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Regulation of cardiovascular cellular processes by S-nitrosylation☆

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Abstract

Background—Nitric oxide (NO), a highly versatile signaling molecule, exerts a broad range of regulatory influences in the cardiovascular system that extends from vasodilation to myocardial contractility, angiogenesis, inflammation, and energy metabolism. Considerable attention has been paid to deciphering the mechanisms for such diversity in signaling. S-nitrosylation of cysteine thiols is a major signaling pathway through which NO exerts its actions. An emerging concept of NO pathophysiology is that the interplay between NO and reactive oxygen species (ROS), the nitroso/redox balance, is an important regulator of cardiovascular homeostasis.

Scope Of Review—ROS react with NO, limit its bioavailability, and compete with NO for binding to the same thiol in effector molecules. The interplay between NO and ROS appears to be tightly regulated and spatially confined based on the co-localization of specific NO synthase (NOS) isoforms and oxidative enzymes in unique subcellular compartments. NOS isoforms are also in close contact with denitrosylases, leading to crucial regulation of S-nitrosylation.

Major Conclusions—Nitroso/redox balance is an emerging regulatory pathway for multiple cells and tissues, including the cardiovascular system. Studies using relevant knockout models, isoform specific NOS inhibitors, and both in vitro and in vivo methods have provided novel insights into NO- and ROS-based signaling interactions responsible for numerous cardiovascular disorders.

General Significance—An integrated view of the role of nitroso/redox balance in cardiovascular pathophysiology has significant therapeutic implications. This is highlighted by human studies where pharmacologic manipulation of oxidative and nitrosative pathways exerted salutary effects in patients with advanced heart failure. This article is part of a Special Issue entitled Regulation of Cellular Processes by S-nitrosylation.

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Keywords

Nitric oxide; Nitrosative and oxidative stress; Excitation; contraction coupling; Adrenergic contractility; Angiogenesis; Inflammation

1. Introduction

Nitric oxide (NO) is a labile free radical gas that functions as a highly versatile and ubiquitous signaling molecule exerting a broad range of regulatory influences in the cardiovascular and renal systems, ranging from control of systemic and microvascular tone to platelet function, myocardial contractility, calcium (Ca^{2+}) cycling, vascular inflammatory and growth responses, renal sodium excretion, and cellular energy metabolism [1–7]. Much attention has been paid to deciphering the mechanisms underlying such diversity in signaling.

In addition to cGMP-dependent signaling [8], S-nitrosylation of specific cysteine thiol residues or metal centers is a major signaling pathway through which NO modifies protein activity and thereby exerts its widespread and diverse effects [9,10]. An emerging concept of NO pathophysiology is that the interplay between NO and reactive oxygen species (ROS), the nitroso/redox balance, is an important regulator of cardiovascular homeostasis [11,12]. ROS readily react with NO and limit its bioavailability and also compete with NO for binding to the same sites in effector molecules. Accumulating evidence shows that nitrosative stress, an impairment in NO signaling caused by increased amounts of reactive nitrogen species (RNS), is caused by or associated with a disturbance in the cellular redox state. RNS of biological significance include NO, low and high molecular weight S-nitrosothiols (SNO), and peroxynitrite. This review addresses the role of S-nitrosylation in cardiovascular cell function and the significant interplay between oxidative stress and S-nitrosylation-based signaling in cardiovascular health and disease.

2. NO based signaling

2.1. Sources of NO

NO is produced from the amino acid L-arginine by the enzymatic action of NO synthases (NOS) or by the breakdown of nitrite or other compounds [13–16]. NOS generated NO is under complex, tight control to dictate specificity of its signaling and to limit toxicity to other cellular components, due to its potent chemical reactivity and high diffusibility. There are three major NOS isoforms in mammalian systems: Neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3), each of which oxidizes the terminal guanidino nitrogen of L-arginine to form NO and the amino acid L-citrulline. They share a common basic structural organization and requirement for substrates (arginine and NADPH) and cofactors (tetrahydrobiopterin, heme, calmodulin, FAD, and FMN) for enzymatic activity. The binding of calmodulin is triggered by transient elevations in intracellular Ca^{2+} levels and serves as an allosteric modulator of the three NOS isoforms. NOS1 is expressed in neural tissues, myocardium, skeletal muscle, and macula densa as well as other renal tubule segments and is a Ca^{2+}/calmodulin-dependent enzyme that is also

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subject to transcriptional and other post-translational controls [16–19]. Transcription of NOS2 is induced in nearly all tissues in response to cytokines, endotoxin, or other proinflammatory stimuli. NOS2 is less responsive to intracellular Ca^{2+} transients owing to tight calmodulin binding at ambient intracellular Ca^{2+}levels [20]. NOS3 is expressed in the endothelium and myocardium and plays a central role in the regulation of systemic blood pressure, cardiovascular remodeling, myocardial contractility, and angiogenesis [16,19,21,22]. NOS3 is subject to rapid regulation by calcium Ca^{2+}/calmodulin as well as a variety of transcriptional, post-transcriptional, and post-translational controls. A major structural difference between NOS1 and NOS3 is that the NOS1α isoform has an N-terminal PDZ domain that is crucial in regulation of its spatial localization and protein–protein interactions [23]. Furthermore, we have shown that NOS1 and NOS3 reside in precise subcellular organelles in cardiac myocytes and interact with oxidative enzymes in a spatially confined manner, as discussed in more detail later [19,24–26].

2.2. cGMP pathway

NO activates soluble guanylate cyclase (sGC), a heterodimer with an α subunit and a β subunit,to generate the second messenger 3′,5′-cyclic guanosine monophosphate (cGMP) [8]. Activation of this enzyme is mediated by the binding of NO to the heme moiety of sGC to form the nitrosyl-heme adduct of sGC [27]. As a result, the heme iron is shifted out of the plane of the porphyrine ring configuration, initiating binding of GTP and the formation of cGMP. Cyclic GMP activates cGMP-dependent protein kinase (PKG), which in turn phosphorylates a number of proteins involved in vascular smooth muscle relaxation, the proliferative process, adhesion molecule expression, and platelet aggregation [28]. cGMP signaling is terminated by the action of the cyclic nucleotide-hydrolyzing phosphodiesterases (PDE). PDE5 is spatially localized within cells in proximity to NOS. In the case of the cardiac myocyte, PDE5 is found at the cell membrane associated with caveolae [29]. cGMP mediated signaling has been extensively reviewed elsewhere and is not the focus of this review (Fig. 1A) [8].

