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Modulation of cardiac ryanodine receptor activity by ROS and RNS

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1. ABSTRACT

Calcium release through cardiac ryanodine receptors (RyR2) triggers heart muscle contraction. Reactive oxygen/nitrogen species (ROS/RNS), normally produced in the heart, promote endogenous RyR2 S-nitrosylation and S-glutathionylation. These reversible redox modifications increase RyR2 activity *in vitro*, and presumably also *in vivo*. RyR2 S-glutathionylation increases under physiologically relevant conditions (tachycardia and exercise), suggesting that cardiac cells utilize this redox modification to increase RyR2 activity under increased demand. In contrast, *in vivo* changes in RyR2 S-nitrosylation in response to physiological stimuli remain uncharacterized. The number and identity of the highly reactive RyR2 cysteine residues and the nature of the redox modification they undergo are presently unknown. Likewise, the physiological sources of ROS/RNS responsible for functionally relevant RyR2 redox modifications have not been completely identified. The redox state of RyR2 is altered in heart failure leading to enhanced RyR2 activity, which presumably contributes to decrease SR calcium content and induce other calcium release abnormalities observed in heart failure. Greater understanding of RyR2 redox modulation is necessary to counteract the deleterious consequences of RyR2 activity deregulation caused by oxidative stress.

2. INTRODUCTION

Every heart beat is initiated by a small entry of calcium ions (Ca^{2+}) from the extracellular medium; Ca^{2+} enters cardiac myocytes through voltage-dependent calcium channels that open in response to the action potential that propagates along the cardiac cell membrane. This Ca^{2+} entry current activates the opening of cardiac Ryanodine receptors (RyR2), which are large-conductance sarcoplasmic reticulum (SR) calcium channels that allow calcium release from the SR to activate contraction. This process, known as calcium induced calcium release (CICR), is the basis of excitation-contraction coupling in the heart (1).

Ryanodine receptors are homotetrameric proteins of 2.2 MDa. Mammalian tissues express three RyR isoforms, RyR1, RyR2 and RyR3, which share 70% homology. Adult skeletal muscle expresses mainly the RyR1 isoform, which is probably the isoform most studied, plus some minor RyR3 expression, while cardiac muscle expresses only the RyR2 isoform. All three RyR isoforms are activated by micromolar Ca^{2+} and millimolar adenine nucleotides and inhibited by sub-mM or mM Ca^{2+} or Mg^{2+} concentrations and by acid pH (2, 3). In addition, phosphorylation of a limited number of serine residues modulates RyR activity; immunoprecipitation studies have

shown that RyR2 co-immunoprecipitates with kinases and phosphatases suggesting a permanent association *in vivo* (4-7). There is also evidence for the modulation of RyR2 by the association of several other proteins such as calmodulin, sorcin, and the 12.6 KDa isoform of the immunophilin FK506-binding protein (FKBP) to the cytoplasmic face (3, 8). The regulation of RyR2 activity by some of these proteins however, remains highly controversial (9-11). Similarly, the association of the RyR2 luminal domain with proteins present in the SR lumen, like triadin, junctin and calsequestrin, has been proposed to modulate RyR2 activity (3, 12, 13).

Both RyR1 and RyR2 channels are sensitive to changes in the redox potential of the environment and to redox agents, and several reviews on the redox regulation of the different RyR isoforms have been published (14-20). From the early 80's calcium flux measurements in vesicles isolated from cardiac sarcoplasmic reticulum and single channel experiments in planar bilayers showed that oxidizing agents stimulate RyR2 channel activity whereas reducing agents are inhibitory. From the initial studies on the effects of exogenously applied redox reagents, current attention has shifted to determine the effects of endogenously produced reactive oxygen and nitrogen species (ROS/RNS) on RyR2 activity. In this review we will attempt to summarize and discuss critically the accumulated evidence for the effects of ROS and RNS on RyR2 activity, with emphasis on those reversible redox modifications that occur *in vivo* and the most relevant sources of ROS/RNS that produce them under physiological conditions. In addition, we will address the effects of ROS and RNS on RyR2 activity in some pathophysiological conditions, albeit this is not the central focus of this review article.

3. MOLECULAR BASIS OF RyR2 REDOX REGULATION

As a result of aerobic metabolism, cells continuously produce ROS, including superoxide anion ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). Mitochondria are important sources of ROS and the amount of partially reduced oxygen that escapes the electron transport chain is estimated as 2 % of the total oxygen consumed (21). Other important sources of ROS in the heart are xanthine oxidase, which produces hydrogen peroxide as a by-product in the oxidation reaction of xanthine or hypoxantine (22), and NADPH oxidase. This last enzyme produces superoxide anion in a reaction that utilizes NADPH as electron donor (23), and its up regulation has been linked to the pathogenesis of cardiac hypertrophy and heart failure (24, 25). Other metabolic pathways - including peroxisomal oxidation of fatty acids (26), monoamine oxidase (27) and cyclooxygenase (28) - are also sources of ROS in the heart. In addition, cardiac cells produce RNS including nitric oxide (NO) and species generated from its reaction with ROS such as peroxynitrite ($ONOO^-$) and higher oxides of nitrogen (29). Furthermore, under certain non physiological conditions uncoupling of nitric oxide synthase also generates ROS (30). In high concentrations ROS and RNS will damage cellular components but ample evidence indicates that in physiological conditions ROS act as cellular second messengers (31). Accordingly, controlled and localized increases in ROS might serve a physiological role and are not synonymous with oxidative stress (32, 33).

