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Forum Review

S-Nitrosylation: NO-Related Redox Signaling to Protect Against Oxidative Stress

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ABSTRACT

Nitric oxide (NO) plays an important role in the regulation of cardiovascular function. *S*-nitrosylation, the covalent attachment of an NO moiety to sulfhydryl residues of proteins, resulting in the formation of *S*-nitrosothiols (SNOs), is a prevalent posttranslational protein modification involved in redox-based cellular signaling. Under physiologic conditions, protein *S*-nitrosylation and SNOs provide protection preventing further cellular oxidative and nitrosative stress. However, oxidative stress and the resultant dysfunction of NO signaling have been implicated in the pathogenesis of cardiovascular diseases. *Antioxid. Redox Signal.* 8, 1693–1705.

INTRODUCTION

Oxidative stress in vivo can result from a reduction in endogenous antioxidant, burst formation of reactive oxygen species (ROS), or other imbalances between antioxidants and ROS. Increasing data suggest that physiologic levels of ROS may play an important role in normal cell signaling. In contrast, under pathophysiologic conditions, such as myocardial ischemia–reperfusion injury and cardiomyopathy, ROS production increases and exceeds the antioxidant defense of the cell. Thus, a large transient increase or a moderate sustained increase in ROS is suggested to be detrimental and to contribute to heart dysfunction and myocyte death (47, 77).

Nitric oxide (NO) plays an important role in the regulation of cardiac function (7, 22, 47). In addition to activating cyclic guanosine monophosphate (cGMP)-dependent signaling pathways, NO can directly modify sulfhydryl residues of proteins through *S*-nitrosylation, which has emerged as an important posttranslational protein modification based on prototypic redox mechanisms in signal transduction (12, 48, 68, 82). Under physiologic oxidative stress, NO might provide protection to cells by *S*-nitrosylation of some critical protein thiols, preventing them from further oxidative modification by ROS (Fig. 1). Conversely, increased oxidative stress and the resultant dysregulation of NO are implicated in the pathogenesis of cardiovascular diseases. Nitrosative stress occurs with an increase in reactive nitrogen species (RNS) and ROS formed from oxidative stress. For example, the peroxynitrite ($OONO^-$), generated from NO and superoxide, is a very strong cytotoxic oxidant, which can irreversibly damage cells by oxidation of free thiols, nitration of tyrosine residues, and lipid peroxidation (100, 112). In cardiac myocytes, ROS and RNS induce stress-signaling pathways involved in mitochondrial dysfunction, intracellular Ca^{2+} overload, hypertrophy and heart failure, and apoptosis and necrosis (122).

Redox and NO

Physiologic levels of ROS and NO can interact and modulate the signaling of one another. The redox status of the cell influences NO signaling in two major ways. First, the balance between NO, molecular oxygen (O_2), superoxide anion (O_2^-) radical, and antioxidants determines what products are made. As shown in Fig. 1, depending on the localization and level of enzymes that produce or consume O_2^-, NO can (a) activate guanylyl cyclase and mediate cGMP-dependent signaling; (b) form N_2O_3 by autooxidation via the reaction with O_2 and lead to protein *S*-nitrosylation; (c) generate GSNO in the presence of GSH, which can mediate transnitrosylation reactions;

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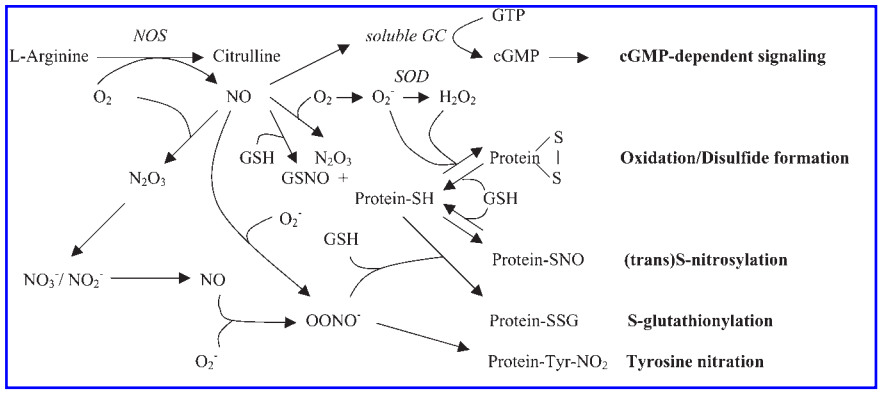

FIG. 1. Redox-based NO-related signaling. Under physiologic condition, NO is produced from NOS and mediates cGMP-dependent and/or cGMP-independent signaling, which is dependent on the (sub)cellular redox status. NO can also be produced by nitrate/nitrite in nonenzymatic (such as low pH) and enzymatic ways (such as by XOR) in ischemic or anoxia conditions. The formation of N_2O_3 by autooxidation of NO or the formation of LMW-SNOs, such as GSNO, leads to protein *S*-nitrosylation by direct SNO formation or transnitrosylation. The ROS (O_2^-, H_2O_2, etc.) causes the oxidation of protein such as disulfide formation. Low physiologic levels of $ONOO^-$ via the reaction of NO and O_2^- can interact with GSH, resulting in reversible *S*-glutathiolation of proteins; however, high concentrations or sustained formation of NO and O_2^- increase $ONOO^-$ formation, leading to irreversible tyrosine nitration of proteins.

