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Chapter 7

Cardioprotective Effects of S-Nitrosothiols in Ischemia-Reperfusion: Role for Mitochondria and Calcium Channels

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Additional information is available at the end of the chapter

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Abstract

The most important clinical consequence of coronary disease is acute myocardial infarction caused by an occlusion that limits the irrigation to the heart. Although the gold standard treatment is to restore blood flow, this reperfusion causes inherent damage by increasing the size of the infarcted area primarily through the opening of the mitochondrial permeability transition pore (MPTP). The cardioprotective effect of nitric oxide (NO) has been described to operate through S-nitrosylation of several important proteins in the cardiomyocytes such as the calcium channels RyR2 and the L-type Ca^{2+} channel and mitochondrial proteins, including the MPTP. In this sense, an attractive strategy to prevent the ischemia-reperfusion damage is to increase the bioavailability of endogenous S-nitrosothiols. S-nitrosoglutathione reductase (GSNOR) is an enzyme involved in the metabolism of NO through denitrosylation, which would limit the cardioprotective effect of NO. Although inhibition of GSNOR has been studied in different organs, its effects on myocardial reperfusion have not yet been fully elucidated. In this chapter, we review the pathophysiology underlying myocardial reperfusion injury and the opening of the MPTP along with the cardioprotective role of S-nitrosothiols and the potential role for GSNOR.

Keywords: permeability transition pore, heart, GSNOR, nitric oxide, S-nitrosylation, Ca^{2+}

1. Introduction

Coronary heart disease is the leading cause of death worldwide [1]. According to the World Health Organization, in 2008, more than 7 million of deaths worldwide were the result of this

disease (12.8% of all deaths) [2]. The main clinical manifestation of this condition is acute myocardial infarction, which is characterized by a coronary occlusion, most frequently produced by the rupture of an unstable atherosclerotic plaque [3]. Ischemic damage in the myocardium produced by this pathology requires immediate restoration of blood flow to the affected heart area. The injury size of the resulting infarction depends on: (1) the ischemic area at risk, (2) the duration and intermittency of the coronary occlusion and (3) the magnitude of the residual collateral flow and the degree of microvascular dysfunction [4].

The preservation of myocardial functionality after ischemia is critical for the viability of the damaged heart, and although reperfusion is necessary for the survival of the myocardium, paradoxically this reperfusion itself can generate injury or even induce the death of cardiomyocytes. The damage can range from a reversible effect such as "myocardial stunning" to an irreversible one, increasing the size of the infarcted area or increasing microvascular alterations [5]. This phenomenon is known as reperfusion myocardial damage [6, 7].

To combat this condition, most of the current pharmacological strategies of cardioprotection converge to the mitochondria, particularly, to prevent the opening of mitochondrial permeability transition pore (MPTP), which is a large conductance channels that forms during stressing conditions for the heart such as reduction in ATP synthesis, increased Ca^{2+} and increased ROS production [8]. Nitric oxide (NO), a potent signaling molecule with pleiotropic effects in the heart, has long been studied to evaluate its cardioprotective effects. Agents that promote continuous NO releases are among the most commonly used drugs to treat cardiovascular disease [9]. Although its effects on vascular tone and cyclic GMP are well studied [10], S-nitrosylation, a posttranslational modification in which an NO group is added to a cysteine

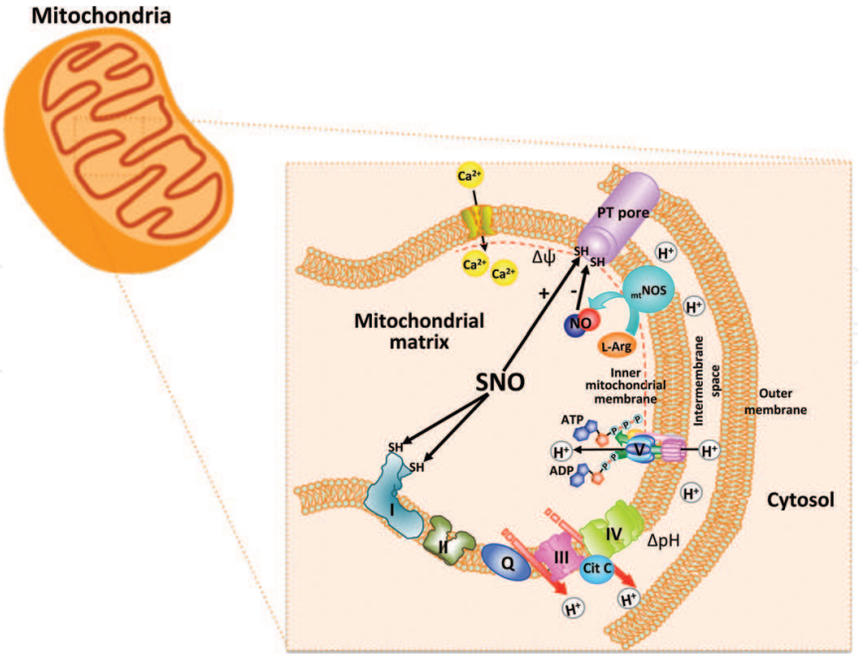

Figure 1. S-nitrosylation in mitochondria. The figure depicts two important targets where S-nitrosothiols (SNO) exert cardioprotective effects within the mitochondria. S-nitrosylation of complex I prevents the burst of reactive oxygen species during reperfusion. CyclophylinD, which interacts with mitochondrial permeability transition pore is also S-nitrosylated, reducing its opening.

thiol, has showed remarkable cardioprotective effects altering the function of different proteins important for the regulation of cell function and viability.