Several amino acids are oxidized by ROS or RNS *in vivo*; among them, cysteine residues are of special interest since they can experiment reversible redox modifications under physiological conditions. Considerable evidence accumulated at present indicates that redox modification of cysteine residues modulates RyR2 activity, as detailed below. Although methionine residues also undergo reversible redox modifications *in vivo*, there is no evidence, until now, that they modulate RyR activity. Cytoplasmic proteins maintain most of their cysteine residues in the reduced state under resting physiological conditions; the high intracellular concentration of reduced glutathione (GSH), which ranges between 0.5 and 10 mM (34), plus the activity of two enzymatic systems: the thioredoxin and the glutaredoxin systems (35, 36), contribute to keep cysteine residues in the reduced state. Both systems reduce protein disulfides bridges, employing the reducing equivalents generated from NADPH in the thioredoxin pathway in a reaction catalyzed by thioredoxin reductase, or from GSH in the glutaredoxin pathway. This last reaction produces oxidised glutathione (GSSG) that is reduced back to GSH by the action of glutathione reductase at the expense of NADPH oxidation (37). Glutathione reductase activity keeps the cytoplasmic GSH/GSSG ratio around 100/1 (38). As a consequence, most cytoplasmic proteins possess free sulfhydryl (SH) residues while disulfide bond formation is favoured in other compartments such as the lumen of the endoplasmic reticulum, where the GSH/GSSG ratio is around 4/1. The reported values for GSH concentration in the heart, as for other tissues, are highly variable and range from 0.6 mM in rat (39) to 33 mM in guinea pig heart (40). Notwithstanding, most reported values lie in the range of 1-4 mM (41-43). These variations may arise from species differences, the different methodological approaches used for the preparation of the samples (total homogenates vs subcellular fractions) or from the method of quantification used (enzymatic determination vs fluorescent probes measurements). Similarly, in physiological conditions reported values for GSH/GSSG ratio vary from 300/1 (40) to 30/1 (39). As described above, the differences may arise from methodological reasons since different cellular compartments maintain different GSH/GSSG ratios. In physiological conditions, this ratio may also change transiently in different microdomains (44). Therefore RyR2 redox modifications could occur locally in the absence of detectable changes in global GSH concentration and/or of GSH/GSSG ratio.

Due to their high pKa (> 8.5), the cysteine thiol residues (SH) of cytoplasmic proteins are mostly protonated under physiological conditions. This feature hinders their reaction with redox agents. Depending on the functional groups of the surrounding amino acids and the hydrophobic conditions of the environment, some cysteines display lower pKa values (around 7.5). Therefore, at

physiological pH the non-dissociated cysteinyl form (Cys-SH) is at equilibrium with the dissociated form (Cys-S^-), allowing these low pKa or "hyper-reactive" cysteines to react more readily with oxidants. Hyper-reactive cysteines are good targets of ROS and RNS, which promote reversible covalent modifications such as *S*-nitrosylation, which entails the formation of a covalent bond between the cysteinyl and nitrosyl residues (Cys-S-NO), or *S*-glutathionylation, with the formation of a covalent bond with the glutathionyl residue (Cys-S-SG). Since neither GSH nor NO can directly react with cysteine thiols, both *S*-nitrosylation and *S*-glutathionylation require previous SH oxidation by superoxide anion, hydrogen peroxide or other ROS or RNS, or the formation of a suitable oxidizing species by the reaction of ROS/RNS with nitric oxide (NO) or GSH. Superoxide anion and hydrogen peroxide react with cysteine residues producing, in sequential steps, -sulfenic (R-SOH), sulfinic (RSO_2^-) and sulfonic ($R\text{-}SO_3^{2-}$) acids. Sulfonic acids are irreversible oxidation products of SH groups and therefore do not have a role in the reversible regulation of protein activity. Sulfenic and sulfinic acids are highly reactive but under certain microenvironmental conditions can be stabilized by electrostatic interactions. Recent advances in detection methods have revealed their role as modulators of the activity of a great number of proteins (45), but most often sulfenic acid reacts with another thiol of the same protein to form a stable disulfide bridge or with GSH to form a mixed disulfide, giving rise to an S-glutathionylated protein. The reaction of NO with superoxide anion generates peroxinitrite and other RNS, which readily modify critical thiols and produce nitroso thiol derivatives. The possible molecular mechanisms by which protein redox modifications occur *in vivo* have been recently addressed in several reviews (29, 46-52).

The cardiac RyR2 channel has ~ 90 cysteine residues per monomer, 21 of which are in the free thiol state (53). Unlike RyR1 (see below), the number of RyR2 hyperactive cysteines has not been determined, but presumably only a small number of these 21 cysteines are hyper-reactive and amenable to reversible modification by ROS/RNS. As detailed below, *S*-nitrosylation is believed to be an important mechanism of modulation of RyR2 activity under both physiological and pathological conditions. RyR2 are also endogenously *S*-gluthationylated and this redox modification increases after physiologically relevant stimuli such as exercise or tachycardia. We will review next the evidence supporting a role of these redox modifications as important modulators of RyR2 activity under physiological conditions.

3.1. Nitric Oxide Synthases and RyR2 *S*-Nitrosylation

Nitric oxide is synthesized from L-arginine in a reaction catalyzed by nitric oxide synthase (NOS). Three isoforms of NOS are expressed in the heart, namely neuronal (nNOS or NOS1), endothelial (eNOS or NOS3) and inducible (iNOS or NOS2) NOS. In this review we will use indistinctively either name, according to the way the authors cited the corresponding NOS isoform in their papers. Both, nNOS and eNOS are constitutively expressed in the heart and synthesize NO at low rates in contrast to the higher rate of NO production exhibited by iNOS, which also contributes to NO generation in the heart in response to inflammatory and immunological stimuli (54, 55). Both constitutive isoforms, nNOS and eNOS, are regulated by calcium-dependent calmodulin binding and via phosphorylation by different kinases (56-58). Interestingly, while phosphorylation at some sites stimulates NOS activity, phosphorylation at other sites is inhibitory because it reduces the affinity of Ca^{2+}-CaM binding to the protein. This is the case for PKC or AMPK-dependent eNOS phosphorylation on treonine-495 (57, 59) and for CaMKII-dependent nNOS phosphorylation on serine-847 (58). The concentration of NO in the beating heart, measured with fast responding microsensors, shows cyclical changes during the cardiac cycle with the highest levels coincident with late diastole (60). It is important to note that NO is produced in this case by endothelial and endocardial cells, rather than by cardiomyocytes, since most of the measurable increase in NO disappears when hearts are denuded of these cells (60).

