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S-Nitrosylation in Cardiovascular Signaling

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Abstract

Well over two decades has passed since the endothelium-derived relaxing factor (EDRF) 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, EDRF activity—i.e. 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. Further, 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.

Keywords

Nitric oxide; S-nitrosylation; denitrosylation; cysteine; redox; angiogenesis; apoptosis; inflammation; atherogenesis; electrophysiology; excitation-contraction coupling; heart failure; β-adrenergic receptor; hemoglobin

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This Review is in a thematic series on **Novel Post-translational 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 [please fill in pub. info from 2/5/10 issue]
Protein S-Nitrosylation and Cardioprotection [please fill in pub. info from 2/5/10 issue]
Sent to Destroy: The Ubiquitin Proteasome System Regulates Cell Signaling and Protein Quality Control in Cardiovascular Development and Disease [please fill in pub. info from 2/19/10 issue]
S-Nitrosylation in Cardiovascular Signaling
S-Nitrosylation and Cardiac Ischemia
Glycosylation and Cardiovascular Signaling
Ubiquitination and Cardiovascular Signaling
Elizabeth Murphy, Guest Editor

NO as the Third Respiratory Gas

Molecular oxygen (O_2) and carbon dioxide (CO_2) 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 O_2 or CO_2 processing are both causative and indicative of pathophysiology[1]. 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 O_2 or CO_2. These observations have lead to the proposal that NO is the "third gas" of the respiratory cycle[2–4].

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 three isoforms may be constitutive or inducible. NOS's are heme- and flavin-containing enzymes that employ NAPDH, tetrahydrobiopterin and O_2 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 G (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 (Cys) residues (to generate an S-nitrosothiol, SNO), designated S-nitrosylation (Figure 1A).

Cys is a unique amino acid due to its thiol side chain. This functional group is nucleophilic, acidic ($pK_a \sim 8$) and redox active due to its hybridized *p*- and *d*-orbitals, which together underlie the large range of reactivities for Cys residues within proteins. Within the realm of redox chemistry (i.e., transfer of electrons and consequent change in atomic oxidation state), numerous reactions are known to occur on Cys thiol side chains that affect protein structure and function. Of particular physiological significance is the redox reaction between NO and a Cys thiol leading to S-nitrosylation (forming a protein SNO). In contrast to the cGMP axis that employs a single principal molecular effector (i.e., 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 [8, 9]. 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.

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 HSP90[13] and Akt/protein kinase B [14]. PKG has regulatory thiols as well, which may be susceptible to S-nitrosylation. Further, 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 (e.g. hemoglobin)17, or participate in protein trans-nitrosylation (i.e., NO group transfer between proteins)18. More recently, however, protein denitrosylation has been shown to play a major role in controlling cellular S-nitrosylation19–21 (similar to the role of phosphatases in protein phosphorylation), and has been shown to operate on hundreds of proteins in intact cells21, 22. To date, two 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-nitrosoglutathione (GSNO) reductase (GSNOR)23, 24. This enzyme employs the reducing power of NADH to convert GSNO to glutathione S-hydroxysulfenamide (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 GSNO25). Although GSNOR acts only on GSNO, i.e. SNO-proteins are not substrates, it governs protein S-nitrosylation by influencing the cellular equilibrium between SNO-proteins and GSNO26, 27 (Figure 1B). Importantly, GSNOR has been shown to play a role in regulating SNO signaling downstream of the β-adrenergic receptor28, 29, and is therefore operative in cellular signal transduction (discussed further below). Pharmacological inhibition or genetic deletion of GSNOR leads to enhanced vasodilation19, 30, 31, consistent with a role for GSNO 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 methylotropic bacteria, GSNOR also metabolizes formaldehyde. A recent report indicates that another NADPH-oxidoreductase (carbonyl reductase 1) possesses GSNOR activity32. In addition, xanthine oxidase metabolizes GSNO, but the K_m is high and its physiological relevance is therefore not clear33. 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 enzyme family —originally described as protein disulfide reductases34, 35—are also bona fide intracellular denitrosylases36, 37. In contrast to the strict substrate specificity of GSNOR for GSNO, a small-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 employs a thioredoxin 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 (i.e. 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 transduction8,21. 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 (e.g., 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[38].

Roles of S-Nitrosylation in Vascular Signaling

EDRF 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 hypotension19. Thus, to the extent that peripheral vasodilation by eNOS is identified with 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 pathways39, 40. Further, 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 511. 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 over one hundred proteins[41]. Furchgott's EDRF was not generated by shear but rather by acetylcholine, a G protein-coupled receptor (GPCR) that activates 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 β_2-adrenergic receptor, is regulated critically by and very likely dependent in large part upon GSNO-mediated S-nitrosylation of a set of proteins that includes G protein-coupled receptor kinase GRK2[28, 29, 42]. Inhibition of GRK2 by S-nitrosylation prevents receptor densensitization. Thus, the available evidence is consistent with a prominent role for SNO in conveying NO-based vasodilatory signals[43–45].

Substantial evidence supports a model in which endothelial dysfunction contributes to evolution of vascular disease, including hypertension, diabetes and atherosclerosis. A signature feature of this model is the excess production of superoxide, which eliminates NO[43]. Endothelial sources of superoxide include NADPH oxidases and eNOS itself, through a process known as "uncoupling" that results from co-factor or substrate deficiency. It is therefore of interest that S-nitrosylation of NADPH oxidase may preserve NO bioavailabilty in healthy endothelium by inhibiting production of superoxide[44]. By contrast, excessive S-nitrosylation of arginase has been implicated in the uncoupling eNOS that is characteristic of arteriosclerotic vessels[45]. It has been recently suggested that impaired S-nitrosylation of endothelial clock-related proteins may be linked to hypertension[46], emphasizing the emerging theme that hypo- and hyper-nitrosylation of specific protein targets, rather than a general NO/superoxide imbalance that may be interpreted principally in terms of altered levels of NO, may correlate best with pathophysiology.

