donor pharmacotherapy, however, results in a diminished clinical effect. Several potential mechanisms to account for this observation have been theorized, including, among many others, depletion of reactive sulfhydryl groups necessary for enzymatic release of NO[•] from nitrovasodilators and increased endothelial production of free radicals, such as superoxide ($\text{O}_2^{\cdot-}$) that oxidizes the heme ligand of sGC to decrease NO[•]-sensing by sGC (20, 35, 86, 107, 128, 132, 148).

S-nitrosylation of sGC may be involved in the development of NO[•] donor tachyphylaxis. sGC contains three functional cysteinyl thiols on the β_1 -subunit (Cys78, Cys122, Cys214) that regulate NO[•]-sensing by sGC (39). Of these, Cys122 is an established target of S-nitrosylation; when formed, S-nitrosylation of this cysteine diminishes sGC activity. In cultured aortic smooth muscle cells exposed to S-NO-cysteine to induce S-nitrosylation at Cys122, NO[•]-induced stimulation of sGC (by heme group nitrosylation) is decreased by 60% compared to cells exposed to NO[•] alone (115). Conversely, substitution of cysteine for alanine at position 122 prevents S-nitrosylation and partially restores NO[•]-stimulated sGC activation. The complete three-dimensional structure of mammalian sGC is unresolved, which hinders efforts to determine the precise mechanism accounting for this observation. Nevertheless, the possibility exists that S-nitrosylation of Cys122, which is situated near the NO[•]-sensing heme ligand of sGC, results in a conformational change in sGC that prevents normal NO[•] sensing by the enzyme.

Hemoglobin

S-nitrosylation of hemoglobin at Cys93 of the hemoglobin β -subunit (SNO-Hb) occurs in a predictable pattern according to fluctuations in the local partial pressure of oxygen (pO_2). Under hypoxic conditions, hemoglobin assumes a tense (T) allosteric conformation. This conformation impairs the stability of covalent attachment of NO[•] to Cys93 owing to externalization of S-nitrosylated Cys93, which promotes its interaction with factors that induce denitrosylation. Conversely, at high pO_2 levels, the relaxed state (R) of hemoglobin affords S-NO formation, since in this state Cys93 resides internally within the tertiary structure of hemoglobin (44, 65). The relationship between pO_2 levels, hemoglobin allosteric conformation, and Cys93 (de)nitrosylation governs, in part,

the phenomenon of hypoxic vasodilation. Sonveaux *et al.* proved this postulate by examining the effects of changes in the fraction of inspired O₂ (FiO₂) on NO[•] bioactivity and tumor perfusion in rats under conditions of normoxia or hyperoxia. In the former, tumor perfusion was decreased (consistent with vasodilation upstream of the tumor inducing a vascular steal phenomenon), whereas in the latter, NO[•] bioactivity from SNO-Hb was decreased, tumor perfusion was maintained, and blood pressure was increased (122).

Whether SNO-Hb regulates vascular tone by endocrine or paracrine signaling *in vivo* remains debatable. On the one hand, it is postulated that binding of NO[•] to deoxygenated heme in the circulation (forming Fe²⁺-NO) facilitates trans-S-nitrosylation to Cys93; upon deoxygenation, hemoglobin is converted from the R to the T state resulting in the release of NO[•] that may diffuse through the vascular endothelium and into smooth muscle cell layers to induce cGMP-dependent vasodilation (43, 127). On the other hand, although SNO-Hb is stable in purified solutions and erythrocyte lysates, experiments performed in erythrocytes acquired from human subjects has yielded SNO-Hb levels (69 nM in arterial samples, 46 nM in venous samples) far below that believed necessary for hemoglobin to function as a NO[•] reservoir in the circulation (44). Moreover, SNO-Hb levels tend to fall rapidly irrespective of oxygen tension or hemoglobin saturation, while ferricyanide stabilizes SNO-Hb levels, suggesting that the intrinsically reductive microenvironment within erythrocytes prevents long-term storage of NO[•] as SNO-Hb (43).