Single RyR2/calcium release channels incorporated in lipid bilayers are either activated by NO donors (61) or inhibited by NO generated *in situ* from L-arginine (62). A direct effect of NO on RyR2 was first demonstrated by Meissner and co-workers (53), who showed that the RyR2 channel protein purified from canine cardiac SR has low levels of endogenous *S*-nitrosylation and is activated by NO donors when incorporated in planar bilayers. These authors reported the endogenous association of 74 pmol of SNO groups per mg of purified calcium release channel. Assuming a molecular weight of 2.24×10^6 Da per channel tetramer, this very low level of endogenous *S*-nitrosylation is equivalent to approximately one *S*-nitrosylated cysteine residue per every 6 tetrameric channels (or one *S*-nitrosylated residue per 24 monomers). Exogenously applied *S*-nitrosylating agents sequentially modify RyR2 SH groups; *S*-nitrosoglutathione (GSNO) causes *S*-nitrosylation of 3 SH residues per RyR2 monomer and produces reversible channel activation whereas SIN-1 oxidizes seven or more SH residues and causes irreversible channel activation (53). Table 1 summarizes these and other reported RyR2 redox modifications. The number of SH residues that have to be modified *in vitro* to activate these channels, 3 per monomer, suggests that the reported low levels of endogenous *S*-nitrosylation (53) do not modulate channel activity under basal conditions. More recently, this same group (63) reported a higher level of endogenous *S*-nitrosylation (1-2 SH modified per channel tetramer) in a partially purified RyR2 fraction, both at low (tissue) and high (ambient) oxygen tension. These values are still below the threshold for activation since, depending on the oxygen tension, 3 to 4 thiols per monomer should be modified *in vitro* to activate RyR2 channels (63). Furthermore, only the NO donors GSNO and SIN-1, but not NO itself, *S*-nitrosylate RyR2 *in vitro*, increasing the binding of [^{3}H]-Ryanodine and the opening probability of single RyR2 channels incorporated in lipid bilayers (63).

Following the 1998 report showing that RyR2 was endogenously *S*-nitrosylated and susceptible to modifications by NO donors (53), most of the subsequent work on the effects of *S*-nitrosylation on RyR function was

Table 1. Redox modifications of cardiac Ryanodine receptor thiols

Preparation	Stimulus	RyR2 Modification	Effect	Ref
Purified RyR (dog)	GSNO SIN-1	S-nitrosylation of ~3 SH/subunit Oxidation of 7 SH/subunit	Increased Po in planar bilayers Irreversible activation	53
SR vesicles (dog)	Pacing-induced heart failure	Increased oxidation	Increased calcium leak in SR vesicles corrected by edaravone	125
SR vesicles (dog)	Five 5-min period of stimulation at 220 bpm	Increased S-glutathionylation	Increased calcium release rates	89
Cardiac myocytes isolated from nNOS-/- mice	none	Decreased S-nitrosylation; increased oxidation	Increased diastolic calcium leak	78
Isolated heart (rat)	SNAP	Dose dependent increase in S-nitrosylation	Increased contractility	80
SR vesicles (dog)	Five 5-min period of high intensity exercise	Increased S-glutathionylation	Increased calcium release rates	90
SR vesicles (dog)	Pacing-induced heart failure	Increased oxidation	Increased leak from cardiomyocytes and increased channel Po in planar bilayers due to increased Ca^{2+} sensitivity; both reversed by DTT	126
RyR2 enriched fraction (dog)	GSNO SIN-1	Increased S-nitrosylation Oxidation of 7-9 SH/subunit	Increased Po of channels in planar bilayers Increased [^{3}H]-Ryanodine binding	63
SR vesicles (dog)	Ventricular fibrillation secondary to myocardial ischemia	Increased oxidation	Increased diastolic calcium leak reversed by DTT	127

Abbreviations: GSNO, *S*-nitrosogluthatione; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-N-acetylpenicillamine.

performed on RyR1, the skeletal muscle isoform that shares 70% homology with RyR2. In the heart, research on this subject shifted its focus from *S*-nitrosylation to the elucidation of the effects of NO on cardiac contractility in isolated cardiomyocytes or whole hearts, and in the identification of the sources of NO responsible for RyR2 modification.

It is necessary to consider, however, that in addition to modifying RyR2 NO interacts directly or indirectly with almost every protein involved in excitation-contraction coupling in the heart, such as L-type calcium channels, the sarcoplasmic reticulum calcium ATPase (SERCA) and phospholamban (64, 65). Consequently, the effects of NO on cardiac contractility are quite complex; moreover, as discussed below, reports on the effects of genetic deletion of one particular NOS isoform are contradictory. As already mentioned, effective *S*-nitrosylation of SH residues requires simultaneous NO and ROS generation to oxidize the residue or to generate an effective *S*-nitrosylating agent. Therefore the identification of the sources of ROS is another relevant issue to be considered and which will be addressed below.

