Reviews

This Review is in a thematic series on Novel Posttranslational Modifications of Proteins and Their Cardiovascular Significance, which includes the following articles:

- The Emerging Characterization of Lysine Residue Deacetylation on the Modulation of Mitochondrial Function and Cardiovascular Biology [2009;105:830–841]
- Protein Acetylation in the Cardiorenal Axis: The Promise of Histone Deacetylase Inhibitors [2010;106:272–284]
- Protein S-Nitrosylation and Cardioprotection [2010;106:285–296]
- Sent to Destroy: The Ubiquitin Proteasome System Regulates Cell Signaling and Protein Quality Control in Cardiovascular Development and Disease [2010;106:463–478]

S-Nitrosylation and Cardiovascular Signaling

S-Nitrosylation and Cardiac Ischemia
Glycosylation and Cardiovascular Signaling

Elizabeth Murphy, Guest Editor

S-Nitrosylation in Cardiovascular Signaling

Brian Lima, Michael T. Forrester, Douglas T. Hess, Jonathan S. Stamler

Abstract: Well over 2 decades have passed since the endothelium-derived relaxation factor was reported to be the gaseous molecule nitric oxide (NO). Although soluble guanylyl cyclase (which generates cyclic guanosine monophosphate, cGMP) was the first identified receptor for NO, it has become increasingly clear that NO exerts a ubiquitous influence in a cGMP-independent manner. In particular, many, if not most, effects of NO are mediated by S-nitrosylation, the covalent modification of a protein cysteine thiol by an NO group to generate an S-nitrosothiol (SNO). Moreover, within the current framework of NO biology, endothelium-derived relaxation factor activity (ie, G protein–coupled receptor–mediated, or shear-induced endothelium-derived NO bioactivity) is understood to involve a central role for SNOs, acting both as second messengers and signal effectors. Furthermore, essential roles for S-nitrosylation have been implicated in virtually all major functions of NO in the cardiovascular system. Here, we review the basic biochemistry of S-nitrosylation (and denitrosylation), discuss the role of S-nitrosylation in the vascular and cardiac functions of NO, and identify current and potential clinical applications. (Circ Res. 2010;106:633-646.)

Key Words: angiogenesis ■ apoptosis ■ atherosclerosis ■ cardiac electrophysiology ■ excitation-contraction coupling ■ adrenergic contractility

Molecular oxygen (O₂) and carbon dioxide (CO₂) are critical components of cardiovascular physiology (and pathophysiology). These gaseous molecules are central to tissue physiology and cellular respiration, and it has long been understood that disturbances in O₂ or CO₂ processing are both causative and indicative of pathophysiology. Over time, however, it has become increasingly clear that nitric oxide (NO) is also an endogenous regulator in cardiovascular physiology and cellular respiration, operating at considerably lower concentrations than O₂ or CO₂. These observations have led to the proposal that NO is the “third gas” of the respiratory cycle.

The major sources of NO in vivo are the NO synthase (NOS) isoforms. These include predominantly the neuronal
(nNOS/NOS1), inducible (iNOS/NOS2) and endothelial (eNOS/NOS3) enzymes. It is worth noting that this naming system is primarily of historical significance; NOS tissue expression is far less strict than implied by this nomenclature, and all 3 isoforms may be constitutive or inducible. NOSs are heme- and flavin-containing enzymes that use NADPH, tetrahydrobiopterin, and O₂ to convert L-arginine to L-citrulline with concomitant release of NO.5

**NO-Based Signaling: The Roles of cGMP and S-Nitrosylation**

One of the earliest described intracellular receptors for NO is the soluble guanylyl cyclase (sGC).6,7 Binding of NO to the heme group of sGC leads to increased conversion of GTP to cGMP, which in turn activates protein kinase (PKG). Despite the wealth of studies that have focused on sGC, it has become increasingly clear that NO exerts most of its cellular influence in a cGMP-independent manner. More generally, it is now appreciated that hemes in proteins do not generally mediate NO-based signaling that involves posttranslational protein modification, but rather serve to promote the requisite redox chemistry of NO. These observations led to the exploration of alternative molecular mechanisms through which NO might regulate cellular function, which culminated in the discovery of NO-mediated modification of protein cysteine residues (to generate an S-nitrosothiol [SNO]), designated S-nitrosylation (Figure 1A).8,9

Cysteine is a unique amino acid because of its thiol side chain. This functional group is nucleophilic, acidic (pKa 8) and redox active because of its hybridized p- and d-orbitals, which, together, underlie the large range of reactivities for cysteine residues within proteins. Within the realm of redox chemistry (ie, transfer of electrons and consequent change in atomic oxidation state), numerous reactions are known to occur on cysteine thiol side chains that affect protein structure and function. Of particular physiological significance is the redox reaction between NO and a cysteine thiol leading to S-nitrosylation (forming a protein SNO). In contrast to the