One of the main cardioprotective effects of S-nitrosylation is the avoidance of the opening of MPTP. The opening of the MPTP triggers a proapoptotic response mediated by the release of cytochrome C and other factors, as well as necrotic cell death. Agents that induce mitochondrial S-nitrosylation of this pore may reduce the infarcted area after ischemia and to maintain the functionality of the heart (**Figure 1**).

2. Reperfusion damage

Myocardial reperfusion injury was first described by Jennings et al. in 1960, using a model of coronary occlusion in dogs [11]. They observed alterations in the sarcolemma and in mitochondria, which accelerated the process of cardiomyocyte necrosis. This reperfusion injury in animal models of acute myocardial infarction can result in up a 50% of infarcted area [1]. During reperfusion, inflammatory mediators are released, such as cytokines, which recruit neutrophils to the affected endothelium, permeabilizing it for the entrance to the injured myocardium of more inflammatory cells. In fact, decreasing this influx of neutrophils confer cardioprotection in animal models [12]. In addition, during the period of ischemia, molecules called "danger signals" are released from the extracellular matrix and damaged cells such as fragments of fibronectin, hyaluronic acid, heat shock proteins and high-mobility group box-1 (HMBG1). HMBG1 is able to interact with toll-like receptors (TLR) such as TLR2, TLR4, TLR9 and the receptor for advanced glycation end products (RAGE). These ligand-receptor interactions lead to the nuclear translocation of NF-kappa B, triggering the transcription of proinflammatory genes [13].

One of the main organelles affected by reperfusion injury is mitochondria, which in pathological conditions may mediate detrimental effects to the cell such as apoptosis or cell necrosis [14].

After ischemia-reperfusion, mitochondria undergo structural as well as functional alterations. One of the most important alterations is the formation of MPTP, a pore of nonselective permeability, stimulated by high concentrations of mitochondrial Ca^{2+}, adenosine and reactive oxygen species (ROS) [15]. The function of this pore is thought to be the regulation of Ca^{2+} concentrations, preventing the overload of this ion at the mitochondrial level [16]. In fact, inhibition of the mitochondrial Ca^{2+} exchanger showed cardioprotective effects in isolated hearts of newborn [17] as well as in adult rabbits [18].

3. Mitochondrial permeability transition pore

In situations such as Ca^{2+} overload, alterations in ATP production, mitochondrial membrane depolarization, or inhibition of respiration, mitochondria may undergo a swelling process that represents a permeabilization of the internal membrane, product of the opening of nonspecific channels known as the mitochondrial permeability pore [19]. This pore, which allows the passage of molecules up to 1.5 kDa, mediates the rupture of the outer mitochondrial membrane

and the release of proapoptotic substances such as endonuclease G and the mitochondrial apoptosis inducing factor (AIF) allowing the exit of elements such as the cytochrome C [20].

What is the function of this mitochondrial pore? The pathological effects of the presence of the pore have been well described and usually associated with long-term openings since short-term openings do not have a major impact on cell viability [21]. On the contrary, it is believed that its transient opening can play a very important role both in the physiological regulation of Ca^{2+} and redox homeostasis in the generation of ROS [22].

3.1. Constituents of the transition pore of mitochondrial permeability

3.1.1. Cyclophilin D

Cyclophilin D (Cyp-D) is an 18 kDa protein encoded by the Ppif gene, synthesized in the cytosol and transported to the mitochondria to form part of the pore. It is inhibited by cyclosporine A, an immunosuppressant, decreasing its sensitivity to elevations in Ca^{2+} concentration [23]. CypD is a member of a family of cyclophilin proteins that have peptidylprolyl isomerase (PPIase) activity, catalyzing the cis-trans isomerization of peptidylprolyl bonds. In studies in knockout mice for the Ppif gene, mitochondria were more resistant to swelling and decreased the ability to open the pore [24]. It was also observed that overexpression of this gene caused an increase in swelling and cell death after ischemia-reperfusion [25]. Canceling the expression of this protein would, in theory, desensitize to the mitochondria for Ca^{2+} and will be protective. Nevertheless, it was described that when Ca^{2+} concentration is high enough, the pore can be opened independent of the presence of CypD [26]. It has been postulated that both CypD and the mitochondrial Ca^{2+} pore opening participate in the regulation of mitochondrial Ca^{2+} homeostasis, since the pore opening directly depends on the Ca^{2+} concentrations reached and its opening would try to reinforce the effect of the Na^{+}/Ca^{2+} through an efflux of Ca^{2+} to prevent the overload of this cation [27].

