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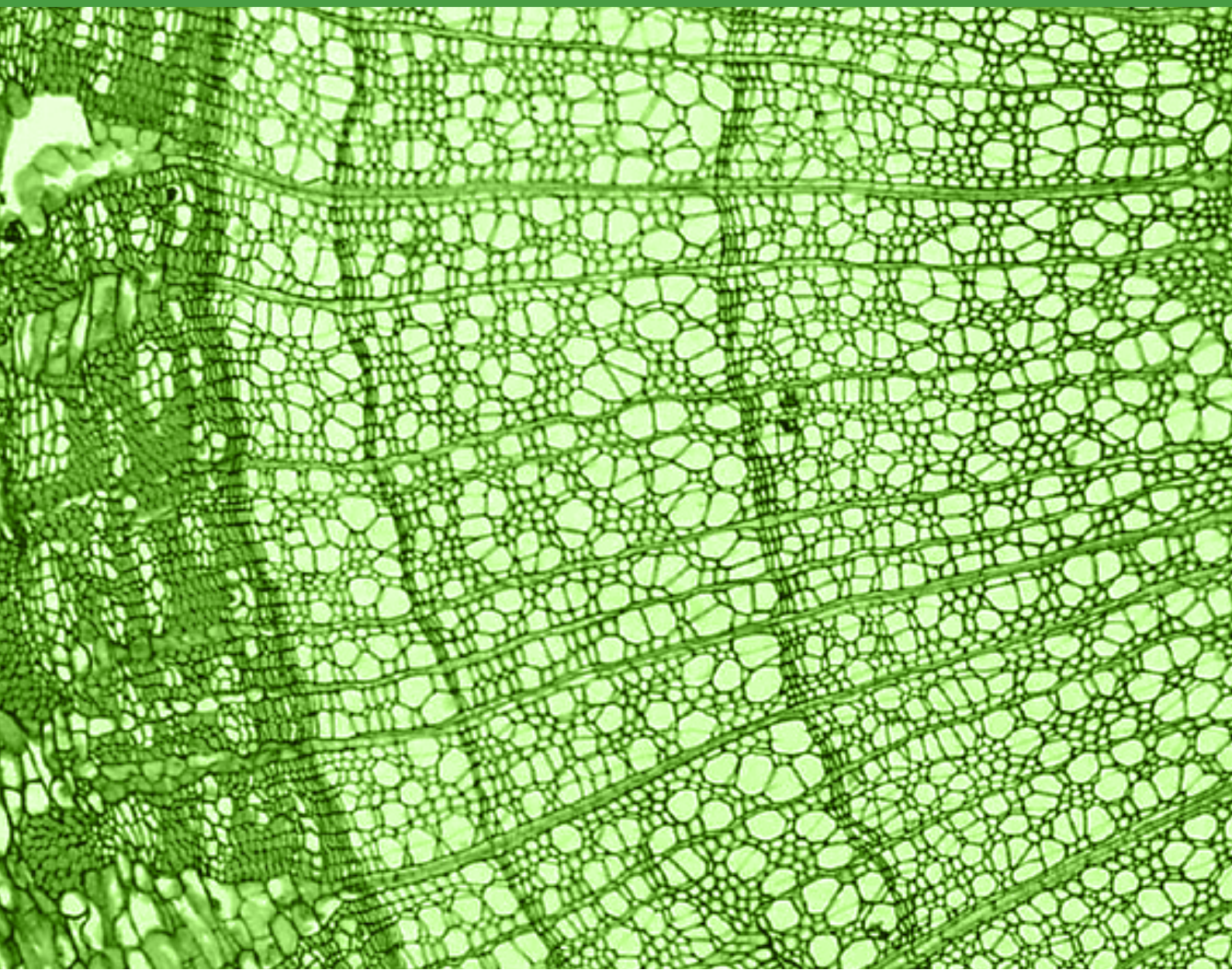

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REDOX REGULATION OF SARCOPLASMIC RETICULUM CALCIUM CYCLING IN THE HEART

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ABSTRACT

The coordinated release and reuptake of calcium into the sarcoplasmic reticulum (SR) is critical to maintain an adequate heart function. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), generated in the heart under normal basal condition, modulate the function of different proteins via the reversible oxidation of critical cysteine residues. Excess ROS/RNS generation has been shown to impair heart function, but extensive evidence indicates that the controlled production of these molecules increases cardiac contractility by targeting SR calcium proteins.