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### Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AR</td>
<td>adrenergic receptor</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-derived relaxation factor</td>
</tr>
<tr>
<td>ECC</td>
<td>excitation–contraction coupling</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial NO synthase</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>GSNOR</td>
<td>S-nitrosoglutathione oxidoreductase</td>
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<tr>
<td>GSSG</td>
<td>oxidized glutathione</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible NO synthase</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
</tr>
<tr>
<td>NOS</td>
<td>NO synthase</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>PK</td>
<td>protein kinase</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>sarcoplasmic reticulum Ca(^{2+}) ATPase</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SNO</td>
<td>S-nitrosothiol</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>thioredoxin reductase</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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**Figure 1.** The roles of cGMP and S-nitrosylation in NO-based signaling (A) and enzymatic protein denitrosylation mediated by the GSNOR and Trx systems (B). A, NOS synthesizes NO, which may activate sGC and thereby enhance production of cGMP (left) or subserve protein S-nitrosylation (right). The cGMP-dependent pathway is deactivated by cGMP-phosphodiesterase, which hydrolyzes cGMP to GMP. (Phosphodiesterase may also be activated allosterically by cGMP.) The SNO-based mechanisms are dynamically regulated via S-nitrosylation and denitrosylation of a multitude of cysteine-containing proteins. In contrast to the multiple elements regulated by S-nitrosylation, the cGMP-based signaling system relies primarily on the cGMP-dependent protein kinase, PKG. B, Proteins undergo reversible S-nitrosylation and denitrosylation (center). Denitrosylation mediated by GSNOR is depicted on the left. Transnitrosylation of glutathione (GSH) by S-nitrosylated proteins generates GSNO and native protein. GSNO undergoes NADH-dependent reduction by GSNOR to generate glutathione S-hydroxy sulfenamide (GSNHOH), which can undergo further reaction with GSH to generate GSSG. The redox cycle is completed by reduction of GSSG to GSH via GSSG reductase. Denitrosylation mediated by the Trx system is depicted on the right. The active site dithiol motif (CXXC) of Trx1 (cytoplasmic) or Trx2 (mitochondrial) undergoes oxidation coupled to denitrosylation of SNO substrate. Oxidized Trx is reduced by the selenoprotein TrxR, which employs the reducing power of NADPH to regenerate active Trx.
**Table 1. Exemplary SNO Proteins in the Cardiovascular System**

<table>
<thead>
<tr>
<th>SNO Protein</th>
<th>Cell/Tissue</th>
<th>Effects of S-Nitrosylation</th>
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<tbody>
<tr>
<td>Albumin</td>
<td>Serum</td>
<td>NO bioactivity reserve [104]</td>
</tr>
<tr>
<td>β-arrestin 2</td>
<td>Endothelium</td>
<td>Enhanced binding of β-arrestin 2 to clathrin and internalization of β-AR [28]</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Endothelium, lymphocytes</td>
<td>Antianapoptosis and preservation of endothelial function [64–67,163]</td>
</tr>
<tr>
<td>Dimethylarginine dimethylaminohydrolase</td>
<td>Endothelium</td>
<td>Accumulation of dimethylarginine and NOS inhibition [16]</td>
</tr>
<tr>
<td>GRK2</td>
<td>Endothelium, myocardium</td>
<td>Attenuation of β-AR desensitization [29]</td>
</tr>
<tr>
<td>Hb</td>
<td>Erythrocyte</td>
<td>Hypoxic vasodilation and regulation of vessel tone [4,17]</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Myocardium</td>
<td>Increased VEGF production and myocardial capillary density [31]</td>
</tr>
<tr>
<td>MKP7</td>
<td>Endothelium</td>
<td>Promotes endothelial cell migration and angiogenesis [14]</td>
</tr>
<tr>
<td>NSF</td>
<td>Platelets</td>
<td>Prevention of platelet activation [73,74]</td>
</tr>
<tr>
<td>RyR2</td>
<td>Cardiac muscle</td>
<td>Enhanced cardiac Ca²⁺ release and contractility [136,137]</td>
</tr>
<tr>
<td>Tissue transglutaminase</td>
<td>Endothelial surface</td>
<td>Inhibition of platelet aggregation [164]</td>
</tr>
<tr>
<td>Metallothionein</td>
<td>Vascular smooth muscle</td>
<td>Myogenic reflex, pulmonary vasorelaxation [170,171]</td>
</tr>
</tbody>
</table>

Examples of S-nitrosylated protein of interest and the general location and overall effect of S-nitrosylation. See Table 2 for dysregulated SNO-proteins in cardiovascular disease. MKP7 indicates mitogen-activated protein kinase phosphatase 7.

cGMP axis that uses a single principal molecular effector (ie, PKG) to carry out the downstream functions of NO. S-nitrosylation allows for a wide range of NO-mediated functions, inasmuch as a plethora of proteins may undergo this modification. S-Nitrosylation therefore helps to explain the wide range of cellular effects of NO in the cardiovascular system, some of which are listed in Table 1. Table 2 provides dysregulated SNO proteins in cardiovascular disease.

In addition, the ongoing delineation of cellular SNO proteins has revealed multiple loci through which S-nitrosylation might influence levels of cGMP. It has been reported that S-nitrosylation inhibits sGC [10] and cGMP phosphodiesterase [11] as well as eNOS itself [12] and eNOS-regulating proteins including heat shock protein 90 [13] and Akt/PKB [14]. PKG has regulatory thiols as well, which may be susceptible to S-nitrosylation. Furthermore, activating S-nitrosylation of arginase [15] and inhibitory S-nitrosylation of dimethylarginine dimethylaminohydrolase [16] would decrease NOS substrate levels and increase levels of endogenous, methylarginine NOS inhibitors, respectively.