3.1.2. Adenine nucleotide traslocase

Adenine nucleotide traslocase (ANT) is a member of the long list of mitochondrial carriers. It shows a tripartite conformation of repeated sequences of 100 amino acids, with ANT1 being the isoform present in the heart [28]. Given the importance of the production of ATP by the mitochondria and its use mainly in the cytosol, a mechanism of fast and effective transport between the two compartments is necessary: the exit of ATP to the cytosol and transport of the ADP to the mitochondria. Therefore, ANT1 is one of the most abundant proteins inside the mitochondria and more than 10% of the energy used by the cardiomyocyte is used to maintain this transport [28]. Regarding to the role of ANT in the pore formation, it has been observed that inhibition of binding of ATP and ADP to ANT increases the sensitivity of MPTP in response to variations in $[Ca^{2+}]_i$ [19]. Evidence shows that ANT has the ability to bind CypD and that this binding is important for pore opening, with ANT acting as a sensor of $[Ca^{2+}]_i$. Although this protein has been categorized as a component of the MPTP, its importance in the pore gating is controversial since inhibition of both ANT 1 and 2 in mouse liver could still constitute the mitochondrial pore, although with a higher increase in the $[Ca^{2+}]_i$ [29].

3.1.3. Mitochondrial phosphate carrier

The mitochondrial phosphate carrier is encoded by the gene SLC25A3 and is a primary transport system of inorganic phosphate (Pi) by a proton transporter or an exchanger with hydroxyl ions [16]. It has been described that Pi is a potent modulator for the MPTP opening. In an energized condition, Pi can induce the opening of the pore. This contrasts with other studies postulating Pi as an inhibitor of pore opening [30]. This carrier could be considered as a structural component of the MPTP, but studies are still lacking to confirm its actual function.

3.1.4. Voltage-dependent anion channel

This is a channel present in the external mitochondrial membrane that serves as a pore for different substances in or out of the mitochondria. Like ANT, voltage-dependent anion channel (VDAC) can be considered a structural element in the conformation of the MPTP. Inhibiting VDAC activity, inhibits Bax (a proapoptotic protein) function and the release of cytochrome C, decreasing the apoptotic process [31].

3.1.5. F_1F_0-ATP synthase

More recent investigations have shown that a dimer of the mitochondrial F_1F_0-ATP-synthase is essential to form the core of the mPTP. F_1F_0-ATP-synthase interacts with CyPD and can reversibly undergo a Ca^{2+}-dependent transition to form a channel with the characteristics of the mPTP [20].

3.2. MPTP opening in ischemia/reperfusion damage

3.2.1. Ischemia

During the period of ischemia, the hypoxic condition makes the mitochondria unable to produce ATP through oxidative phosphorylation, leading to a rapid depletion of cellular ATP reserves, with the concomitant increase of ADP and Pi [15]. There is a change in ATP production from oxidation of fatty acids (most common) to glucose metabolism, but through an anaerobic route, a less efficient mechanism, which leads to increased lactate, NADH and H^+ [32] with a decrease in pH. This inhibits phosphofructokinase and activates the H^+/Na^+ symporter. This compensatory mechanism is unable to deliver enough ATP to supply cardiomyocytes, and specially pumps such as Na^+/K^+ ATPase. The accumulation of Na^+ facilitates the accumulation of Ca^{2+} in the cell because the Na^+/Ca^{2+} exchanger works in a reverse mode [15]. Then, oxidative damage alters the sarcoplasmic reticulum, releasing Ca^{2+}, further contributing to the overload of this ion [1]. The small amount of oxygen that remains serves for the oxidation of xanthine by the enzyme xanthine oxidase, mainly in the endothelium but also in the cardiomyocyte, which produces superoxide and other ROS. The duration of the ischemia period is an important factor; in short periods of ischemia (less than 15 min), reperfusion manages to restore homeostatic values with respect to mitochondrial membrane potential, cardiomyocyte contracture and Ca^{2+} concentration. However, in periods longer than 25 min, all these values are altered in a way that cell death is promoted [33].

3.2.2. Reperfusion

In reperfusion, other factors beside an increase in Pi, ROS and Ca^{2+} and the decrease in ATP come into play. Although all of them contribute to the opening of the mitochondrial pore, the diminished pH would be a "protective" factor in the conformation of this pore [34]. In fact, a decrease in pH inhibits the H^+/Na^+ symporter, which would prevent accumulation of Ca^{2+} in the cell [35].

Recovery of oxygen levels re-energizes the mitochondria to restore its role in the production of ATP via fatty acids metabolism. An alteration of the membrane potential causes Ca^{2+} to enter the mitochondria, while the oxygen entering it contributes to the formation of more ROS due to the reduced respiratory chain [19]. Reperfusion favors the recovery of pH in a period close to 3 min, time in which lactate diffuses. During the first few minutes, the damage caused by reperfusion is small; nevertheless, as reperfusion is delayed, the infarcted area grows. Once the pore has been established as a result of the abovementioned alterations, mitochondria may become swollen and the rupture of the outer membrane occurs, which will trigger the release of cytochrome C and other proapoptotic substances leading to cell death [15, 36–38].