Ryanodine receptors (RyR2) are endogenously *S*-nitrosylated and *S*-glutathionylated and both redox modifications increase the activity of these channels *in vitro*. Moreover, exercise or rapid pacing increases the RyR2 *S*-glutathionylation, a modification that depends on the activation of NADPH oxidase (NOX2). In isolated cardiomyocytes, this enzyme is rapidly activated by stretch, generating an immediate burst of ROS which increases calcium release by RyR2.

Nitroxyl, a particular ROS/RNS, increases cardiac inotropy *in vivo*, by targeting critical thiols in RyR2, the SR Ca^{2+}-ATPase and phospholamban, allowing the simultaneous increase in calcium release and reuptake, required to produce a sustained increase in the calcium transient.

In this minireview we present some of the recent work on the redox regulation of SR calcium cycling proteins.

Keywords: calcium cycling; sarcoplasmic reticulum; reactive oxygen species; reactive nitrogen species.

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Excitation contraction coupling and SR calcium cycling

Cardiac muscle contracts cyclically to pump blood across the body. Each contraction is the response to an action potential that propagates across the surface membrane and towards the centre of the cardiac muscle cell through the transverse tubule system, causing the opening of voltage-dependent calcium channels. The entry of a small amount of calcium ions (Ca^{2+}) from the extracellular space stimulates the opening of the large-conductance calcium channels present in the membrane of the sarcoplasmic reticulum (SR). The opening of these channels, *also known as* ryanodine receptors type 2 (RyR2), causes the release of a much larger amount of to activate the myofilaments and produce contraction. Upon repolarization, RyR2 channels rapidly close and the calcium concentration returns to the resting levels by the action of the SR Ca^{2+}-ATPase (SERCA2a) which transports the calcium back inside the SR [1]. The same amount of calcium that entered the cell via L-type calcium channels during the action potential is extruded out of the cell via Na^{+}-Ca^{2+}-exchanger during each cycle, thus the amount of calcium inside the cell remains constant.
Calcium release and reuptake are modulated by adrenergic stimulation and by calcium itself, via the PKA and CaMKII dependent phosphorylation of both RyR2 and phospholamban (PLB), the inhibitor of SERCA2a. Phosphorylation of PLB in serine 16 by PKA and threonine 17 by CaMKII, relieves the inhibition over SERCA2a, increasing the rate of calcium uptake into the SR and the amount of calcium available for release in the following beats [2]. At the same time, phosphorylation of RyR2 in serine 2808 and 2814 (2809 and 2815 in some species), by PKA and CaMKII respectively, increases the opening probability of the channels, allowing the release of more calcium for contraction [3]. This coordinated regulation of calcium uptake and release is a critical determinant of the cardiac contractile force.