Angiogenesis

Endogenously synthesized NO is an established facilitator of endothelial function and survival: eNOS is both induced and activated following endothelial stimulation with vascular endothelial growth factor (VEGF)[47, 48], a major promoter of vessel growth *in vivo*, as well as by shear

stress[49]. Importantly, eNOS$^{-/-}$ mice are deficient in VEGF responsiveness, thus establishing that NO is indeed a critical element in angiogenesis[50]. It has also been demonstrated that both shear stress41, 51 and VEGF52, [53] regulate protein S-nitrosylation in the vascular endothelium. In a recent specific example, VEGF production downstream of the chemokine-type GPCR CXCR4 was shown to be intimately coupled to S-nitrosylation of MAP kinase phosphatase 7 (MKP7), which facilitates endothelial cell migration via activation of c-Jun N-terminal kinase 3 (JNK3), thus promoting angiogenesis[54]. Promotion of endothelial cell survival and angiogenesis also appears to be mediated via S-nitrosylation and activation of dynamin55, a regulator of endothelial cell endocytosis. Finally, endothelial S-nitrosylation is perturbed by known pathophysiological stimuli including aging56 and hyperglycemic states57, 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 due to rapid proteolytic degradation that is initiated by prolyl hyroxylation. Interestingly, exogenously administered SNO donors exert a hypoxia-mimetic effect59, 60, leading to nuclear accumulation of HIF. HIF stablilization by SNO under conditions of normoxia, observed both *in vitro*[61, 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 anti-apoptotic/protective effects of endogenous NO[63]. These efforts demonstrated that NO S-nitrosylates and inhibits the active site Cys residue of the pro-apoptotic effector caspase-3[64–66]. It was shown subsequently that caspase-3 undergoes stimulus-coupled activation, driven by proapoptotic Fas stimulation, via thioredoxin-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 thioredoxin, 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 anti-inflammatory 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 two key areas of SNO-mediated regulation: control of endothelial protein trafficking and suppression of nuclear factor-κB (NF-κB)-dependent expression of pro-inflammatory 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 (PSGL-1) on the leukocyte surface. P-selectins are transmembrane proteins that reside within resting endothelial cells in granules designated

Weibel-Palade bodies (WPB). Upon 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 Cys residues[73]. The resultant interruption of NSF-mediated disassembly of soluble NSF-attachment protein receptor (SNARE) complexes prevents WPB exocytosis from endothelial cells. Thus, S-nitrosylation of NSF, consequent, for example, upon stimulation with the GPCR agonist thrombin, is identified with the anti-inflammatory activity of eNOS (Figure 2). Similarly, inhibitory S-nitrosylation of NSF in platelets is anti-thrombotic through a similar mechanism[74] (see below).

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 (ICAMs) required for leukocyte adherence75–77. These and other pro-inflammatory effectors, including cytokines and cytokine receptors, are under direct transcriptional control by NF-κB78. Inhibitory S-nitrosylation of both NK-κB79 and its upstream activating enzyme complex, inhibitory κB kinase (IKK)[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 anti-inflammatory 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, re-establishment of vascular blood flow and O_2 delivery causes paradoxical tissue damage referred to as "reperfusion injury"81. 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 COX282, thereby generating cytoprotective prostaglandins. In addition, estrogen appears to exert its cardioprotective effect, at least in part, by augmenting S-nitrosylation of mitochondrial proteins 83–85. 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)86–88, 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 involved89. iNOS also contributes to the protective effects of pre-conditioning (exposure to moderate hypoxia prior to I/R, which attenuates reperfusion injury) that are partly recapitulated by nitroglycerin90, 91. Inasmuch as nitroglycerin bioactivation occurs predominantly in mitochondria and results in accumulation of protein S-nitrosothiols92, it may be suggested that S-nitrosylation plays a protective role85, 89, 93, 94. In support of this idea, SNO-proteins that have been shown to increase in pre-conditioned hearts are also identified following I/R[95].

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[43]. As described above, thioredoxin functions as a critical regulator of cellular

redox status and as an important modulator of S-nitrosylation. Recent studies suggest that statins, frequently employed anti-dyslipidemic and vasculoprotective agents, may exert their effects, at least in part, by inducing both S-nitrosylation and activation of thioredoxin[96].

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 effects97. In addition, accumulating evidence also supports the idea that NO may inhibit platelet aggregation via a cGMP-independent pathway98–102. 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 (i.e., 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 [74](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*[74].

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 control19, 46, 103, and in plasma, SNO-albumin provides a major reservoir of NO bioactivity[104]. 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[103]. Notably, albumin infusions may precipitate elevations in blood pressure by limiting the pool of bioavailable SNO for basal vessel relaxation105. 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 patients106, and misappropriation of NO as SNO-albumin is also directly implicated in the pathogenesis of hypertension in preeclampsia107, [108]. 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 ischaemia/reperfusion-associated heart damage[109, 110], lung injury in sickle cell disease[111] and cardiopulmonary dysfunction in endotoxemia[112, 113].

A number of studies have confirmed that the aging process is accompanied by a progressive decrease in bioavailable NO and concomitant endothelial dysfunction[15, 114–116]. 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 NOS 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[114, 116]. A subsequent study revealed that arginase is activated by S-nitrosylation of a single Cys residue, leading to its stabilization and to substantially enhanced substrate affinity (6-fold reduction in K_m)[15], 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[115].

SNO-Hemoglobin and Hypoxic Vasodilation

Hypoxic vasodilation is an autoregulatory physiological response that maximizes blood flow to regions in the arterial periphery with low hemoglobin (Hb) O_2 saturation, thereby matching perfusion with tissue O_2 demand[4, 17, 117]. The progressive diminution in blood O_2 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[118], and RBCs added to aortic ring bioassays at varying PO_2 actuate graded vasodilation (Figure 3A)17. Moreover, these RBC-induced responses can be replicated by S-nitrosohemoglobin (SNO-Hb), which has a well-documented role in mediating hypoxic vasodilation4, 119–122. Infusion of SNO-Hb (but not unmodified Hb) augments blood flow *in vivo* under normoxic conditions (Figure 3B)[122], and vasodilation is blunted in the setting of supraphysiological PO_2 (e.g., ambient O_2 at 3 atmospheres absolute)[122] (Figure 3C). By contrast, hypoxemia augments vasodilation by SNO-Hb. Changes in peripheral blood flow are predictably correlated with circulating SNO-Hb concentrations[123].