3.1.1. Localization and function of NOS isoforms in cardiac myocytes

It has been proposed that the different NOS isoforms are confined to different cellular microdomains where they convey directly the NO generated to downstream targets. Based on immunoelectron microscopy labelling of nNOS and on its co-immunoprecipitation with RyR2, this isoform was proposed to have an SR location, where it would regulate RyR2 opening probability (66, 67). Yet, other investigators have not found nNOS and RyR2 colocalized in mice hearts (68) or have localized nNOS at the sarcolemma (69, 70). Therefore, although most published reviews and diagrams place nNOS at the SR, attributing to this isoform a special role in RyR2 modulation, this localization remains uncertain. There is also evidence for the regulation of RyR2 by eNOS. This isoform increases RyR2 activity in cardiac myocytes in response to stretch, as indicated by an increase in calcium sparks, which are absent in the eNOS deficient mice (71). Additionally, other authors have localized eNOS at the plasmalemma based on its immunoprecipitation with caveolin (72), have demonstrated its presence in isolated cardiac sarcolemmal membranes (69) and have shown that eNOS co-immunoprecipitates with RyR2 in the cardiac myocyte cell line H9C2 (73). Thus it is not clear if only one or both NOS isoforms regulate RyR2 activity. It is also not settled if they are localized at the SR or the sarcolemmal membrane, since these two membranes are so close, only a few nm apart, that NO generated in the sarcolemma can easily diffuse through this distance (74) and target RyR2 present in the SR membrane.

Functional studies with genetically modified animals have not contributed to resolve this issue. Isolated hearts from eNOS knock out (KO) mice show no change (75) or enhanced contractility (67) in basal conditions and an increased response to beta-adrenergic stimulation (67, 75). In contrast, nNOS KO mice show a decrease in the inotropic response to isoproterenol (67) with either no changes (67) or enhanced basal contractility (76, 77). The interpretation of these studies is hindered by the fact that NO modulates other proteins involved in EC coupling, as well as contractile proteins, so that the effects of NO cannot be attributed solely to changes in RyR2 activity. Moreover, the changes in contractility reported in those experiments were not contrasted with changes in RyR2 *S*-nitrosylation since this redox modification was not explored. But even assuming that RyR2 is a preferential target of nNOS it is not possible to conclude from these reports that nNOS is the only source of NO that activates RyR2.

More recently it has been shown (78) that RyR2 *S*-nitrosylation is strongly decreased in NOS1-/- mutant mice while other undefined oxidative modifications are

increased (Table 1). This effect is specific for the NOS1-/- mutant mice because *S*-nitrosylation is unchanged in NOS3-/- mutants, suggesting that NOS1-generated NO is responsible for the enhanced RyR2 *S*-nitrosylation. Decreased RyR2 *S*-nitrosylation in these KO animals increases diastolic calcium leak and hence decreases SR calcium content and contractility; the authors suggest that RyR2 *S*-nitrosylation protects protein thiols from irreversible oxidation (78).

Besides promoting S-nitrosylation of cellular proteins, NO also activates guanylate cyclase by binding to the heme group of the enzyme, stimulating the synthesis of cyclic GMP, which activates protein kinase G (PKG). By decreasing calcium influx through L-type calcium channels, PKG exerts a negative inotropic effect in cardiac myocytes and hence affects the ensuing RyR2-mediated calcium transients (79). Independent activation of the cGMP-PKG pathway and protein *S*-nitrosylation has been clearly shown recently in isolated perfused hearts (80). In this work, low concentrations of the NO donor SNAP produce a positive inotropic effect and also increase RyR2 *S*-nitrosylation. Both effects are abolished by tempol, a superoxide scavenger, suggesting that the increase in RyR2 activity by *S*-nitrosylation requires ROS production and is part of the inotropic response (80). In the same preparation, higher SNAP concentrations produce negative inotropy by a cGMP-dependent PKG activation mechanism (80). This biphasic response to NO may explain some of the contradictory effects described in the literature when using KO mice for the different NOS isoforms.

Moreover, the proximity of the sarcolemma and the SR membrane and the relatively long half-life of the diffusible NO gas (74) suggests that other factors determine which NOS isoform promotes RyR2 *S*-nitrosylation. For instance, the existence of an actual complex between nNOS and RyR2 could make the product of the former (NO) directly available to specific domains in the RyR2 complex. An associated source of ROS would also be needed to oxidize the target cysteine prior to its *S*-nitrosylation by NO. Examples of such complexes are abundant in intermediate metabolism, where the product of one enzyme is channelled to the active centre of the next enzyme in the metabolic pathway without mixing with the cytoplasm (81, 82). Such association has not been demonstrated between RyR2 and nNOS at the present time.

3.1.2. Effects of nitroxyl on cardiac calcium cycling

Changes in cardiac calcium handling have also been reported after nitroxyl (HNO) administration, a one electron reduced and protonated form of NO (its proper name is nitrosyl hydride), which is much more reactive toward thiol residues than NO. There is no evidence for the generation of HNO *in vivo*, but it can be exogenously supplied by administration of Angeli's salt (sodium trioxodinitrate, $Na_2N_2O_3$) (83). Nitroxyl improves contraction and relaxation in normal and failing hearts *in vivo* (84), and significantly increases Ca^{2+} transients and fractional Ca^{2+} release from the SR in isolated cardiac myocytes while decreasing simultaneously the time constant for relaxation by increasing SERCA activity (85). Although nitroxyl activates purified RyR2 incorporated in planar bilayers, the demonstration of thiol modification in the RyR2 protein by nitroxyl is still lacking. The recent demonstration that nitroxyl activates SERCA by *S*-glutathionylation of a single cysteine (86) indicates that nitroxyl has the potential to promote this redox modification and raises the possibility that *S*-glutathionylation underlies the increased RyR2 channel activity produced by nitroxyl.

3.2. NADPH Oxidase and RyR2 *S*-glutathionylation

As already mentioned, ROS generation is needed for redox modification of cysteine residues. Several enzymes can produce ROS in cardiac myocytes, as a by-product of the reactions they catalyze (87) and some of these ROS may have an important role in pathological conditions (see below). The NADPH oxidase (NOX) is an enzyme whose only known function is to produce ROS. This enzyme catalyzes the transfer of one electron from NADPH to oxygen, producing superoxide anion as its main product. First described in macrophages, it is now know that almost every cell expresses one or more of the 7 isoforms described for this enzyme (88). The phagocytic NOX isoform possesses two integral membrane subunits that form its catalytic core, gp91phox (NOX2) and p22phox, and three cytoplasmic regulatory subunits, p47phox, p67phox, p40phox, which together with the small GTPase Rac conform the active enzyme. Cardiomyocytes express two NOX isoforms, NOX2 and NOX4, and their role in redox signalling in the cardiovascular system in several pathological conditions has been recently reviewed (23, 24).

