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Review

Does nitric oxide modulate cardiac ryanodine receptor function? Implications for excitation–contraction coupling

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Nitric oxide (NO) is a highly reactive, free radical signalling molecule that is constitutively released in cardiomyocytes by both the endothelial and neuronal isoforms of nitric oxide synthase (eNOS and nNOS, respectively). There are increasing data indicating that NO modulates various proteins involved in excitation–contraction coupling (ECC), and here we discuss the evidence that NO may modulate the function of the ryanodine receptor Ca^{2+} release channel (RyR2) on the cardiac sarcoplasmic reticulum (SR). Both constitutive isoforms of NOS have been shown to co-immunoprecipitate with RyR2, suggesting that the channel may be a target protein for NO. eNOS gene deletion has been shown to abolish the increase in spontaneous Ca^{2+} spark frequency in cardiomyocytes exposed to sustained stretch, whereas the effect of nNOS-derived NO on RyR2 function remains to be investigated. Single channel studies have been performed with RyR2 reconstituted in planar lipid bilayers and exposed to various NO donors and, under these conditions, NO appears to have a dose-dependent, stimulatory effect on channel open probability (P_{open}). We discuss whether NO has a direct effect on RyR2 via covalent *S*-nitrosylation of reactive thiol residues within the protein, or whether there are downstream effects via cyclic nucleotides, phosphodiesterases, and protein kinases. Finally, we consider whether the proposed migration of nNOS from the SR to the sarcolemma in the failing heart may have consequences for the nitrosative vs. oxidative balance at the level of the RyR2, and whether this may contribute to an increased diastolic Ca^{2+} leak, depleted SR Ca^{2+} store, and reduced contractility in heart failure.

1. Introduction

A role for the free radical signalling molecule nitric oxide (NO) in modulating cardiac function has been recognised for 15 years or more. The precise actions of NO, however, remain under investigation. This probably stems from the varied (and often opposing) actions of NO in the heart, which are determined by different subcellular compartmentalization and regulation of the NO synthase (NOS) isoforms.[1] Two are now known to be constitutively expressed in cardiomyocytes: 'endothelial' NOS (eNOS or NOS3) mainly localized to invaginations of the plasmalemma called caveolae,[2] and 'neuronal' NOS (nNOS or NOS1), which is mostly found on the sarcoplasmic reticulum (SR)[3] (*Figure 1*). The third isoform is the 'inducible' NOS (iNOS or NOS2), whose expression is stimulated by inflammatory mediators.

NOS catalyses the synthesis of NO by the conversion of L-arginine and oxygen to L-citrulline and NO, in the presence of NADPH and tetrahydrobiopterin (BH4).[4] eNOS and nNOS are both activated by Ca^{2+} and produce NO at different rates (16 nmol of NO min^{-1} mg^{-1} for eNOS vs. 96 nmol of NO min^{-1} mg^{-1} for nNOS).[5,6] A further important difference in the regulation of these two NOS isoforms is that under physiological conditions, eNOS activity is predominantly regulated by phosphorylation,[7,8] whereas nNOS activity and expression are exquisitely regulated by Ca^{2+}.[9] It has been shown that in the left ventricular (LV) myocardium, the concentration of NO changes cyclically and on the same timescale as the heart beat.[10] Moreover, increasing or decreasing the LV preload appears to be associated with parallel changes in intramyocardial NO concentration.[10] Taken together, these findings support the idea that NO is involved in a fast, autoregulatory mechanism that modulates myocardial contraction on a beat-by-beat basis and assists in matching preload with cardiac output.

During excitation–contraction coupling (ECC), depolarization of the cardiomyocyte plasmalemma opens L-type voltage-gated Ca^{2+} channels (LTCC) and Ca^{2+} enters the cell. This Ca^{2+} influx stimulates a larger release of Ca^{2+} through the opening of ryanodine receptor Ca^{2+} release