Of note, S-nitrosylation has been shown to modulate cGMP levels by inhibiting sGC [30] and to inhibit NOS3 [31] and NOS3 regulating proteins, including heat shock protein 90 and Akt [32,33]. PKG has regulatory thiols that may also be susceptible to S-nitrosylation. In addition, S-nitrosylation has been shown to activate arginase and inhibit dimethylarginine dimethylaminohydrolase, which leads to decreased NOS substrate levels and increased levels of methylarginine NOS inhibitors, respectively [34,35].

2.3. S-nitrosylation

Besides the cGMP pathway, NO exerts its actions by the nitrosylation of sulfhydryl groups on proteins and small molecules (Fig. 1A) [36,37]. S-nitrosylation is a ubiquitous post-translational modification that regulates diverse biologic processes [9,38,39]. Nitrosylation of specific cysteine thiol residues or metal centers is a reversible covalent modification that modulates protein activity. Cysteines susceptible to nitrosylation tend to be located between an acidic and a basic amino acid and a consensus motif predictive of sites of S-nitrosylation has been described [37,39].

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Protein S-nitrosylation occurs by transnitrosylation from low-molecular weight SNO, such as S-nitrosoglutathione (GSNO) or S-nitrosocysteine, by transition metal catalyzed addition of NO, or by endogenous NO-mediated nitrosylating agents such as dinitrogen trioxide (N_2O_3), which is formed by the autoxidation of NO and is particularly increased within the hydrophobic interior of biological membranes [39–41]. There is evidence that the redox state and ultrastructural accessibility of cysteine residues determines whether a particular thiol in a protein is S-nitrosylated [42,43]. Increases in cellular ROS and RNS production lead to decreases in the intracellular reduced glutathione (GSH) pool, thereby attenuating GSH-mediated trans- or denitrosylation and stabilizing SNO formation [44]. The stability of SNO is favored by low ambient oxygen, whereas with increasing oxygen S-thiolation is promoted [45,46]. The intermediate thiyl radical may be involved in the decomposition of SNO [47].

As evidenced from studies in the myocardium [2], the sub-cellular localization of NOS determines the local concentration of NO and appears to becritical in the formation of SNO. For example, sarcolemmal NOS3 inhibits the L-type Ca^{2+} channel (LTCC) [48] via S-nitrosylation, whereas NOS1 colocalizes with the sarcoplasmic reticulum (SR) Ca^{2+}-release channel (ryanodine receptor, RYR) and stimulates its activity via S-nitrosylation (Fig. 2) [49,50]. The role of S-nitrosylation in regulating cardiac calcium signaling is discussed in greater detail later.

2.4. Denitrosylation

S-nitrosylation-based signaling is regulated not only by NO production, but also by enzymatic degradation (Fig. 1B). A recent series of reports demonstrates that GSNO reductase (GSNOR) is a key regulator of S-nitrosylation and is important in protecting cells from nitrosative stress, the mechanisms of which have been recently reviewed [9,51]. GSNOR has widespread activity in cells, using NADH to reduce GSNO into glutathione S-hydroxysulfenamide (GSNHOH), which in turn is converted to oxidized glutathione (GSSG) [52]. Reduction of GSSG by glutathione reductase terminates the denitro-sylation reaction (Fig. 1). Although GSNOR selectively metabolizes GSNO, by depleting the pool of GSNO it shifts the equilibrium and therefore limits levels of S-nitrosylated proteins. A knockout mouse ($GSNOR^{-/-}$) has been generated and manifests increased levels of S-nitrosylated proteins despite similar production of NO, suggesting that S-nitrosylation is tightly regulated both at the level of formation and decomposition of SNO bonds [53]. We and others have shown that $GSNOR^{-/-}$ mice have increased mortality following endotoxic challenge and manifest hypotension under anesthesia, whereas they exhibit decreased cardiac infarct size and remodeling and increased cardiac function and survival after myocardial infarction [53,54]. These findings support the notion that hypo- and hyper-nitrosylation of specific protein targets correlate with pathophysiology.

Another major enzymatic system mediating intracellular protein denitrosylation is the thioredoxin system [55]. Unlike GSNOR which specifically denitrosylates GSNO, the cytoplasmic and mitochondrial thioredoxins mediate the denitrosylation of multiple S-nitrosylated proteins in a stimulus coupled, substrate specific, and spatially restricted manner [51,55,56]. Following denitrosylation, the thioredoxin system uses thioredoxin reductase and

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NADPH to regenerate reduced thioredoxin (Fig. 1B). This enzymatic system has been shown to reverse the S-nitrosylation-induced reduction of NOS activity and inhibition of protein kinase C and thioredoxin is itself regulated by S-nitrosylation of multiple residues [57–59]. Other enzymatic systems reported to function as denitrosylases include carbonyl reductase 1 [60], Cu, Zn-superoxide dismutase [61], and xanthine oxidoreductase [62]. All of these denitrosylases are coupled to cellular antioxidant redox systems, highlighting the importance of the cellular redox state in regulating the level of protein S-nitrosylation and thereby cellular signaling.

3. Myocardial cellular processes regulated by S-nitrosylation

NO has both autocrine and paracrine activities within the heart [63], and influences myocardial contractility [25,64,65], ventricular relaxation [66,67], and mitochondrial respiration [68–71]. At a biochemical level, these effects are due to interactions with Ca^{2+} handling proteins, including the L-type Ca^{2+} channel (LTCC), ryanodine receptor/Ca^{2+}-release channel (RYR2), and SR Ca^{2+} ATPase (SERCA) [9,49,72], the contractile myofilaments [73], and respiratory complexes [74], respectively. Extensive study by multiple groups has led to a view of isoform-specific NO signaling in precise subcellular compartments (Fig. 2)[24,25]. NOS knockout mice have been a valuable tool for deciphering these signaling pathways, and overexpression or reconstitution strategies have continued to support isoform specific activity [19,26,50,75,76].

