NITROSO-REDOX BALANCE: A KEY MECHANISM IN THE REGULATION OF THE MYOCARDIAL FUNCTION IN HEALTH AND DISEASE

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ABSTRACT
Given the close interaction between nitric oxide (NO) and reactive oxygen species (ROS) in biological systems and their especial relevance in regulating aspects of the cardiovascular physiology, the concept of "nitroso-redox balance" has arisen as a more comprehensive manner of interpreting the intracellular redox state. Nitroso-redox signaling pathways participate in numerous cardiovascular mechanisms, including myocardial contractility and relaxation, mitochondrial respiration, and endothelial function. Alterations of this balance are involved in numerous aspects of cardiovascular pathophysiology. NO and ROS generating mechanisms play a major role in both regulating and responding to the redox state of the cell, which targets calcium handling, contractile and vasoactive mechanisms. Thus, the nitroso-redox signaling pathway is critically important in cardiac physiology and pathophysiology, and consequently a fundamental therapeutic target. This article briefly addresses the cardiovascular implications of the biological balance between NO and ROS, and their relevance in the development of heart diseases.
Keywords: Nitroso-redox balance, S-nitrosylation, myocardial contractility, calcium handling, oxidative stress.

RESUMEN
Dada la íntima interacción entre el óxido nítrico (NO) y las especies reactivas de oxigeno (ROS) en los sistemas biológicos, y su especial relevancia en la regulación de la fisiología cardiovascular, ha surgido el concepto de "balance nitroso-redox" como una forma más inclusiva para interpretar el estado redox intracelular. Las vías de señalización nitroso-redox participan en numerosos mecanismos cardiovasculares, tales como contractilidad y relajación miocárdica, respiración mitocondrial y función endotelial. Las alteraciones de este balance están involucradas en muchos aspectos de la fisiopatología cardiovascular. Los sistemas que producen NO y ROS juegan un rol clave tanto en la regulación como en la respuesta al estado redox de la célula, el cual afecta el manejo de calcio y mecanismos contráctiles y vasoactivos. Así, las vías de señalización nitroso-redox son claves para la fisiología y fisiopatología cardiaca, y consecuentemente son un blanco terapéutico fundamental. Este artículo aborda brevemente las implicaciones cardiovasculares del balance biológico entre NO y ROS, y su relevancia en el desarrollo de enfermedades cardiacas.
Palabras clave: Balance nitroso-redox, S-nitrosilacion, contractilidad miocárdica, manejo de calcio, estrés oxidativo.

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The cardiomyocyte redox state is tightly regulated by several cellular systems. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) (such as superoxide and nitric oxide (NO), respectively) normally participate in signaling pathways as part of delicate mechanisms that maintain the cardiovascular homeostasis [1]. Given the close interaction between ROS and NO signaling, and the regulation they can reciprocally exert, it is useful to think of the cellular redox state as a "nitroso-redox balance" (Figure 1). Disruption of this equilibrium leads to an intracellular nitroso-redox imbalance, which has the potential to act as a hazardous agent targeting diverse cellular components [2;3]. Specifically, this concept of nitroso-redox balance is characterized by three levels of interaction between ROS and RNS, namely enzymatic production, chemical reaction, and signaling via post-translational modifications.