Hemoglobin exists predominantly in one of two structural states: R (relaxed, high O_2 affinity) and T (tense, low O_2 affinity)[120]. S-nitrosylation of hemoglobin (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 (e.g., hypoxia, low pH), NO groups are released to the surrounding tissues with resultant vasodilation. The duality of SNO-Hb as simultaneous O_2 carrier and NO donor may plausibly be harnessed in creating viable blood substitutes, with some applicability already reported in ischemic myocardium[124]. Additionally, it appears that the coronary vasodilator nitroglycerin improves perfusion of ischemic myocardium by utilizing SNO-Hb-mediated O_2 delivery in concert with NO unloading[125]. Inasmuch as RBCs play a central role in autoregulation of blood flow[4], perturbations in the delivery of SNO by RBCs may underlie a variety of pathophysiological states characterized by microvascular dysfunction[126]. For example, pulmonary hypertension, a clinical entity often triggered by sustained hypoxemia, leads to depletion of RBC SNO-Hb stores and consequently to defective PO_2-coupled vasoregulation and ventilation/perfusion mismatching[127]. Moreover, *in vivo* repletion of SNO-Hb can correct these physiologic 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 [128]. 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[129, 130].

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 Ca^{2+} cycling that underlies excitation-contraction coupling (ECC) is no exception[131]. ECC spans an ordered sequence from electrical excitation of the individual myocyte to heart contraction, subserved by the tightly regulated trafficking of Ca^{2+} flux from one cellular compartment to another[132]. Upon membrane depolarization of the cardiac myocyte generated by voltage-gated Na^+ channels, a cytosolic influx of Ca^{2+} occurs via the plasmalemmal L-type Ca^{2+} channel. Through a process known as Ca^{2+}-induced Ca^{2+} release, this initial Ca^{2+} current triggers a more pronounced Ca^{2+} release from the sarcoplasmic reticulum (SR) through the ryanodine receptor/Ca^{2+} release channel (RyR2). Myocyte contraction proceeds when Ca^{2+} binds to troponin C in myofilaments, activating myosin ATPase. Relaxation of the myocyte entails diastolic reuptake and extrusion of cytosolic Ca^{2+} by way of the SR Ca^{2+} ATPase (SERCA2a) and the sarcolemmal Na^+/Ca^{2+} exchanger[132], 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[85, 131, 133–138], 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 NOSs (NOS1and NOS3), which reside in close proximity to substrates for S-nitrosylation[139](Figure 4). NOS3 is spatially confined to sarcolemmal membrane caveolae and is thus adjacent to the L-type Ca^{2+} channel, whose S-nitrosylation inhibits ion influx[135, 140]. In a similar fashion, NOS1 resides in the SR where it is complexed with RyR2, and S-nitrosylation activates RyR2 (increases channel opening probability, P_o)[136, 141]. Co-localization and targeted S-nitrosylation may also hold true for SERCA2a[142, 143]. Collectively, these observations demonstrate the precise spatiotemporal regulation of S-nitrosylation that underlies control by NO of cardiac ECC.

It has been reported that nNOS re-distributes to the sarcolemma in heart failure, where it may regulate both β-adrenergic responsiveness and Ca^{2+} flux[143–145], 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 Ca^{2+} channels[143]. Thus, inhibitory S-nitrosylation of L-type Ca^{2+} channels by nNOS is likely anti-arrhythmogenic.

More generally, disruption of the NO/redox equilibrium in myocytes, through alteration of either levels or spatiotemporal distribution of NO/ROS, is widely regarded as a *sine qua non* of heart failure[146]. Up-regulation of oxidant (ROS) production, notably by xanthine oxidase, can overwhelm endogenous, NO-based signaling and promote the mechano-energetic uncoupling characteristic of cardiac dysfunction[147]. Therapies directed against xanthine oxidase enable reverse remodeling in rats with dilated cardiomyopathy148. Thus, restoration of NO/redox homeostasis provides a potentially fruitful approach to restoring cardiac contractile function149

β-Adrenergic Receptor System

In cardiac myocytes, eNOS is activated following β-adrenergic receptor stimulation[150], 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[135]. 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[83].

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-adrenergic receptor (β-AR) phosphorylation and desensitization[29], 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[28], leading to enhanced eNOS-dependent receptor trafficking (Figure 5A). Importantly, these studies reported increased protein S-nitrosylation (SNO-GRK2 and SNO-β-arrestin) in $GSNOR^{-/-}$ mice. Finally, S-nitrosylation of dynamin facilitates clathrin-dependent endocytosis of membrane receptors including the β-AR and thereby receptor downregulation [151] (Figure 5C). S-nitrosylation is thus under enzymatic control and GSNO is a central player in β-adrenergic receptor signaling. Other studies have demonstrated that VEGF may regulate GSNOR expression[152]; crosstalk between VEGF (and other pro-vascular signals) and β-adrenergic receptors may be mediated via the GSNOR/S-nitrosylation axis.

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[138] (Figure 6 summarizes findings in the case of the ventricular action potential). For example, the inward-rectifying K current (IK_1) shapes phase 3, and S-nitrosylation of a single cysteine in the relevant channel protein, Kir2.1, shortens the action potential[153]. 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.[141] 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[141], which creates contractile dysfunction and a pro-arrythmogenic state[154, 155]. nNOS mutant mice also exhibit a pro-arrhythmic state following myocardial infarction, which is associated with diminished S-nitrosylation of RyR2, SERCA2a and L-type calcium channel[143].

Mutations in α-syntrophin, a dystrophin-associated protein that acts as a scaffold between nNOS and the plasma membrane Ca-ATPase[156], have been shown to contribute to long-QT syndrome[157]. The Ala^{390}→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[157], a pro-arrhythmic event recapitulating long-QT syndrome.

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 SNO-based cardiovascular function and pathophysiology will undoubtedly yield novel therapeutic targets with great potential to improve clinical outcomes[38].