It has also been reported that the half-life of NO[•] is inversely proportional to the concentration of erythrocytes in a closed experimental system, independent of the hemoglobin concentration (83). Compared to solutions containing free oxyhemoglobin, the disappearance rate of NO[•] in the presence of erythrocytes is 650-fold slower. Since NO[•] is believed to traverse cellular membranes by passive diffusion, these findings suggest that a barrier to diffusion from the extracellular to intraerythrocytic compartment exists that impairs S-nitrosylation of hemoglobin *in vivo*. Collectively, these and other similar findings have raised speculation that despite the undisputed predilection for NO[•] to react with free hemoglobin, this is not a central mechanism for regulating NO[•] bioactivity in the setting of intact erythrocytes *in vivo*.

Clinical implications of SNO-Hb

Preclinical trials in humans have established that intravascular infusion of NO[•] influences blood vessel tone at remote sites; however, it is unresolved as to whether this occurs primarily due to free NO[•], SNO-Hb, or a combination of both (111). In murine models of cerebrovascular injury, S-nitrosylation of hemoglobin is associated with a decrease in subarachnoid hemorrhage size (119) and ischemic stroke severity (69). Owing to the beneficial effect of S-nitrosylation on the affinity of hemoglobin for O₂, Gladwin *et al.* recently investigated the role of point-of-care inhaled NO[•] therapy in the management of sickle cell disease-associated vaso-occlusive crises (42). They tested the hypothesis that inhaled NO[•] therapy, through inducing SNO-Hb formation and/or improvements in local oxygen tension, is an effective strategy by which to attenuate erythrocyte sickling, improve microcirculatory perfusion during vaso-occlusive crises, and reduce patient-reported symptoms. In that study, treatment with

inhaled NO[•] did not meet the predetermined clinical end points, although low plasma levels of the stable NO[•] metabolite, NO₂⁻, observed in patients receiving inhaled NO[•] suggests inefficiency in the method of delivery of inhaled NO[•] as a confounding factor in the study.

Angiotensinogen

Angiotensinogen is an α -2 globulin and member of the serpin family of proteins. By catalyzing the conversion of renin to angiotensin I (Ang I), angiotensinogen is a key determinant of the rate-limiting step for synthesis of the pressor hormones angiotensin II (Ang II) and aldosterone (91, 149). The crystal structure of angiotensinogen was recently resolved to 2.1 Å, allowing for analysis of coordinated changes in angiotensinogen that modulate renin-angiotensin binding, and, thus, angiotensinogen function (149). Zhou *et al.* reported that formation of a disulfide bridge involving Cys18-Cys138, two angiotensinogen cysteines conserved across all species, is a critical determinant for renin binding (41, 149). Reductive cleavage of this disulfide bond mediated by glutathione disulfide or glutathione, for example, changes the structure of the renin-binding pocket of angiotensinogen to a less favorable conformation and, thereby, decreases angiotensinogen activation. Moreover, incubation of reduced recombinant human angiotensinogen with S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (1 mM) resulted in stoichiometric loss of Cys18 and Cys138, suggesting S-nitrosylation at these cysteines. In purified protein samples exposed to iodoacetamide to block free thiols followed by dithiothreitol (DTT) treatment to reduce the Cys18-Cys138 disulfide bond, treatment with SNAP resulted in a progressive decrease in detectable thiols, but an increase in NO[•] per mole of angiotensinogen. The latter is believed to reflect an accumulation of free NO[•] following stoichiometric S-nitrosylation of Cys18 and Cys138. Thus, S-nitrosylation of angiotensinogen at Cys18 and Cys138 is believed to regulate blood vessel tone by preventing the formation of functionally critical disulfide bonds involving these cysteines.