**Protein Denitrosylation: A Critical Regulator of SNO Biology**

Numerous studies have focused on the mechanistic aspects of S-nitrosylation, leading to the identification of proteins that may either catalyze S-nitrosylation (eg, hemoglobin [Hb]) [17] or participate in protein transnitrosylation (ie, NO group transfer between proteins) [17,18]. More recently, however, protein denitrosylation has been shown to play a major role in controlling cellular S-nitrosylation [19–21] (similar to the role of phosphatases in protein phosphorylation), and has been shown to operate on hundreds of proteins in intact cells [21,22]. To date, 2 major enzymatic systems mediating denitrosylation have been described (Figure 1B), and are discussed in greater detail below.

A number of enzymes have been reported to catalyze the reduction of SNOs, and thus may be viewed as candidate denitrosylases. One of the first described is known as S-nitrosothiol reductase (GSNOR). This enzyme uses the reducing power of NADH to convert GSNOR to glutathione S-hydroxy sulfenamide (GSNHOH), which in turn is converted to oxidized glutathione (GSSG); reduction of GSSG by glutathione reductase completes the denitrosylation cycle (GSSG reductase activity is therefore required for physiological denitrosylation of GSNOR). Although GSNOR acts only on GSNO, ie, SNO proteins are not substrates, it governs protein S-nitrosylation by influencing the cellular equilibrium between SNO proteins and GSNO (Figure 1B). Importantly, GSNOR has been shown to play a role in regulating NO signaling downstream of the β-adrenergic receptor (β-AR) and is therefore operative in cellular signal transduction (discussed further below). Pharmacological inhibition or genetic deletion of GSNOR leads to enhanced vasodilation [19,30,31] consistent with a role for GSNOR in conveying the systemic activity of NO derived from eNOS.

GSNOR is an atypical member of the alcohol dehydrogenase family, inasmuch as it has no known alcohol-based substrate. In methylotrophic bacteria, GSNOR also metabolizes formalde-
hyde. A recent report indicates that another NADPH-oxidoreductase (carbonyl reductase 1) possesses GSNOR activity. In addition, xanthine oxidase metabolizes GSNO, but the \( K_m \) is high and its physiological relevance is therefore not clear. Nonetheless, these studies, taken together, raise the idea that multiple enzymes may modulate GSNO levels in vivo.

A new line of investigation has recently revealed that the ubiquitous thioredoxin (Trx) enzyme family (originally described as protein disulfide reductases\(^{34,35} \)) are also bona fide intracellular denitrosylases.\(^{36,37} \) In contrast to the strict substrate specificity of GSNOR for GSNO, a low-molecular-weight SNO, the cytoplasmic and mitochondrial thioredoxins (Trx1 and Trx2, respectively) directly mediate the denitrosylation of multiple substrate SNO proteins. As illustrated in Figure 1B, the Trx system uses a Trx reductase (TrxR) and NADPH to regenerate reduced Trx following denitrosylation. Recent examples demonstrate that, in the context of signal transduction, denitrosylation by Trx/TrxR can be stimulus-coupled, substrate specific and spatially restricted (compartmentalized).\(^{20,21,30} \)

Accumulating evidence indicates that protein S-nitrosylation status in vivo is not determined simply by rates of NO synthesis (ie, NOS activities), but rather involves a precisely regulated equilibrium between S-nitrosylation and denitrosylation pathways, in particular involving transnitrosylation reactions between a variety of peptides and proteins, and that consequently protein denitrosylation is critical in SNO-based signal transduction.\(^{8,21} \) Enzymatic control of both S-nitrosylation and denitrosylation, established by stringent genetic criteria, underlies the spatiotemporal specificity necessary for cellular signaling. In addition, elucidation of the mechanisms of denitrosylation may provide novel genetic and pharmacological tools for manipulating SNO-based signaling in vivo (eg, as revealed in studies of GSNOR\(^{-/-} \) mice; discussed further below) and help identify potential targets for therapeutic intervention in dysregulated SNO processing in the cardiovascular system.\(^{58} \)

### Roles of S-Nitrosylation in Vascular Signaling

#### Endothelium-Derived Relaxation Factor and Systemic Vascular Resistance

NO derived constitutively from eNOS (which mediates endothelium-dependent relaxation) is thought to account for the increase in blood pressure that is produced by both NOS inhibitors and genetic deletion of eNOS. It is worth considering in this light the implications of the findings that inhibitors of GSNOR elicit vasodilation\(^{30} \) and deletion of GSNOR results in lowering of systemic vascular resistance.\(^{19,31} \) GSNOR null mice are in fact highly susceptible to hypotension.\(^{19} \) Thus, to the extent that peripheral vasodilation by eNOS is identified with endothelium-derived relaxation factor (EDRF), analysis in GSNOR mutant mice indicates that GSNO is a major effector of EDRF action.