We have shown that NOX2 colocalizes with RyR2 in canine cardiac muscle and that NOX2 activity increases following exercise in conscious dogs or after tachycardia in anesthetized dog (89, 90). Concurrently with the increase in NOX2 activity, we found an increase in RyR2 *S*-glutathionylation (Table 1, Figure 1), which is prevented by the administration of the NOX2 inhibitor apocynin before exercise or tachycardia (90). Furthermore, incubation of SR vesicles isolated from dog hearts, which are enriched in RyR2 and NOX2, with [^{35}S]-GSH and NADPH to stimulate *in vitro* superoxide generation, results in the covalent binding of a [^{35}S]-glutathionyl moiety to RyR2 (89). In isolated cardiac SR vesicles, under experimental conditions that promote maximal RyR2 phosphorylation in control and experimental vesicles, RyR2 *S*-glutathionylation increases calcium release rates without modifying calcium leak from these vesicles (89, 90). These combined results suggest that *S*-glutathionylation is a physiological mechanism to activate RyR2 channel activity when the heart requires a faster release of calcium such as during exercise or tachycardia.

We have also measured RyR2 *S*-nitrosylation in cardiac SR vesicles obtained after exercise or tachycardia. In contrast to the increase in *S*-glutathionylation produced by these conditions, we found a small but significant decrease in RyR2 *S*-nitrosylation (Figure 1A). The decrease in RyR2 *S*-nitrosylation was not due to *S*-glutathionylation of endogenously *S*-nitrosylated cysteine residues, because

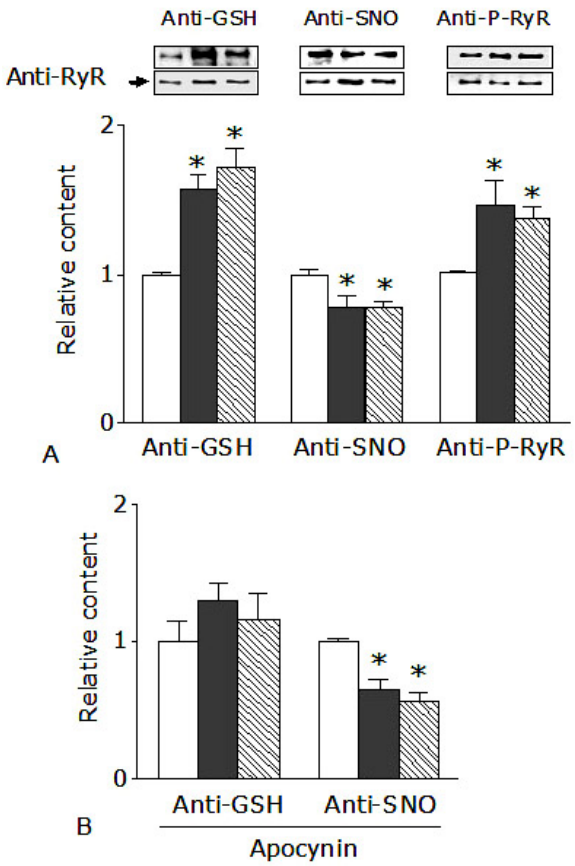

Figure 1. Post-translational modifications of RyR2 induced by exercise or tachycardia. A) A representative Western blot showing RyR2 *S*-glutathionylation, *S*-nitrosylation and phosphorylation in Serine-2808, detected with anti-GSH, anti-nitrocysteine or anti P-serine 2808 antibodies in SR fractions isolated from control dog hearts (empty bars), after tachycardia (grey bars), or exercise (hatched bars). The graph bar shows the quantification of Western blots by densitometry, normalized by the corresponding immunoreactivity against anti-RyR2 (90). Except from the anti-GSH data, which were taken from (90), all other data represent unpublished experiments. Anti-nitrosocysteine antibody was from AG Scientific (San Diego, CA) and anti P-serine 2808 was from Badrilla (UK). Details of the electrophoretic conditions are given in (90). B) Quantification of RyR2 *S*-glutathionylation and *S*-nitrosylation in cardiac SR vesicles isolated from dogs treated with apocynin (10 mg/k body weight). Results were normalized with respect to RyR2 content, determined with anti-RyR2 antibody. Control: empty bars; tachycardia: grey bars; exercise: hatched bars. Anti-GSH data were taken from previously published results (90). *: $P<0.05$, determined by ANOVA followed by Tukey's test.

apocynin administration, which suppressed the increase in RyR2 *S*-glutathionylation produced by exercise or tachycardia, did not increase *S*-nitrosylation to control values (Figure 1B). We also found that tachycardia and exercise increased RyR2 endogenous phosphorylation at Ser-2808 (Figure 1A), a residue which is phosphorylated by both CaMKII and PKA (5). This result is consistent with the activation of these kinases during exercise and tachycardia (mostly CaMKII in this case). It is therefore conceivable that enhanced RyR2 phosphorylation after exercise or tachycardia may hinder RyR2 *S*-nitrosylation, a possibility worth testing. Alternatively, increased NOS phosphorylation may decrease NOS activity during exercise or tachycardia, resulting in reduced RyR2 *S*-nitrosylation. As mentioned above, phosphorylation of nNOS in serine-847 by CaMKII decreases its affinity for calcium-calmodulin and decreases NOS activity (58); similarly, eNOS phosphorylation on treonine-495 by PKC or AMPK also inhibits its activity (57, 59).