S-Nitrosylation in Cardiovascular Diseases

Vascular injury and ischemic heart disease

Pathological remodeling of atherosclerotic blood vessels following vascular injury may be attenuated through the effects of NO[•] on promoting vasodilation, inhibiting platelet activation, and limiting vascular smooth muscle cell proliferation (140). For example, the administration of NO[•] donor pharmacotherapy to patients for 6 months following coronary balloon angioplasty is associated with an increase in the luminal diameter of the treated atherosclerotic blood vessels (78). To explain this effect, it is believed that S-nitrosothiol denitrosylation offsets maladaptive cellular responses to balloon-induced arterial injury that contribute to vessel restenosis, including endothelial dysfunction, vascular smooth muscle cell migration, and proliferation, and extracellular matrix production. In support of this hypothesis is the observation that S-nitrosothiols exhibit high affinity for endothelium-denuded blood vessels. In a rabbit model of balloon arterial injury of the femoral artery, locally administered S-NO-BSA increased by 26-fold vascular accumulation of S-NO-BSA compared to uninjured vessels (90). Moreover, a polythiolated form of BSA enhanced further the favorable

effects of S-NO-BSA on the intimal/medial ratio and extent of platelet deposition in the femoral artery following balloon-induced injury (90).

Hyperhomocysteinemia is believed to promote atherogenesis, in part, by increasing ROS-dependent endothelial cell apoptosis *via* upregulation of p53-dependent Noxa expression (79, 130). S-nitroso-homocysteine generated following induction of iNOS, however, exhibits decreased homocysteine-dependent ROS formation, p53-dependent Noxa expression, and endothelial apoptosis. Taken together, these data suggest that factors which impair S-nitrosylation of homocysteine may also contribute to the pathogenesis of hyperhomocysteinemia-associated vascular dysfunction.

Ischemic preconditioning is the development of tolerance to ischemia-induced myocardial injury following brief periods of impaired coronary arterial blood flow (101). Numerous myocytes, vascular endothelial, and vascular smooth muscle receptor-dependent molecular mechanisms, which regulate initiation of protective cell signaling pathways necessary for the beneficial consequences of preconditioning, have been described previously (29). A key cell surface receptor-independent determinant of ischemic preconditioning is endogenously synthesized NO[•]. The precise role of NO[•] in the biology of ischemic preconditioning remains unresolved: although there is evidence to suggest that high concentrations of NO[•] are toxic in various animal models of ischemia/reperfusion injury, the net effect of NO[•] is likely tied to its function as an intermediary that participates in various protective processes of ischemic preconditioning (23).

Early preconditioning is characterized by a large increase in bioavailable NO[•] owing to the robust activation of iNOS in the myocardial tissue that is stimulated by a decrease in local (tissue) oxygen levels following microcirculatory hypoperfusion. In contrast, acute reperfusion injury is coupled with an increase in (predominantly) mitochondrial-derived ROS formation (*e.g.*, [•]O₂⁻ and hydrogen peroxide) (7). Elevated levels of ROS may (i) oxidatively modify key protein cysteinyl thiols involved in NO[•]-dependent vasodilatory signaling; (ii) decrease bioavailable levels of NO[•] by depleting key cofactors necessary for normal NOS activity (*e.g.*, tetrahydrobiopterin [BH₄]); and/or (iii) react with NO[•] to generate other reactive oxidative intermediates, such as ONOO⁻ (80, 92, 149). Thus, it has been hypothesized that NO[•] derived from iNOS during preconditioning leads to the formation of S-nitrosothiols (and perhaps *via* acidified NO₂⁻ under conditions of extreme acidity, pH ≤ 3), which preserves cardiovascular performance during subsequent time periods of ischemia by protecting functional cysteinyl thiols from ROS-induced oxidative modification(s) and minimizing oxygen-free radical-mediated depletion of NO[•]. This hypothesis assumes, however, that the half-life of S-nitrosothiols is sufficient to accommodate potentially lengthy periods of time between ischemia, and, thus, does not account for the effects of degradation of S-nitrosothiols, which for CysNO may decrease levels by 90% within 120 min, as an example (89).