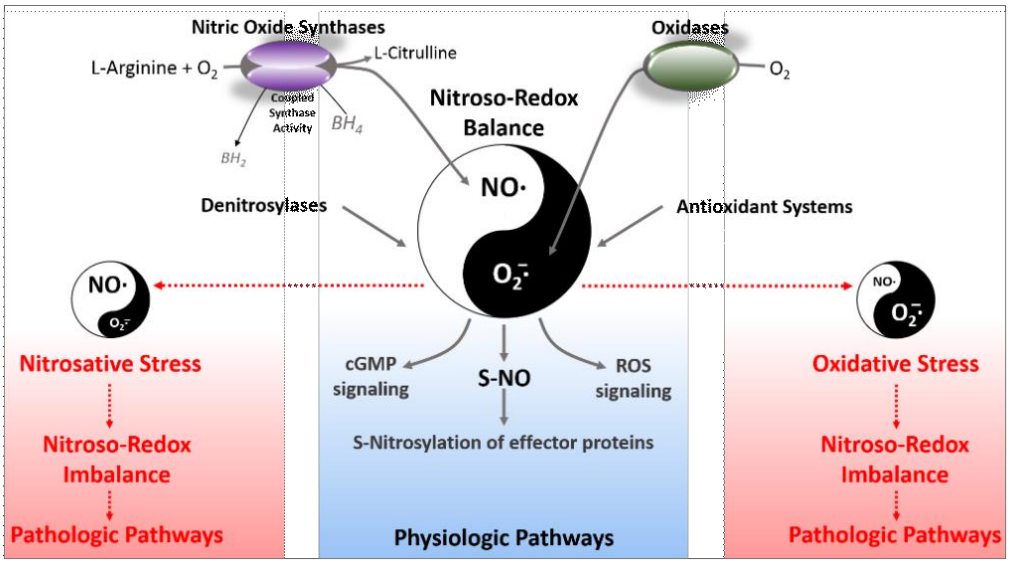

Figure 1. Schematic overview of the nitroso-redox balance. Nitric oxide synthases-mediated NO production and the superoxide (O_2^{-}) generated by proteins with oxidase activity as well as by the electron transport chain, are the main sources of chemical species which determine the nitroso-redox state of the cell. The equilibrium nitroso-redox is tightly maintained by a number of interacting enzymatic systems with regulatory function, including antioxidant and denitrosylation systems. A shift toward higher abundance of NO (left of the scheme) may induce a state of nitrosative stress with detrimental functional effects. Similarly, dysregulated O_2^{-} (right) production induces oxidative stress with consequent cellular damage. Both alterations of the equilibrium are considered as "disrupted nitroso-redox balance".

NO sources and NO-based signaling

NO production is enzymatically mediated by nitric oxide synthases (NOS) of which three isoforms have been identified: NOS1 (or neuronal NOS), NOS2 (or inducible NOS) and NOS3 (or endothelial NOS). Extensive study has led to a view of isoform-specific NO signaling in precise subcellular compartments. NOS3 plays a critical role in regulation endothelial function in the vasculature. In the myocardium, NOS3 is localized primarily to caveolae of the sarcolemma and t-tubules, where is linked to multiple cell surface receptors, including muscarinic, β-adrenergic, and bradykinin receptors. NOS1 localizes to the SR, in close interaction with xanthine oxidoreductase (XOR) and ryanodine receptor (RyR2) where it influences Ca^{2+} cycling and thereby exerts positive inotropic effects in the heart. NOS1 and NOS3 are constitutively expressed and their activity are Ca^{2+}-dependent, while NOS2 is inducible under certain stressing conditions or inflammation, and it is Ca^{2+}-independent.
NO activates numerous downstream pathways. A classical pathway is the activation of soluble guanylyl cyclase (sGC) to produce cGMP which is able to activate cGMP-dependent protein kinase (PKG). Dysfunctional NO-sGC-cGMP signaling is observed in several diseases

undergoing oxidative stress, including heart failure [4], which would explain the poor response to NO-based therapies in these pathologies.