Non-standard abbreviations and acronyms

EDRF	endothelium-derived relaxation factor
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione reductase
SNO	S-nitrosothiol

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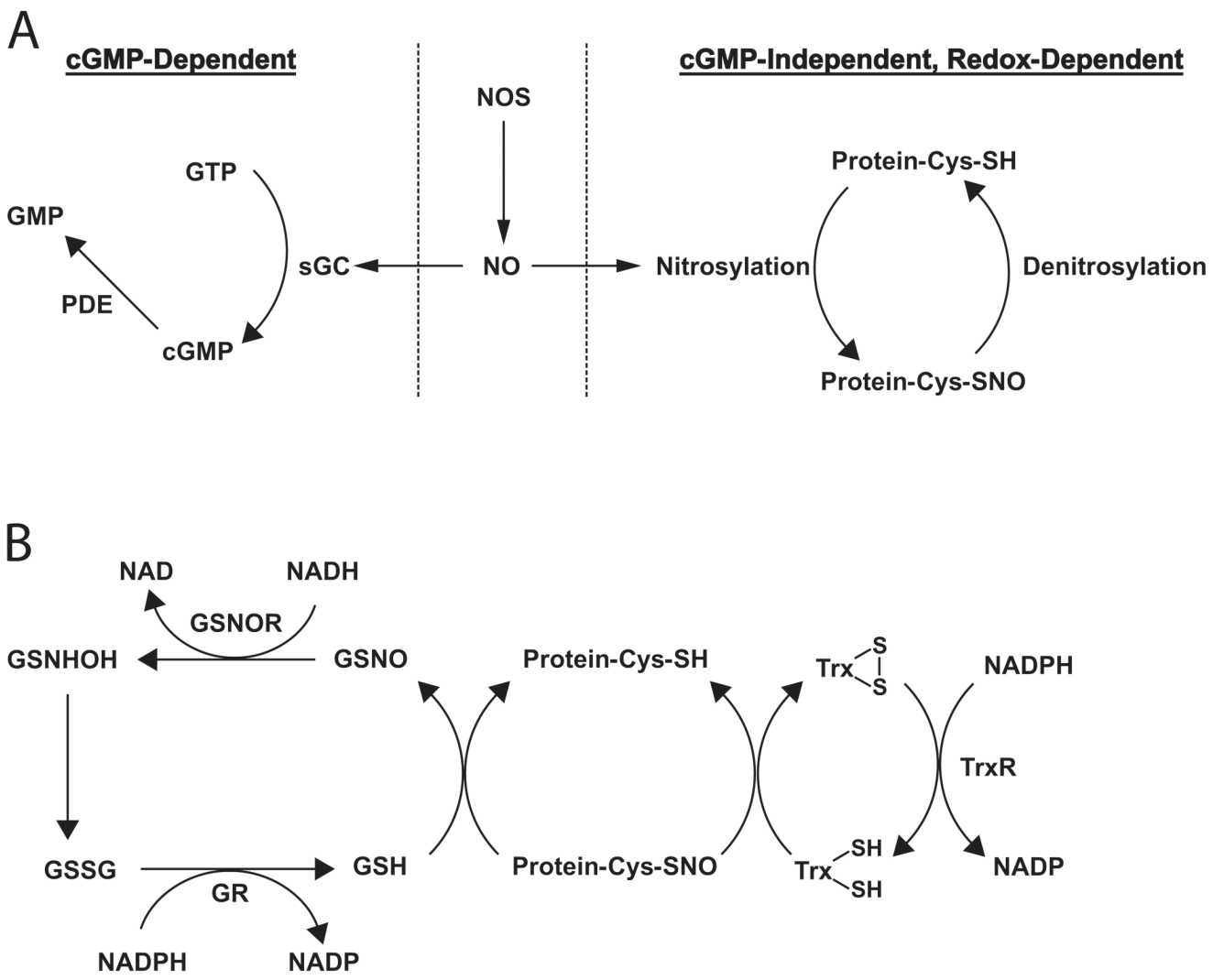

Figure 1.

The roles of cGMP and S-nitrosylation in NO-based signaling (A) and enzymatic protein denitrosylation mediated by the S-nitrosoglutathione reductase (GSNOR) and thioredoxin (Trx) systems (B). (A) NO synthase (NOS) synthesizes NO, which may activate soluble guanylyl cyclase and thereby enhance production of cGMP (left) or subserve protein S-nitrosylation (right). The cGMP-dependent pathway is deactivated by cGMP-phosphodiesterase (PDE), which hydrolyzes cGMP to GMP (PDE 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-hydroxysulfenamide (GSNHOH), which can undergo further reaction with GSH to generate oxidized glutathione (GSSG). The redox cycle is completed by reduction of GSSG to GSH via GSSG reductase. Denitrosylation mediated by the thioredoxin (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 thioredoxin reductase (TrxR), which employs the reducing power of NADPH to regenerate active Trx.