3.1. Spatial localization of NO production

In the cardiac myocyte, NO activity is predominantly determined by its site of production, which in turn is controlled by spatial localization of the NOS enzymes (Fig. 2) [24,25]. NOS3 is localized primarily to caveolae of the sarcolemma and t tubules, where its function is regulated by interaction with caveolin-3 and is linked to multiple cell surface receptors, including muscarinic, β-adrenergic, and bradykinin receptors [65]. Activation of NOS3 results in negative inotropy and chronotropy, an effect that is enhanced by β-adrenergic activation, suggesting that NO acts as a negative feedback mechanism over contractile reserve [77]. NOS1 has been localized to the SR, where it influences Ca^{2+} cycling and thereby exerts positive inotropic effects in the heart.Of note, immunoprecipitation studies have demonstrated that NOS1 binds to RyR2 [25,49,50,72,78]. There is also evidence of a mitochondrial NOS expressed in the inner mitochondrial membrane or matrix [79], although this remains controversial [80,81]. Another source of mitochondrial NO may be NOS1 associated with the SR, since the SR membrane has been shown to be attached to the outer mitochondrial membrane [82]. Importantly, this specificity of NOS localization is critical for the subcellular organelle generation of NO because it provides a localized signal for protein S-nitrosylation (Fig. 2).

3.2. Alteration in subcellular localization following tissue injury

NOS enzyme activity and subcellular localization are altered after myocardial infarction and in heart failure (Fig. 2C) [83–85]. In this regard, it has been shown that NOS1 expression is not only increased post-myocardial infarction, but NOS1 is shifted in localization within the cell to the level of the sarcolemma with interactions with caveolin-3, in contrast to its

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location at the SR in mice without myocardial infarction. Furthermore, NOS1 accounted for the majority of the NO produced within the cell, as NOS3 expression and activity decreased post-myocardial infarction. This translocation of NOS1 to the sarcolemma may explain the observation that NOS1 signaling inhibits myocardial β-adrenergic contractility after myocardial infarction [86]. This likely represents an adaptive response since others and we have shown that NOS1$^{-/-}$ mice exhibit increased mortality after myocardial infarction (Fig. 3) [87,88]. Indeed, the cardioprotective effect of NOS1 post-myocardial infarction was associated with increased S-nitrosylation of the LTCC leading to decreased Ca^{2+} influx [89]. In turn, the reduced Ca^{2+} entry within the cardiac myocyte prevented Ca^{2+} overload-induced injury. NOS1 has also been shown to protect against the development of myocardial remodeling after infarction [87,88]. In summary, based on their specific spatial localization within the cardiac myocyte, NOS1 and NOS3 play a specific and unique role in cardiac biology and pathophysiology (24).

3.3. Calcium signaling

Depolarization of the cardiac myocyte plasma membrane triggers a cascade of events leading to a rapid increase in cytosolic Ca^{2+} and resulting in muscle contraction, termed excitation–contraction coupling [2]. S-nitrosylation modulates the function of ion channels that regulate excitation–contraction coupling and therefore normal systolic and diastolic function [9,11].

In the normal heart, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is tightly regulated throughout the cardiac cycle. In excitation–contraction coupling, a small amount of Ca^{2+} enters through the LTCC during membrane depolarization. This influx triggers massive Ca^{2+} release from the SR,mainly trough theCa^{2+}release channel RyR2. Thiselevated $[Ca^{2+}]_i$ causes the binding of Ca^{2+} to troponin C in the myofilaments, which activates the contraction. Relaxation is initiated when Ca^{2+} is transported out of the cytosol, which is achieved by SERCA in the SR and the sarcolemmal sodium–calcium exchanger (NCX).

Defects in intracellular Ca^{2+} handling, such as reductions in the systolic $[Ca^{2+}]_i$, increase in diastolic $[Ca^{2+}]_i$, and impairments in diastolic Ca^{2+} re-uptake, have an important role in the depressed contractility and cardiac reserve observed in heart failure [90]. The down-regulation of SERCA and up-regulation of NCX that occurs in heart failure act in concert to shift Ca^{2+} out of the cell and reduce SR Ca^{2+} content, leaving less Ca^{2+} available for contraction. Beside the changes in SERCA and NCX expression, there is evidence for diastolic Ca^{2+} leak in heart failure [90]. Redox modifications of RyR2 (S-nitrosylation, oxidation) in the setting of heart failure play an important role in the activity of the channel [91–95]. Due to its high number of cysteines (89 cysteine residues per subunit), it is susceptible to S-nitrosylation, S-glutathionylation, and disulphide oxidation [49,96]. A few of these cysteines can be rapidly oxidized by ROS and RNS, including H_2O_2, O_2^-, NO, and GSNO [42,96–98].An increased oxidation of the RyR2 channel has been demonstrated in animal models of heart failure [92,94,95]. This was restored by β-adrenergic-blockers and antioxidants, associated with improved cardiac function, although it was not investigated whether the change in free thiols was due to increased S-nitrosylation, glutathionylation, or further oxidation (disulphide bonds, sulphenic or sulphonic acids). Our group has shown that

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with a low concentration of NO donor, the increase in S-nitrosylation of the channel is associated with an increase in contractility and that loss of S-nitrosylation decreases inotropic responsiveness [50,99–101]. This highlights the importance of determining the redox state of RyR2 in disease.

ROS and RNS species play a significant pathophysiological role in heart failure. The cardiac RyR2 has low-level basal SNO. S-nitrosylation of additional cysteines leads to further activation of the channel [49]. This is a highly reversible modification that can occur on a time scale proportionate to excitation–contraction coupling. On the other hand, oxidation of cysteine residues on RyR2 leads to irreversible activation of the channel, leading to SR leak and resulting in SR Ca^{2+} depletion (Fig. 2C). We have shown that cardiac RyR2 is hyponitrosylated in heart failure, due to nitroso-redox imbalance (Fig. 4) [50,101]. We evaluated excitation–contraction coupling and nitroso-redox balance in spontaneously hypertensive-heart failure (SHHF) rats with dilated cardiomyopathy and age matched Wistar–Kyoto rats. SHHF cardiomyocytes were characterized by depressed contractility, increased diastolic Ca^{2+} leak, hyponitrosylation of RyR2, and enhanced xanthine oxidase (XO)-derived superoxide [101]. Global S-nitrosylation was decreased in failing hearts compared to non-failing. XO inhibition restored global and RyR2 nitrosylation and reversed the diastolic SR Ca^{2+} leak, improving Ca^{2+} handling and contractility. Together these findings demonstrate that nitroso-redox imbalance causes RyR2 oxidation, hyponitrosylation, and SR Ca^{2+} leak, a hallmark of cardiac dysfunction. The reversal of this phenotype by inhibition of XO has important pathophysiological and therapeutic implications [102,103].