4. Mitochondrial ROS release and reperfusion injury

ROS release by mitochondria is one of the major events that trigger ischemia-reperfusion injury. Although this event is thought to be produced by an alteration in the mitochondrial electron transport chain during reperfusion, it is further believed that these reactive species can be produced by an altered metabolic process during ischemia, whose consequences appear in the reperfusion period. For example, in a study by Couchani et al., they observed the accumulation of succinate, a metabolic intermediate of the Krebs cycle, during ischemia, as product of the reversal activity of succinate dehydrogenase. During reperfusion, the accumulated succinate is rapidly re-oxidized by succinate dehydrogenase, driving extensive ROS generation by reverse electron transport at mitochondrial complex I [39].

4.1. Inhibition of mitochondrial pore opening protects the heart against ischemia-reperfusion injury

The importance of MPTP opening in myocardial damage makes it an excellent target for therapies aimed to reduce reperfusion injury. In studies in animal models in which the mitochondrial pore opening was inhibited, a reduction of the infarcted area was observed in more than 50% [1, 40], highlighting the impact of the presence of this pore on the magnitude of myocardial damage. These types of treatments are currently in development and in the search for safe and specific pore opening inhibitors [1, 38, 41].

In this context, different mechanisms have been developed to mitigate cell damage induced by reperfusion, and although improvements have been achieved with regard to the response of the infarcted region, there is no effective therapy yet to completely avoid reperfusion injury [2].

An interesting target to achieve inhibition of the opening of the pore is cyclophilin D. As mentioned earlier, the inhibition of CypD with cyclosporine in isolated hearts has shown

protective effects in ischemia-reperfusion [42]. A decrease in infarcted area was also demonstrated in knockout mice for CypD compared control mice [43].

Interestingly, CypD can be S-nitrosylated on cysteine 203. This S-nitrosylation reduces the activity of the MPTP [23].

There are other constituents of the mitochondrial pore that, being inhibited, contribute to avoid the gating of the pore. Unfortunately pore elements such as ANT and the carrier of mitochondrial phosphate are indispensable for other vital cellular functions, and therefore their inhibition would add the cell an additional risk after ischemia-reperfusion. The opening of the pore can also be avoided indirectly. By decreasing the influx of Ca^{2+} from the extracellular space, the overload of this cation is avoided and thus the pore opening.

5. Cardioprotective function of nitric oxide

Nitric oxide is a molecule that participates in different processes, including neurotransmission, vasodilatation, defense against pathogens and depending on cellular conditions, in apoptosis [44–46]. This molecule is produced by the conversion of L-arginine to L-citrulline, a reaction catalyzed by one of three types of enzyme nitric oxide synthase (NOS) [47]. Neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are constitutively expressed enzymes and inflammatory nitric oxide synthase (iNOS) is inducible. The activity of NO as a cell signaling molecule via cyclic GMP is well known. The cGMP formed by NO has three targets: protein kinase G (PKG), cyclic nucleotide-dependent channels and phosphodiesterase (PDE) [48] achieving well-studied vasoactive effects. However, recent studies suggest that NO can modify proteins posttranslationally, via a nitrosative pathway, known as S-nitrosylation [49]. S-nitrosylation consists in the addition a NO group to a cysteine residue generating nitrosothiols (SNO). Different studies have shown that this nitrosative modification of proteins alters their function, reversibly [49]. In addition, the S-nitrosylated cysteines of proteins are in a chemical equilibrium with S-nitrosoglutathione (GSNO), a low molecular weight nitrosothiol. In this manner, GSNO functions as a reserve of NO that can be exchanged with GSH in a reaction termed transnitrosylation and in this way participates in part of NO bioactivity [50, 51].

5.1. S-nitrosylation and cardioprotection

Although the cardioprotective effects of NO by their dependent cGMP pathway are known, recent research suggests a relationship between increased SNO formation and cardioprotection [52]. In cardiomyocytes, S-nitrosylation occurs in a large number of proteins [53].

In ischemia-reperfusion studies, S-nitrosylation has been associated with an increase in the recovery of developed ventricular pressure and ventricular work [54]. This has been observed in both preconditioning and ischemic postconditioning studies [55], resulting in an increase in S-nitrosylation associated with an improvement in contractile activity. This recovery of functionality has also been appreciated with the use of S-nitrosothiols donors such as Mito-SNO (targeted to mitochondria) or S-nitroso-N-acetylpenicillamine (SNAP) [56]. Using GSNO to generate a preconditioning effect in an ischemia-reperfusion experiment in mice resulted in a greater