4. IDENTIFICATION OF REDOX-MODIFIED RyR CYSTEINES

Many studies on the regulation of RyR activity by ROS or RNS have been performed *in vitro*, applying exogenous SH modifying agents. The role and physiological relevance of these effects is questionable since the reaction of a protein with a redox reagent *in vitro* does not necessarily imply that this protein is amenable to redox modification *in vivo*. One example is the *S*-glutathionylation of both RyR2 and RyR3 in endoplasmic reticulum vesicles isolated from rat brain cortex induced by ROS *in vitro*, whereas *in vivo* brain cortex ischemia increases only RyR2 *S*-glutathionylation (91). These findings indicate that cells possess mechanisms that under certain conditions target selectively a specific RyR isoform for redox modifications. Therefore, a valid approach to study the functional effects of RyR2 *S*-nitrosylation and *S*-glutathionylation in cardiac muscle would be to identify the specific RyR2 cysteines modified *in vivo* by different physiological (or pathological) stimuli.

Facing the lack of information about the identity of the cysteine residues modified and the kind of redox modification produced, some investigators have quantified changes in the number of RyR free thiols rather than investigating one particular redox modification (Table 1). In the absence of better methods to identify the precise redox modification produced, this is a good approximation, albeit one has to be aware that the loss of free thiols does not give information about the type or the reversibility of the redox modification. Furthermore, the nitrosyl residue (-NO) is small and lipophilic, while the glutathionyl residue is a negatively charged hydrophilic tripeptide. Accordingly, from a theoretical point of view the effects of *S*-nitrosylation or *S*-glutathionylation on the conformation and function of the modified protein are expected to be different and different also from other redox modifications such as the formation of disulfide bridges. In fact, different redox modifications produce different effects on the modified protein. For instance, *S*-nitrosylation of the skeletal RyR1 isoform increases the affinity for calcium activation while *S*-glutathionylation decreases the inhibitory effect of Mg^{2+} on RyR1-mediated CICR (92).

More detailed knowledge of the type of redox modifications that RyR can undergo has been obtained for the skeletal muscle RyR1 isoform, where hyperreactive

Table 2. Hyperreactive cysteines in Ryanodine receptors

RyR1, Rabbit Skeletal muscle					RyR2, Cardiac muscle		
Cysteine residue		Redox modification			Cysteine residue		
Ref 93	Ref 94	R-SNO	R -GSH	R -S-S-R	Dog	Human	Rat
	36		x	x	30	36	36
	253	x	x				
	315	x	x				
	811	x	x		816	822	815
	906	x	x		911	917	910
1040	1040	x					
1303	1303	x					
	1591		x		1576	1582	1576
	2326		x	x			
	2363		x	x	2324	2330	2320
2436					2396	2402	2392
2565					2525	2531	2521
2606					2566	2572	2562
2611					2571	2577	2567
	3193		x		3152	3158	3158
3635	3635	x	x	x	3599	3602	3580

Hyper-reactive cysteines residues identified in RyR1 (93, 94) and the redox modifications they undergo as described in 94 are shown. The equivalent cysteine residues in RyR2 for the species indicated are also shown.

cysteines have been identified by mass spectroscopy (93, 94). Under conditions favouring RyR1 closed state, seven hyperreactive cysteines were identified on the basis of their reactivity with 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin: Cys-1040, Cys-1303, Cys-2436, Cys-2565, Cys-2606, Cys-2611, and Cys-3635 (Table 2) (93). The specific modifications that RNS or ROS may produce on these cysteines were not explored in this study. In conditions favouring the open state of the channel, 9 endogenously modified cysteines were found (94): Cys-36, Cys-315, Cys-811, Cys-906, Cys-1591, Cys-2326, Cys-2363, Cys-3193, and Cys-3635, while three other cysteines were modified by exogenous redox agents: Cys-253, Cys-1040, and Cys-1303. The modifications of these cysteine residues by ROS/RNS and the equivalent cysteines present in dog, rat and human RyR2 are shown in Table 2. The amino acidic sequence around these cysteines in these species is shown in Figure 2. The high degree of homology among the skeletal and cardiac RyR isoforms suggests that ROS/RNS could modify equivalent cysteines in RyR2. Yet, five RyR1 cysteine residues, Cys-253, Cys-315, Cys-1040, Cys-1303 and Cys-2326, do not have an equivalent in RyR2.

5. REDOX MODIFICATION OF RyR2-ASSOCIATED PROTEINS

Redox regulation of RyR2 could also affect the binding of the associated proteins and vice versa. Changes in these interactions could be an indirect way of modulating RyR activity. Several of the proteins that supposedly modulate RyR2 are also targets of redox regulation. For instance, calmodulin binding to RyR2 at a domain localized between amino acid residues 3583 and 3603 inhibits the channel at all Ca^{2+} concentrations (95, 96). The affinity for calmodulin binding decreases under oxidizing conditions (97) and therefore the increase in RyR2 activity produced by oxidation could be caused in part by a decrease in calmodulin inhibition. The interaction between FKBP12.6 and RyR2 is redox sensitive (98) and oxidants promote dissociation which may contribute to the abnormal calcium release associated to redox stress (99). S100A1, a member of a family of ubiquitous calcium binding proteins of 16-26 kDa (100) binds to RyR2 in ventricular muscle in a calcium-dependent manner, increasing RyR2 activity at micromolar calcium concentrations (101) and promoting channel closure at nanomolar calcium concentrations (102). S-glutathionylation increases S100A1 calcium affinity from the micromolar to the nanomolar range (101, 103) and may contribute to increase RyR2 activity at low calcium concentrations. CLIC-2 (chloride intracellular channel type 2) is another protein that could potentially regulate RyR2 activity. Its binding to RyR2 is redox sensitive; moreover, CLIC-2 inhibits RyR2 under oxidizing conditions but activates it under reducing conditions (104-106). Future research is needed to clarify the effects of these and other proteins on RyR2 function *in vivo*.