(d) produce $ONOO^-$ by reacting with O_2^-, which was originally suggested to be a toxic end product of high levels of NO and O_2^-, leading to irreversible nitration of proteins; however, recent data suggest that low physiologic levels of $ONOO^-$ can interact with reduced glutathione (GSH), resulting in reversible *S*-glutathiolation of proteins, such as the cardiac sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (*i.e.,* SERCA2a) (1). In some disease states, the thiols in SERCA2a that are the target of *S*-glutathiolation are irreversibly oxidized, thereby blocking the NO-dependent activation of SERCA2a (1). It is also interesting that neuronal NO synthase (nNOS) and xanthine oxidoreductase (XOR), an enzyme that produces O_2^-, have been reported to colocalize in the cardiac SR. Furthermore, inhibition or deletion of nNOS results in an increase in XOR-mediated O_2^- production, suggesting that NO produced by nNOS inhibits the activity of the colocalized XOR (57). Taken together, these studies suggest that the alterations in the regulation of NO- and ROS-generating enzymes, or the levels of antioxidants such as superoxide dismutase (SOD) and GSH, will alter NO and ROS signaling and the resulting protein modifications. Also consistent with this theme, endothelial NOS (eNOS) and extracellular SOD (ecSOD) have been reported to localize in the sarcolemma of ventricular myocytes (11). The efficiency of NO synthase (NOS) can also be a factor, because it has been shown that NOS can produce O_2^- if the substrate L-arginine or other cofactors such as tetrahydrobiopterin are limiting (19).

ROS and NO can both interact with thiol groups, and this is a second mechanism by which redox and NO signaling interact. It has been reported that *S*-nitrosylation of thiol groups in proteins can protect these proteins against irreversible oxidative stress (41, 128). In contrast, as mentioned earlier, irreversible oxidation of thiols can block the physiologic modification by *S*-nitrosylation or *S*-glutathiolation and thereby interfere with normal physiologic signaling (1). It has been suggested that NO can protect cells from oxidative stress, whereas loss or inhibition of NOS enhances oxidative stress. Hare and Stamler (47) also suggested that ROS can alter the balance between phosphorylation and *S*-nitrosylation of key signaling molecules.

Protein S*-nitrosylation and its detection*

The *S*-nitrosylation reaction can be mediated through NO carriers such as *S*-nitrosothiols (SNOs), NO complexed with transition metals, or a direct reaction between NO and thiols in the presence of electron acceptors. NO is unable to react with nucleophiles under oxygen-free conditions, suggesting that its higher oxides, possibly N_2O_3, are actually the nitrosylating agents. It has been found that oxidation of NO to N_2O_3 is facilitated by micellar catalysis, which is mediated within the hydrophobic pocket of proteins (73, 97). The protein *S*-nitrosylation is redox reversible with high spatial and temporal specificity.

In most cases, the specificity of *S*-nitrosylation is governed by consensus acid–base motifs controlling targeted thiol pKa and nucleophilicity, and physiologic concentrations of NO can lead to *S*-nitrosylation of only a single cysteine thiol. However, there is complexity in the acid–base motif (*i.e.*, that the target cysteine may not have to be juxtaposed with acidic and/or basic residues with respect to primary sequence, but such a juxtaposition may emerge in three-dimensional protein structure) (48). In addition, the acid–base motif may be limited in its application to hydrophilic environments, whereas NO-related signals may originate in membranes and other hydrophobic environments that facilitate protein *S*-nitrosylation (73, 97). Thus, the relative hydrophobicity of the region surrounding the target thiol may provide a "hydrophobic motif" for protein *S*-nitrosylation (48). A further determinant that governs the specificity of posttranslational protein modification by NO is provided by the colocalization of NO sources and targets proteins, which is based at least in part on specific protein–protein interactions with NO synthases. Conversely, the *S*-nitrosylation also is a temporal signaling event, which depends on the formation of NO by NOS and other nitrosylating equivalents. A subsequent transnitrosylation reaction may occur once a protein within a signaling complex or low-molecular-weight thiols such as GSH becomes *S*-nitrosylated, which may serve to deliver NO sequentially to its neighboring proteins within the complex, potentially creating a cascade of spatial and temporal *S*-nitrosylation–based NO signaling (15, 48).

Thus, a number of models have been proposed to provide for targeting *S*-nitrosylation to specific proteins, including (a) the consensus *S*-nitrosylation acid–base motif controlling targeted thiol pKa and nucleophilicity; (b) hydrophobic compartmentalization facilitating the reaction of NO and O_2; (c) spatial subcellular compartmentalization of NOS and proximity to potential targets; (d) allosteric regulation of thiol accessibility and reactivity by cellular redox, oxygen, metal ions, and nitrosonium (NO^+) addition reaction; and (e) subsequent transnitrosylation reactions (15, 48).

The multiplicity of effects of protein *S*-nitrosylation has prompted the development of reliable techniques for detection of SNO, including immunoassay with antibody against *S*-nitrosocysteine (119), NO-based (after breakdown of SNO by mercury) chemical reactions such as Saville–Griess colorimetric method (49) or 2,3-diaminonaphthylene (DAN) fluorescence assay (38, 85), and a photolytic/ozone chemiluminescence technique (31). A newly developed biotin switch method (54, 55) has become a widespread technique in combination with proteomic approaches (44, 65, 76, 81). However, a recent study showing that ascorbic acid is not a selective reductant for *S*-nitrosothiols raises concerns about the specificity of the biotin switch method (67).