Redox regulation of proteins and relevant sources of ROS and RNS in the heart

In basal, non-stimulated conditions, cells produce small amounts of very reactive molecules by the partial reduction of molecular oxygen or nitrogen. The term reactive oxygen species (ROS) is used to collectively designate molecules such as superoxide anion ($O^{\bullet -}_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$). Reactive nitrogen species (RNS) include nitric oxide (NO) and molecules derived from NO such as peroxinitrite ($ONOO^{-}$), nitroso glutathione (GSNO) and other nitrogen containing species such as nitroxyl (HNO). ROS and RNS can react with almost every cellular component and, their excessive generation can produce cell damage, but under normal physiological conditions oxidation-reduction (redox) reactions are tightly controlled and redox modification of proteins play important signalling roles in the cell.
Intracellular RNS derive from nitric oxide (NO), a gas second messenger synthesized by nitric oxide synthase (NOS), from L-arginine. The heart constitutively expresses two of the three known isoforms of NOS i.e. the neuronal (nNOS or NOS1) and the endothelial (eNOS or NOS3). Both are regulated by calcium-calmodulin binding and by phosphorylation by different kinases. The inducible (iNOS or NOS2) isoform, that generates NO at a higher rate in a calcium independent fashion, is also expressed in the heart in response to inflammatory and immunological stimulus [4]. In spite of being a free radical, NO is relatively unreactive towards cellular proteins and its main targets are heme proteins such as guanylate cyclase and myoglobin. NO reacts with superoxide anion , another free radical, in a diffusion controlled reaction that leads to the generation of peroxinitrite, a far more potent oxidant than NO that can react with most biomolecules and,

probably, is the responsible for most of the NO-induced posttranslational modifications of proteins.

Cells generate ROS during mitochondrial electron transport and other enzymatic systems such as xanthine oxidase or "uncoupled" NOS [5,6], but in physiological conditions the only enzyme dedicated exclusively to produce ROS is NADPH oxidase (NOX). Most cells express one or more isoforms of this seven member family of enzymes called NOX or DUOX depending on their final product. NOXs (NOX1 to NOX5) generate superoxide anion and DUOXs (DUOX 1 and 2) generate hydrogen peroxide. The heart expresses NOX2 and NOX4. In cardiomyocytes, NOX2 is located at the plasma membrane. The catalytic core of the enzyme is constituted by two membrane integral subunits, p22phox and gp91phox (gp91phox is also called simply NOX2; the term phox comes from phagocytic oxidase, since phagocytic cells express the same isoform). The generation of superoxide anion by NOX2 is low under basal conditions and increases by the recruitment to the membrane, upon appropriate stimuli, of the cytosolic subunits p47phox, p67phox, p40phox and the small G protein rac. The isoform NOX4, also forms a complex with p22phox, resides at the membrane of intracellular compartments such as the endoplasmic reticulum and the mitochondria, and is constitutively active [7].