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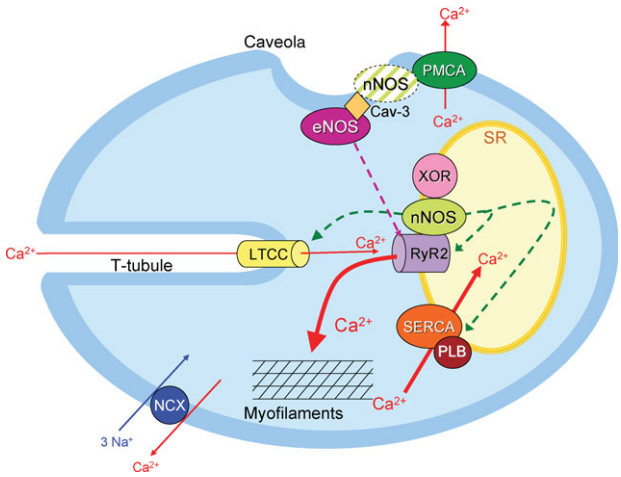

Figure 1 Cartoon showing cardiomyocyte excitation–contraction coupling (ECC) and its modulation by constitutive NO production. The 'endothelial' isoform of NO synthase (eNOS) is preferentially localized to caveolae in the sarcolemmal membrane and is expressed in association with the scaffolding protein caveolin-3 (Cav-3). eNOS gene disruption has been shown to abolish the stretch-dependent increase in Ca^{2+} spark frequency, suggesting that eNOS-derived NO may increase RyR2 P_{open}. In the healthy heart, the 'neuronal' NOS isoform (nNOS) is predominantly localized to the sarcoplasmic reticulum (SR) where it has been shown to co-immunoprecipitate with both RyR2 and xanthine oxidoreductase (XOR). In the failing heart, nNOS has been shown to partially translocate to the sarcolemmal membrane and co-immunoprecipitate with Cav-3 (shown with hatched shading). nNOS is now thought to modulate various components of ECC, including Ca^{2+} influx via the L-type Ca^{2+} channel (LTCC), Ca^{2+} release from the SR via RyR2, and Ca^{2+} reuptake into the SR via the SR Ca^{2+} ATPase (SERCA2a). nNOS has also been shown to have an inhibitory effect on XOR production of superoxide (O_2^-), which in turn might irreversibly increase the P_{open} of the RyR2 channel and decrease myofilament Ca^{2+} sensitivity. Other abbreviations used in the figure: PLB, phospholamban; PMCA, plasmalemma Ca^{2+} ATPase; T-tubule, transverse tubule.

channels (RyR2) on the SR, a process termed Ca^{2+}-induced Ca^{2+} release (CICR).[11] Ca^{2+} binding to troponin C produces a conformational change in the troponin I—tropomyosin complex, which permits the initiation of myosin—actin cross-bridge cycling and contraction. Relaxation occurs when the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) is returned to resting levels either by reuptake into the SR via the SR Ca^{2+} ATPase (SERCA2a) or by extrusion from the cell via the Na^+/Ca^{2+} exchanger (NCX1) and, to a lesser extent, the plasmalemma Ca^{2+} ATPase (PMCA4b) (for an overview of ECC, see Bers[12]) (*Figure 1*).

It is now well established that constitutive NO production modulates the function of many ion channels and transporters involved in cardiac ECC (reviewed in references[9,13–15]). Here we discuss the evidence suggesting that NO may modulate RyR2 function both through classic cGMP-signalling and via direct redox modification of specific thiol residues in the RyR2 protein.

2. What are the functional effects of an increase in RyR2 open probability?

The function of the RyR2 is influenced by multiple factors including cytosolic Ca^{2+} concentration, SR Ca^{2+} content, and binding of accessory proteins [triadin, junctin, calsequestrin,[16] FK-506 binding protein-12.6,[17] protein kinase A (PKA),[18] protein phosphatases 1 and 2A (PP1 and PP2A),[19] and calmodulin[20]]. NO has been reported to both increase and decrease the open probability (P_{open}) of the RyR2. To understand the possible functional correlates of these changes, it is useful first to consider the effects of *selectively* increasing RyR2 P_{open}. At first sight, one might assume that this would increase the amplitude of the Ca^{2+} transient. However, experimental work in rat ventricular myocytes shows that the increased Ca^{2+} transient amplitude produced by low-dose caffeine (a compound that increases the P_{open} of the RyR2) disappears after a few electrical stimulation pulses and in the steady state the Ca^{2+} transient is the same size as before adding caffeine.[21,22] This occurs because in the steady state, during each heartbeat, the efflux of Ca^{2+} from the cell (largely via NCX) must exactly equal the influx (largely via LTCC). The amount of Ca^{2+} pumped out of the cell on NCX depends on both the properties of NCX and the amplitude and duration of the systolic Ca^{2+} transient. If we assume that neither the properties of NCX nor the duration of the Ca^{2+} transient is affected, a constant Ca^{2+} efflux per beat can occur only if the amplitude of the Ca^{2+} transient is constant. In other words, an increase in RyR2 P_{open} would *not* be expected to cause a sustained increase in the amplitude of the systolic Ca^{2+} transient. Indeed there are two situations in which a *decreased* Ca^{2+} transient may result. First, sufficient opening of the RyR2 may decrease the SR Ca^{2+} content to such a low level that even if all free Ca^{2+} were released, the Ca^{2+} transient may still be smaller than in control. Under these conditions, the decay of the Ca^{2+} transient will be slowed, as the SR would make a smaller contribution to relaxation. Second, if the opening of the RyR2 were increased during diastole, there might be a net diastolic leak of Ca^{2+} leading to a decrease in SR Ca^{2+} content and therefore a decrease in the amplitude of the Ca^{2+} transient. Such a diastolic Ca^{2+} leak has been suggested to contribute to decreased contractility in heart failure (HF) (Marx *et al.*[17] and see below).