S-Nitrosylation. Alternatively, NO exerts signaling via post-translational modifications of sulfhydryl groups of proteins and small molecules, a reaction termed S-nitrosylation [5]. Protein S-nitrosylation occurs by transnitrosylation from low molecular weight S-nitrosothiols (S-NO, Figure 1), 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 [6] (Figure 2). Cysteines susceptible to S-nitrosylation are generally located in a predictive consensus motif, between an acidic and a basic amino acid [6]. Physiologic superoxide and NO production as well as low ambient oxygen stabilizes SNO formation, favoring S-nitrosylation of proteins [7] , whereas increasing oxygen drives the reaction towards S-thiolation [8;9]. Pathologically elevated superoxide generation leads to oxidative stress, an altered intracellular redox state which disrupts the NO-signaling mechanisms. Superoxide reacts with NO, yielding peroxynitrite and preventing NO-triggered signaling [2]. S-nitrosylation is a labile and reversible covalent redox-sensitive modification, which regulates diverse biologic processes [6;10]. The effect of S-nitrosylation depends on the target protein, subcellular location and the extent of nitrosylation. S-nitrosylation competes with other post-translational modification of protein thiols, such as S-glutathionylation and irreversible oxidations. In turn, superoxide targets cysteine thiol moieties in proteins that could be S-nitrosylated, thus inhibiting these proteins from being reversibly regulated [11].

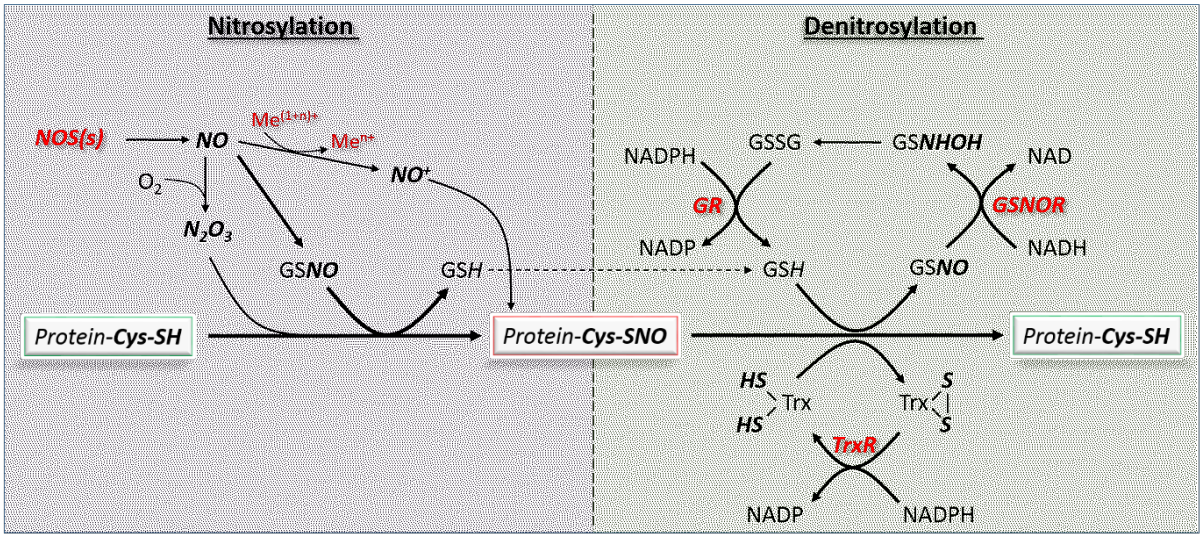

Figure 2. Mechanisms of nitrosylation/denitrosylation of redox-sensitive proteins. The left side of the image depicts some representative NO-mediated mechanisms by which certain proteins carrying cysteine (Cys) residues surrounded by an appropriate amino acidic environment can be S-nitrosylated. The right side of the image summarizes the main mechanisms enzymatically regulated of denitrosylation of modified proteins. GSNO: S-nitrosoglutathione; GSH: glutathione; N_2O_3: dinitrogen dioxide; Me: transition metal; GSSG: oxidized glutathione; GSNHOH: glutathione S-hydroxysulfenamide; Trx: thioredoxine; NOS(s): nitric oxide synthases; GR: glutathione reductase; GSNOR: S-nitrosoglutathione reductase; TrxR: thioredoxine reductase.