Vasodilation by EDRF may be mediated by cGMP or may be cGMP-independent, depending on the animal species and vessel type. In the classic Furchgott bioassay of rabbit thoracic aorta, the EDRF response is equally dependent on cGMP- and non-cGMP-regulated pathways.\(^{39,40} \) Furthermore, increases in cGMP in and of themselves provide little insight into the nature of the NO-based effector, because both NO and GSNO can increase cGMP levels. In addition, cGMP elevations may either result not only from NO binding to heme in sGC but also from inhibitory S-nitrosylation of phosphodiesterase 5.\(^{5,11} \) GSNO-based EDRF activity would be fully consistent with these data. GSNO is in equilibrium with protein SNO, and it has recently been reported that shear-induced activation of endothelial cells is associated with S-nitrosylation of more than 100 proteins.\(^{41} \) Furchgott's EDRF was not generated by shear but rather by acetylcholine, a G protein-coupled receptor (GPCR) agonist that both activates eNOS and releases NO from SNO-protein reservoirs.\(^{172} \) As discussed in more detail below, it has been shown recently that GPCR-mediated vasodilation via a different, eNOS-coupled GPCR, the \( \beta_2 \)-AR, is regulated critically by and very likely dependent in large part on GSNO-mediated S-nitrosylation of a set of proteins that includes G protein-coupled receptor kinase (GRK)2.\(^{28,29,42} \) Inhibition of GRK2 by GSK3 (GSK-3) \( \beta \) and MKP7 (mitogen-activated protein kinase phosphatase 7), thus establishing that NO is indeed a critical element in angio-gene- sis.\(^{54} \) Promotion of endothelial cell survival and angiogenesis...
also appears to be mediated via S-nitrosylation and activation of dynamin,59 a regulator of endothelial cell endocytosis. Finally, endothelial S-nitrosylation is perturbed by known pathophysiological stimuli including aging56 and hyperglycemic states,57 clearly linking defective S-nitrosylation to vascular disease.

It is well recognized that hypoxia stimulates angiogenesis primarily via the transcription factor hypoxia-inducible factor (HIF), which augments VEGF expression.58 Under normoxic conditions, HIF is typically undetectable because of rapid proteolytic degradation that is initiated by prolyl hydroxylase. Interestingly, exogenously administered SNO donors exert a hypoxia-mimetic effect,59,60 leading to nuclear accumulation of HIF. HIF stabilization by SNO under conditions of normoxia, observed both in vitro61,62 and in vivo,31 is mediated by S-nitrosylation of HIF itself. Specifically, HIF is constitutively S-nitrosylated in normoxic GSNOR−/− mice, with increased binding of S-nitrosylated HIF to the gene for VEGF.31 These mice also exhibited increased myocardial capillary density, lending further support for an integral role of S-nitrosylation in promoting angiogenesis.

Apoptosis
Some of the earliest studies examining the functions of S-nitrosylation focused on the antiapoptotic/protective effects of endogenous NO.63 These efforts demonstrated that NO S-nitrosylates and inhibits the active site cysteine residue of the proapoptotic effector caspase-3.64–66 It was shown subsequently that caspase-3 undergoes stimulus-coupled activation, driven by proapoptotic Fas stimulation, via Trx-mediated denitrosylation.20 Importantly, this mechanism has been shown to operate in endothelial cells,67 suggesting that the S-nitrosylation/denitrosylation equilibrium of caspase-3 may be a critical determinant of endothelial cell survival and vessel function. Furthermore, the oxidoreductase function of Trx, a vital element in preserving endothelial redox equilibrium and protecting against the deleterious effects of oxidative and/or nitrosative stress, is itself stimulated by S-nitrosylation.68

Inflammation
The robust antiinflammatory attributes of NO were first appreciated in experimental observations of diminished leukocyte adherence to vascular endothelium in the presence of exogenous NO donors.69 Administration of NOS inhibitors predictably results in increased leukocyte rolling along the endothelium. Studies in knockout mice lacking a specific NOS isoform also underscore the contribution of endogenous NO sources in mitigating leukocyte adherence: compared to wild-type, eNOS−/−, nNOS−/−, and iNOS−/−/− mice exhibit increased leukocyte adherence to endothelium.70,71

The molecular bases of these findings have been elucidated in part, and encompass 2 key areas of SNO-mediated regulation: control of endothelial protein trafficking and suppression of nuclear factor κB–dependent expression of proinflammatory cytokines and adhesion molecules.72 During the initial phase of an inflammatory response, leukocyte rolling requires interactions between P-selectins on the endothelial cell surface with the cognate P-selectin glycoprotein ligand-1 on the leukocyte surface. P-Selectins are transmembrane proteins that reside within resting endothelial cells in granules designated Weibel–Palade bodies. On endothelial cell activation by an inflammatory stimulus, these granules translocate to the cell surface, resulting in exposure of P-selectin to the vessel lumen. N-Ethylmaleimide-sensitive factor (NSF), a principal component of this exocytic trafficking machinery, is subject to direct inhibition by S-nitrosylation of critical cysteine residues.73 The resultant interruption of NSF-mediated disassembly of soluble NSF-attachment protein receptor (SNARE) complexes prevents Weibel–Palade body exocytosis from endothelial cells. Thus, S-nitrosylation of NSF, consequent, for example, on stimulation with the GPCR agonist thrombin, is identified with the antiinflammatory activity of eNOS (Figure 2). Similarly, inhibitory S-nitrosylation of NSF in platelets is antithrombogenic through a similar mechanism (see below).74