recovery of developed ventricular pressure and a smaller infarcted area compared to controls [54]. Furthermore, it was observed that the inhibition of caspase-3 by S-nitrosylation decreases its proapoptotic activity [57]. In a study in mice undergoing cardiac ischemia-reperfusion, it was observed that an increase in S-nitrosylation of the cardiac L-type Ca^{2+} channel reduced Ca^{2+} entry, thereby reducing Ca^{2+} overload, subsequently reducing cardiac damage by reperfusion [58]. On the other hand, in transgenic mice with iNOS overexpression submitted to cardiac ischemia-reperfusion, a smaller infarcted area was observed in addition to a lower Ca^{2+} overload and a smaller opening of the mitochondrial pore [59]. Mito-SNO (5-(2-acetylamino-3-methyl-3-nitrosothiobutyrylamino)-pentyl]-triphenylphosphonium methanesulfonate) was used in a study in mice and it was observed that the application of this donor was able to improve cardiac function and also to decrease the infarcted area compared to controls [60]. This cardioprotective effect was due to the reversible S-nitrosylation of the mitochondrial complex I. This effect would slow mitochondrial activation in the first few minutes of reperfusion, a crucial moment in ROS release, decreasing it and limiting reperfusion injury [61]. Similar effects were found in ischemia-reperfusion in rat hearts, where another S-nitrosothiol donor, S-nitroso-2-mercaptopropionyl glycine (SNO-MPG) was used, resulting in inhibition of the complex I, mainly during the late stage of ischemia and early reperfusion, thereby reducing ROS production by mitochondria, similar to the previous case. In addition, it was observed that in hearts treated with the nitrosylating agent they were more resistant to the opening of the transition pore [62]. In studies of ischemic preconditioning in mouse hearts, a relationship between increased S-nitrosylation and cardioprotection was observed, where an elevation of the nitrosothiols with GSNO treatment, developed positive ventricular pressure and reduced total infarcted area [54].

In mitochondria, complex I is the primary site of ROS production, with the generation of superoxide anion. S-nitrosylation of complex I was shown to inhibit its activity, which reduces ROS production during reperfusion [63]. However, SNAP reversibly inactivated the mitochondrial complex I, resulting in an increase in H_2O_2 production [64]. On the other hand, S- nitrosylation decreased the Ca^{2+} elevation produced during reperfusion through the modification of the L-type Ca^{2+} channel [54], which prevented the opening of the mitochondrial pore.

An interesting question arises as whether nitrosylation occurs equally in all mitochondria. Sun et al. found higher S-nitrosylation in the sub sarcolemmal mitochondria than in the interfibrillar in mice hearts subjected to ischemic preconditioning. Both types of mitochondria differ in protein and lipid composition, in addition to the capacity for protein synthesis and oxygenation [65]. This suggests that subsarcolemmal mitochondria are more susceptible to ischemia-reperfusion injury, with the MPTP more prone to ischemia-reperfusion injury and also being exposed to a higher oxygen gradient during reperfusion [66]. This difference in mitochondrial response has already been observed with the use of other cardioprotective agents such as diazoxide [67].

5.2. S-nitrosoglutathione reductase

The cellular levels of S-nitrosothiols are governed by the enzyme S-nitrosoglutathione reductase (GSNOR), an alcohol dehydrogenase type III enzyme, whose function is to remove NO groups from the cysteine thiols in proteins [68] through denitrosylation, degrading GSNO and

increasing levels of glutathione (GSH), thereby reducing nitrosylation [69]. This enzyme is highly conserved from bacteria to mammals and has the function of modulating the S-nitrosylating function of NO and thus avoiding for example nitrosative stress in the presence of excessive NO.

Although this enzyme does not act directly on SNO-protein substrates, its deficiency causes an increase in the intracellular concentrations of nitrosylated proteins (**Figure 2**). This means that there is an equilibrium based on the transfer of SNO groups between low molecular weight nitrosothiols and protein cysteines. It is also thought that the role of GSNO is not only a transitrosilation (from one thiol to another) but also function as a source of NO independent of the action of NOS.