The introduction of SNO resin-assisted capture- and 2D-DIGE protein screening techniques for the determination of novel S-nitrosylation targets has expanded broadly the range of proteins known to undergo S-nitrosylation in responses to ischemia/reperfusion (77, 82). For example, Lin *et al.* used 2D-DIGE methodologies to demonstrate that compared to control, stimulation of eNOS with a selective estrogen receptor- β

(ER- β) agonist or 17 β -estradiol induced *de novo* S-nitrosylation of 11 proteins present in the hearts of ovariectomized mice following ischemia/reperfusion injury (82, 103). Of the proteins found to undergo ER- β receptor-dependent S-nitrosylation, four were known previously to be associated with the protective effects of ischemic preconditioning in other experimental models: malate dehydrogenase, creatine kinase M-type, heat shock protein (60 kDa), and myosin light chain 1 (131). The precise mechanism(s) by which these proteins and their S-nitroso-derivatives modulate the salutary effects of ischemic preconditioning has not yet been fully determined.

S-Nitrosylation may also affect favorably the function of mitochondrial proteins involved in ROS generation during ischemia/reperfusion. Murray *et al.* reported findings from a large-scale survey of candidate S-nitrosylated proteins harvested from rat cardiac mitochondria following exposure to GSNO. They described 83 S-nitrosylated cysteine residues on 60 proteins (99), including the reversible S-nitrosylation of mitochondrial complex I, the entry point of electrons into the respiratory chain and the key contributor to the synthesis of ROS in mitochondria (15, 22). S-Nitrosylation of mitochondrial complex I may be protective against reperfusion injury by limiting the generation of oxygen-derived free radicals. Other nonmitochondrial ROS-generating proteins are S-nitrosylated, including the cell membrane-bound NADPH oxidase (NOX) complex subunit p47^{phox}. During ischemia/reperfusion, S-nitrosylation of p47^{phox} is associated with maintaining the cellular redox potential *via* suppressed NOX-dependent vascular ROS generation (118).

S-Nitrosylation of cyclooxygenase-2 (COX-2) with iNOS-derived NO $\bullet$ is associated with increased synthesis of prostaglandin I₂ (an eicosanoid that promotes vasodilation and inhibits platelet aggregation) and myocardial protection following ischemia/reperfusion injury in rats. The stimulatory effect of the HMG-coA-reductase inhibitor, atorvastatin, on iNOS activity, COX-2 synthesis, and COX-2 S-nitrosylation has led some investigators to propose S-nitrosylation as a potential mechanism by which statins protect against various forms of vascular injury, including that following ischemia/reperfusion (4).

Congestive heart failure

S-Nitrosylation-induced alterations in L-type Ca²⁺ channel, ryanodine receptor 2 (RyR2), and SERCA2a function have been reported in animal models of congestive heart failure. Neuronal nitric oxide synthase-deficient mice (nNOS^{-/-}) demonstrated decreased S-nitrosylation of L-type Ca²⁺ channels following myocardial infarction, resulting in increased L-type Ca²⁺ channel activity and elevated levels of intracellular Ca²⁺ in ventricular myocytes. Calcium overload as a consequence of decreased S-nitrosylation of L-type Ca²⁺ channels, in turn, was linked to disturbances of the cardiac membrane potential and potentiation of fatal arrhythmias, such as ventricular fibrillation (13, 131).