Other phases of the inflammatory response and leukocyte trafficking are impacted by S-nitrosylation. Specifically, NO has been shown to limit the expression of integrins and intracellular adhesion molecules required for leukocyte adherence.75–77 These and other proinflammatory effectors, including cytokines and cytokine receptors, are under direct transcriptional control by nuclear factor κB.78 Inhibitory S-nitrosylation of both nuclear factor κB79 and its upstream activating enzyme complex, inhibitory κB kinase,80 has been demonstrated in multiple studies. Taken together, these demonstrations of multiple loci of S-nitrosylation in the inflammatory signaling cascade support a comprehensive and multifaceted regulatory scheme akin to that subserved by phosphorylation/dephosphorylation. It may be anticipated that the antiinflammatory actions of NO via S-nitrosylation will be relevant across a range of vascular pathologies from atherosclerosis to vasculitis and septic shock.
Reperfusion Injury

Following a period of transient tissue ischemia, reestablishment of vascular blood flow and 
O2 delivery causes paradoxic tissue damage referred to as “reperfusion injury”.

Elucidating the biochemical and molecular mechanisms of reperfusion injury has been an active area of investigation, inasmuch as amelioration would be of significant benefit during both percutaneous and pharmacological reperfusion techniques. Altered S-nitrosylation is intimately linked with reperfusion injury, helping to explain the salutary actions of statins, estrogen, and mitochondrial respiratory chain inhibitors. In particular, atorvastatin stimulates iNOS-mediated S-nitrosylation of cyclooxygenase 2, thereby generating cytoprotective prostaglandins. In addition, estrogen appears to exert its cardioprotective effect, at least in part, by augmenting S-nitrosylation of mitochondrial proteins. Whereas dysregulated S-nitrosylation appears to facilitate injury via irreversible inhibition of mitochondrial complex I (necessary for converting electrons from NADH to an ATP-producing proton gradient), targeted delivery of nitrosylating agents to mitochondria is protective in ischemia/reperfusion (I/R), and inhibition of reactive oxygen generation by complex I may be involved. iNOS also contributes to the protective effects of preconditioning (exposure to moderate hypoxia before I/R, which attenuates reperfusion injury) that are partly recapitated by nitroglycerin. Inasmuch as nitroglycerin bioactivation occurs predominantly in mitochondria and results in accumulation of protein S-nitrosothiols, it may be suggested that S-nitrosylation plays a protective role. In support of this idea, SNO proteins that have been shown to increase in preconditioned hearts are also identified following I/R.

Atherogenesis, Risk Factors, and Circulating SNO

Defective S-nitrosylation may contribute significantly to the pathophysiology of atherosclerosis. This condition reflects a contribution from myriad factors, including disruptions in the NO/redox equilibrium, immunologic/inflammatory stresses, platelet activation, aberrant vessel tone, and age-associated endothelial dysfunction. A major role in atherosclerosis for oxidative stress with resultant NO/redox disequilibrium is well characterized. As described above, Trx functions as a critical regulator of cellular redox status and as an important modulator of S-nitrosylation. Recent studies suggest that statins, frequently used antidislipidemic and vasculoprotective agents, may exert their effects, at least in part, by inducing both S-nitrosylation and activation of Trx.

Platelet activation is highly relevant in atherosclerosis. The role of NO in platelet biology has been complicated in recent years by findings that both eNOS and iNOS may contribute to platelet cGMP production, and that cGMP may exert both inhibitory and stimulatory effects. In addition, accumulating evidence also supports the idea that NO may inhibit platelet aggregation via a cGMP-independent pathway. Platelet aggregation is the third and final stage of the platelet activation process, preceded by platelet adherence and granule secretion (exocytosis). The contents of the various platelet granules (ie, dense, α-, and lysosomal granules) figure prominently in platelet recruitment, rolling, adherence, and aggregation. As in the case of endothelial exocytosis discussed above, S-nitrosylation of NSF also exerts an inhibitory effect on exocytosis in platelets, thereby suppressing thrombosis and vascular inflammation (Figure 2). These effects are mediated by endogenously generated NO, inasmuch as platelets from eNOS−/− mice exhibit increased rolling on venules, increased arteriolar thrombosis, and increased exocytosis in vivo.

Hypertension is a primary risk factor for progression of atherosclerotic disease and cardiovascular morbidity and mortality. Endogenous SNOs are implicated as key mediators of vasodilation and blood pressure control and in plasma, SNO-albumin provides a major reservoir of NO bioactivity. However, albumin can also serve as a deleterious NO sink, whereby excessive sequestration of endogenous NO as S-nitrosoalbumin (SNO-albumin) negatively impacts vascular homeostasis in a variety of pathophysiological states. Notably, albumin infusions may precipitate elevations in blood pressure by limiting the pool of bioavailable NO for basal vessel relaxation. Similarly, increased plasma SNO levels, suggestive of impaired NO delivery or excessive NO sequestration, are associated with adverse cardiovascular outcomes and hypertension in end-stage renal disease patients, and misappropriation of NO as SNO-albumin is also directly implicated in the pathogenesis of hypertension in preeclampsia. It is important to emphasize, however, that exogenously administered SNO-albumin has been shown to serve as an effective therapeutic agent in a number of animal models including I/R-associated heart disease, lung injury in sickle cell disease and cardiopulmonary dysfunction in endotoxemia.