Importantly, physiological RyR2 activation requires calcium entry through L-type calcium channels, which are also modulated by redox changes. Consequently, redox modification of L-type channels is bound to affect RyR2 channel activity. Similarly RyR2 activity is modulated by luminal calcium, which is turn dependent on SERCA activity. Redox dependent regulation of SERCA, therefore, will also influence indirectly RyR2 activity. The regulation of these proteins by redox agents has been reviewed recently (15, 107-109).

6. RELEVANCE OF RyR2 REDOX MODULATION IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

6.1. Physiological conditions

As discussed above, RyR2 is endogenously *S*-nitrosylated (53) and *S*-glutathionylated (89, 90). *S*-

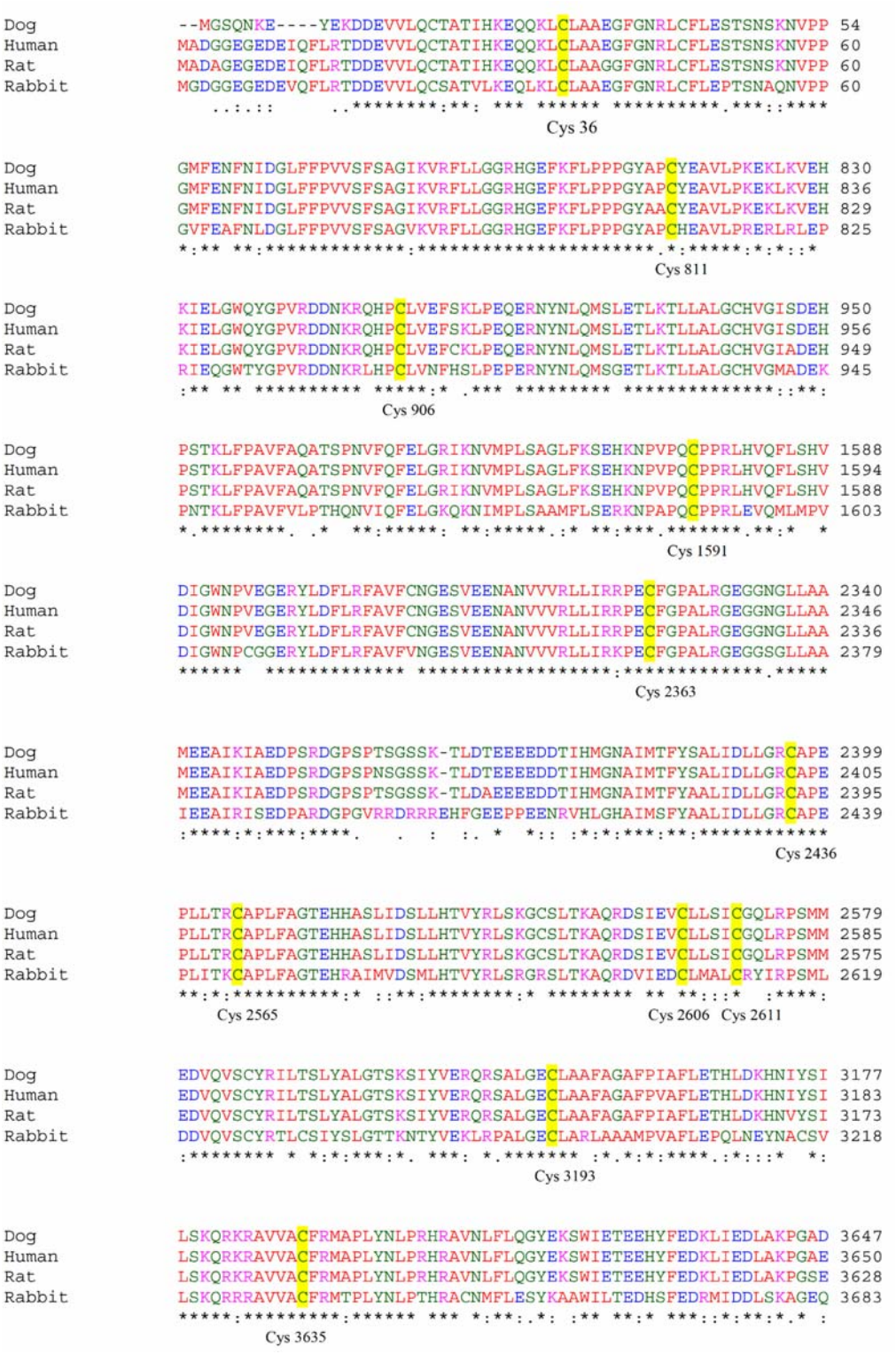

Figure 2. Sequence alignment of the hyper-reactive cysteine residues present in RyR1 (rabbit) with RyR2 from dog, human or rat. Multiple alignments of the four RyR2 protein sequences were performed using ClustalW2 tool. Amino acid residues are colored according to the following criteria: AVFPMILW are shown in red, DE in blue, RHK in magenta and STYHCNGQ in green. Hyper-reactive RyR1 cysteines and their corresponding RyR2 counterparts are highlighted in yellow. The symbol *, indicates the same residue in all the aligned sequences, the symbol **:** indicates a conserved substitution, and the symbol **.** indicates a semi conserved substitution.

glutathionylation increases with tachycardia (89) or with exercise (90). *S*-nitrosylation, however, did not increase but rather decreased after tachycardia or exercise (Figure 1A). RyR2 *S*-glutathionylation increases RyR2 activity *in vitro*, suggesting that this modification may be a physiological mechanism to increase calcium release *in vivo* under conditions of increased heart rate. The increase in RyR2 activity is unlikely to have by itself a sustained effect on the size of the calcium transient due to auto regulation of SR calcium content (110). The recent demonstration that nitroxyl-mediated *S*-glutathionylation of SERCA increases calcium transport activity in cardiac myocytes (86) raises the possibility that simultaneous redox modulations of RyR2 and SERCA operate in concert to increase cardiac inotropism independently of beta-adrenergic signalling. The positive inotropic effects of nitroxyl described above (85) suggest that this is a reasonable proposal.