3. Evidence that eNOS-derived NO modulates RyR2

There is evidence suggesting that stretch-dependent stimulation of NO synthesis by myocardial eNOS may increase the RyR2 P_{open}. In particular, the stretch-dependent increase in the spontaneous Ca^{2+} spark rate (which is often used as an indirect measurement of RyR P_{open}) was abolished in eNOS-deficient cardiomyocytes (*Figure 2*).[23] Furthermore, sustained stretch of wild-type cardiomyocytes was associated with Akt-dependent phosphorylation of eNOS and increased release of NO, which was abolished in eNOS-deficient mice.[23] On the basis of these data, the authors suggested that eNOS-derived NO enhanced RyR2 P_{open} in response to stretch, thereby contributing to the increase in Ca^{2+} transient amplitude and developed contractile force that accompanies a sustained change in muscle length, known as the Anrep effect. However, it is not clear whether the increased spark frequency and transient amplitude produced by eNOS-derived NO are solely due to effects on RyR2 or whether other components of the Ca^{2+} handling system are involved. As mentioned above, selective stimulation of RyR2 would not be expected to cause a sustained increase in Ca^{2+} transient amplitude. The observation that SR Ca^{2+} content is unchanged under these conditions[23] suggests that other

Ca^{2+} fluxes might have been affected by stretch. For instance, previous work has shown that stretch activates the Na^+/H^+ exchanger (NHE)[24] and this activation increases intracellular Na^+, which in turn stimulates Ca^{2+} influx via the reverse mode of NCX.[25,26]

The pathway by which physical stretch is transduced into Akt activation and eNOS phosphorylation is not well defined. However, it has been proposed that the location of eNOS in plasmalemmal caveolae and its association with the scaffolding protein caveolin-3[2] may make eNOS particularly well placed to participate in this transduction process. Recent immunoprecipitation and immunofluorescence studies by Martinez-Moreno *et al.*[27] suggest that a fraction of the total eNOS protein (63%) directly associates with RyR2 (*Figure 3A*), and that they may functionally interact. Evidence for the role of eNOS in modulating RyR function is not restricted to the heart. eNOS-derived NO from endothelial cells has been shown to increase Ca^{2+} spark frequency in smooth muscle cells from rat cerebral artery.[28] Spark frequency decreased by about 50% following inhibition of eNOS or denudation of the endothelium, and increased 3- to 4-fold following application of the NO donor *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (DEA-NONOate).