A number of studies have confirmed that the aging process is accompanied by a progressive decrease in bioavailable NO and concomitant endothelial dysfunction. Explanations include increased superoxide production and elevated levels of naturally occurring NOS inhibitors. Upregulation of arginase activity in aging vasculature has also been espoused as a predominant mechanism for age-related endothelial dysfunction. Elevated levels of arginase, which competes directly with NO for the common substrate L-arginine, would theoretically limit the amount of NO synthesized. Indeed, it was reported that in vitro inhibition of arginase activity restores (NO-based) vasodilation in aortic rings derived from aged rats. A subsequent study revealed that arginase is activated by S-nitrosylation of a single cysteine residue, leading to its stabilization and to substantially enhanced substrate affinity (6-fold reduction in $K_{m}$), which might enhance its ability to compete with NOS. Moreover, S-nitrosylation of arginase was increased in blood vessels from aging rats and was mediated by iNOS, previously shown to be expressed in aging vasculature.

SNO-Hb and Hypoxic Vasodilation

Hypoxic vasodilation is an autoregulatory physiological response that maximizes blood flow to regions in the arterial periphery with low Hb O2 saturation, thereby matching perfusion with tissue O2 demand. The progressive diminution in blood O2 content accompanying the decline in arteriolar diameter within the microcirculation results in...
graded vasodilation. Autoregulation of blood flow occurs within seconds or less (A-V transit times) and is recapitulated by direct intra-arterial infusion of variably deoxygenated but not of oxygenated RBCs, and RBCs added to aortic ring bioassays at varying PO2 actuate graded vasodilation (Figure 3A). Moreover, these RBC-induced responses can be replicated by S-nitrosohemoglobin (SNO-Hb), which has a well-documented role in mediating hypoxic vasodilation.4,119–122 Infusion of SNO-Hb (but not unmodified Hb) augments blood flow in vivo under normoxic conditions (Figure 3B), and vasodilation is blunted in the setting of supraphysiologic PO2 (eg, ambient O2 at 3 atmospheres absolute) (Figure 3C). By contrast, hypoxemia augments vasodilation by SNO-Hb. Changes in peripheral blood flow and PO2 are predictably correlated with circulating SNO-Hb concentrations.123

Hb exists predominantly in one of 2 structural states: R (relaxed) (high O2 affinity) and T (tense) (low O2 affinity).120 S-Nitrosylation of Hb (to generate SNO-Hb) occurs at Cys93 of the β subunit (Cys β93).120 The allosteric conformation of the Hb molecule governs reactivity of the Cys β93 residue and thus the propensity for NO binding (Figure 3D). SNO-Hb formation is favored in the oxygenated (R) structure, whereas in the T configuration and Cys β93-SNO is exposed to solvent. Furthermore, in venous blood a population of deoxygenated (T-state) Hb reacts with NO to produce nitrosylated heme in the β-chain (bottom left). Transition to R-state draws Cys β93 close to the nitrosylated heme (top left) with a subsequent transfer of NO from heme to Cys β93, forming a SNO (top right). Deoxygenation of Hb favors the T conformation (bottom right), allowing SNO-Cys β93 to react with other cellular thiols and thereby facilitating release of NO/SNO from the RBC (adapted from Sonveaux et al).126

**Figure 3.** SNO-Hb subserves hypoxic vasodilation. A, PO2 determines the ability of RBCs to constrict or relax aortic ring preparations on a second-by-second time scale. PO2 is indicated for each curve, which illustrates a graded response. B and C, O2-dependent effects of SNO-Hb and Hb on local cerebral blood flow are shown in normoxia and hyperoxia. SNO-Hb infusion in vivo (1 μmol/kg over 3 minutes, beginning at time 0) immediately increases local cerebral blood flow in the caudate–putamen nucleus of rats breathing 21% O2 at 1 atmosphere absolute (ATA), where tissue PO2 ranges from 19 to 37 mm Hg. Thus, SNO-Hb appropriately increases blood flow in relatively hypoxic tissue; however, nonnitrosylated Hb decreases perfusion. In 100% O2 at 3 atmospheres absolute, where tissue PO2 ranges from 365 to 538 mm Hg, vasodilation is abrogated because SNO-Hb cannot allosterically dispense NO bioactivity (adapted from Allen et al).126 D, Allosteric transitions of circulating Hb regulate delivery of NO bioactivity to preserve vascular O2 homeostasis. Hb in RBCs senses [O2] and responds through allosterically controlled NO binding, SNO formation, and NO group release. At high O2 in the pulmonary venous system, Hb is in the R-state, Cys β93 is reactive and Cyst3-SNO is shielded in a hydrophobic pocket. On partial RBC deoxygenation in the periphery, Hb adopts the T configuration and Cys β93-SNO is exposed to solvent. Furthermore, in venous blood a population of deoxygenated (T-state) Hb reacts with NO to produce nitrosylated heme in the β-chain (bottom left). Transition to R-state draws Cys β93 close to the nitrosylated heme (top left) with a subsequent transfer of NO from heme to Cys β93, forming a SNO (top right). Deoxygenation of Hb favors the T conformation (bottom right), allowing SNO-Cys β93 to react with other cellular thiols and thereby facilitating release of NO/SNO from the RBC (adapted from Sonveaux et al).126
play a central role in autoregulation of blood flow, perturbations in the delivery of SNO by RBCs may underlie a variety of pathophysiological states characterized by microvascular dysfunction. For example, pulmonary hypertension, a clinical entity often triggered by sustained hypoxemia, leads to depletion of RBC SNO-Hb stores and consequently to defective Po2-coupled vasoregulation and ventilation/perfusion mismatching. Moreover, in vivo repletion of SNO-Hb can correct these physiological deficits. Defective production of SNO-Hb by sickle RBCs has been implicated in impaired vasoregulation in sickle cell disease; the severity of symptoms is correlated with the degree of impairment of SNO-Hb processing and of RBC-mediated vasodilation, and these deficits can be ameliorated by repletion of SNO-Hb. In diabetes, derangements of SNO delivery by RBCs, resulting from glycosylation of Hb, which promotes the R configuration and thereby limits NO delivery, may exacerbate the vasculopathy associated with this disease.