SNO storage and transportation

NO is a lipophilic and short-lived free radical. In the cardiovascular system, NO can produce remote or long-lasting effects by formation of various SNOs, serving as "NO carriers" to store and transport NO *in vivo*, which induces slower but much more persistent effects than does pure NO (96). SNOs derived from proteins, peptides, and amino acids supply cellular compartments and extracellular fluids with reservoirs of NO bioactivity, playing key roles in human health and disease (34).

Low-molecular-weight SNO. Intracellular NO may be buffered by low-molecular-weight (LMW)-SNOs, such as *S*-nitrosoglutathione (GSNO), after the reaction of NO with LMW thiols like GSH. These LMW-SNOs have been proposed to function as major physiologic mediators of the actions of NO (96). GSNO is able to modify protein thiols via *S*-nitrosylation or glutathionylation, which is dependent on the surrounding redox equilibrium. Regulation of the cardiovascular system by GSNO appears to be of particular physiologic interest, because GSNO is the most abundant endogenous SNO and has been suggested to be a potential NO-storage and -transport species. GSNO decomposes slowly to generate NO, a reaction catalyzed by LMW thiols and trace metal ions (130). In addition, denitrosylation can also be achieved by GSNO reductase. An elevated level of SNOs in the plasma of GSNO reductase–deficient mice suggests that SNO proteins and GSNO are in a dynamic equilibrium and that transnitrosylation via GSNO is an integral mechanism for SNO-protein formation *in vivo* (72).

SNO-albumin. Albumin is the most abundant transport and storage protein in the mammalian vasculature. At physiologic NO concentrations, plasma albumin accelerates formation of LMW-SNOs *in vitro* and *in vivo* via a mechanism of micellar catalysis of NO oxidation in the albumin hydrophobic core and subsequent transfer of NO^+ to LMW thiols. The albumin-mediated LMW-SNO production, which is directly dependent on the concentration of circulating LMW thiols, contributes to vasodilatory vascular control (106). Therapeutically, inhaled NO enhances SNO-albumin formation and has been found to decrease ischemia–reperfusion injury (98).

SNO-hemoglobin. In red blood cells, it has been found that the binding of O_2 to heme iron in hemoglobin (Hb) promotes NO binding to a particular cysteine residue (β chain-Cys93), forming SNO-Hb. Deoxygenation leads to an allosteric transition in SNO-Hb that releases the NO group in the microcirculation and regulates vascular tone (23, 114). The exportation of NO bioactivity from red blood cells is through transnitrosylation from SNO-Hb to vicinal cysteine residues in the cytoplasmic domain of anion-exchange protein AE-1, also known as Band 3 (51). Thus, red blood cell–derived SNO-Hb regulates the vascular response to changes of tissue oxygen tension, thereby matching regional blood flow with local metabolic demands (113).

SNO-myoglobin. Myoglobin (Mb) is a key element influencing redox pathways in cardiac muscle to protect the heart functionally and metabolically from oxidative damage (32). Mb has been suggested to be a scavenger of cellular NO in myocardium (33), which can protect the heart from iNOS-mediated nitrosative stress (40). The observed impairment of cardiac function and exercise endurance in $Mb^{-/-}$ mice can be partly attenuated by NO inhibition (90). Although direct evidence is still lacking, *S*-nitrosylation of Mb to form SNO-Mb is thought to be one of the molecular mechanisms for its NO-scavenging function, given the similarities in the kinetic and thermodynamic properties of NO interactions with Hb and Mb (33).

Nitrite. Although SNO-proteins have been found to exist in the human circulation, their role in the regulation of basal vascular tone has been challenged because of the presence of other bioavailable NO sources, such as nitrite. Nitrite, generated from the reaction of NO and oxygen, can be converted to NO by protons or via enzymatic conversion by XOR (9, 56, 127, 139). Hemoglobin also has been shown to function as a nitrite reductase (52). It has been found that circulating nitrite is bioactive and provides a source of intravascular NO (15, 39, 123). In models of heart and liver ischemia–reperfusion injury, nitrite has been shown to reduce infarct size dramatically and to exert NO-dependent protective effects on cellular apoptosis and necrosis. Thus, tissue nitrite can serve as a significant extravascular pool and biologic storage reserve of NO during a period of hypoxia, subserving a critical function in tissue protection from ischemic injury (28, 53, 121, 125).

Regulation of cardiac function by protein S*-nitrosylation*

NO plays an important role in modulating myocardial function in both health and disease (7, 47). Increasing evi-

dence suggests that nitrosative and oxidative stress play important roles in the regulation of cardiac myocyte function and survival (47, 111). Under physiologic oxidative stress, NO might provide protection to cells by *S*-nitrosylation of some critical protein thiols, preventing them from further oxidative damage.

Intracellular Ca^{2+} handling

Ca^{2+}-induced Ca^{2+} release (CICR) is a well-known molecular mechanism of excitation–contraction (e-c) coupling in cardiac myocytes. In brief, membrane depolarization leads to Ca^{2+} entry via the sarcolemmal L-type Ca^{2+} channel, which then stimulates a larger Ca^{2+} release from the SR through the cardiac isoform of the ryanodine receptor (RyR2), ultimately activating systolic myocyte contraction. Diastolic myocyte relaxation is mediated by Ca^{2+} uptake via SERCA2a and Ca^{2+} efflux via the sarcolemmal sodium–calcium exchanger. Oxidative stress could impair intracellular Ca^{2+} regulation, and dysregulation of intracellular Ca^{2+} homeostasis is thought to be an important mechanism in many acute and chronic cardiovascular diseases (27).