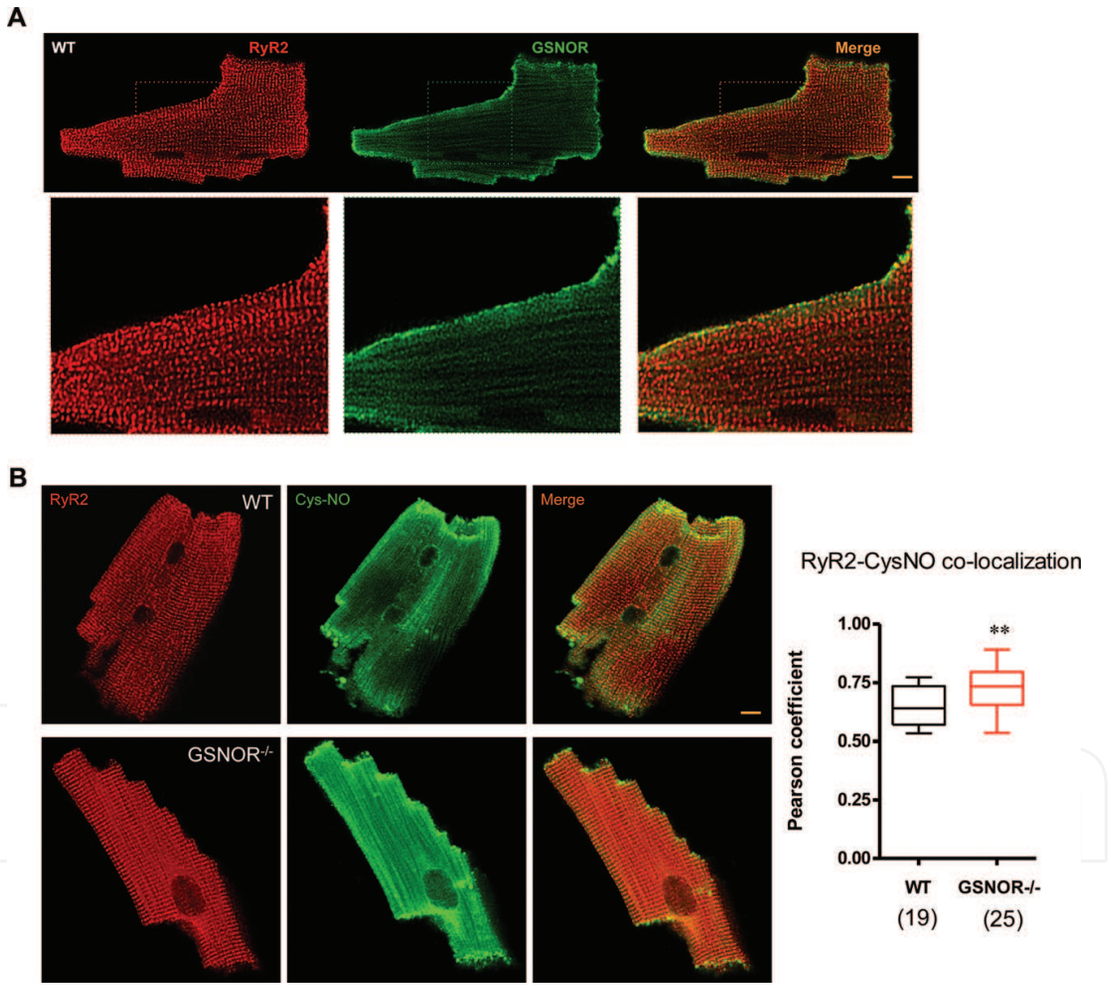

Figure 2. GSNOR in the cardiac cell. Immunocytochemical analysis of S-nitrosoglutathione reductase in cardiac myocytes. (A) The pictures depicts the localization in a mouse cardiomyocytes of GSNOR (green) compared to that of the ryanodine receptor (RyR2, red) using confocal microscopy. The sections demarked by dotted lines are amplified below. Notice that GSNOR partially co-localizes with RyR2, suggesting association in the sarcoplasmic reticulum. (B) Co-localization analysis for RyR2 (red) and S-nitrosylated thiols (Cys-NO, green) using a specific antibody against Cys-NO in wild type (WT) and GSNOR-deficient ($GSNOR^{-/-}$) mice. The bar graph shows the Pearson analysis for co-localization of RyR2 and CysNO. ** indicates $p < 0.001$ using T test. Number of cells analyzed is indicated between parentheses. The scale bar indicates 10 μm.

While most proteins have cysteine residues and NOS is expressed in almost all cells, substrate specificity is an important feature of endogenous S-nitrosylation of proteins. Moreover, in all proteins possessing more than one S-nitrosylation site, the single modification of one cysteine thiol can modify their function under physiological conditions [70].

Apparently, the activity of GSNOR is increased lung endothelial cell of females compared to males [71]. In the heart the results are similar. Females possess a higher activity of GSNOR than males [72], without differences in the activity of this enzyme in sexually immature mice. Although there are no significant differences in the expression of GSNOR for either sex, estrogens stimulate the activity of eNOS, which would lead to increased S-nitrosylation, an important factor that would stimulate GSNOR activity. On the other hand androgens may play a role, since in neutered male mice the activity of the GSNOR seems to be similar to that of the females. This mechanism of GSNOR activation is not known [71].

5.3. The complex role of GSNOR

Given the anti-inflammatory and muscle relaxant properties of NO, inhibition of GSNOR is already being tested for the treatment of associated pathologies, mainly respiratory [73]. For example, in bronchoscopy samples from different subjects, GSNOR activity was increased in certain types of asthma [74], which makes it a good target for the treatment of this pathology. In a mice model of asthma, the GSNOR inhibitor SPL-334 was evaluated, resulting in a reduction of bronchial inflammation, airway hyperreactivity, mucus production and eosinophil accumulation and allergen-specific T [75]. Also, in mice lung smooth muscle, inhibition of GSNOR decreased the contractile response in the methacholine test, in addition to anti-inflammatory effects [76]. On the other hand, GSNOR appears to protect lung immune cells from nitrosative stress, since its deficiency tends to increase lung susceptibility for *Klebsiella pneumoniae* infection [77].