**Roles of S-Nitrosylation in Cardiac Signaling**

**Electrophysiology**

Within the heart, S-nitrosylation has emerged as a ubiquitous signaling modality, impacting virtually every facet of cardiac function and dysfunction. The elaborate cascade of Ca2+ cycling that underlies excitation–contraction coupling (ECC) is no exception. ECC spans an ordered sequence from electric excitation of the individual myocyte to heart contraction, subserved by the tightly regulated trafficking of Ca2+ flux from one cellular compartment to another. On membrane depolarization of the cardiac myocyte generated by voltage-gated Na+ channels, a cytosolic influx of Ca2+ occurs via the plasmalemmal L-type Ca2+ channel. Through a process known as Ca2+-induced Ca2+ release, this initial Ca2+ current triggers a more pronounced Ca2+ release from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR)/Ca2+ release channel (RyR2). Myocyte contraction proceeds when Ca2+ binds to troponin C in myofilaments, activating myosin ATPase. Relaxation of the myocyte entails diastolic reuptake and extrusion of cytosolic Ca2+ by way of the SR Ca2+-ATPase (SERCA2a) and the sarcoplasmal Na+/Ca2+ exchanger, respectively.

The ion channels participating in ECC, as well as those that determine the shape and duration of the action potential (see below), are modulated by S-nitrosylation, which thereby exerts effects on both contractility and arrhythmogenesis. The specific effects exerted by NO in the myocyte are dictated in part by the subcellular compartmentalization of NOs (NOS1and NOS3), which reside in close proximity to substrates for S-nitrosylation (Figure 4). NOS3 is spatially confined to sarcoplasmic membrane caveolae and is thus adjacent to the L-type Ca2+ channel, whose S-nitrosylation inhibits ion influx. In a similar fashion, NOS1 resides in the SR where it is complexed with RyR2, and S-nitrosylation activates RyR2 (increases channel opening probability). The S-nitrosylation may also hold true for SERCA2a. Collectively, these observations demonstrate the precise spatio-temporal regulation of S-nitrosylation that underlies control by NO of cardiac ECC.

It has been reported that nNOS redistributes to the sarcomlemma in heart failure, where it may regulate both β-adrenergic responsiveness and Ca2+ flux, and the deleterious consequences of myocardial infarction in mice (ventricular arrhythmia and mortality) are significantly more severe in nNOS−/− animals than in wild-type animals, in association with decreased S-nitrosylation of L-type Ca2+ channels. Thus, inhibitory S-nitrosylation of L-type Ca2+ channels by nNOS is likely antiarrhythmogenic. More generally, disruption of the NO/redox equilbrium in myocytes, through alteration of either levels or spatiotemporal distribution of NO/reactive oxygen species, is widely regarded as a sine qua non of heart failure. Upregulation of oxidant production, notably by xanthine oxidase, can overwhelm endogenous, NO-based signaling and promote the mechanoenergetic uncoupling characteristic of cardiac dysfunction. Therapies directed against xanthine oxidase enable reverse remodeling in rats with dilated cardiomyopathy. Thus, restoration of NO/redox homeostasis provides a potentially fruitful approach to restoring cardiac contractile function.
Figure 5. Schematic summary of the regulation of agonist-induced \( \beta_2 \)-AR trafficking by \( S \)-nitrosylation/denitrosylation of \( \beta \)-arrestin 2 (\( \beta \)-Arr2), GRK2, and dynamin. A, \( \beta \)-Arrestin 2 serves as a scaffold that functionally colocalizes eNOS and \( \beta \)-ARs (as well as other GPCRs). Ligand (isoproterenol) stimulation results in activation of eNOS and \( S \)-nitrosylation of \( \beta \)-arrestin 2. \( S \)-Nitrosylation of \( \beta \)-arrestin 2 promotes its dissociation from eNOS and its association with clathrin heavy chain/\( \beta \)-adaptin (AP-2), which facilitates routing of the \( \beta_2 \)-AR into the clathrin-based endocytotic pathway, and \( \beta \)-arrestin 2 is subsequently denitrosylated. B, Inhibition of GRK2 by ligand-coupled \( S \)-nitrosylation suppresses agonist-stimulated \( \beta \)-AR phosphorylation, \( \beta \)-arrestin 2 recruitment, and receptor desensitization and downregulation (schematic at top). Bottom, Desensitization (decline in cardiac contractility in the continued presence of ISO) is enhanced by inhibiting NO production. C, After GPCR activation, eNOS-mediated \( S \)-nitrosylation of dynamin promotes multimerization and GTPase activity, as well as relocation to the plasma membrane, which facilitates scission of endocytotic vesicles and receptor internalization. Adapted from Ozawa et al\(^{28} \) and Whalen\(^{29} \) et al.
**β-AR System**

In cardiac myocytes, eNOS is activated following β-AR stimulation, and important roles have been demonstrated for S-nitrosylation in transducing adrenergic signals. For example, S-nitrosylation of the L-type Ca\(^{2+}\) channel increases following isoproterenol stimulation in an eNOS-dependent manner. Interestingly, a difference in protein S-nitrosylation appears to explain, at least in part, the gender disparity in I/R injury: females exhibit higher SNO levels and improved protection.