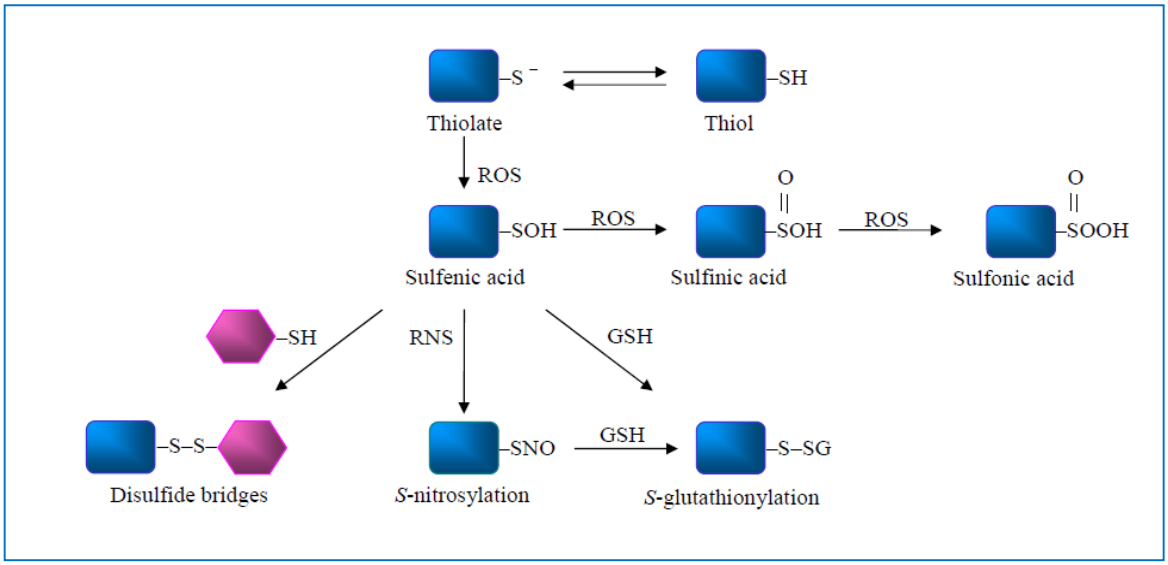

Figure 1. Protein cysteine residues (R-SH/R-S-) are oxidized by ROS, generating sulfenic acid (R-SOH) which can react with another thiol, of the same or another protein, to form a disulfide bridge (R-S-S-R), or with glutathione (GSH) to become S-glutathionylated (R-S-S-G) or with RNS (or NO) to become S-nitrosylated (R-SNO). Under stronger oxidative conditions sulfenic acid can be further oxidized to sulfinic ($R\text{-}SO_2H$) and sulfonic acid ($R\text{-}SO_3H$).

ROS and RNS can modify the lateral chain of several amino acid residues which, in turn, can change the function of the protein bearing that amino acid. Among the amino acids modified by ROS/RNS, cysteines are unique because their side chain possesses a thiol (-SH) group that can be reversible oxidized and reduced. Cysteines differ in their reactivity against ROS/RNS. Those cysteines, whose thiol group exists as thiolate (i.e. dissociated, $-S^-$) at physiological pH, tend to react more rapidly with ROS or RNS. These "hyperreactive" cysteines are oxidized by superoxide anion or hydrogen peroxide producing, in sequential steps, -sulfenic (R-SOH), sulfinic ($R\text{-}SO_2^-$) and sulfonic ($R\text{-}SO_3^{2-}$) acids (**Figure 1**). Sulfenic acid, the first stage of oxidation of a thiol, is highly unstable and can react either with another thiol to form a disulfide bridge or with GSH to form a mixed

disulfide, a modification called S-glutathionylation. RNS such as NO or peroxinitrite, also react with sulfenic acids, producing S-nitrosylation of cysteine residues (Cys-S-NO). Further oxidation of a sulfenic residue produces a sulfinic derivative; this was previously believed to be an irreversible redox modification, but it is now known that it can be enzymatically reversed by sulfiredoxin in an ATP-dependent reaction. The oxidation to sulfonic acid is irreversible and therefore is unlikely that this modification operates in cell signalling. It is possible that sulfenic, and even sulfinic, acid derivatives may have a role *per se* in the redox regulation of protein activity but there are no evidences at present for these modifications in the regulation of the SR cardiac calcium handling proteins.

To operate as cellular signals, oxidative modifications of thiol residues, such as disulfide bridges, S-glutathionylation or S-nitrosylation, should be rapidly reversed when the stimulus that produced them comes to an end and conditions return to the basal level. Reversion is catalyzed mainly by two enzymatic pathways: the thioredoxin and the glutaredoxin systems [8]. Both, glutaredoxin and thioredoxin are small protein oxido-reductases that reduce protein disulfides bridges and mixed disulfides. Besides, thioredoxin is also able to reduce S-nitrosylated cysteine residues [9]. Glutaredoxin utilizes GSH as a reducing agent and generates oxidised glutathione (GSSG) that is reduced back to GSH in a reaction catalyzed by glutathione reductase which requires NADPH as a reducing agent (**Figure 2**). Glutathione reductase maintains the GSH/GSSG ratio around 100/1 and this high ratio keeps the SH residues of cytoplasmic proteins in the reduced state, limiting the half-life of oxidative modifications. Oxidized thioredoxin is reduced back by thioredoxin reductase and NADPH as reducing coenzyme (**Figure 2**).