XO is an important source of ROS in the cardiovascular system and our studies have provided significant new insights into the mechanism of cardiac XO signaling and its interaction with NOS1. We and others have demonstrated that NOS1 deficiency increases mortality, remodeling, and ventricular arrhythmia after myocardial infarction, associated with increased XO activity and decreased S-nitrosylation of Ca^{2+} handling proteins, while specific myocardial NOS1 overexpression has been shown to protect from remodeling by preserving Ca^{2+} cycling components [26,87,88,104,105]. Increased expression of NOS1 in the caveolae is associated with increased S-nitrosylation of the LTCC leading to decreased Ca^{2+} current. In turn, the reduced Ca^{2+} influx within the cardiac myocyte prevented Ca^{2+}overload-induced injury [69,89,106]. This is a protective mechanism, since it has been recently shown that increasing Ca^{2+} influx through the LTCC channel after myocardial infarction prevents depressed myocyte contractility but increases the risk of ischemic injury, precipitates sudden death, and exacerbates depressed cardiac pump function [107].

We have shown that XO inhibition preserves the expression of components of Ca^{2+} handling, such as SERCA, phosphorylated phospholamban (PLB) and NCX [108]. In this regard, it has been described that NOS1 influences PLB phosphorylation, probably through phosphatase activity [66]. Interestingly, it has been proposed that physiologically, this NOS1-dependent effect on PLB phosphorylation is mediated by peroxynitrite, formed from the concerted action of NOS1 and XO [109]. More recently, phospholamban has been shown to be S-nitrosylated in the heart, leading to activation of SERCA [67]. In NOS1 deficient mice, after myocardial infarction, we have shown that XO inhibition prevented remodeling

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and contractility by decreasing ROS/RNS damage and preserving the components of Ca^{2+} cycling [26].

NO activity has been shown by some [106,110], but not all [42,111] investigators to inhibit SR Ca^{2+} uptake. S-nitrosylation reactions have the potential to modify SERCA via thiol reactions [106,111–113]. Previous studies have shown that thiol-oxidation reduces Ca^{2+} pump activity. To the extent that nitrosative stress and ROS compete for the same thiol [39,114], it is possible that reversible S-nitrosylation of SERCA may also modulate pump activity to coordinate with S-nitrosylation regulation of the RYR2 [50]. It is also conceivable that oxidant signaling may directly regulate cross-bridge cycling kinetics, thereby modulating the efficiency of contraction [115,116]. In so far as modulation of proteins via thiol S-nitrosylation and oxidation is a general phenomenon, many potential proteins involvedincardiac Ca^{2+} signaling have the potential to be influenced by NOS, XO, and other oxidase signaling [11,72]. Whether these proteins are modulated reversibly so as to preserve physiologic signaling or irreversibly so as to cause toxicity is determined by nitroso/redox balance.

3.4. Voltage-gated potassium and sodium channel function

Cardiac voltage-gated potassium (K^+) and sodium (Na^+) channels, important in the regulation of the cardiac action potential, have been shown to be subject to regulation by S-nitrosylation [72]. Voltage-gated K^+ channels determine the resting membrane potential and the duration of the cardiac action potential. The delayed rectifier K^+ current is one of the major components that determines the timing of repolarization of cardiac myocytes and consists of a rapidly activating (I_{Kr}) and a slowly activating component (I_{Ks}). It has been reported that S-nitrosylation of cysteine 445 in the pore-forming subunit KCNQ1 increases I_{Ks} in a NOS3 dependent manner [117–119]. This activation resulted in shortening of the action potential duration. S-nitrosylation of a cysteine in the Kir2.1 channel protein has also been shown to shorten the action potential by increasing the inward-rectifying K^+ current [120].

In cardiomyocytes, the voltage-gated Na+channels are responsible for fast depolarization. The main Na^+ channel expressed in the mammalian myocardium is encoded by the gene SCN5A. Although Na+channels typically inactivate very quickly, in cardiomyocytes a late current is observed that has been shown to be dependent on S-nitrosylation coupled to NOS activity [121]. The Na^+ channel is rich in cysteines that could also be subject to oxidation under oxidative stress conditions [122,123]. Of note, a mutation in α-syntrophin associated with a form of the long QT syndrome results in aberrant S-nitrosylation of the Na^+ channel [124]. α-syntrophin, a dystrophin-associated protein, normally serves as scaffold protein for NOS1 and the plasma membrane CA^{2+}-ATPase, an interaction that results in inhibition of NO production [125]. The α-syntrophin mutation results in a disruption of the CA^{2+}-ATPase-NOS1 complex and favors interaction of NOS1 with the Na^+ channel, promoting S-nitrosylation and increased late Na^+ currents [124]. Although the impact of this process on the action potential duration was not investigated, it has been shown by others that mutations of the Na^+ channel that lead to similar increased late currents prolong the duration of the action potential [126].

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3.5. β-adrenergic receptor signaling

In the heart, NOS3 is activated by coupling to numerous receptors, including the β-adrenergic, muscarinic, and bradykinin receptors. The prototypic mode of activation appears to occur via agonist-stimulated increase in Ca^{2+}, leading to Ca^{2+}/calmodulin activation of NOS3. NOS3 is localized to caveolae of the sarcolemma and t-tubules, where the scaffolding protein caveolin-3 inactivates it until displaced by Ca^{2+}/ calmodulin [65,127]. NOS3 can also be activated directly by Akt phosphorylation without intracellular increases in Ca^{2+}[128,129].

NO exerts negative inotropic effects, which are more marked when contractility is stimulated by either β-adrenergic activation or heart rate, a finding reminiscent of the phenomenon of "accentuated antagonism" that is observed with vagal nerve stimulation [130]. The observation that NO inhibition of contractility is more apparent during β-adrenergic stimulation has led to the proposal that NO serves as a negative feedback mechanism over contractile reserve, a notion supported by the finding that adrenergic agonists directly stimulate NO production [77]. We have shown that the β3-adrenergic receptor, present in myocardium [131], is linked to NO production [132]. Studies performed using $NOS^{-/-}$ mice recapitulate central features of NO biology. For example, $NOS3^{-/-}$ mice have an enhanced β-adrenergic inotropic response [133], and some [134], but not all [135], patch clamp experiments demonstrate a requirement for NOS3 in cholinergic inhibition of intracellular Ca^{2+}.