Densensitization of GPCRs is a characteristic feature of disease, as is a deficiency of NO bioactivity. Recent studies have helped to connect these phenomena by demonstrating that GRK2 undergoes agonist-coupled, inhibitory S-nitrosylation (Figure 5B). GRK2 activity is a molecular correlate of receptor densensitization. Thus, S-nitrosylation leads to decreased β-AR phosphorylation and desensitization, and absent S-nitrosylation, cardiac contractility declines rapidly during maintained adrenergic stimulation (Figure 5B). β-Arrestin 2, a scaffolding protein that targets receptors for stimulus-coupled internalization, has also been shown to undergo S-nitrosylation, leading to enhanced eNOS-dependent receptor trafficking (Figure 5A). Importantly, these studies reported increased protein S-nitrosylation (SNO-GRK2 and SNO-β-arrestin) in GSNO-treated mice. Finally, S-nitrosylation of dynamin facilitates clathrin-dependent endocytosis of membrane receptors including the β-AR and thereby receptor downregulation (Figure 5C). S-Nitrosylation is thus under enzymatic control and GSNO is a central player in β-AR signaling. Other studies have demonstrated that VEGF may regulate GSNO expression; crosstalk between VEGF (and other provascular signals) and NO targets for stimulus-coupled internalization, facilitating clathrin-dependent endocytosis of membrane proteins, and NO plays an important role in virtually all aspects of cardiac and vascular physiology. However, the molecular details are understood in only very few instances. The emergence of SNOs as second messengers and of NO-based cardiovascular function and pathophysiology will undoubtedly yield novel therapeutic targets with great potential to improve clinical outcomes.

**Arrhythmogenesis**

As indicated above, the shape and duration of the cardiac action potential are regulated by multiple ion channels that are subject to regulatory S-nitrosylation. (Figure 6 summarizes findings in the case of the ventricular action potential.) For example, the inward-rectifying K current (IK1) shapes phase 3, and S-nitrosylation of a single cysteine in the relevant channel protein, Kir2.1, shortens the action potential. Chronic atrial fibrillation was associated with decreased Kir2.1 S-nitrosylation, as assessed in human atrial samples.

A role for dysregulated S-nitrosylation in the development of a number of cardiac arrhythmias is supported by additional studies. Gonzalez et al. demonstrated that nNOS-mediated S-nitrosylation of RyR2 is critical for maintaining intracellular Ca\(^{2+}\) homeostasis (Figure 4). Mice deficient in nNOS exhibit a diastolic calcium leak, which creates contractile dysfunction and a proarrhythmicogenic state. nNOS mutant mice also exhibit a proarrhythmic state following myocardial infarction, which is associated with diminished S-nitrosylation of RyR2, SERCA2a, and L-type calcium channel.

Mutations in α-syntrophin, a dystrophin-associated protein that acts as a scaffold between nNOS and the plasma membrane Ca-ATPase, have been shown to contribute to a long-QT syndrome. The Ala390→Val mutation in α-syntrophin alters the inhibitory interaction between nNOS and the plasma membrane Ca-ATPase, and the resultant S-nitrosylation of the Na\(^{+}\) channel SCN5A enhances Na\(^{+}\) influx, as well as Kir2.1, and thus the ultra-rapid delayed rectifier current. Adapted from Gonzalez et al.

**Figure 6. S-Nitrosylation of channel proteins regulates all phases of the ventricular action potential.** S-Nitrosylation of SCN5A channels enhances the Na\(^{+}\) current (I_Na), whereas S-nitrosylation of the α1C subunit of the L-type Ca\(^{2+}\) channel inhibits the L-type Ca\(^{2+}\) current (I_CaL). Among voltage-gated potassium channels, S-nitrosylation of the KCNQ1 subunit facilitates the slowly activating component of the delayed rectifier K\(^{+}\) current (I_Ks). whereas S-nitrosylation exerts an inhibitory influence on Kv4.3 and thus the transient outward potassium current (I_to) as well as Kir2.1, and thus the ultra-rapid delayed rectifier current.

**Conclusions**

NO plays an important role in virtually all aspects of cardiac and vascular physiology. However, the molecular details are understood in only very few instances. The emergence of SNOs as second messengers and of S-nitrosylation as the preeminent NO-based signal presages a new era in cardiovascular biology. Unraveling the molecular underpinnings of NO-based cardiovascular function and pathophysiology will undoubtedly yield novel therapeutic targets with great potential to improve clinical outcomes.

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**Disclosures**

None.

**References**