Skeletal muscle tissue has also been evaluated for GSNOR function. With the use of GSNOR knockout mice, the tibialis anterior muscle of $GSNOR^{-/-}$ mice was found to be more resistant to fatigue, with no alterations in mitochondrial function or in capillary density, associated with hypernitrosylation ryanodine receptor (RyR1), which could increase skeletal muscle contractility without altering mitochondrial function [78]. However, another study using $GSNOR^{-/-}$ mice showed the opposite effect, since the silencing of GSNOR revealed a muscular atrophy and loss of muscle mass, associated with increased S-nitrosothiols, along with mitochondrial fragmentation and depolarization [79].

GSNOR also appears to have a role in breast cancer. The study by Cañas et al. indicates that the antiproliferative action exerted by trastuzumab on breast cancer cells overexpressing HER2 is suppressed when GSNOR is inhibited. This indicates that an increase in SNOs would provide a survival advantage for cancer in HER2 + tumors and may constitute a mechanism of resistance to this targeted treatment in breast cancer [80]. On the other hand, other studies have shown that inhibition of GSNOR can be detrimental. Rizza et al. observed that the silencing of GSNOR was shown to protect SH-SY5Y cells from toxins characteristic of Parkinson's disease. However, it was also observed that overexpression of this enzyme is a resistance factor for the treatment of amyotrophic lateral sclerosis [81]. It has also been shown in other investigations

that the absence of GSNOR may be harmful and that this increase in S-nitrosylation that would cause its absence would be pathological. Rizza et al. observed that hepatocytes from GSNOR-deficient mice had mitochondrial alterations, characterized by an increase in the levels and activity of the enzyme succinate dehydrogenase, mediating this alteration in a greater proclivity to the development of hepatocarcinoma [82]. This relationship had already been studied previously by Wei et al. who found that human hepatocarcinoma cells showed less GSNOR activity than noncancerous liver cells [83], inferring that the absence of GSNOR can alter DNA repair and thus induce tumor growth. In lung cancer, studies show that GSNOR is reduced in lung cancer samples relative to normal lung tissue [84]. This emphasizes the relationship between nitrosative stress and the development of neoplasia and how this enzyme can participate in its development.

GSNOR also appears to be important in the immune system. A study in GSNOR-deficient mice shows that the absence of this enzyme would cause a pathological nitrosylation causing apoptosis in thymus cells and a reduction of B and T lymphocytes. All this was mediated by the uncontrolled activity of iNOS [85].

5.4. GSNOR and cardioprotection

Given the large number of benefits delivered by S-nitrosylation in the heart, there is currently a search for therapies that pursue a greater availability of S-nitrosothiols in order to reduce cardiac damage and improve ventricular performance after ischemia. Therefore, NO donor agents, ischemic pre- and postconditioning, and modulation of GSNOR activity have been tested.

In the cardiomyocyte of knockout mice for GSNOR, a decrease in the response to a β-adrenergic agonist and a decrease in cytosolic Ca^{2+} concentration were observed [86], highlighting the role of endogenous S-nitrosothiols in cardiac function. In these GSNOR it was observed S-nitrosylation of β-arrestin and G protein receptor kinase 2, two proteins involved in the regulation of the β-adrenergic receptors pathway [87], besides the nitrosylation of different Ca^{2+} channels, such as the L-type Ca^{2+} channel, ryanodine receptor RyR2 and the pump SERCA [88] (**Figure 3**). Lima et al., using a model of myocardial infarction in GSNOR-deficient mice, observed a reduction in the infarcted area, maintenance of ventricular function, with an increase in vascular density compared to wild type mice. This proangiogenic effect of GSNOR inhibition was explained as a result of increased activity of transcription factor hypoxia inducible factor-1 (HIF-1 α) due to increased S-nitrosylation in normoxic conditions, which resulted in increased binding to the VEGF gene promoter [50].

In a similar study in GSNOR-deficient mice that underwent myocardial infarction, the improved contractile function and increased angiogenesis were confirmed. Importantly, these authors reported that after myocardial infarction, the GSNOR knockout mice hearts showed increased proliferation of endogenous cardiac stem cells and increased mitosis in cardiac myocytes compared to wild type mice [89].

On the other hand, pharmacological inhibition of GSNOR has been shown to improve endothelial function in rats. In a study by Chen et al. it was observed that using the GSNOR