Similar to the findings in L-type Ca²⁺ channels, S-nitrosylation of the RyR2 receptor, which contains 89 free thiols (143), appears protective against sarcoplasmic reticulum Ca²⁺ leak that in heart failure is associated with impaired myocardial contractility and electromechanical instability (46). Gonzalez *et al.* explored the relationship between oxypurinol and RyR2 receptor S-nitrosylation as a mechanism by which

to explain the beneficial clinical effects of *xanthine oxidase* inhibitors observed in clinical studies of patients with congestive heart failure (45). In cardiac myocytes of spontaneously hypertensive rats with dilated cardiomyopathy, decreasing *xanthine oxidase*-derived $\bullet\text{O}_2^-$ with pharmacologic *xanthine oxidase* inhibition restored the intracellular nitroso-redox balance, increased levels of S-nitrosylated RyR2, and resulted in the attenuation of RyR2-mediated diastolic Ca²⁺ leak and improvements in myocardial contractility (46). Conversely, S-nitrosylation may contribute to the pathophysiology of congestive heart failure by inducing a loss of function of myocyte contractile proteins. For example, compared with normal controls, left ventricular biopsy samples from explanted failing human hearts express elevated levels of S-nitrosylated tropomyosin (18). Although the effect of tropomyosin S-nitrosylation on actomyosin ATPase function has not been fully characterized, these observational data raise speculation that S-nitrosylation of tropomyosin may modulate the adverse downstream actions of iNOS-derived NO $\bullet$ on myocyte contractility that has been observed previously in certain animal models of coronary ischemia (57).

Pulmonary hypertension

S-Nitrosothiols play a regulatory role in the pulmonary vascular response to alterations in blood oxygen levels. Erythrocytes exposed to chronic hypoxia exhibit increased levels of iron-heme nitrosyl, which impairs S-nitrosylation of hemoglobin, is associated with disruption of oxygen-mediated pulmonary vascular relaxation, and promotes the development of pulmonary hypertension (94). Impaired trans-S-nitrosylation is also implicated in the pathobiology of pulmonary arterial hypertension: increased right ventricular systolic pressure and the development of negative pulmonary vascular remodeling is observed in male mice administered N-acetylcysteine *in vivo*. In this model, maladaptive structural and functional changes to pulmonary blood vessels are linked to the formation of S-nitroso-N-acetylcysteine, which, by scavenging free NO $\bullet$, limits trans-S-nitrosylation of hemoglobin to decrease levels of S-nitrosohemoglobin (105). Interestingly, female or castrated male mice are protected against the pathological effects of N-acetylcysteine on pulmonary arterial pressure. This observation may reflect the beneficial effects of estrogen (and/or deleterious effect of androgens) on the synthesis of NOS-dependent (vascular protective) S-nitrosothiols prior to treatment with N-acetylcysteine (12).

Cardiac arrhythmias

Nitric oxide and S-nitrosylation of specific cardiomyocyte proteins are at times opposing regulatory determinants of cardiac electrophysiology by virtue of their influence on various phases of the cardiomyocyte action potential. For example, in atrial and ventricular myocytes, NO $\bullet$ promotes activation of the intermediate conductance potassium channel (I_{K1}), the late inward Na⁺ current (I_{NaL}), the slowly activating delayed rectifier current (I_{Ks}), and the adenosine triphosphate (ATP)-sensitive K⁺ channel (I_{K-ATP}) (133). In sinoatrial nodal tissue, NO $\bullet$ exerts a cGMP-dependent stimulatory effect on the pacemaker current (I_f) that is associated with increases in heart rate (133). S-Nitrosylation of cardiac Na⁺ SNC5a *via* nNOS-derived NO $\bullet$ is one mechanism associated with

prolongation of the QT interval in the inherited long QT syndrome (LQTS) (*i.e.*, LQTS due to a missense mutation of the SNTA1 gene [A390V-SNTA1]) (138), whereas S-nitrosylation of the I_{Ks} channel at Cys445 shortens the cardiac action potential to modulate chronotropic responses to autonomic stimulation (1, 3, 53). In nNOS deficient rats, decreased bioavailable $\text{NO}^{\bullet}$ is associated with impaired S-nitrosylation of the voltage-dependent L-type Ca^{+2} channel that destabilizes the cardiomyocyte membrane potential and decreases the threshold for ventricular arrhythmias (13).