Both eNOS and nNOS are constitutively expressed in cardiomyocytes. A recent study suggested that NO regulates cardiac function by spatial confinement of NOS isoforms (*i.e.*, eNOS is localized in caveolae where it regulates the L-type Ca^{2+} channel in the sarcolemma, and nNOS is located in the SR where it regulates Ca^{2+} release from the SR) (5, 131). However, the molecular mechanisms responsible for spatial and temporal specificity of NO-mediated regulation of intracellular Ca^{2+} and myocardial function are still not clear. Although NO-mediated regulation is dependent at least in part on the activation of guanylyl cyclase and the subsequent modification of the phosphorylation state of channels, NO could also participate in the regulation of e-c coupling through cGMP-independent redox mechanisms, because all of the major Ca^{2+}-handling proteins possess multiple free cysteine residues and are subjected to redox regulation (46, 95, 124).

L-type Ca^{2+} channel

The L-type Ca^{2+} current ($I_{Ca\text{-}L}$) has been shown to be reversibly regulated by redox, with both activation (18) and inhibition (50, 91, 105) reported. Using the biotin switch method, Sun *et al.* (118) have found that the L-type Ca^{2+} channel α1 subunit is the predominant *S*-nitrosylated protein in the membrane fractions. Protein *S*-nitrosylation of the L-type Ca^{2+} channel in heart occurs endogenously *in vivo*, and the levels of the *S*-nitrosylation are significantly increased after β-adrenergic stimulation and ischemia–reperfusion, with the level of this increase being significantly higher in female than in male subjects. The higher level of SNO in females is due to a greater production of NO from constitutive isoforms of NOS. Compared with males, female mice have more caveolin-3–associated eNOS and a greater translocation of nNOS to the sarcolemma after β-adrenergic stimulation and ischemia–reperfusion (118). This increase in SNO of the L-type Ca^{2+} channel in females is correlated with reduced ischemia–reperfusion injury under β-adrenergic stimulation. Isoproterenol treatment before ischemia and reperfusion results in higher levels of *S*-nitrosylation of the L-type Ca^{2+} channel, lower cardiomyocyte $I_{Ca\text{-}L}$, a smaller Ca^{2+} transient, less SR Ca^{2+} loading, less ischemic injury, and better functional recovery after reperfusion in females (Fig. 2), suggesting that the inhibition of $I_{Ca\text{-}L}$ by *S*-nitrosylation of the L-type Ca^{2+} channel may play a cardioprotective role in hypercontractile hearts, such as with β-adrenergic stimulation (137) and ischemia–reperfusion (6, 75).

RyR2

It has been found that physiologic concentrations (submicromolar) of NO *S*-nitrosylate and activate the skeletal muscle isoform of RyR (RyR1) at a single cysteine (Cys3635, within the hydrophobic calmodulin-binding domain) from among ~50 free thiols in each homotetrameric subunit, which is allosterically dependent on the presence of physiologic muscle O_2 tension (31, 119, 120). A comparable study of cardiac RyR2 suggests that rather than direct NO-mediated specific *S*-nitrosylation, transnitrosylation via LMW-SNO may play a major role in regulation of RyR2 channel function (Sun and Meissner, unpublished study). It has been previously found that SNO compounds could poly-*S*-nitrosylate RyR2 and activate the channels *in vitro* (132). In addition, Petroff *et al.* (103) reported that stretching of cardiac muscle modulates Ca^{2+} release from RyR2, Ca^{2+} sparks, and the electrically stimulated Ca^{2+} transient via activation of PtdIns-3-OH kinase (PI3K)/Akt/eNOS. The resultant production of NO exerts its action independent of cGMP, most probably through *S*-nitrosylation of the RyR2 or one of its regulatory components (103).

SERCA2a

Sequestration of Ca^{2+} by SERCA2a mediates cardiac muscle relaxation. NO has been reported to modulate the activity of SERCA2a (58, 131), but the molecular mechanism is not

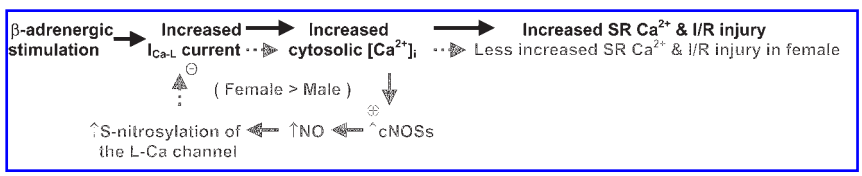

FIG. 2. Cardioprotection role of *S*-nitrosylation of L-type Ca^{2+} channel in ischemic reperfused heart under adrenergic stimulation. An increase in Ca^{2+} before the ischemia, as occurs under β-adrenergic stimulation or other hypercontractile conditions, causes Ca^{2+} overload and an increased ischemia–reperfusion (I/R) injury (text in *black*). However, the increased cytosolic Ca^{2+} leads to a greater increase (+) in NO production and protein *S*-nitrosylation in females, because of increased constitutive NOS (cNOSs), eNOS, and nNOS association with caveolin-3 in females. The increase in *S*-nitrosylation of the L-type Ca^{2+} channel in females reduces (–) Ca^{2+} entry and SR Ca^{2+} loading at the start of ischemia, thereby reducing Ca^{2+} overload during ischemia and reperfusion and thus reducing ischemia–reperfusion injury (text in *grey*).

clear. Protein modification mediated by NO carriers could result from *S*-nitrosylation or from other secondary oxidative modifications (102). Recently, a dual role of NO/O_2^- in regulating SERCA2a has been elucidated, in which SERCA2a is activated by reversible *S*-glutathiolation at Cys674 via the formation of peroxynitrite in the presence of GSH (1). Chronically elevated levels of oxidative stress in some disease states, such as atherosclerosis, could irreversibly oxidize the responsible thiols and block NO-induced *S*-glutathiolation (1). Peroxynitrite has been found to be a mediator of cytotoxic damage during inflammation, by inducing irreversible tyrosine nitration and dysfunction of proteins (100, 112). However, the study from Adachi *et al.* (1) suggests that under certain redox conditions, peroxynitrite can readily react with reactive thiols to form *S*-glutathione adducts.