Reversible redox modifications of RyR2, SERCA2a and PLB modulate their activity. We present below the most relevant data that suggests that redox regulation of these proteins has an important role in physiological conditions.

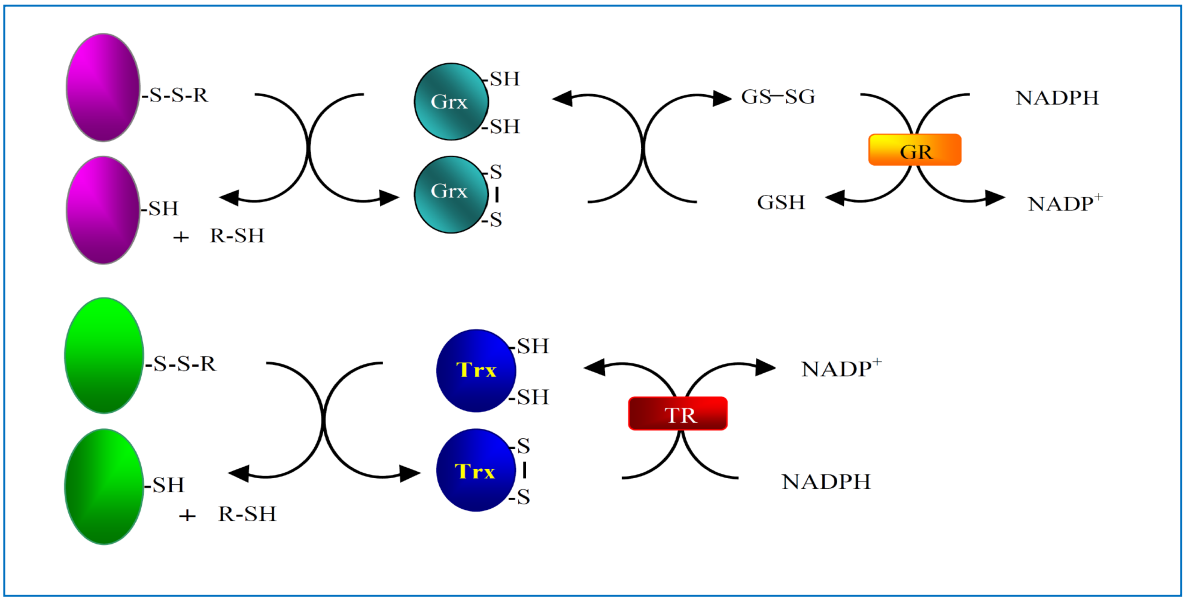

Figure 2: The Glutaredoxin and the Thioredoxin systems reduce disulfide bridges (in the scheme), becoming oxidized in the reaction. Glutaredoxin also reduces *S*-glutathionylated cysteines and thioredoxin reduces both *S*-glutathionylated and *S*-nitrosylated cysteines. Glutaredoxin is reduced back by GSH, producing GSSG which is reduced to GSH by the action of Glutathione reductase (GR) and NADPH as reducing agent. Thioredoxin is reduced by Thioredoxin reductase (TR) and NADPH.

Redox modifications of Ryanodine receptors

Ryanodine receptors are homotetrameric proteins of 2.2 MDa, expressed in different mammalian tissues. There are three isoforms, product of different genes, that share 70 % homology among them, and the heart expresses the RyR2 isoform. About 10% of the carboxy terminus of each RyR2 monomer spans the membrane and forms the channel pore; the remaining 90% of the protein constitutes the amino-terminal cytoplasmic portion. Interactions within each subunit and inter subunits modulate the opening and closing of the channel [10, 12].