6.2. Pathological conditions

Although the present review addresses mainly the effects of redox agents on RyR2 activity in physiological conditions, we will briefly discuss RyR2 redox modifications in pathological conditions.

Heart failure is the inability of the heart to adequately pump blood to meet the demands of the body. Many models of experimental heart failure have shown that failing hearts display little contractile force because their myocytes produce lower calcium transients due to a reduction in SR calcium content. This reduction may be due to both a decrease in calcium transport activity by the SERCA (111) and to an increase in diastolic calcium leak through RyR2 (112-114). Oxidative stress, a rupture in the balance between ROS and RNS generation and cellular antioxidant activities, is another feature of several heart pathological conditions (30, 64, 115, 116). The enzymes most likely responsible for oxidative stress are NOX2, xanthine oxidase and NOS. They are all activated in the diseased heart: NOX2 is upregulated after myocardial infarction (117) and in experimental models of hypertrophy leading to heart failure (118). Xanthine oxidase (XO) originates from xanthine dehydrogenase (XDH) in a reversible, redox-dependent reaction, or is irreversibly generated by proteolysis (119). Both XO and XDH catalyze the conversion of hypoxanthine into xanthine and of xanthine into uric acid, the last two stages in purine metabolism. These two enzymes are especially abundant in the heart, where they are localized to the SR (120). XDH reduces NAD to produce NADH and XO reduces O_2 to produce superoxide anion, constituting an important source of ROS in cardiac myocytes. Noteworthy, XO expression is increased in human heart failure (121) and in dog models of experimental heart failure (122, 123). Inhibition of XO activity by allopurinol improves cardiac function in heart failure and reverses oxidative stress (121-123). Furthermore, NO appears to down-regulate XO activity since NOS1 KO mice exhibit higher xanthine-dependent superoxide anion generation than normal mice and both enzyme activities co-immunoprecipitate from total heart lysates, suggesting that they can interact *in vivo* (120). In fact, NO-dependent XO inhibition has been reported in endothelial cells (30) and this mechanism could also function in cardiac cells. Uncoupled NOS due to limited supply of its cofactor tetrahydrobiopterin (BH4) is another source of superoxide anion in the heart (30). Besides, several cardiac pathological conditions display increased iNOS expression (74); changes in the activity of all these ROS, RNS generating enzymes may contribute to decrease the GSH/GSSG ratio as it happens after ischemia-reperfusion (42) and in diabetes (124). The ensuing decrease in antioxidant defences may produce harmful (irreversible) redox modifications in many proteins including RyR2.

Reports that relate this increase in ROS/RNS generation with oxidation of RyR2 thiols in pathological conditions have only recently emerged. In a canine model of rapid pacing-induced heart failure, a decrease in the number of RyR2 free thiols (Table 1) correlates with an increased Ca^{2+} leak measured *in vitro*, which is prevented by the antioxidant edaravone (125).

Further evidence supporting RyR2 oxidation-dependent Ca^{2+} leak has been provided by Gyorkes's group (113, 126). In normal cardiomyocytes, RyR2 channels respond to a decrease in luminal calcium with a decrease in their opening probability, which contributes to terminate calcium release. In cardiomyocytes isolated from hearts after the induction of heart failure by rapid pacing, oxidized RyR2 shows a high opening probability at low luminal calcium producing Ca^{2+} leak and SR Ca^{2+} depletion. Reducing agents normalize this abnormal response to low luminal calcium preventing calcium leak (126). Yet, the precise nature of RyR2 redox modifications was not described. Oxidation of RyR2 may also have an important role in the generation of arrhythmias. As recently shown, cardiomyocytes isolated from hearts after myocardial infarction-induced ventricular fibrillation are more prone to produce calcium alternans, i.e. the generation of small and large calcium transient in alternating manner. Calcium alternans is an important cause of cardiac arrhythmias and sudden cardiac death. Again RyR2 oxidation seems to be determinant in the production of calcium alternans, since treatment with antioxidants normalized calcium release in isolated cardiomyocytes (127).

7. SUMMARY

The evidence presented in this review indicates that redox modifications of RyR2 play an important role in the regulation of calcium release in the heart. We are just starting to understand which redox modifications occur in the RyR2 molecule and how they affect calcium release. Future work should be done to identify the specific cysteine residues that are modified, the type of redox modification they undergo and their effect on RyR2 channel activity. The identification of the enzymatic sources of RNS and ROS that modify RyR2 under both physiological and pathological conditions is also necessary for future therapeutical approaches. Another important issue to investigate concerns the characterization of the endogenous mechanisms that recover the basal redox state of the RyR2 protein after it has been redox-modified.

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Abbreviations: AMPK: AMP-activated protein kinase; CaMKII: Ca2+/calmodulin dependent protein kinase type II; CICR: Ca2+-induced Ca2+ release; FKBP : FK506-binding protein; GSH: reduced glutathione; GSSG: oxidised gluthatione; GSNO: S-nitrosoglutathione ; eNOS or NOS3: endotelial nitric oxide synthase; iNOS or NOS2: inducible nitric oxide synthase; nNOS or NOS1: neuronal nitric oxide synthase; NO: nitric oxide; NOX: NADPH oxidase; PKA: cAMP-dependent protein kinase; PKC: protein kinase C; PKG: cGMP-dependent protein kinase; ROS: reactive oxygen species; RNS: reactive nitrogen species; RyR2: ryanodine receptor type 2; SERCA: sarco/endoplasmic reticulum Ca2+ ATPase; SR: sarcoplasmic reticulum; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine; XDH: xanthine dehydrogenase ; XO: Xanthine oxidase

Key words: Heart; calcium release channel; reactive oxygen species; reactive nitrogen species; redox regulation; Ryanodine Receptor; nitric oxide synthase; NADPH oxidase; *S*-nitrosylation; *S*-glutathionylation, Review

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