Denitrosylation. NO is removed from proteins by the action of denitrosylases. S-nitrosoglutathione (GSNO) reductase (GSNOR) was the first enzyme discovered that metabolized S-nitrosothiols and is important in protecting cells from nitrosative stress, mechanisms which have been extensively reviewed [10]. A second major enzymatic system mediating intracellular protein denitrosylation is the thioredoxin system [12]. Unlike GSNOR which specifically denitrosylates GSNO, thioredoxins denitrosylate several S-nitrosylated

proteins in a stimulus-coupled, substrate specific, and spatially restricted manner [12] (Figure 2).

Heart Failure

Heart failure (HF) has been classically defined as a complex clinical syndrome characterized by an inadequate pumping of blood to meet the metabolic demands of the body. Recently, HF has been subdivided into heart failure with reduced ejection fraction (HFrEF) courses with evident systolic dysfunction (left ventricular ejection fraction <50%), and heart failure with preserved ejection fraction (HFpEF, ejection fraction>50%) exhibit signs of diastolic dysfunction [13;14]. In the early stages of HF, a compensatory response arises, involving structural and molecular remodeling of the heart to preserve circulatory integrity, but later on, this leads to further deterioration. Several defects in cardiac excitation-contraction (EC) coupling have been identified in failing hearts, including altered intracellular Ca^{2+} handling. In the cardiomyocyte, the final common feature in heart failure, regardless of the cause, is an increased diastolic sarcoplasmic reticulum (SR) Ca^{2+} leak and reduced SR Ca^{2+} content, which diminish the effectiveness of EC coupling. Changes in the expression of NOSs have been described in heart failure, involving disruption of the isoforms compartmentalization, although these changes remains controversial. It is mostly accepted that there is a reduced endothelial NO production, which affects myocardial perfusion and, in turn, contributes to circulatory dysfunction. In general, there is a consensus about limited NO (or associated derivatives) bioavailability due to increased superoxide. In addition, reduced NOS activity contributes to the disrupted nitroso-redox balance in heart failure leading to contractile dysfunction, hypertrophy and adverse remodeling.

Cardiomyocytes from spontaneously hypertensive-heart failure (SHHF) rats were characterized by depressed contractility and increased SR Ca^{2+} leak, and exhibited hyponitrosylated and oxidized RyR2 due to the nitroso-redox imbalance in the SR microdomain [15] (Figure 3). Consistently, XOR expression and activity were increased. Despite an increase in total NOS1 abundance in heart failure, a portion has been demonstrated to translocate to the sarcolemma [16], and the remaining NOS1 on the SR might undergo enzyme uncoupling due exacerbated XOR-derived superoxide generation. XOR inhibition restores RyR2 nitrosylation and improves Ca^{2+} handling and contractility [15], and improves cardiac function and reverses remodeling [17]. However, most of clinical studies using XOR inhibitors in heart failure have shown little or none benefit [18-20]. Another therapeutic strategy, which combines hydralazine plus isosorbide dinitrate (an organic nitrate which releases NO), has been tested in clinical trials in patients with congestive heart failure, yielding successful outcomes in self-identified African-American patients [21]. Interestingly, it has been shown that treating SHHF cardiomyocytes with a combination of organic nitrates and hydralazine improves contractile performance by restoring the efficiency in Ca^{2+} handling [22]. Reduced levels of the NOS cofactor tetrahydrobiopterin (BH_4), observed in heart failure due to oxidation into dihydrobiopterin, is suggested to favor NOS uncoupling leading to NO signaling disruption, further oxidative stress and worsening the nitroso-redox imbalance (Figure 3B). In a model of pressure overload, BH_4 supplementation prevented cardiac remodeling and progression to heart failure likely associated with conserved Ca^{2+} handling [23] by enhancing NOS1 activity, favoring phospholamban phosphorylation and accelerating relaxation. S-nitrosylation of phospholamban is required for its proper phosphorylation [24], and disruption of S-nitrosylation of sensitive Cys residues also contribute to dysfunctional myocyte relaxation. In this sense, GSNOR deficient mice which undergo hyper S-nitrosylation, exhibit improved recovery after myocardial infarction associated to faster Ca^{2+} decline [25], suggesting a role for S-nitrosylation on Ca^{2+} re-uptake. However, it has not been characterized if this effect is mediated by the sarcoplasmic reticulum calcium ATPase