Studies have shown that NOS3-induced S-nitrosylation of β-arrestin 2, dynamin, and G protein coupled receptor kinase (GRK2) regulates agonist-induced β2-adrenergic receptor trafficking by promoting receptor internalization, promoting endocytosis, and decreasing receptor phosphorylation and desensitization, respectively [136–139]. β-arrestin 2 serves as a scaffold that functionally colocalizes NOS3 and β2-adrenergic receptor. Isoproterenol stimulation results in activation of NOS3 and S-nitrosylation of β-arrestin 2, which promotes its dissociation from NOS3 and its association with clathrin heavy chain/β-adaptin. This facilitates routing of the β2-adrenergic receptor into the clathrin-based endocytotic pathway, and β-arrestin 2 is subsequently denitrosylated. Inhibition of GRK2 by isoproterenol induced S-nitrosylation suppresses β2 adrenergic receptor phosphorylation, β-arrestin 2 recruitment, and receptor desensitization and downregulation. On the other hand, desensitization is enhanced by inhibiting NO production. NOS3 also mediates S-nitrosylation of dynamin, which promotes clathrin-dependent endocytosis and internalization of the β2 adrenergic receptor.

3.6. Mitochondrial function

Many studies indicate that mitochondrial NO regulates energy metabolism. In studies measuring muscle O_2 consumption, NO donors and agonists suppress tissue O_2 consumption in a fashion that could be attenuated by NOS inhibitors [140]. Myocardial O_2 consumption is also physiologically inhibited by NO in a manner that improves mechanical efficiency [68]. It has been demonstrated in both anesthetized and conscious animals, that myocardial oxygen consumption (MVO_2) increases in response to NOS inhibition [68,141,142]. Thus, NO regulates not only the major energy consuming process of the heart, contraction, but also

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mitochondrial energy production. Collectively, these studies suggest that NO promotes mechanoenergetic coupling and thereby enhances myocardial mechanical efficiency.

There is evidence that S-nitrosylation of proteins is involved in the regulation of mitochondrial energetics [69]. S-nitrosylation inhibits the activity of Complex 1 and F1F0ATPase, thereby attenuating ROS generation and reducing ATP consumption, respectively, during ischemia–reperfusion [69]. Cytochrome c oxidase activity is also inhibited by S-nitrosylation leading to decreased oxygen consumption [70]. S-nitrosylation of creatine kinase inhibits its activity and suppresses contractility under stress [71]. On the other hand, the activity of α-KGDH is increased by S-nitrosylation, which may prevent oxidative inactivation upon ischemia–reperfusion [69].

4. Vascular cellular processes regulated by S-nitrosylation

4.1. Vasodilation

In addition to being the largest reservoir of oxygen (O_2), hemoglobin is a major NO donor that vasodilates blood vessels in response to low oxygen tension, thereby matching perfusion with tissue O_2 demand, a process termed hypoxic vasodilation [143]. Hemoglobin is a tetramer of 2 alpha and 2 beta chains that exhibit cooperative binding of O_2 and exists in one of 2 structural states, R (relaxed, high O_2 affinity) and T (tense, low O_2 affinity). NO is carried both by binding to hemes in a manner similar to O_2 and by S-nitrosylation of Cys93 of the β subunit. S-nitrosohemoglobin (SNO-Hb) has been shown to mediate hypoxic vasodilation [120]. SNO-Hb formation is favored in the oxygenated R structure, whereas in hypoxia or low Ph the T structure releases NO and S-nitrosothiols to the surrounding tissues with resultant vasodilation. There is evidence that the coronary vasodilator nitroglycerin improves myocardial perfusion by utilizing SNO-Hb mediated O_2 delivery in concert with NO release [121].

A recent clinical study has shown that inorganic nitrate capsules or a dietary nitrate load results in dose-dependent increases in plasma nitrite concentration via bioconversion in vivo [15]. This bioactive nitrite, after reduction to NO, causes dose-dependent decreases in blood pressure and prevents ischemia–reperfusion-induced endothe-lial dysfunction in healthy volunteers. Nitrite, within the realm of physiological concentrations, vasodilates both the arterial and venous sides of the forearm circulation and systemic nitrite application decreases blood pressure in humans [13,14]. It is thought that these effects of nitrite are mainly because of its reduction to NO within the blood vessel wall and within the red blood cell. Of note, nitrite infusions have been shown to be associated with rapid formation of iron-nitrosylated hemoglobin and, to a lesser extent, S-nitroso-hemoglobin [13]. Therefore, red blood cells play key role in autoregulation of blood flow and disturbances in nitroso-redox balance may underlie vascular dysfunction in a variety of disease states, including heart failure [122], pulmonary hypertension [123], sickle cell disease [144], and diabetic cardiovascular disease [145].

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4.2. Angiogenesis

Vascular endothelial growth factor (VEGF), a major promoter of angiogenesis, stimulates NOS3 production of NO [146,147]. NOS3$^{-/-}$ mice are deficient in VEGF responsiveness, supporting the important role of NO in angiogenesis [148]. Accumulating evidence suggests that S-nitrosylation mediates the pro-angiogenic effects of NO. VEGF has been shown to induce S-nitrosylation of mitogen activated protein kinase phosphatase 7 (MKP7), which facilitates endothelial cell migration [149]. In addition, S-nitrosylation-mediated activation of dynamin, a regulator of endocytosis, promotes endothelial cell survival and angiogenesis [138]. In vascular diseases associated with aging [150] and diabetes mellitus [151], alterations in endothelial cell protein S-nitrosylation have also been reported.

Hypoxia stimulates angiogenesis via the transcription factor hypoxia-inducible factor (HIF), which increases VEGF expression [152]. SNO donors have been shown to exert an effect similar to hypoxia, leading to increased HIF nuclear expression and S-nitrosylation-mediated HIF stabilization [153–156]. Furthermore, studies in normoxic GSNOR$^{-/-}$ mice demonstrated constitutively S-nitrosylated HIF with increased binding to the VEGF gene [54]. These mice also manifested cardioprotection after myocardial infarction, associated with increased myocardial capillary density. Collectively, these studies support the notion that NO promotes angiogenesis via protein S-nitrosylation.