In the sarcoplasmic reticulum, nNOS couples with the ROS generating enzyme *xanthine oxidoreductase* (XOR), which may serve as an alternate mechanism by which to account for decreased protein S-nitrosylation in the setting of diminished nNOS activity. Specifically, nNOS deficient myocytes demonstrate significantly increased XOR-derived $\text{O}_2^{\bullet-}$ accumulation, which, in turn, may consume $\text{NO}^{\bullet}$, thereby decreasing levels of the primary substrate for the S-nitrosylation reaction (*i.e.*, $\text{NO}^{\bullet}$) (74).

Nitric oxide is also involved in ion channel-independent mechanisms of cardiac conduction, such as those that involve calcium/calmodulin-dependent calcium ATPase and SERCA (14, 45, 117); however, there are conflicting data on the net effect of S-nitrosylation on ion channel-independent regulatory mechanisms of electrophysiologic stability. In one study, myocytes from genetically engineered iNOS $^{-/-}$ and double iNOS $^{-/-}$ /eNOS $^{-/-}$, but not eNOS $^{-/-}$, mice demonstrated decreased S-nitrosylation of the RyR, resulting in diastolic Ca^{2+} leak and a proarrhythmic substrate (45, 46). In another study involving a murine model of Duchenne muscular dystrophy in which *elevated* levels of iNOS are reported (11), S-nitrosylation of the RyR2 receptor in combination with depletion of the key RyR2 receptor subunit calstabin 2 was mechanistically linked to ventricular arrhythmias (34).

Thrombosis and stroke

Translational studies have defined a clear role for S-nitrosylation in at least three pathobiological aspects of thrombosis and associated brain injury: platelet aggregation, MMP-dependent neurodegeneration, and neurotransmitter-mediated response(s) to cerebrovascular injury. As discussed earlier, $\text{NO}^{\bullet}$ -dependent inhibition of platelet activation and aggregation by S-nitrosothiols (S-NO-N-acetyl-cysteine and GSNO) is among the original observations to suggest the antithrombotic potential of S-nitrosylation (128). Work from Lowenstein and colleagues developed this observation further to demonstrate that exposure of human platelets to the $\text{NO}^{\bullet}$ donor diethylamine NONOate results in S-nitrosylation of NSF, which inhibits NSF attachment receptors, thereby blocking the release of platelet granules (*e.g.*, ATP, ADP, serotonin) responsible for inducing platelet recruitment and subsequent thrombosis (97). S-Nitrosylation of NSF at Cys91 and Cys264 in vascular endothelial cells is likewise associated with inhibition of Weibel-Palade body exocytosis, a process that otherwise promotes vascular inflammation and thrombosis (93).

In the brain, MMPs promote pathologic extracellular matrix remodeling. During cerebral ischemia, nNOS-dependent $\text{NO}^{\bullet}$ synthesis is associated with S-nitrosylation-mediated activation of MMP-9 that induces neuronal apoptosis and promotes neuronal tissue injury (50, 87). Interestingly, ischemia-mediated S-nitrosylation of MMP-9 is reversible, with the

S-NO-derivative serving as a cysteinyl thiol reactive intermediate. Further, cysteinyl thiol oxidation of the S-nitrosylated thiol results in the formation of higher cysteinyl thiol oxidative species, including sulfinic acid ($-\text{SO}_2\text{H}$) and sulfonic acid ($-\text{SO}_3\text{H}$), that are ultimately responsible for the pathophysiological activation of MMP-9 (125). S-nitrosylation of pre-synaptic proteins is also implicated in poststroke changes to neuronal function. By inhibiting vesicular glutamate release that induces long-term inhibition of synaptic signal transmission (113), abnormal trans-synaptic signaling occurs and is a contributing factor to neuronal injury following cerebrovascular infarction.