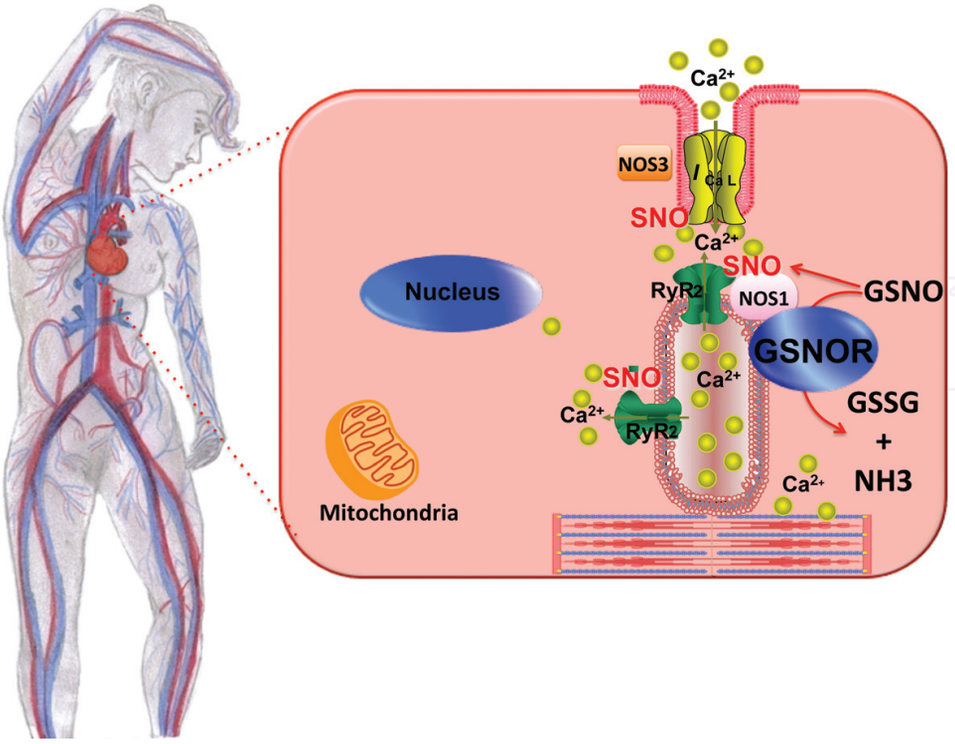

Figure 3. Regulation of intracardiac S-nitrosothiols by GSNOR. Schematic model of the regulation of S-nitrosylation within the cardiac cell by GSNOR. S-nitrosoglutathione reductase is able to metabolize endogenous S-nitrosothiols by controlling the fate of S-nitrosoglutathione (GSNOR). The RyR2 and the L-type (*I* Ca) calcium channels are nitrosylated (SNO) by the activity of the nitric oxide synthases 1 (NOS1) and 3 (NOS3) located in the sarcoplasmic reticulum and plasmalemma, respectively. SNO are in equilibrium with nitrosoglutathione (GSNO). Thereby, by controlling the fate of GSNO, GSNOR regulates the levels of S-nitrosylation of these channels, important for the reperfusion damage. GSSH, oxidized glutathione, NH_3, ammonia.

inhibitor N6338 at a single dose maintained flow-mediated arterial dilation versus inhibition of NOS. In hypertensive rats, application of N6338 was shown to decrease blood pressure and vascular resistance, as well as to restore altered flow-mediated dilatation [90].

Then, the question arises whether inhibition of GSNOR is cardioprotective. Much of the evidence suggests that this would be the case. However, the protective role of GSNOR in preventing pathological S-nitrosylation should be considered, as there are studies that suggest that the enzyme is essential for cardiovascular homeostasis. For example, Sips et al. used mice that overexpressed GSNOR specifically in cardiomyocytes and observed an improvement in myocardial properties post induction of myocardial damage after sepsis, a condition that is associated with nitrosative stress derived from the activity of iNOS [91]. Nevertheless, in conditions where increased bioavailability of S-nitrosothiols is required, such after ischemia, the inhibition of GSNOR appears as an attractive therapeutic strategy. This would increase the S-nitrosylation for example, of calcium-handling proteins, which, in a setting of ischemia-reperfusion would prevent the calcium overload. In addition, increased S-nitrosylation of mitochondrial proteins may prevent the generation of ROS and a reduction in mitochondrial permeability transition pore activity, reducing cell death. Nevertheless, these effects remain to be probed experimentally.

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6. Conclusions

Although reperfusion is essential for the recovery of the heart after ischemia, it can itself initiate myocardial damage and cause death of the cardiomyocyte. A key event in this cellular disorder of this alteration is the opening of the MPTP, which induces mitochondrial dysfunction and ultimately leads to cardiomyocyte death. For this reason, therapies are being developed in order to limit the opening of the pore and reduce the damage by reperfusion. One important mechanisms of cardioprotection is S-nitrosylation, which consists of adding NO groups to cysteine residues of proteins, modifying their function. S-nitrosylation of mitochondrial complex I and MPTP using NO donors and S-nitrosylating agents has shown to induce cardioprotection. It is known that agents or genetic alterations that increase SNO are cardioprotective and in this context the modulation of GSNOR is of relevance, since this protein is a critical regulator of endogenous SNOs. This enzyme participates in the redox balance, regulating the levels of nitrosylation/denitrosylation and its inhibition can increase the bioavailability of S-nitrosothiols, being this a potential cardioprotection element. So far, in the heart, genetic cancelation of GSNOR has indeed shown positive results in the context of damage by infarction. For this reason, more studies are needed in order to assess the impact of inhibition of GSNOR on global cellular functioning. This will indeed help in the development of new knowledge for the treatment and prevention of myocardial diseases.

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