(SERCA2a)/phospholamban or the cardiac sodium/calcium exchanger (NCX1), since S-nitrosylation of this ion exchanger also accelerates Ca^{2+} decline [26]. Cardiac overexpression of NOS1 prevented contractile dysfunction by increasing phospholamban phosphorylation in a model of transverse aortic constriction [27].

At the sarcolemma, NOS3-dervived NO plays an important role in regulating L-type Ca^{2+} channels (LTCC) by depressing Ca^{2+} inward current. NOS3 is sensitive to the nitroso-redox state of the cell and it can be either S-nitrosylated or S-glutathionylated in different redox circumstances, involving specific cysteine residues and leading to enzyme inhibition [28]. In turn, NOS3 is coupled to β3-adrenergic receptors (β3-AR). Although it is still controversial, there is increasing evidence suggesting that β3-adrenergic signaling would be a physiological brake to reduce the effects of sympathetic overstimulation in overt heart failure, by reducing Ca^{2+} entrance. β3-AR are upregulated in failing hearts and some studies overexpressing β3-AR mice showed attenuated left ventricular remodeling with chronic treatment of isoprenaline [29]. In this sense, specific stimulation of β3-adrenergic receptors protects against cardiac hypertrophy and failure due to pressure overload, involving a mechanism of NOS1-NOS3 interaction, which prevents nitroso-redox dysregulation and preserves NOS3 coupling and signaling [30], favoring the hypothesis of a cardioprotective role for the β3 signaling.

NOS2 is known to be upregulated in myocytes from patients with heart failure, and the Ca^{2+} handling mechanisms are severely affected by the unregulated NO generation. The septic shock leads to worsening of the nitroso/redox disequilibrium, where increased NOS2-derived NO and XOR-derived superoxide lead to profound disturbances, particularly in diastolic Ca^{2+} leakage [31], likely by oxidation of cysteine residues of RyR2 [32] by ROS or peroxynitrite.

There is evidence of a mitochondrial NOS (mtNOS) expressed in the inner mitochondrial membrane or matrix, although its remains controversial. It seems likely that mtNOS is NOS1 or a spliced variant of it (likely NOS1α or NOS1μ). In mitochondria, NO regulates electron transport chain and ROS production. It can inhibit Complex IV or, in combination with high $[Ca^{2+}]$ in the matrix, inhibits Complex I (which can also be S-nitrosylated) and so, favoring superoxide and peroxynitrite formation. Peroxynitrite at the mitochondria, in turn, can also inhibit Complex III and V, leading to additional ROS production and contributing to the opening of the permeability transition pore and cellular apoptosis or necrosis [33]. Thus, although NO can be protective against mitochondria-mediated cell death, dysregulated mtNOS-derived NO is likely to contribute and aggravate the nitroso-redox imbalance in pathological myocardial conditions.

Failing cardiomyocytes also exhibit disrupted myofilament cross-bridge kinetics, which has been associated with nitroso-redox imbalance, involving oxidation of sensitive cysteine residues of myofibrillar proteins (Figure 3). In this sense, it remains unknown whether uncoupled NOS-derived superoxide also contributes to these effects, but the evidence indicates that BH_4 improves diastolic dysfunction by reversing oxidative changes in myofibrillar proteins [34].