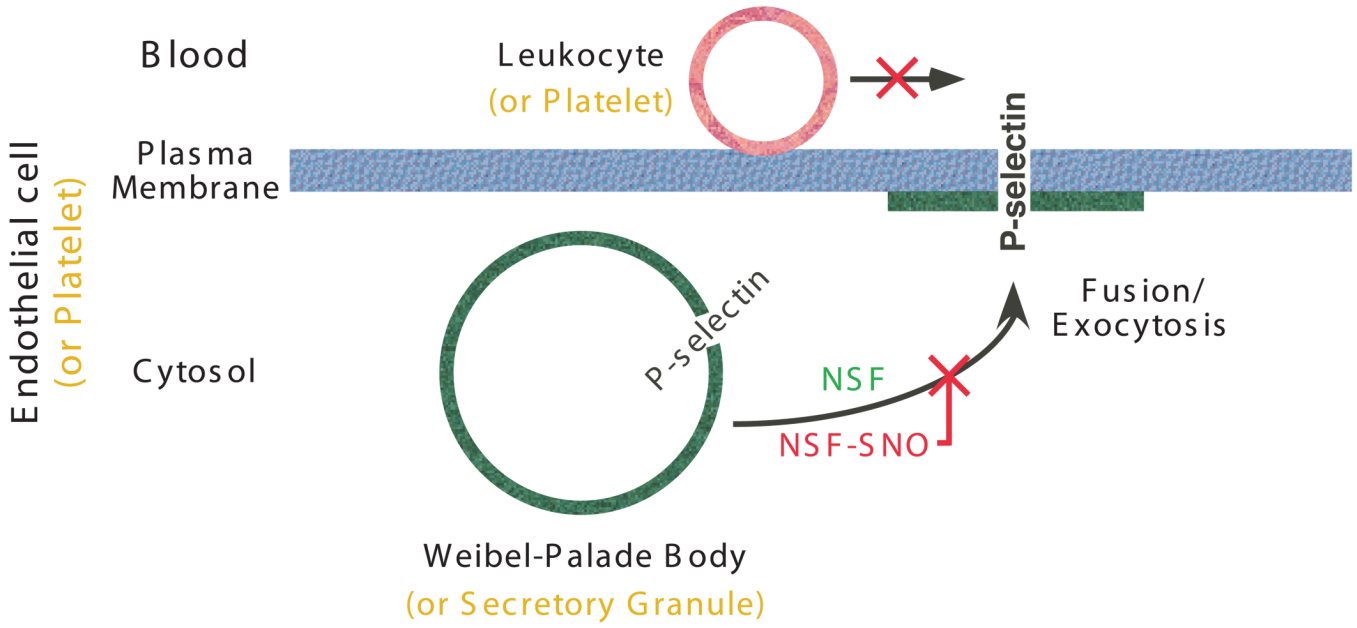

Figure 2.

S-nitrosylation of NSF is anti-inflammatory and anti-thrombotic[73, 74]. In endothelial cells, inhibitory S-nitrosylation of NSF suppresses exocytosis of Weibel-Palade bodies and thereby externalization of P-selectin, which inhibits leukocyte rolling and thus vascular inflammation. Similarly, in platelets (labeling in parentheses), inhibitory S-nitrosylation of NSF suppresses exocytosis of secretory granules and thereby externalization of P-selectin (and other adhesive molecules), which reduce platelet activation, adhesion, aggregation and rolling on the endothelium. These effects are anti-thrombotic.

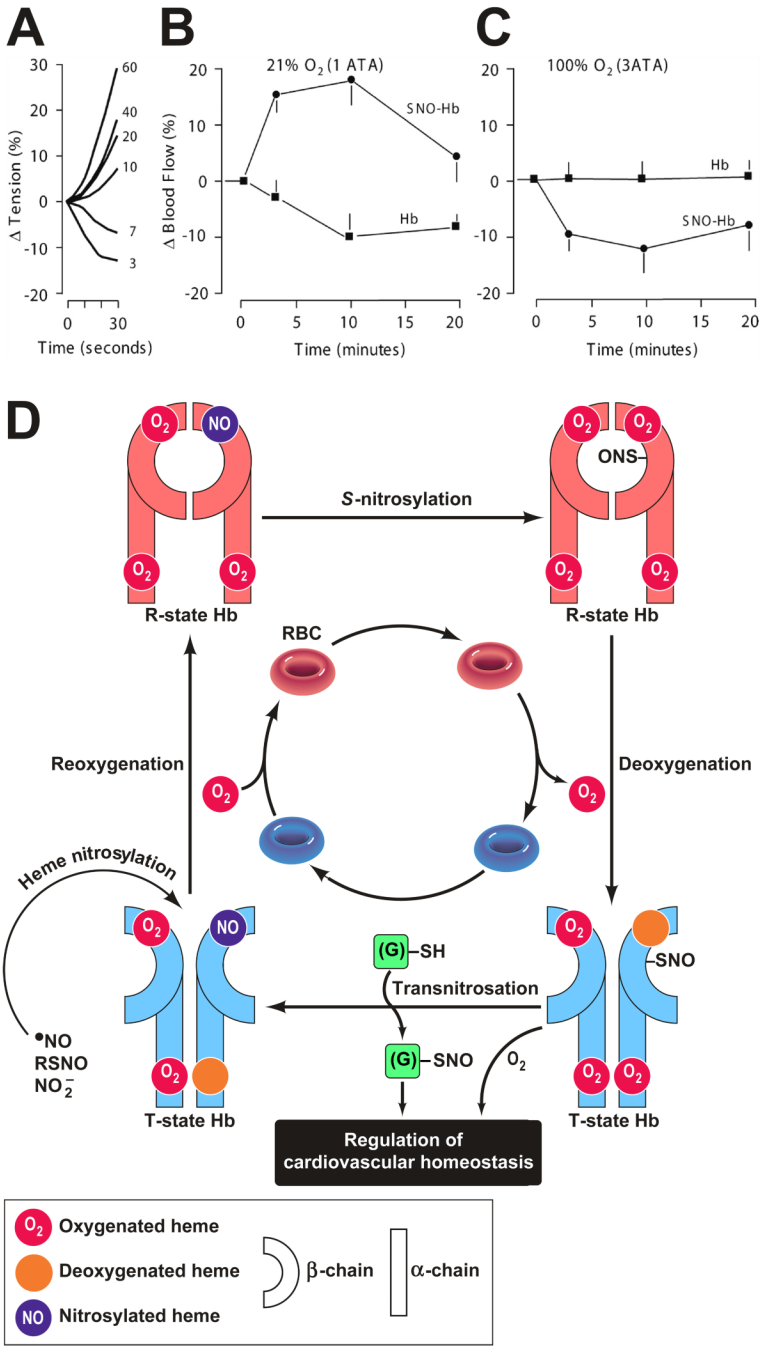

Figure 3.

SNO-Hb subserves hypoxic vasodilation. (A) PO_2 determines the ability of RBCs to constrict or relax aortic ring preparations on a second-by-second time scale. PO_2 is indicated for each curve, which illustrate a graded response. (B) and (C) O_2-dependent effects of SNO-Hb and Hb on local cerebral blood flow are shown in normoxia and hyperoxia. SNO-Hb infusion in vivo (1μmol/kg over 3min, beginning at time 0) immediately increases local cerebral blood flow in the caudate-putamen nucleus of rats breathing 21% O_2 at 1 atmosphere absolute (ATA), where tissue PO_2 ranges from 19 to 37 mmHg. Thus, SNO-Hb appropriately increases blood flow in relatively hypoxic tissue; however, non-nitrosylated Hb decreases perfusion. In 100% O_2 at 3 ATA, where tissue PO_2 ranges from 365 to 538 mmHg, vasodilation is abrogated

because SNO-Hb cannot allosterically dispense NO bioactivity (Adapted from Allen et al.) [158]. (D) Allosteric transitions of circulating hemoglobin (Hb) regulate delivery of NO bioactivity to preserve vascular O_2 homeostasis. Hb in RBCs senses [O_2] and responds through allosterically controlled NO binding, SNO formation, and NO group release. At high O_2 in the pulmonary venous system, Hb is in the R-state, Cys β93 is reactive and Cys93-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. Further, 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 126).