The redox sensitivity of RyR2 has been known for almost three decades but evidence for a physiological role of the redox modifications of this channel protein is just emerging. Experiments *in vitro* have shown that oxidation of SH residues increases the activity of RyR2 channels incorporated in planar bilayers and the rate of calcium fluxes in isolated SR vesicles, while reduction decreases them [13]. Excessive oxidation, however, causes irreversible activation of the channels. Increased oxidation has been proposed to be the cause of calcium leak and arrhythmias in the failing heart, a condition characterized by increased oxidative stress. In cardiomyocytes isolated from failing hearts, antioxidants normalize calcium release and decrease the occurrence of arrhythmias [14, 15]

RyR2 are endogenously S-nitrosylated and NO or NO donors such as GSNO or SIN-1, increase both the opening probability of RyR2 channels and the number of S- nitrosylated cysteines in RyR2 [16]. Since S-nitrosylation, as other redox modifications, is believed to proceed in the absence of catalyzing enzymes, specificity should be provided by the proximity of the protein to the source of ROS/RNS. It is generally assumed that nNOS colocalizes with RyR2 in the SR membrane and that this isoform provides the NO that modulates RyR2 activity; but there are also studies that show that eNOS colocalize with RyR2 and the fact is that any of these isoforms can be responsible for RyR2 S-nitrosylation. Furthermore, both isoforms have been shown to increase the activity of RyR2 either in isolated cardiomyocytes or in subcellular cardiac muscle fractions [17].

RyR2 are endogenous S-gluthationylated as well, and this modification also increases the opening probability of the channel in vitro. S-glutathionylation of RyR2 increases significantly after exercise in conscious dogs or after rapid pacing in anesthetized animals concomitantly with the activation of NOX2 [18, 19]. The administration of apocynin prior to exercise or rapid pacing prevents the activation of NOX2, prevents RyR2 S-glutathionylation and decreases the rate of calcium release in isolated SR vesicles [18]. In contrast, neither rapid pacing nor exercise increases RyR2 S-nitrosylation, suggesting that S-glutathionylation and not S-nitrosylation was responsible for the observed increase in channel activity [16].

When the heart is stretched during diastole, such as when the blood venous return increases, the force of contraction in the following systole increases. This effect, known as the Frank–Starling law of the heart, is explained by an increase in the number of cross bridges produced between actin and myosin as the myofilaments overlap and by the increased calcium sensitivity of troponin C which increases the rate of cross-bridge cycling and the tension developed by the heart. The initial fast increase in the contraction force is followed by a slower increase in force generation (the slow force response or Anrep effect) that depends on an increased calcium transient [20]. The stretch-dependent activation of NOX2 may have a role in both the immediate increase in force and the slower force response, through redox modifications of RyR2. Recent observations in isolated cardiomyocytes show that when the cells are mechanically stretched, within a physiological sarcomeric length, there is an immediate increase in ROS generation and in the number of calcium sparks, the elementary calcium release events that underlie the cell

wide calcium transient. The response requires an intact microtubule network and the inhibition of NOX2 prevents both the increase in ROS and calcium sparks. Because it was elicited by the activation of NOX2, the response was termed X-ROS signalling [21]. Therefore, it seems that X-ROS signaling links mechanical stress to changes in [Ca^{2+}] in the heart under normal physiological conditions [21]. Interestingly, X-ROS signaling is turned off as soon as the cell returns to the pre-stretch length. The mechanisms of this rapid deactivation are not understood at the moment.

Stretching the heart also activates NOS and increases NO generation [22, 23]. Since NO is unlikely to nitrosylate proteins unless a more oxidizing specie is produced, it is possible that the concomitant activation of NOS and NOX are required to modulate RyR2.