Thus, NO signaling mostly mediated by S-nitrosylation is believed to play a protective role, preventing or delaying the progress toward failure after myocardial injury by modulating Ca^{2+} cycling kinetics and myofilament responsiveness.

Therapeutic approaches for heart failure targeting NO signaling

Conventional pharmacological therapies for heart failure include neurohumoral blockade, statins and diuretics (Table 1). Thus, myocardial dysfunction is battled by these indirect antioxidant strategies, which attenuates the symptoms and progression of heart failure, by targeting directly or indirectly the nitroso-redox state of cardiomyocytes, but do not reverse the condition. Importantly, these therapeutic strategies are ineffective against the progression of HFpEF, which represent about 50% of the cases of HF [35]. Thus, the development of novel

specific therapeutics for HFpEF is a priority since this proportion is predicted to continue increasing relative to HFrEF.

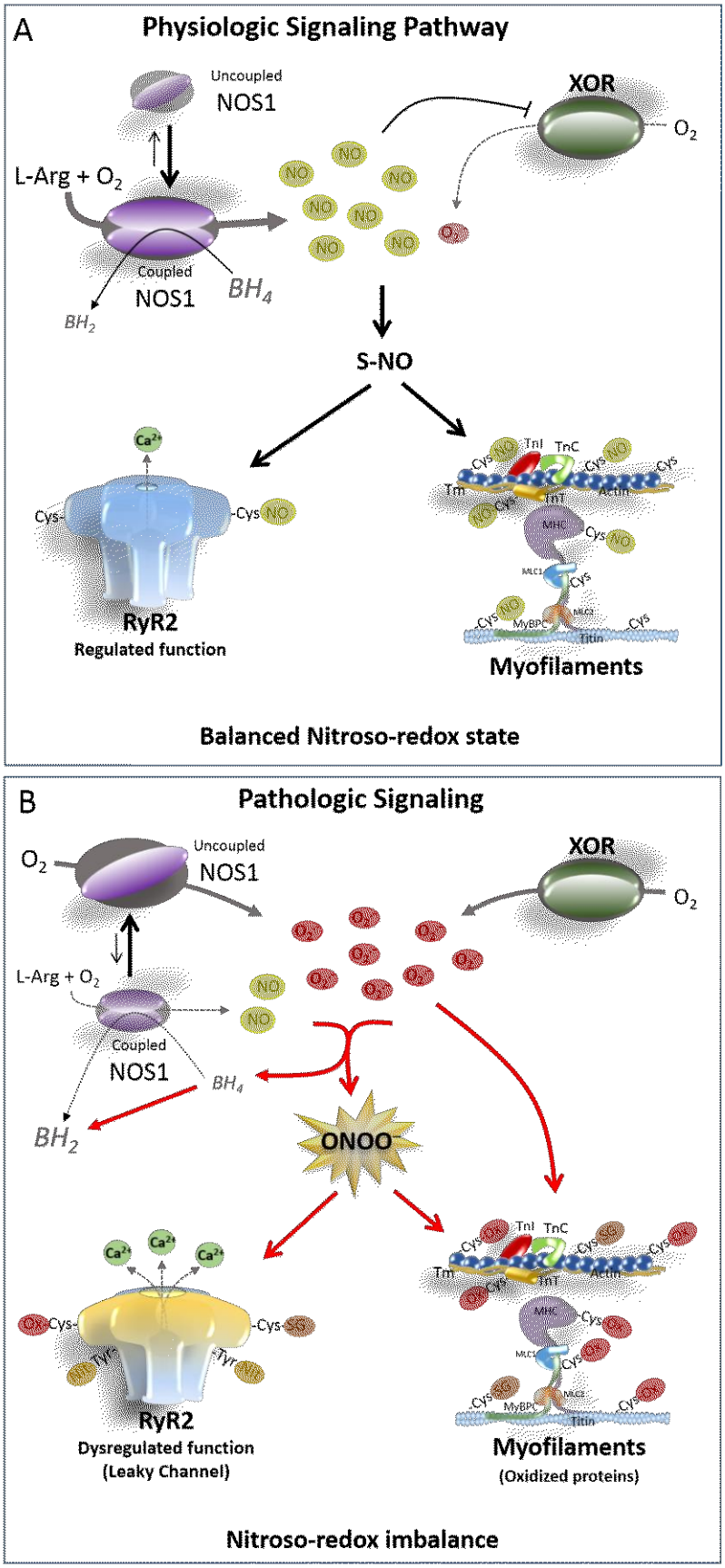

Figure 3. Schematic representation of myocardial processes regulated by the nitroso-redox state. (A) With normal nitroso-redox balance, NOS1 finely interacts with XOR in the SR microenvironment, thus regulating the NO and superoxide ($O_2^{\cdot-}$) production and S-nitrosothiols formation. This physiologic condition allows the signaling pathway to be regulated by dynamic S-nitrosylation of susceptible cysteine residues on effector proteins such as ryanodine receptor (RyR2) or myofibrillar proteins (actin, troponins (TnI, TnC, TnT), myosin heavy chain (MHC), myosin binding protein C (MyBPC), titin). **(B)** A shift toward oxidative stress dysregulates NOS1/XOR interaction and promotes uncoupling of NOS which causes a switch from NO to ROS production, further disrupting the nitroso-redox balance and thereby potentiating the oxidative stress. This condition favors generation of peroxynitrite (ONOO-, a harmful agent for the integrity of the cell) and oxidative modifications of sensitive aminoacids, such as sulfonation (-Ox), nitration (-Nit) and S-glutathionylation (-SG), affecting protein structure and function.