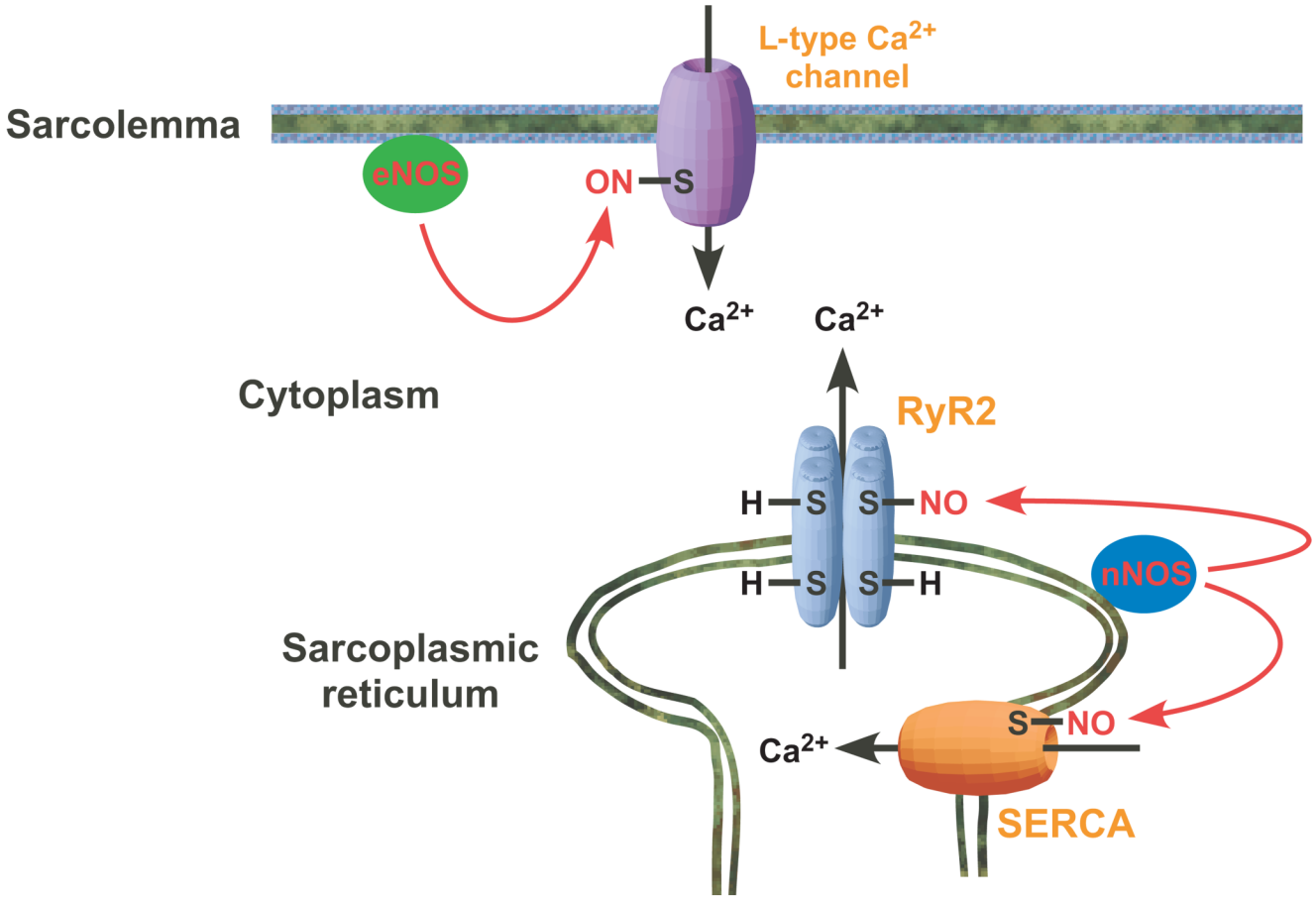

Figure 4.

S-nitrosylation regulates myocardial Ca^{2+} handling and thereby excitation-contraction coupling. RyR2, the cardiac form of the tetrameric ryanodine receptor/Ca^{2+} release channel, is localized to the SR membrane in proximity with the plasma membrane L-type calcium channel (LTCC), which provides the substrate for calcium-mediated calcium release from SR to cytosol. The SR-localized Ca^{2+}-ATPase (SERCA) replenishes SR Ca^{2+}. RyR2 co-localizes with nNOS in the SR, and S-nitrosylation of RyR2 (mediated by GSNO) potentiates Ca^{2+} release. As in skeletal muscle RyR1, physiological S-nitrosylation of one or a few Cys within each RyR2 monomer is likely to be the case. S-nitrosylation of the LTCC (α_{1C} subunit; resulting in, for example, attenuated β-AR-dependent contractility) and of SERCA is inhibitory. Hypo-S-nitrosylation of RyR2 is associated with diastolic Ca^{2+} leakage and arrhythmia characteristic of sudden cardiac death. S-nitrosylation of the LTCC has been associated with ischemic preconditioning that reduces reperfusion injury, whereas hyper-S-nitrosylation of the LTCC has been associated with atrial fibrillation. Note in addition that aberrant S-nitrosylation can result from the translocation of nNOS to the plasma membrane that is seen in association with myocardial infarction and cardiomyopathy. Further, aberrant and in particular hyper-S-nitrosylation can result in irreversible oxidative modification of S-nitrosylated proteins in concert with reactive oxygen species produced by endogenous enzymes including xanthine oxidase.