4.3. Inflammation and apoptosis

Extensive evidence has demonstrated that NO exerts anti-inflammatory effects in the vasculature. NO donors decrease, whereas NOS inhibitors increase, leukocyte-endothelial adherence [6,157]. Studies in mice lacking specific NOS isoforms have further supported this notion [158] [159]. S-nitrosylation has been shown to be the NO-based signaling mechanism regulating endothelial protein trafficking and suppression of nuclear factor κB (NFκB)-dependent expression of proinflammatory cytokines and adhesion molecules [160]. In regard to protein trafficking in endothelial cells, S-nitrosylation of N-ethylmaleimide sensitive factor suppresses exocytosis of granules (i.e. Weibel–Palade bodies) and thereby externalization of the adhesion molecule P-selectin [161]. This inhibits leukocyte rolling and thus vascular inflammation. A similar mechanism is operative in platelets, reducing activation, adhesion, aggregation, and thrombosis [162]. Studies have demonstrated inhibitory S-nitrosylation of both NFκB and its activating enzyme complex, inhibitoryκB kinase [163,164]. Thus, the S-nitrosylation mediated anti-inflammatory actions of NO are relevant to a wide range of cardiovascular disease processes, including atherosclerosis, sepsis, and autoimmune disorders.

The antiapoptotic effects of NO have been shown to be mediated, at least in part, by S-nitrosylation of caspase-3 [165–167]. A cysteine residue on the active site of caspase-3 is S-nitrosylated thereby inhibiting its proapoptotic effects. A recent study has reported that thioredoxin-mediated denitrosylation is the mechanism by which caspase-3 undergoes stimulus-coupled activation [55]. To the extent that these mechanisms are operative in endothelial cells, the balance between S-nitrosylation and denitrosylation may play a pivotal role in endothelial cell survival.

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5. Renal cellular processes regulated by S-nitrosylation

NO plays a key role in regulating the capability of the kidneys to excrete sodium, an important determinant of arterial blood pressure [5,168]. Studies evaluating the intrarenal effects of NO donors and NOS inhibitors indicated that NO serves as a diuretic and natriuretic agent [5]. Experimental evidence from proximal tubule and cortical collecting duct cells and isolated proximal tubule and collecting duct segments demonstrated that this effect of NO is mediated by direct inhibition of epithelial transport mechanisms [4]. NO inhibits the sodium/hydrogen (Na^+/H^+) antiporter on the luminal membrane of the proximal tubule and attenuates the sodium/potassium (Na^+/K^+) ATPase activity on the basolateral membrane of the proximal tubule and collecting duct segments [169]. However, the signaling mechanisms mediating the inhibitory effects of NO on renal epithelial ion channels have not been fully elucidated. There is also evidence suggesting that the effects of intrarenal NOS inhibitors and NO donors on tubular reabsorptive function are mediated indirectly by the associated changes in peritubular hemodynamicsor interstitial pressure [5,170].

NO has been shown to be abundantly produced in the renal medulla. Renal medullary cells adapt to the hyperosmotic interstitial environment by increased expression of osmoprotective genes, which is driven by the transcriptional activator, tonicity-responsive enhancer I.H. Schulman, J.M. Hare / Biochimica et binding protein (TonEBP) [171]. A recent study addressed the effect of NO on the expression of osmoprotective genes and TonEBP activation in Madin–Darby Canine Kidney (MDCK) epithelial cells. NO donors blunted tonicity-induced up-regulation of TonEBP target genes. 8-bromo-cGMP and peroxynitrite failed to reproduce the inhibitory effect of NO, indicating that NO acts directly on TonEBP. S-nitrosylation of TonEBP was found to correlate with reduced DNA binding and transcriptional activity. Thus, this study demonstrated a novel SNO-mediated inhibitory effect on TonEBP, a mechanism relevant for regulation of osmoprotective genes in the renal medulla.

Ecto-5′-nucleotidase (5′-ribonucleoside phosphohydrolase, 5′-NU) is a membrane-bound glycoprotein that hydrolyzes extracellular nucleotides into membrane-permeable nucleosides [172]. In the kidney, 5′-NU is expressed mainly in plasma membranes of proximal tubular cells and, to a lesser extent, in glomerular mesangial cells, interstitial fibroblasts, and intercalated cells of the collecting tubule. It has been shown that NO inhibits 5′-NU activity in a cGMP- and protein synthesis-independent manner, most likely through S-nitrosylation of the enzyme [172]. The inhibition of 5′-NU activity by NO affected renal proximal phosphate reabsorption.

In glomerular mesangial cells, NO can modulate cell migration, cell proliferation, and the expression of extracellular matrix (ECM) proteins, degrading proteases, and intrinsic protease inhibitors [173]. The regulatory effects of NO on the expression pattern of cytokine-inducible genes that contribute to ECM homeostasis are considered to be a critical step in the development and progression of fibrotic processes within the kidney [174]. The role of protein S-nitrosylation in mediating the effects of NO on mesangial cells has not been extensively studied. However, using the biotin-switch method combined with two-

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dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and peptide mass fingerprinting, 31 novel protein targets of S-nitrosylation in NO-treated and cytokine-activated murine mesangial cells have been identified, including signaling proteins, receptors and membrane proteins, cytoskeletal and cell matrix proteins, and cytoplasmic proteins [175]. More recently, PARP-1, a trans-activator of the NOS2 promoter in mesangial cells, has been shown to be a target of NO-mediated S-nitrosylation [176]. This modification limits its DNA binding activity and ability to trans-activate the NOS2 promoter. This NO-mediated negative feedback regulation of PARP-1 binding and action at the NOS2 promoter represents an endogenous mechanism to limit excessive NO generation in pathological states. Further research is warranted to elucidate the pathophysiological role of S-nitrosylation and denitrosylation signaling pathways in the kidney.

6. Summary

NO plays a central role in cardiovascular physiology. Protein S-nitrosylation, a reversible, thiol-based, and redox-sensitive post-translational modification, has emerged as a crucial and ubiquitous NO-based signal. The regulatory effects of S-nitrosylation involve altering protein structure and function by modifying specific thiols and shielding modified thiols from further irreversible modification under oxidative/nitrosative stress. In addition, the spatial localization of NO and SNO signaling, the level of protein S-nitrosylation, and the interaction with other signaling pathways determines whether the overall effect of S-nitrosylation is protective or detrimental. Future research on the mechanisms of S-nitrosylation and denitrosylation and interactions with ROS-based signaling pathways may help identify potential therapeutic targets in cardiovascular diseases.