Antioxidant defense. *S*-nitrosylation is modulated by the cellular redox status; its formation is dependent on the state of redox equilibrium and is prevented by high levels of antioxidants (8, 17, 21). NO has been found to block cell death after GSH depletion by preserving the redox status of mitochondrial protein thiols, probably by a mechanism that involves *S*-nitrosylation of mitochondrial protein thiols (128). This may represent an endogenous protective mechanism for the mammalian cell against nitrosative/oxidative stress when intracellular thiols or other redox constituents have decreased below a critical concentration. In addition, NO generated by the coronary vasculature may serve as one of the antioxidant defenses in the heart, as blocking NO generation causes an increased oxidative stress in the heart (111).

Many redox-related enzymes contain active cysteine(s), which are capable of undergoing NO-mediated *S*-nitrosylation. Among these are catalase (35), glutathione peroxidase (3), glutathione reductase (17), glutathione transferase P1–1 (74), and thioredoxin (41), with the *S*-nitrosylation of thioredoxin being best characterized (please see Haendeler's review in this issue). Thioredoxin and thioredoxin reductase are ubiquitously expressed antioxidant enzyme systems. Studies from Dimmeler's group (41, 49) suggest that thioredoxin is essential for maintaining the content of *S*-nitrosylated molecules in endothelial cells. Thioredoxin itself is *S*-nitrosylated at Cys69 under basal conditions, and this *S*-nitrosylation is required both for scavenging ROS and for preserving its own redox regulatory activity. *S*-nitrosylation of thioredoxin also contributes to its antiapoptotic function, possibly by transnitrosylation of proteins such as caspases, thereby inhibiting their activity (41). Shear stress increases the *S*-nitrosylation and the reductase activity of thioredoxin in endothelial cells (49). The antioxidant effect of statins is partially mediated via *S*-nitrosylation and activation of thioredoxin in endothelial cells (42). Conversely, it has been found that *S*-nitrosylation of thioredoxin at active-site Cys32/Cys35 leads to the dissociation and activation of apoptosis signal-regulating kinase 1 (ASK1), suggesting that *S*-nitrosylation of thioredoxin may also play a role in proapoptotic signaling under certain oxidative stresses (116, 134).

Cell death and survival. Apoptosis is characterized by an energy-dependent process of cell shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation. Apoptosis can be initiated by binding of ligands such as tumor necrosis factor-α (TNF-α) and CD95/Fas to specific death receptors on the cell surface, leading to the formation of a death-inducing signaling complex (DISC). DISC then recruits and activates the protease zymogen procaspase-8, initiating a caspase cascade (26). Alternatively, apoptosis can be initiated by a mitochondrial pathway, which results in release of cytochrome *c*. Under some conditions, cytochrome *c* release is via the mitochondrial permeability transition pore (PTP), which undergoes a Ca^{2+}-dependent transition that disrupts membrane potential and releases apoptogenic proteins. The pore is protected from opening by low pH, ADP, and a high electrochemical proton gradient ($\Delta\Psi$), while pore opening is enhanced by depleting ADP, by Pi, or by low $\Delta;\Psi$. PTP opening is promoted by specific oxidative stress targeted on a critical protein thiol. NO has been proposed to enhance the open probability of the PTP by *S*-nitrosylation on this specific cysteine residue, resulting in the release of cytochrome *c* and endonuclease G from mitochondria (104).

NO is implicated in both apoptotic and necrotic cell death, depending on the biologic milieu, such as the cellular redox state, NO concentration and exposure time, iron mobilization within the cell, and the combination with oxygen and other ROS (13, 61, 89). In addition, ATP depletion might modulate NO and affect cell death (69). On stimulation, NO can be either an antiapoptotic or a proapoptotic regulator, depending on the point in the pathway at which it interacts (20, 61). Protein *S*-nitrosylation may simultaneously inactivate several parts of the apoptotic machinery and serve to balance apoptosis and necrosis.

Akt/PKB. The serine/threonine kinase Akt/protein kinase B (PKB) is believed to play a crucial role in apoptosis and the insulin-signaling cascade in the cardiovascular system (99). The antiapoptotic effect of Akt/PKB has provided an intriguing therapeutic strategy for protecting against myocardial ischemia–reperfusion injury. In cardiovascular endothelial cells, it has been found that shear stress increases NO formation by Ca^{2+}-independent activation of eNOS via Akt/PKB phosphorylation (25). However, a recent study suggests that NO might also inactivate Akt/PKB, providing negative feedback. In mouse C2C12 myoblasts, it has been found that *S*-nitrosylation of Cys296 of Akt/PKB blocks disulfide bond formation between Cys296 and Cys310 and suppresses the biologic effects of Akt/PKB (76). The involvement of *S*-nitrosylation of Akt/PKB in insulin signaling is addressed in the energy metabolism section.