Summary

Cumulative studies in the field of S-nitrosoproteins in the past two decades have established that: (i) S-nitrosylation influences the activity of proteins involved in cardiovascular (patho)physiology; (ii) S-nitrosylation, denitrosylation, and trans-S-nitrosylation are interrelated biochemical reactions that modulate the functional consequences of $\text{NO}^{\bullet}$ -cysteine interactions; and (iii) the potential roles for S-nitrosothiols in the pharmacotherapy of cardiovascular diseases remains not yet fully realized. Novel experimental techniques, including contemporary permutations of the biotin-switch method, LC-MS technology, and 2D DIGE analysis, have afforded proteome-wide assessments of S-nitrosoprotein formation in vascular tissue. These advances have significantly expanded the breadth of known protein cysteinyl thiols targeted for the synthesis of their S-nitroso-counterparts.

In the future, the number of clinical studies designed to test S-nitrosothiol-based treatments in patients with cardiovascular diseases will likely increase, particularly in populations with essential hypertension, sickle cell anemia, and pulmonary hypertension. The success of these endeavors, however, is likely to hinge on a more complete understanding of S-nitrosothiol biochemistry to predict better the downstream effects of S-nitrosothiols on fundamental elements of cardiovascular physiology, particularly vascular reactivity, myocyte contractility, and cardiac conduction.

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Abbreviations Used

ADP = adenosine diphosphate
 Ang I = angiotensin I
 Ang II = angiotensin II
 ATP = adenosine triphosphate
 BH₄ = tetrahydrobiopterin
 BSA = bovine serum albumin
 cGMP = cyclic guanosine monophosphate
 COX-2 = cyclooxygenase-2
 CyDye = cyanide dye-labeling
 Cys = cysteine
 cystMT = cysteine mass tag
 DIGE = difference gel electrophoresis
 DTT = dithiothreitol
 ELISA = enzyme-linked immunosorbent assay
 eNOS = endothelial nitric oxide synthase

Abbreviations Used (Cont.)

ER = estrogen receptor
 ESI = electrospray ionization
 FiO₂ = fraction of inspired O₂
 GSH = reduced glutathione
 GSNO = S-nitrosoglutathione
 GSNOR = S-nitroso-glutathione reductase
 GTP = guanosine triphosphate
 HNO₂ = nitrous acid
 HPDP = N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide
 HSA = human serum albumin
 ICAM-1 = intercellular adhesion molecule-1
 iNOS = inducible nitric oxide synthase
 LC-MS = liquid chromatography-mass spectrometry
 LQTS = long QT syndrome
 MALDI = matrix-assisted laser desorption
 MMP = matrix metalloproteinase
 MMTS = methyl-methanethiosulfonate
 N₂O₃ = nitrogen trioxide
 NADH = nicotinamide adenine dinucleotide
 NADPH = nicotinamide adenine dinucleotide phosphate
 nNOS = neuronal nitric oxide synthase

NO• = nitric oxide
 NO⁺ = nitrosonium
 NO₂⁻ = nitrite
 NO₃⁻ = nitrate
 NOS = nitric oxide synthase
 NOX = NADPH oxidase
 NSF = N-ethylmaleimide-sensitive factor
 •O₂⁻ = superoxide
 ONOO⁻ = peroxynitrite
 PDI = protein disulfide isomerase
 PKG = protein kinase G
 ReTOF = ionization reflection time-of-flight
 ROS = reactive oxygen species
 RyR = ryanodine receptor
 SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 Se = selenium
 sGC = soluble guanylyl cyclase
 SNAP = S-nitroso-N-acetyl-D,L-penicillamine
 SNO-Hb = S-nitrosylated hemoglobin
 SNO-SA = S-nitroso-serum albumin
 TrxR = thioredoxin reductase
 XOR = xanthine oxidoreductase