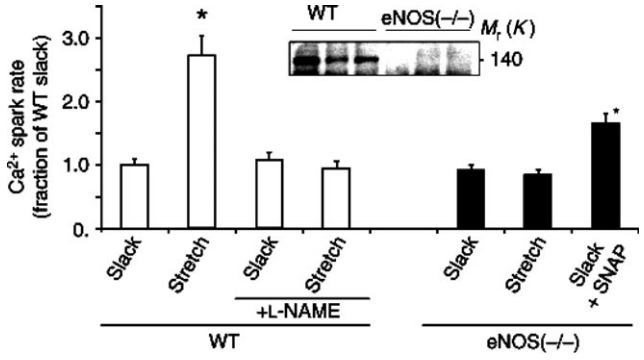

Figure 2 eNOS gene deletion ($eNOS^{-/-}$) abolished the stretch-dependence of the Ca^{2+} spark frequency seen in fluo-4-loaded cardiomyocytes from wild-type (WT) mice ($n = 38$). Furthermore, the increased Ca^{2+} spark frequency in response to stretch is abolished in WT cells exposed to the NOS inhibitor L-NAME (1 mM, $n = 8$); while in the $eNOS^{-/-}$ cells, application of the NO donor SNAP (10 μM, $n = 18$) produced a significant increase in the Ca^{2+} spark frequency. These data indicate that the stretch-dependent increase in the spontaneous Ca^{2+} spark rate requires synthesis of NO by eNOS. $^*P <$ 0.05 vs. respective controls at slack cell length. *Inset*. A western blot analysis for eNOS of protein extracts from suspensions of cardiomyocytes isolated from WT and $eNOS^{-/-}$ mice. From reference;[23] used with permission.

4. Evidence that nNOS-derived NO modulates RyR2

In 1999, Xu *et al.*[3] first demonstrated the presence of a NOS1 gene product in cardiac SR vesicles and suggested that NO produced by this isoform may inhibit SR Ca^{2+} reuptake by SERCA2a. Subsequent work by Barouch *et al.*[1] showed that murine heart protein extracts immunoprecipitated with the anti-RyR2 antibody exhibited immunoreactivity for nNOS, but not eNOS. A protein–protein link between nNOS and RyR2 has also been observed in the rat[29] (*Figure 3*) and human[30] myocardium. It has therefore been postulated[1] that NO may achieve specificity of action by being released in the proximity of its target proteins. This concept may be especially pertinent to the myocardium where cytoplasmic diffusion of NO is likely to be limited as a result of scavenging by myoglobin.[31] The fact, therefore, that nNOS and RyR2 co-immunoprecipitate suggests that the two proteins may form an interactive complex and that nNOS-derived NO could modulate RyR2 function. Equally, as nNOS is a Ca^{2+}-activated enzyme,[5] CICR from RyR2 would be expected to stimulate nNOS production of NO. Pinsky *et al.*'s[10] work using a porphyrinic microsensor to measure the rise and fall of intracellular NO on a beat-by-beat basis supports this idea. Indeed, as NOS quickly desensitizes to sustained elevations in $[Ca^{2+}]_i$, transient increases in $[Ca^{2+}]_i$, such as during ECC, would maintain cyclical NOS activation.

Overall, studies in $nNOS^{-/-}$ mice suggest that, under basal conditions, nNOS-derived NO has negative or neutral inotropic action on the LV myocardium and a positive lusitropic effect.[1,32–35] On the other hand, the positive inotropic response to β-adrenergic stimulation has been shown to be