Caspases. As cysteine aspartyl proteases, caspases are categorized into initiator (caspase-8, -9, -10) and executioner (caspase-3, -6, -7) subtypes. Most caspases contain a single cysteine at the catalytic site, which is subjected to redox modification and can be S-nitrosylated by NO (70). Inactive procaspases exist as a latent zymogen. Upon apoptotic stimulation, these procaspases are cleaved into active forms. *S*-nitrosylation of the redox-sensitive thiol in the catalytic site of caspases plays an essential role in the apoptotic signal cascade by inhibiting apoptotic cell death (61, 88). The activation of cas-

pase-8 is known to involve sequential activation of other caspases and functions primarily upstream of Bcl-2 and cytochrome *c* (60). The activity of caspase-8 can be suppressed by NO-mediated *S*-nitrosylation, which inhibits the cleavage of Bid and Bcl-2 and blocks release of mitochondrial cytochrome *c* (62).

In lymphocytes and endothelial cells, *S*-nitrosylation of caspase-3 (Cys163 in p17 subunit) keeps the zymogen in an inactive state, which protects cells from unwanted apoptosis, whereas Fas activation results in denitrosylation of the catalytic cysteine as well as proteolytic cleavage of caspase-3 and induces apoptosis (43, 49, 110). *S*-nitrosylation of caspases is also dependent on subcellular localization. It has been reported that *S*-nitrosylation occurs frequently to mitochondrial, but not cytoplasmic caspase-3. Also, inhibition of endogenous NOS potentiates ischemia–reperfusion-induced myocardial apoptosis via a caspase-3–dependent pathway (126). A recent study using neonatal rat cardiomyocytes has demonstrated that the inhibition of apoptosis by *S*-nitrosylation of caspase-3 plays an important role in cardiomyocyte apoptosis (78). The ability of NO to inhibit downstream caspase-3 suggests that NO may be able to rescue cardiomyocytes from apoptosis even after the caspase cascade has been activated. In rat heart, 90% of caspase-9 zymogens are mitochondrial (64). In addition, the majority of mitochondrial caspase-9 is also *S*-nitrosylated (79). It would be interesting to know whether mitochondrial caspase-9 in heart undergoes *S*-nitrosylation as it does in other cells. Finding an appropriate dose of NO to affect caspase targets may provide a promising therapeutic strategy for preventing myocyte cell death.

Cyclooxygenase-2. Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme in prostaglandin synthesis, which is induced in response to stress. It has been demonstrated that ischemic preconditioning upregulates the expression and activity of COX-2 in the heart, which mediates the protective effects of the late phase of ischemic preconditioning against both myocardial stunning and myocardial infarction (10). Recently, Atar *et al.* (4) found in rat heart that atorvastatin-induced cardioprotection is mediated by increasing inducible NOS (iNOS), which activates COX-2 in the heart by *S*-nitrosylation.

eNOS and Hsp90. Vascular endothelial cells express high levels of eNOS, and a recent study showed that eNOS is reversibly regulated by *S*-nitrosylation of its zinc-tetrathiolate cysteines (Cys96 and Cys101) and that the intracellular redox milieu mediates eNOS denitrosylation on enzyme activation (29, 30, 107). Thus, the dynamic receptor-mediated regulation of *S*-nitrosylation of eNOS provides a potentially important mechanism for the control of NO signaling pathways in the vascular wall (29, 30). In addition, eNOS specifically interacts with scaffolding proteins such as caveolin and heat shock protein 90 (Hsp90). It has been shown in endothelial cells that *S*-nitrosylation of Hsp90 not only abolishes the positive regulation of eNOS activity mediated by native Hsp90, but also inhibits its ability to hydrolyze ATP and disables its intrinsic properties as a chaperone (84).

Energy metabolism

The heart is capable of altering its metabolic rate during exercise or ischemia. The cytosolic and mitochondrial redox state is important in the regulation of oxidative phosphorylation and glycolysis under physiologic and pathophysiologic conditions. NO-mediated protein *S*-nitrosylation has been found to be involved in the regulation of energy metabolism.

Aldehyde dehydrogenase. Mitochondrial class 2 aldehyde dehydrogenase (ALDH2) is one of the key enzymes in the NAD^+-dependent oxidation of various aldehydes produced during intermediary metabolism. Inactivation of ALDH2 is likely to cause marked accumulation of toxic aldehydes, leading to increased susceptibility to irreversible damage. It has been shown that *S*-nitrosylation of mitochondrial ALDH2 in intact cells leads to reversible inhibition of ALDH2 activity (94).

Creatine kinase. Creatine kinase (CK) plays a crucial role in energy metabolism and exists in both cytoplasmic and mitochondrial compartments. In myocytes, the CK system buffers ATP levels when oxygen supply is limited, such as during ischemia–reperfusion. It has been reported that GSNO dose-dependently inhibits CK, possibly via transnitrosylation (129). In adult rat ventricular myocytes, CK has been shown to be reversibly regulated by NO, possibly through *S*-nitrosylation of Cys283 (2). In addition, the concentration of GSH in myocytes seems to be an important determinant of the extent of *S*-nitrosylation of CK *in situ*. *S*-nitrosylation of CK with subsequent loss of enzyme activity may have important implications in heart during inotropic stimulation or under severe oxidative stress.