Conclusion

ROS and NO signaling pathways interplay to maintain an intracellular nitroso-redox balance, which plays a central role in cardiovascular physiology. The regulation exerted by S-nitrosylation affects protein structure and function by modifying specific thiols and shielding modified thiols from irreversible alteration by oxidative stress. Remarkably, the spatial localization of NO and SNO signaling in coordination with the dynamic balance of protein S-nitrosylation/denitrosylation, and their interaction with ROS determine whether the overall effect of S-nitrosylation is protective or detrimental. Currently, ongoing research on the interacting nitroso-redox mechanisms are being conducted to identify novel therapeutic targets in cardiovascular diseases.

Table 1. NO-related therapeutic drugs

Treatment	Examples	Mechanism	Uses
Organic nitrates	Nitroglycerin Isosorbide dinitrate	NO donors	Ischemic heart disease
Antioxidants	Vitamin C Vitamin E XOR inhibitors	Reduces superoxide	Cardiovascular diseases
Antioxidants/NO donors combo	Hydralazine/Isosorbide dinitrate EMEPO (new compound)	-Antioxidant plus NO supply -Superoxide scavenger + NO release	Heart failure
Statins	Simvastatin, Atorvastatin	Enhances NOS3-derived NO	Hyperlipidemia
ACE inhibitors	Lisinopril Benazepril	Side effects on nitroso-redox balance	Hypertension Heart failure
AT1 antagonists	Losartan Valsartan	Side effect on NOS3-NO production	Hypertension Heart failure
3rd Gen β-blockers	Nebivolol Carvedilol	β1-blocker + enhances NO production	Heart failure
Synthetic BH_4	BH_4	Favors coupling of NOS3 (and/or NOS1)	Phenylketonuria Cardiovascular diseases
Nitroxyl (HNO) donors	Angeli's salt	PKA-independent inotropy and lusitropy enhancer	Heart failure
NOS3 downstream signaling enhancers	Cinaciguat (BAY 58-2667) PDE5 inhibitors	Non-NO sGC activator Prolongs cGMP life	Heart failure Erectile dysfunction Pulmonary hypertension
Thiol-reducing agents	-H_2S	Restores reduced thiol moieties	Myocardial infarction

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