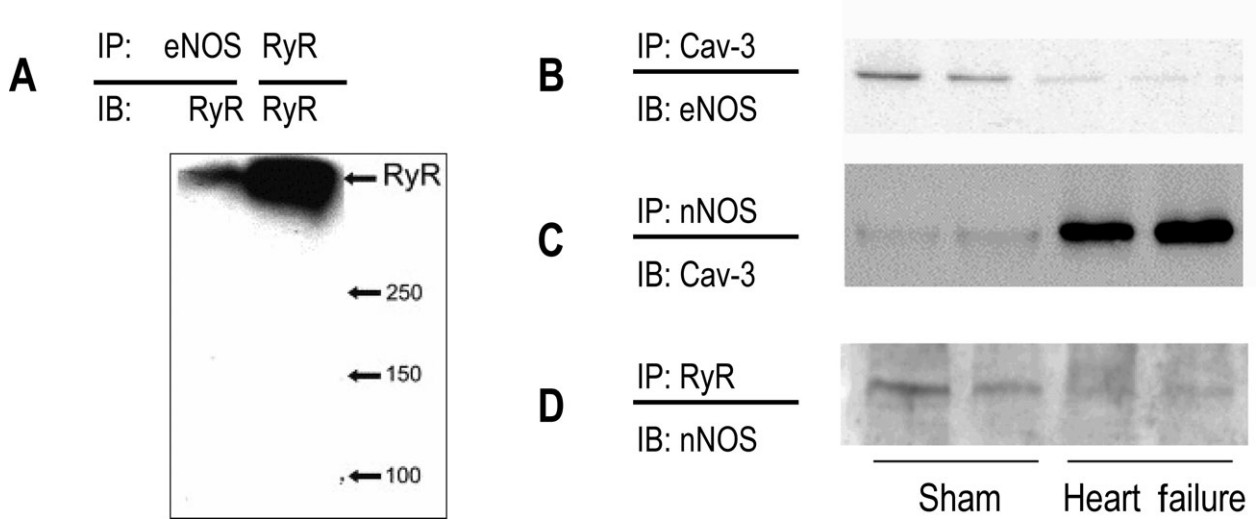

Figure 3 RyR2 partially co-immunoprecipitates with eNOS in the cardiac myocyte cell line H9C2 (*A*). In the LV myocardium of sham-operated rats, caveolin-3 (Cav-3) coimmunoprecipitates with eNOS (*B*) but not with nNOS (*C*). The pattern changes in heart failure, where a much greater proportion of nNOS coimmunoprecipitates with Cav-3 (C). This is associated with some reduction in the interaction between nNOS and RyR2 (*D*). IP, immunoprecipitate; IB, immunoblot. (A) Modified from reference;[27] (B–D) modified from reference.[29]

impaired in nNOS$^{-/-}$ mice *in vivo*.[1,36] It should be noted, however, that such findings could be complicated by concomitant nNOS gene deletion from the central and peripheral nervous systems[37,38] and from blood vessels.[39,40] Loss of nNOS from sympathetic nerves, for example, may affect noradrenaline release and thus the β-adrenergic inotropic response, independent from the autocrine actions of nNOS in cardiomyocytes.[38] That said, in isolated cardiomyocytes nNOS-derived NO might have a biphasic effect; i.e. to diminish the positive inotropic response to submaximal β-adrenergic stimulation and to enhance or maintain inotropy in response to high concentrations of β-adrenergic receptor agonists.[1,32,35] Whether changes in RyR2 function play a role in the myocardial phenotype of mice with nNOS gene deletion or pharmacological inhibition remains to be assessed.

5. Direct modulation of RyR2 by *S*-nitrosylation

S-nitrosylation is the direct, covalent attachment of a NO moiety to a reactive thiol side chain on a cysteine amino acid, which can cause conformational and functional changes in proteins. *S*-nitrosylation is now thought to be involved in dynamic post-translational modification of most or all major classes of protein, including enzymatic proteins (metabolic enzymes, protein kinases and phosphatases, oxidoreductases, and proteases), those involved in cell signalling (receptors, ion channels and transporters, transcription factors, and G-proteins), and structural proteins (cytoskeletal elements); for reviews, see Stamler *et al*.[41,42] Jaffrey *et al*.[43] have identified a population of proteins that are endogenously *S*-nitrosylated, but which lose this post-translational modification in nNOS$^{-/-}$ mice. These proteins, which have structural, metabolic, and signalling functions, are therefore thought to represent targets for *S*-nitrosylation by nNOS-derived NO.

In 1997, Stoyanovsky *et al*.[44] found that exogenous NO applied using the NO donor *S*-nitroso-*N*-acetyl penicillamine (SNAP) increased the P_{open} of RyR2 (and of the skeletal isoform, RyR1) reconstituted in synthetic planar lipid bilayers (*Figure 4A* and *B*). They found that this was a reversible process and that sulphydryl reducing agents promoted channel closure. Their results must, however, be interpreted with caution because application of NO donors does not confer the subcellular, local signalling of endogenously produced NO as described above. Furthermore, it is difficult to estimate whether the concentrations being applied are comparable to physiological levels. These complicating factors may help to explain the conflicting finding of Zahradnikova *et al*.[45] who showed that addition of L-arginine to stimulate production of NO by eNOS (which co-purified with RyR2) *reduced* P_{open} of single RyR2 fused into planar lipid bilayers (*Figure 4E* and *F*). It is also possible that exposure of isolated RyR in lipid bilayers to NO donors may over-emphasise direct (cGMP-independent) effects of NO, whereas most of the well-defined actions of NO described to date are cGMP-dependent and require an intact intracellular milieu. Furthermore, the regulatory effects of both cytoplasmic and luminal Ca^{2+}, kinases, phosphatases, phosphodiesterases (PDEs), and FKBP-12.6 may be disrupted in this preparation of RyR, and this in turn may affect the action of NO.