Glyceraldehyde-3-phosphate dehydrogenase. An essential glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been reported to bind to membranes of diverse organs including the heart. The binding of GAPDH to the membrane not only appears to be a direct regulatory mechanism for cell metabolism but also may prevent further oxidative modification of its active thiol. The most abundant *S*-nitrosylated protein in the resting endothelial cell is GAPDH, suggesting a regulatory function for NO/*S*-nitrosylation in glycolysis (133). It has been reported that NO or GSNO inhibits GAPDH activity by *S*-nitrosylation of Cys149; this *S*-nitrosylation is reversed by LMW thiols such as GSH (92, 93). *S*-nitrosylation of GAPDH is responsible for reversible enzyme inhibition, which initiates subsequent modification by the pyridinium cofactor NADH. The attachment of NADH causes enzyme inactivation. Thus, *S*-nitrosylation may serve to protect GAPDH from oxidant inactivation and to regulate glycolysis (92). In addition, *S*-nitrosylation of GAPDH has been found to decrease the binding affinity of GAPDH for the red blood cell membrane (36). Moreover, it has been shown that GSNO induces *S*-glutathionylation of GAPDH and inactivates the enzyme in ischemic myocardium (63). Using HEK293 cells, Hara *et al.* (45) demonstrated that *S*-nitrosylation of GADPH initiates apoptosis by nuclear translocation after Siah1 (an ubiqutin E3 ligase) binding.

Mitochondrial respiratory chain components. It has been reported that NO inhibits mitochondrial ATP generation via inhibition of the mitochondrial respiratory chain, mainly at complex I and IV, leading to a switch from apoptosis to necrosis (69). In murine macrophage J774 cells, Clementi *et al.* (21) found that long-term exposure to NO leads to persistent inhibition of complex I, which appears to result from *S*-nitrosylation of complex I. A recent study using isolated rat heart mitochondria has shown that the 75-kDa subunit of complex I is *S*-nitrosylated by exogenously added GSNO, which results in significant inhibition of the complex. Furthermore, SNOs can be detected in mitochondria isolated from hearts subjected to ischemic preconditioning (16). Another study using endothelial cells has reported that mitochondrial complex IV/cytochrome *c* oxidase could also be persistently inhibited by *S*-nitrosylation at two active cysteine (Cys196 and Cys200) residues (138).

Insulin regulation. Oxidative and/or nitrosative stress has been implicated in many human diseases including insulin resistance. Insulin is an important metabolic regulator. A study using pancreatic β cells suggests that *S*-nitrosylation of glucokinase may play an important role in glucose-stimulated insulin secretion (109). Yasukawa *et al.* (136) have shown that *S*-nitrosylation of Akt/PKB at Cys224 in skeletal muscle leads to its inactivation. In addition, an increase is found in the level of *S*-nitrosylation and inactivation of Akt in diabetic mice *versus* wild-type mice. These results suggest that *S*-nitrosylation–mediated inactivation of Akt/PKB may contribute to the pathogenesis of insulin resistance (136).

Transcription factors

Some redox-sensitive transcriptional factors (71) crucial for cell death and survival have been reported to be *S*-nitrosylated, including the following.

Estrogen receptor. Estrogen classically exerts its genomic effects by modifying gene expression through the activation of estrogen receptors (ERs). However, a rapid nongenomic action of estrogen in vascular cells appears to play a major cardioprotective role, mainly through NO production by activation of eNOS, which triggers downstream signaling cascades (59). NO-induced *S*-nitrosylation of the ER at cysteine residues in its zinc-finger domain, results in selective inhibition of DNA-binding at specific estrogen-responsive elements (EREs), which may favor activation of rapid nongenomic signaling pathways and subsequent modulation of downstream genomic activity (37).

Hypoxia-inducible factor-1. Hypoxia-inducible factor-1 (HIF-1) is a transcription regulator that responds to oxygen. HIF-1 is a heterodimer composed of subunits HIF-1α and HIF-1α. Under normoxic condition, HIF-1α is hydroxylated at proline and arginine residues, which promotes ubiquitination and inhibits transactivation. Hypoxia impairs these hydroxylations and leads to HIF-1 accumulation and translocation to the nucleus, where it turns on hypoxia-responsive genes. During hypoxia, NO has been found to inhibit HIF-1 activation by the competitive inhibition of mitochondrial respiration, leading to increased oxygen availability (82). In normoxia, NO *S*-nitrosylates HIF-1α, which promotes HIF-1α stabilization, DNA binding, and activation of downstream target-gene expression (14, 101, 117, 135).

Nuclear factor-κB. Nuclear factor κB (NF-κB) is a transcription factor that plays a pivotal role in inflammation, cell survival, and cell proliferation. NF-κB, a heterodimer composed of p50/p65 subunits, is expressed constitutively in most mammalian cells. It has been shown that S-nitrosylation of NF-κB at Cys62 of the p50 subunit inhibits NF-κB–dependent DNA binding and gene transcription (80, 87). In addition, it has been established that NF-κB is complexed with and sequestered in the cytoplasm by NF-κB inhibitor (I-κB), which is phosphorylated by I-κB-kinase complex (IKK-a, -b, and -γ), initiating I-κB ubiquitination, releasing NF-κB which translocates to the nucleus. A recent study has shown that S-nitrosylation of Cys179 of the catalytic IKK-β subunit inhibits the IKK kinase complex and subsequent phosphorylation of I-κB, providing a mechanism for S-nitrosylation to be involved in the upstream regulation of NF-κB–mediated inflammatary responses (108).