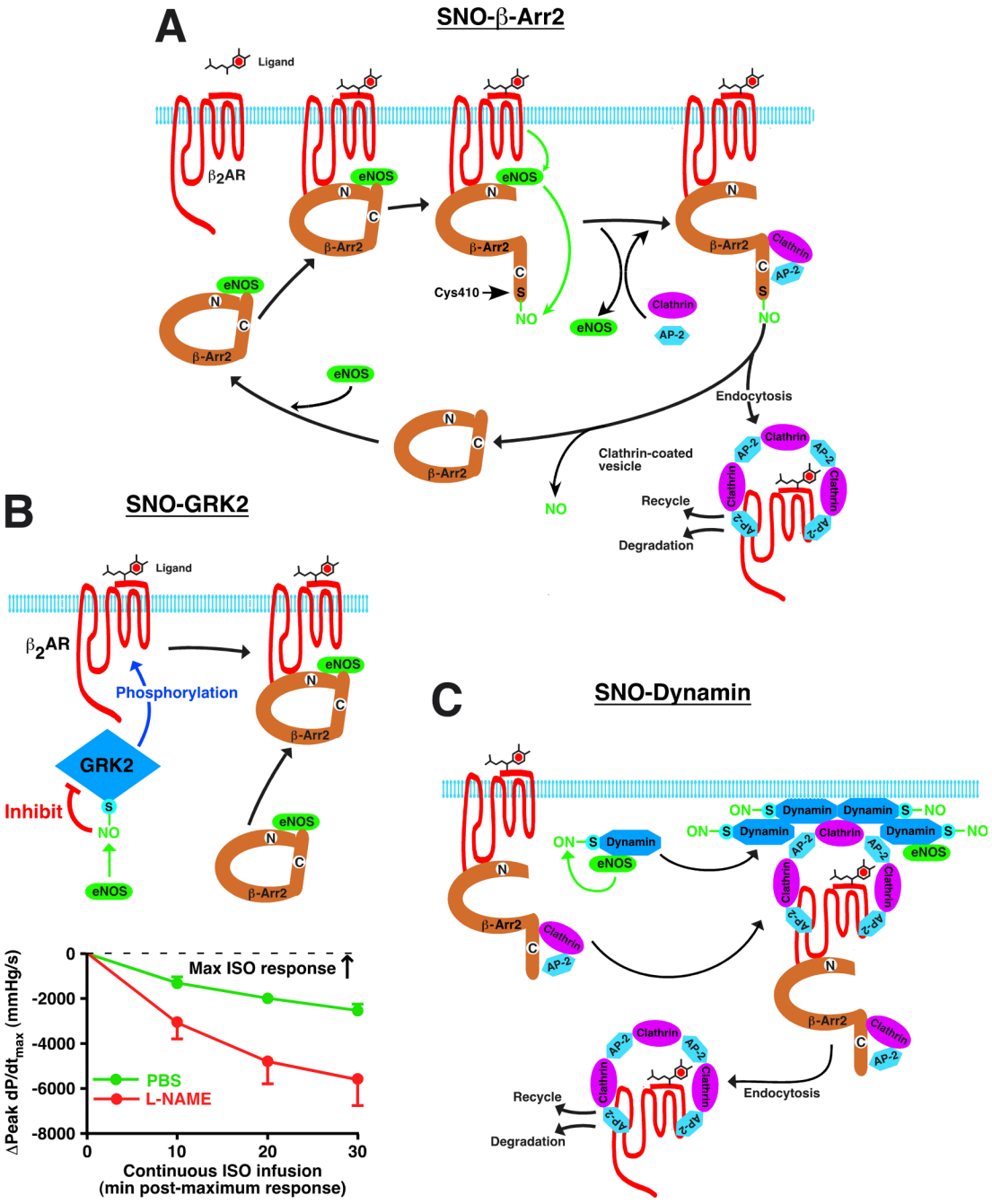

Figure 5.

A Schematic summary of the regulation of agonist-induced β_2-adrenergic receptor trafficking by S-nitrosylation/denitrosylation of β-arrestin 2 (β-Arr 2), G protein-coupled receptor kinase 2 (GRK2), and dynamin. (A) β-Arr 2 serves as a scaffold that functionally colocalizes eNOS and β-ARs (as well as other G protein-coupled receptors [GPCRs]). Ligand (isoproterenol) stimulation results in activation of eNOS and S-nitrosylation of β-Arr 2. S-nitrosylation of β-Arr 2 promotes its dissociation from eNOS and its association with clathrin heavy chain/β-adaptin, which facilitates routing of the β_2-AR into the clathrin-based endocytotic pathway, and β-Arr 2 is subsequently denitrosylated. (B) Inhibition of GRK2 by ligand-coupled S-nitrosylation suppresses agonist-stimulated β-AR phosphorylation, β-Arr 2 recruitment, and

receptor desensitization and downregulation (schematic at top) At 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 from28, 29).