A molecule related to NO, but with different properties and reactivity towards thiols is nitroxyl (HNO). In recent years nitroxyl has attracted increasing attention as a potential therapeutic agent against heart failure, due to its positive inotropic and lusitropic effects in normal and failing heart [24]. Nitroxyl (the correct name is nitrosyl hydride) is the one electron reduced (NO^-) and protonated form of NO, but it is not produced from nitric oxide directly, due to the high reduction potential of NO. Although some biochemical pathways can hypothetically produce HNO, it is not known whether this molecule is generated in vivo [24, 25]. The cardiovascular effects of nitroxyl have been observed after the administration of donors such as Angeli's salt ($Na_2N_2O_3$). In isolated cardiomyocytes Angeli's salt increases the calcium transient by increasing the fractional SR calcium release, with no change in calcium leak and without any increase in calcium entry through L-type calcium channels. This effect is due to the redox dependent activation of RyR2 since it can be reversed by the reducing agent dithiothreitol [24].

In conscious dogs HNO, donated by Angeli'salt (AS), produce a rapid increase in myocardial contractility together with an increase in the rate of relaxation, an effect that is not produced by NO or other RNS [26]. Therefore, as discussed below, nitroxyl enhances calcium cycling across the SR by targeting both RyR2 and SERCA activity.

Redox modification of Ca^{2+}-ATPase and phospholamban

SERCA2 plays a key role in cardiac contractility. It returns the calcium concentration to the basal diastolic level after contraction, producing relaxation and it also replenishes the SR calcium stores to make it available for release in the following contraction. The main isoform of the sarco-endoplasmic reticulum calcium ATPase expressed in the heart is SERCA2a, although low amounts of SERCa2b and 2c, originated by alternative splicing, are also found [27].SERCA2ais also sensitive to ROS and its catalytic activity is dependent on the redox state of some cysteine residues [28]. SERCA2 activity is depressed in some pathological conditions associated with increased oxidative and nitrosative stress such as atherosclerosis [29], metabolic syndrome [30] or diabetes [31] due to irreversible oxidative modifications of cysteines and of other aminoacids as well. In contrast, evidence obtained in vascular smooth muscle microsomes shows that controlled oxidation of some SH residues increases SERCA2 activity [32]. Incubation of smooth muscle microsomes with a small concentration of peroxinitrite in the presence of glutathione increases SERCA2b activity through S-glutathionylation of a single cysteine residue (Cys 674) of the protein. Vascular smooth muscle expresses the SERCA2b isoform, identical to SERCA2a except for a sequence of 49 (or 50, depending on the species) aminoacids at the C-terminus that replace the last 4 aminoacids at the C-terminus of SERCA2a.The high similarity between these two isoforms prompted similar experiments in isolated cardiomyocytes, where it was found that nitroxyl increases SERCA2a activity by exactly the same mechanism [33].

Although these experiments have been challenged, the subject deserves further investigation [34].
Redox regulation also influences the inhibition of SERCA2a by PLB. Under physiological conditions PLB inhibits SERCA2a by decreasing its affinity for calcium. PLB is a pentamer stabilized by interactions among the transmembrane hydrophobic domains, but the monomeric PLB is the actual inhibitory form. It has been recently shown that nitroxyl promotes stabilization of the oligomeric PLB, resulting in less monomeric PLB and decreased inhibition of SERCA2a activity [34].
Nitroxyl also increases the myofilaments responsiveness to calcium increasing the contractile force and exerts an unloading effect on the heart via vasorelaxation. These effects together with its ability to increase the activity of RyR2 and SERCA make nitroxyl a promising therapeutic agent to treat heart failure.
In summary, the present evidence indicates that redox modifications of RyR2, SERCA2a and PLB play an important role in the regulation of the calcium transient in the heart. Much is still needed to know about the molecular details of the redox regulation of these proteins. It would be necessary to know, for instance, the number of cysteines modified in RyR2 and the type of modification that increase its activity, if the S-glutathionylation of cysteine 674 in SERCA2a plays a role in the activity of this pump or if the cysteines targeted by nitroxyl in transmembrane domain are the only cysteines modified in this complex. There is still much to know about the sources and the interplay of ROS/RNS, the signalling pathways that activate them and the molecular mechanisms of reversion. The mode of action of nitroxyl is especially relevant given the beneficial effects observed in heart failure.

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