SUMMARY

In summary, SNOs and protein *S*-nitrosylation can exert important effects and mediate redox signaling in the cardiovascular system (Table 1), and the accumulating evidence suggests that SNOs and *S*-nitrosylation play key roles in human health and disease (34, 83). Changes in the levels of SNOs depend on both enzymatic and nonenzymatic mechanisms of SNO formation, processing, and degradation. The *S*-nitrosylation of cysteine residues is redox reversible with high spatial and temporal specificity (48). The redox environment of targeted cysteine residue(s) in a protein influences the efficiency of *S*-nitrosylation and denitrosylation, and other allosteric effects can impose further control.

The redox reversibility of *S*-nitrosylation provides two possible mechanisms of signal transduction [*i.e.*, (a) *S*-nitrosylation of the specific active cysteine residue(s) not only leads to changes of protein structure and function, but also prevents these thiol(s) from further oxidative modification; and (b) release of NO can activate other signaling pathways]. Thus, SNOs and protein *S*-nitrosylation may serve multiple roles to mitigate oxidative stress. Further investigation of the biologic functions of endogenous SNOs and protein *S*-nitrosylation *in vivo* will help to understand better the molecular mechanisms of NO signaling and provide new therapeutic opportunities and targets for intervention in cardiovascular diseases.

ABBREVIATIONS

Akt/PKB, protein kinase B; ADP, adenosine 5′-diphosphate; ALDH2, mitochondrial class 2 aldehyde dehydroge-

TABLE 1. *S*-NITROSYLATED PROTEINS IN CARDIOVASCULAR SYSTEM

S-Nitrosylated proteins	*Regulatory effects of S-nitrosylation*	*Cell/tissue type (species)*	*References*
I. Inhibition by *S*-nitrosylation			
Akt/PKB	Suppresses the biologic effects of Akt/PKB	C2C12 myoblasts (mouse)	136
Caspase-3	Keeps the zymogen in an inactive state, which protects cells from unwanted apoptosis (antiapoptosis)	Neonatal cardiomyocytes (rat)	78
		Umbilical vein endothelial cells (human)	49, 110
Creatine kinase	Suppresses myocardial contractility	Ventricular myocytes (rat)	2
eNOS	eNOS is tonically *S*-nitrosylated in resting basal level, which is denitrosylated on activation	Aortic endothelial cells (bovine)	29, 30, 107
GAPDH	Decreases GAPDH binding affinity of cell membrane and inhibiting the enzyme activity	Aortic endothelial cells (human, bovine)	133
		Red blood cells (human)	36
HSP90	Inhibits ATP hydrolyzing ability, disables the chaperone property, and abolishes the positive regulation of eNOS	Endothelial cells (EA.hy926 cell line)	84
L-type Ca^{2+} channel $\alpha 1$ subunit	Inhibits $I_{Ca\text{-}L}$ in females under β-adrenergic stimulation	Ventricular myocytes (mouse)	118
Mitochondrial complex	Inhibits complex I	Isolated heart mitochondria (rat)	16
	Persistently inhibits complex IV	Pulmonary artery endothelial cells (human)	138
NF-κB	Inhibits gene transcription and antiinflammation	Alveolar type II epithelial cells (mouse)	108
N-ethylmaleimide-sensitive factor	Inhibits disassembly of SNARE and antiinflammation	Aortic endothelial cell (human)	86
Tissue-type plasminogen activator	Inhibits platelet aggregation	Aortic endothelial cell (bovine)	115
	Attenuates necrosis after ischemia–reperfusion injury	Perfused heart *in vivo* (cat)	24
Tissue transglutaminase	Inhibits enzyme activity and antiapoptosis	Aortic endothelial cell (bovine)	66
II. Activation by *S*-nitrosylation			
COX-2	Elicits preconditioning effect	Perfused hearts *in vitro* (rat)	4
HIF-1	Provokes HIF-1β stabilization in normoxia	Pulmonary artery endothelial cells (bovine)	101
RyR2/SR Ca^{2+} release channel	Increases channel activity by poly-*S*-nitrosylation	Cardiac SR vesicles (dog)	132
Thioredoxin	Increases reductase activity and antiapoptosis	Vascular endothelial cells (human)	41

nase; ASK1, apoptosis signal-regulating kinase 1; ATP, adenosine 5′-triphosphate; cGMP, cyclic guanosine monophosphate; CK, creatine kinase; COX-2, cyclooxygenase-2; Cys, cysteine residue; eNOS, endothelial isoform of NOS; ER, estrogen receptor; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GSH, reducing glutathione; GSNO, *S*-nitrosoglutathione; GTP, guanine 5'-triphosphate; Hb, hemoglobin; HIF-1, hypoxia-inducible factor-1; HSP90, heat shock protein 90; $I_{Ca\text{-}L}$, L-type Ca^{2+} current; iNOS, inducible isoform of NOS; LWM SNO, low-molecular-weight SNO; Mb, myoglobin; mitochondrial PTP, mitochondrial permeability transition pore; NAD^+/NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide; NF-κB, nuclear factor κB; nNOS, neuronal isoform of NOS; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; RyR2, cardiac isoform of ryanodine receptor/SR Ca^{2+} release channel; SERCA2a, cardiac isoform of SR Ca^{2+}-ATPase; SNO, *S*-nitrosothiol; SOD, superoxide dismutase; SR, sarcoplasmic reticulum; XOR, xanthine oxidoreductase.

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