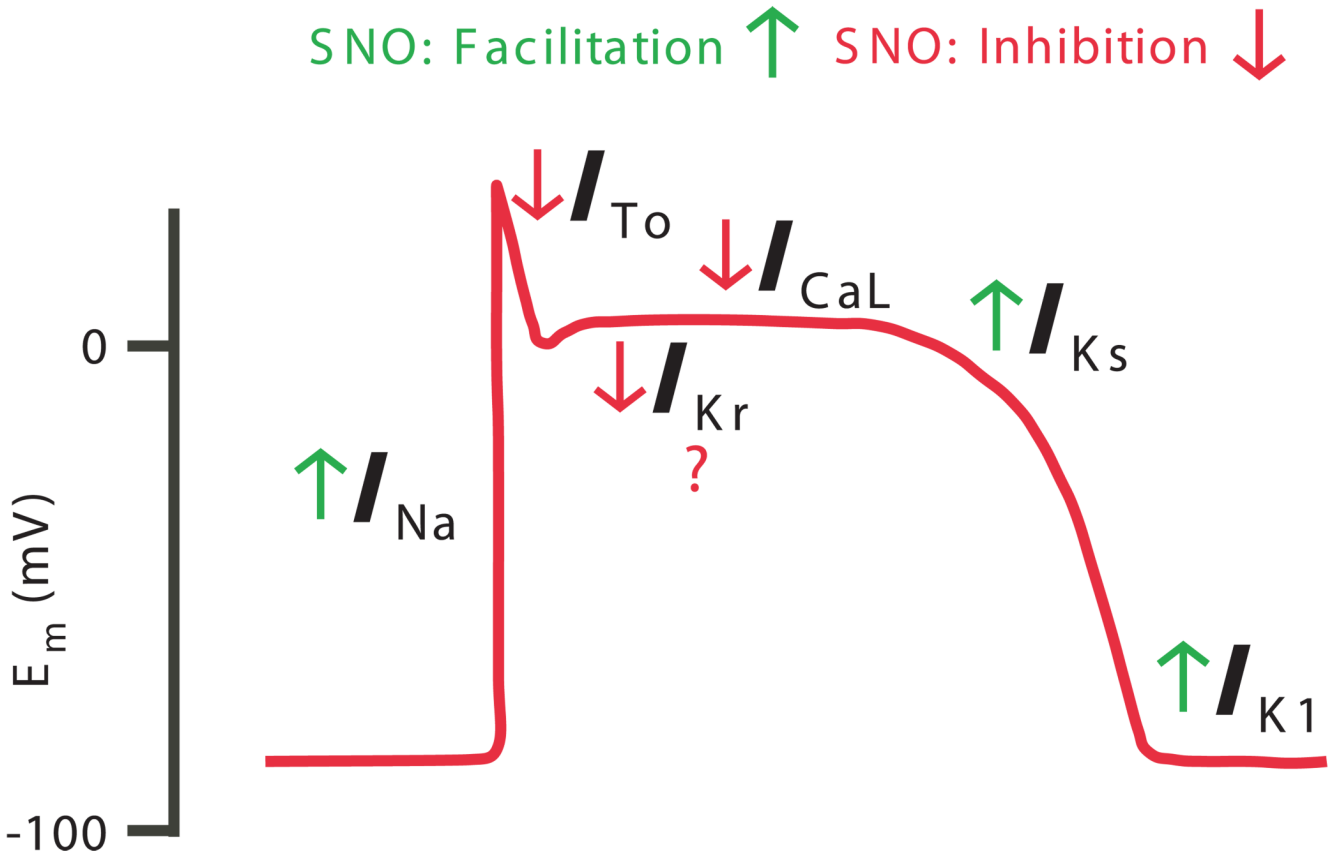

Figure 6.

S-nitrosylation of channel proteins regulates all phases of the ventricular action potential. S-nitrosylation of SCN5A channels enhances the Na^+ current (I_{Na})[157, 159], whereas S-nitrosylation of the α1C subunit of the L-type Ca^{2+} channel inhibits the L-type Ca^{2+} current (I_{CaL})[135, 140]. Among voltage-gated potassium channels, S-nitrosylation of the KCNQ1 subunit facilitates the slowly activating component of the delayed rectifier K^+ current (I_{Ks})[133], whereas S-nitrosylation exerts an inhibitory influence on Kv4.3 and thus the transient outward potassium current (I_{To})[160], as well as Kir2.1[153], and thus I_{K1} (phase 4). In addition, heterologously expressed human ether-a-go-go-related gene 1 (hERG1) potassium channels, which mediate the rapidly activating delayed rectifier K+ channel (I_{Kr}) in their native environment, are inhibited by NO in a cGMP-independent fashion161. Note that, in the atrium, S-nitrosylation inhibits hKv1.5 and thus the ultra-rapid delayed rectifier current 162 (Adapted from138).

Table 1

Exemplary SNO-proteins in the Cardiovascular System

SNO-protein	Cell/Tissue	Effects of S-nitrosylation
Albumin	Serum	NO bioactivity reservoir[104]
β-arrestin 2	Endothelium	Enhanced binding of β-Arr 2 to clathrin and internalization of β-adrenergic receptor[28]
Caspase 3	Endothelium, lymphocytes	Anti-apoptosis and preservation of endothelial function[64–67, 163]
Dimethylarginine dimethylaminohydrolase	Endothelium	Accumulation of dimethylarginine and NOS inhibition[16]
G-protein-coupled receptor kinase 2	Endothelium, Myocardium	Attenuation of β-adrenergic receptor desensitization[29]
Hemoglobin	Erythrocyte	Hypoxic vasodilation and regulation of vessel tone[4, 17]
Hypoxia-inducible factor 1 α	Myocardium	Increased VEGF production and myocardial capillary density[31]
MAP kinase phosphatase 7	Endothelium	Promotes endothelial cell migration and angiogenesis[54]
N-ethylmaleimide-sensitive factor	Platelets	Prevention of platelet activation[73, 74]
Ryanodine receptor 2	Cardiac muscle	Enhanced cardiac Ca^{2+} release and contractility[136, 137]
Tissue transglutaminase	Endothelial surface	Inhibition of platelet aggregation[164]
Metallothionein	Vascular smooth muscle	Myogenic reflex, pulmonary vasoconstriction[170,171]

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. MPK7 indicates mitogen-activated protein kinase phosphatase 7.

Table 2

Aberrant S-nitrosylation in Cardiovascular Disease

SNO-protein	Disease State
Serum albumin	Pre-eclampsia[107, 108] Ischemic coronary syndromes[106]
Hemoglobin	Congestive heart failure[165] Pulmonary arterial hypertension[127] Sickle cell disease[128] Diabetes (Type 1) [129, 130] Septic shock[19]
Hif-1α	Pulmonary arterial hypertension[60]
Matrix metalloproteinase 9	Stroke[166]
Ryanodine receptor 2	Arrhythmogenesis, Heart failure [141]
L-type Ca2+ channel (α_{1C} subunit)	Atrial fibrillation/arrhythmia[135, 143, 167]
Cardiac Na^+ channel SNC5a	Long Q/T syndrome[157]
Slowly activating delayed-rectifier K^+ channel	Atrial fibrillation/arrhythmia [133, 134]
Insulin receptor β	Diabetes (Type 2)[168,169]
Insulin receptor substrate 1	Diabetes (Type 2)[168,169]
Akt (protein kinase B)	Diabetes (Type 2)[14,168,169]

Examples of proteins for which hypo- or hyper-S-nitrosylation has been implicated in the mechanism of disease. Note in addition that S-nitrosylation of multiple substrates including Cox2, Hif-1α, the L-type Ca^{2+} channel, RyR2 and SERCA2 is implicated in the cardio-protective effects of both statins and ischemia-or drug-induced preconditioning, and in amelioration of the effects of myocardial infarction (see text).