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Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
cGMP	3′,5′-cyclic guanosine monophosphate
ECM	Extracellular matrix
GRK2	G protein coupled receptor kinase
GSH	Glutathione
GSNHOH	Glutathione S-hydroxysulfenamide
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione reductase
GSSG	Oxidized glutathione
HIF	hypoxia inducible factor
K^+	potassium
LTCC	L-type Ca^{2+} channel
MKP7	mitogen activated protein kinase phosphatase 7
MVO_2	Myocardial oxygen consumption
Na^+	sodium

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NCX	Sodium-calcium exchanger
NFkB	nuclear factor kB
NO	Nitric oxide
N_2O_3	dinitrogen trioxide
NOS	Nitric oxide synthases
NSF	N-ethylmaleimide sensitive factor
5′-NU	Ecto-5′-nucleotidase
O_2	Oxygen
PDE	Phosphodiesterase
PKG	cGMP-dependent protein kinase
PLB	Phospholamban
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RYR2	Ryanodine receptor/Ca^{2+}-release channel
SERCA	Sarcoplasmic reticulum Ca^{2+} ATPase
sGC	Soluble guanylate cyclase
SHHF	Spontaneously hypertensive-heart failure
SNO	S-nitrosothiols
SNO-Hb	S-nitrosohemoglobin
SR	Sarcoplasmic reticulum
TonEBP	Tonicity-responsive enhancer binding protein
VEGF	Vascular endothelial growth factor
XO	Xanthine oxidase

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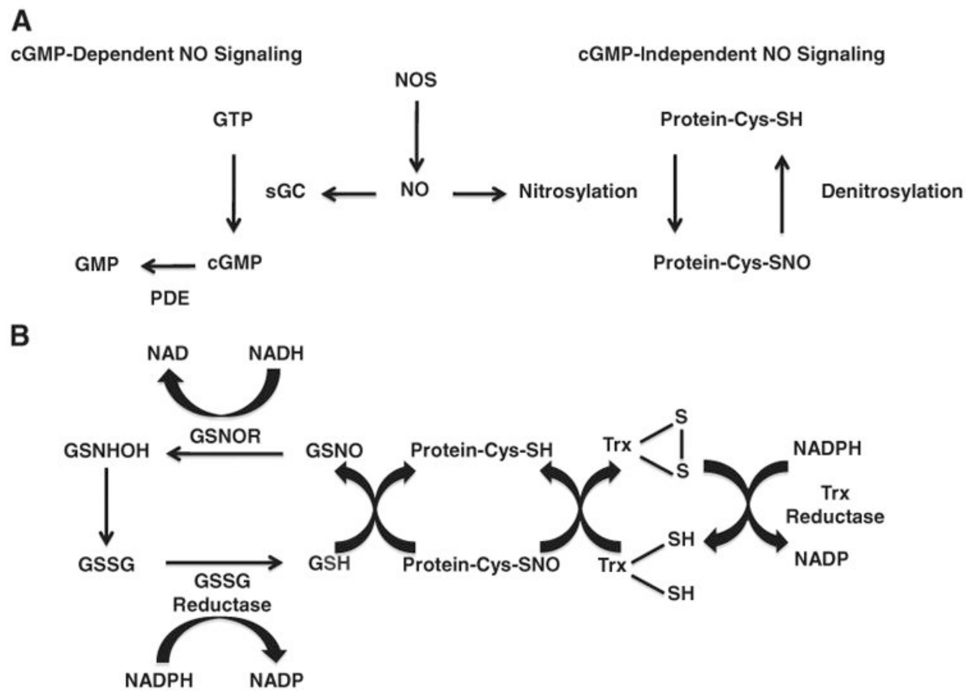

Fig. 1.

Panel A shows cGMP-dependent and independent nitric oxide (NO) signaling pathways. NO synthase (NOS) produces NO, which activates soluble guanylate cyclase (sGC) to generate the second messenger 3′,5′-cyclic guanosine monophosphate (cGMP). cGMP signaling is terminated by cyclic nucleotide-hydrolyzing phosphodiesterases (PDE). NO also exerts its actions independently of the cGMP pathway by the nitrosylation of sulfhydryl groups on proteins. Panel B shows denitrosylation pathways through the S-nitrosoglutathione (GSNO) reductase (GSNOR) and thioredoxin (Trx) systems. GSNOR uses NADH to reduce GSNO into glutathione S-hydroxysulfenamide (GSNHOH), which in turn is converted to oxidized glutathione (GSSG). Reduction of GSSG by glutathione reductase terminates the denitrosylation reaction. Trx mediates the denitrosylation of S-nitrosylated proteins. Following denitrosylation, the Trx system uses Trx reductase and NADPH to regenerate reduced Trx. Adapted from Lima, B. et al., Circulation Research 2010;106:633–646.

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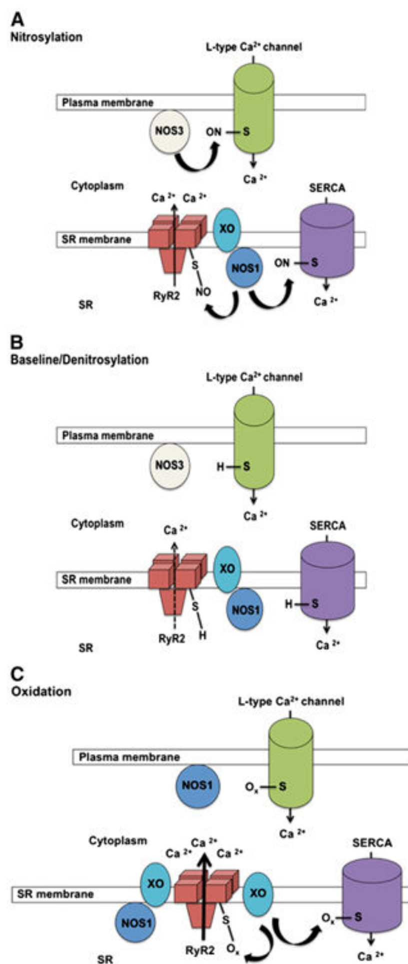

Fig. 2.