More recently, Stoyanovsky *et al*.'s[44] findings have been supported by quantitative data from Xu *et al*.,[46] who applied the NO donor *S*-nitrosoglutathione (GSNO) to RyR2 in planar lipid bilayers (*Figure 4C* and *D*). They calculated that RyR2 had 364 cysteine residues (89 per monomeric subunit, plus 2 per FKBP-12.6), of which ∼84 (or ∼21 per subunit) were free. They found that RyR2 activation required *S*-nitrosylation of many thiol residues. Modification of ∼2 sites per RyR2 was associated with minimal activation, while modification of ∼4 sites caused modest (∼50%) activation. Modification of ∼11 thiol sites per RyR2 (∼3 per subunit) was required to generate a 2- to 3-fold increase in channel activation. They concluded that RyR2 P_{open} was progressively increased as up to ∼3 thiol groups per RyR2 subunit were *S*-nitrosylated and that this was a reversible process during the cardiac cycle.[46] Furthermore, they found that when the reducing agent dithiothreitol (DTT) was eliminated from the standard buffers used in the purification of RyR2 from canine hearts, the isolated channels contained endogenous *S*-nitrosothiol groups; whereas inclusion of DTT resulted in almost complete loss of *S*-nitrosylation of the protein.[46] These findings strengthen the hypothesis that constitutive *S*-nitrosylation of RyR2 can occur and lead to modification of channel P_{open} under physiological conditions.

Subsequent work on skeletal muscle RyRs (RyR1) in synthetic lipid bilayers suggested that NO may have a concentration-dependent effect on RyR P_{open}.[47] Hart and Dulhunty[47] found that 10 μM SNAP caused a 2-fold increase in RyR channel P_{open}, whereas 1 mM SNAP, under the same conditions, resulted in a 2-fold reduction in P_{open} (but with a 1.5-fold increase in mean open time). Furthermore, Ziolo, *et al*.[48] have shown that the level of PKA activation can also determine the effect of NO on RyR2 activity. Importantly, this was the first time that the action of NO on RyR2 P_{open} had been assessed in intact ventricular myocytes. They found that application of 300 μM of the NO donor spermine NONOate following low levels of PKA activation induced by 0.01 μM isoprenaline increased the spontaneous Ca^{2+} spark frequency. Conversely, exactly the same concentration of NO donor following 1 μM isoprenaline (i.e. high PKA activation) caused a reduction of the spontaneous Ca^{2+} spark frequency.[48] The same results were obtained in the presence of a guanylate cyclase inhibitor, indicating that the effects of NO on RyR2 are cGMP-independent, and therefore likely to be via direct *S*-nitrosylation of the channel protein. The authors speculate that the PKA phosphorylation state of RyR2, together with PKA-dependent changes in SR Ca^{2+} load, will be important determinants of the functional effect of RyR2 *S*-nitrosylation. This may also be influenced by other important targets for NO and PKA, including phospholamban and LTCC.

Taken together, these data indicate that NO can have complex and dynamic effects on RyR2 P_{open}, depending on various other factors, such as the concentration of NO present and the level of β-adrenergic PKA activation. At low levels of PKA activation, NO increases RyR2 P_{open} and enhances the inotropic response; but at high PKA activation, NO diminishes RyR2 P_{open}—a mechanism that could be cardioprotective.

Few studies have investigated the action of nitroxyl (HNO) species, the 1-electron reduction product of NO (for review, see Fukuto *et al*.[49]). NO and HNO exist in chemical