Nitroso-redox regulationof cardiac excitation–contraction coupling. Depicted Is NOS isoform-specific signaling in subcellular compartments as well as potential mechanisms for S-nitrosylation and oxidation regulation of the ryanodine receptor/Ca^{2+}-release channel (RyR2). The specificity of NOS localization is critical for the subcellular generation of NO because it provides a localized signal for protein S-nitrosylation (Panels A and B). NOS3 is localized primarily to the plasma membrane and t tubules. NOS1 colocalizes with the tetrameric RyR2 in the sarcoplasmic reticulum (SR) but translocates to the plasma membrane under conditions of stress (Panel C), including myocardial infarction and heart failure. RyR2 is closely associated with the plasma membrane L-type Ca^{2+} channel (LTCC), facilitating Ca^{2+}-mediated Ca^{2+} release from the SR. Panels A and B illustrate the physiological and specific S-nitrosylation and denitrosylation that occur on a millisecond time scale and therefore participate in the regulation of contraction in systole and relaxation in diastole. Panel C shows oxidation of cysteine thiols on RyR2, which leads to irreversible channel activation with maladaptive loss of regulatory control. Oxidation may occurat thiols that are nitrosylated or at other sites, which could change permissiveness to S-nitrosylation via allosteric effects. Reactive oxygen species (ROS) leading to cysteine oxidation are derived from multiple sources, including xanthine oxidase (XO). In heart failure, the excess of ROS derived from xanthine oxidase (XO) impairs the normal nitrosylation of cysteines,

which become oxidized. In this condition, the activity of RyR2 increases, leading to diastolic leak that reduces the Ca^{2+} content of the SR. The LTCC and SR Ca^{2+} ATPase (SERCA) are similarly regulated by redox mechanisms at cysteine thiols.

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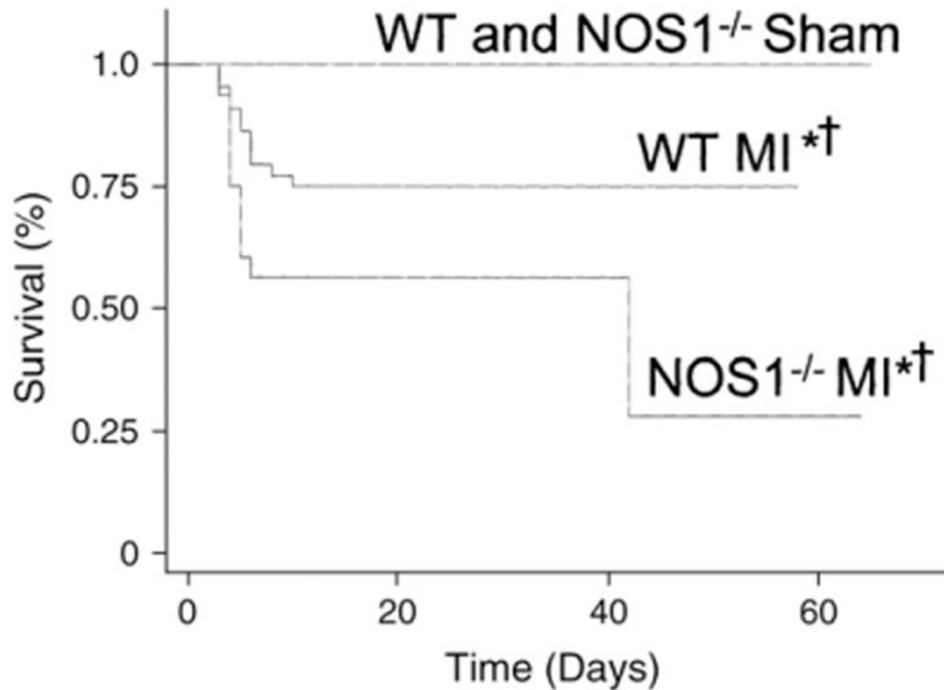

Fig. 3.

Survival curves after myocardial infarction (MI) in wild type (WT) and NOS1$^{-/-}$mice followed up for 60 days. Post-MI survival was significantly reduced in NOS1$^{-/-}$ vs. WT mice (*P=0.036). Both MI groups had reduced survival in relation to their respective sham-operated controls (†P=0.014 between WT subgroups and P=0.0001 between NOS1$^{-/-}$ subgroups). There was no significant difference in survival after sham operation between WT and NOS1$^{-/-}$ mice.

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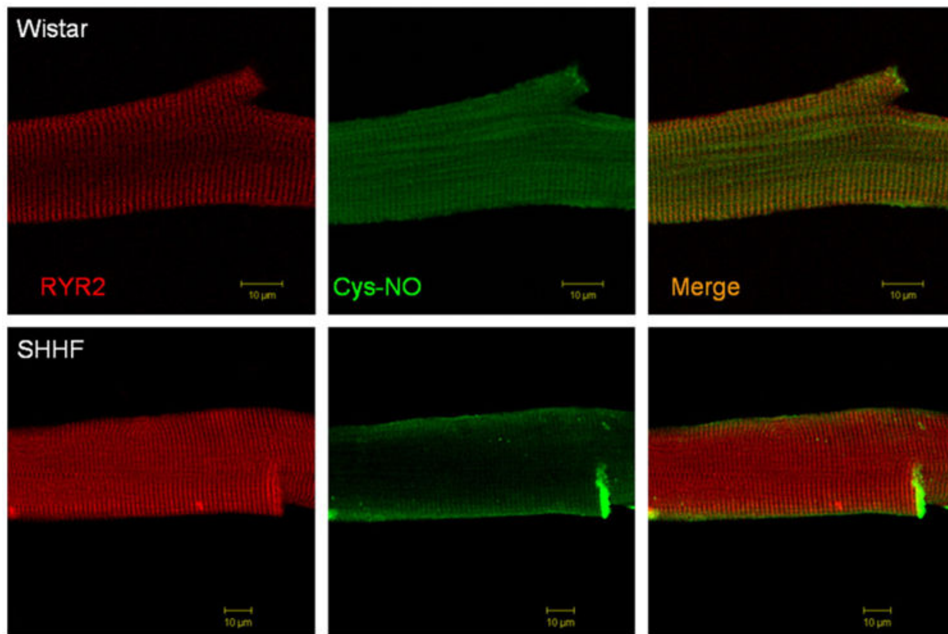

Fig. 4.

Decreased S-nitrosylation of ryanodine receptor/Ca^{2+}-release channel (RyR2) in isolated cardiomyocytes from spontaneously hypertensive-heart failure (SHHF) rats. Confocal microscopy showing red staining for RyR2 (left panels), green staining for nitrosylated cysteines (middle panels) and the combination of both (merge), right panels. The upper panels show a sequence of images of cardiac myocytes from a non-failing heart (Wistar–Kyoto rat). The lower panels show the same sequence for myocytes from a failing heart (SHHF rat). Note the degree of colocalization of RyR2 with the S-nitrosocysteines (Cys-NO) in the non-failing heart, which is lost in the failing myocardium.