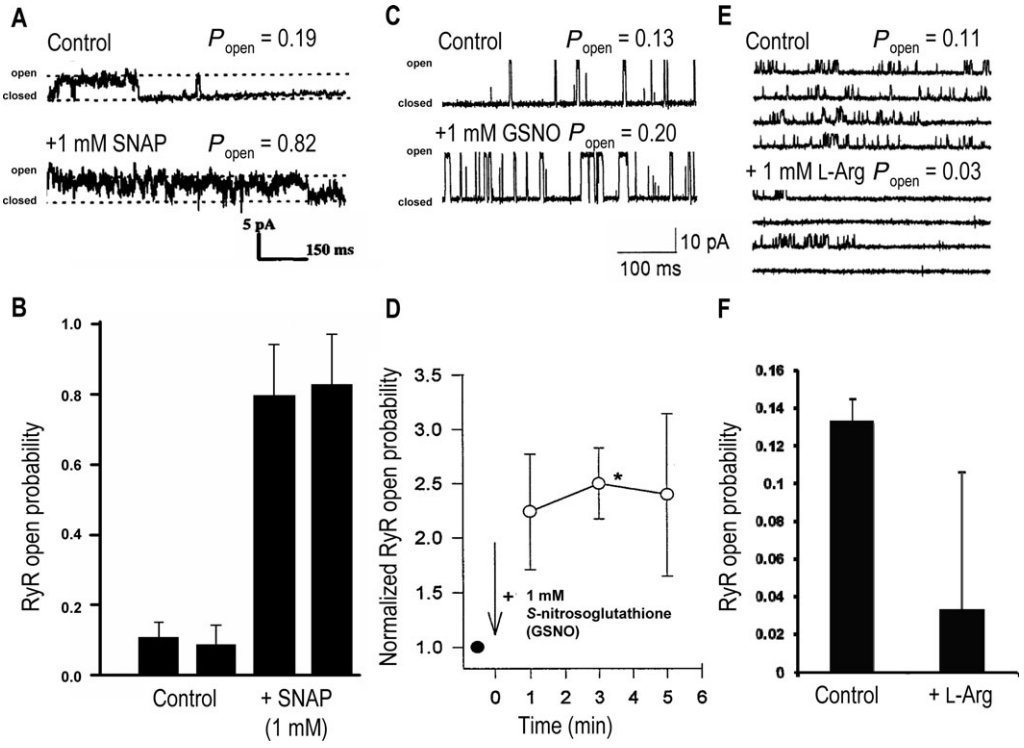

Figure 4 Application of high concentrations of NO donors (SNAP and GSNO, 1 mM) to single RyR2 reconstituted in planar lipid bilayers produces a significant increase in channel P_{open}. Addition of SNAP to the *cis*-side increased P_{open} from 0.19 to 0.82: single channel recording (*A*) and mean P_{open} from 5 min recording (*B*). Similarly, addition of GSNO to the *cis*-side increased P_{open} from 0.13 to 0.20 (*C*). This increase in P_{open} was sustained up to 5 min after application of the NO donor (data are shown mean ± SE from $n = 7$ experiments; * $P < 0.05$ vs. before addition of GSNO) (*D*). However, in other experiments, addition of the NOS substrate L-arginine (L-Arg, 1 mM) resulted in a reduction in P_{open} from 0.11 to 0.03 (single channel recording (*E*); mean data ± SE from $n = 14$ control experiments and $n = 5$ L-Arg experiments, *F*). (A and B) Modified from reference;[44] (C and D) modified from reference;[46] (E) modified from reference.[45]

equilibrium, but oxidise thiols by different reaction mechanisms (*S*-nitrosylation vs. disulphide bond formation, respectively). Recent data indicate that HNO is an even more potent activator of RyR2 than NO (having significant effects at picomolar concentrations), and may contribute *in vivo* to NO's effects on RyR2 P_{open}.[50] HNO has also been shown to accelerate Ca^{2+} reuptake by SERCA2a and therefore to be effective at enhancing Ca^{2+} cycling with significant positive inotropic effects.[51]

6. Nitrosative vs. oxidative modulation of RyR2

In their paper from 1998, Xu *et al.*[46] did not only show that increasing *S*-nitrosylation of up to 12 thiol sites per RyR2 produced progressive increases in channel P_{open}; they also demonstrated that oxidation of seven or more thiols per subunit generated irreversible channel activation. The interaction between reactive oxygen species (ROS) and reactive nitrogen species (RNS) has become a recurring theme in recent literature, to the extent that authors have referred to a ROS/RNS signalling axis.[52]

The enzyme xanthine oxidoreductase (XOR) can be a significant source of ROS, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which XOR produces as byproducts of the terminal steps of purine metabolism (reviewed by Harrison[53]). Scavenging of NO by O_2^- not only decreases the bioavailability of NO itself, it also generates a potent non-radical oxidant species, peroxynitrite ($ONOO^-$). Recent work has shown that XOR can be a contributory source of NO, especially when hypoxic conditions prevent NOS from generating NO.[54] It has been shown that NO production occurs in hypoxic rat myocardium in the presence of NOS inhibitors but not allopurinol (a XOR-selective inhibitor), implicating XOR in the synthesis of NO. XOR can generate NO by the reduction of both organic and inorganic nitrates and nitrites. It should be noted, however, that XOR enzyme activity appears to be much lower in humans than in many other mammals used in experiments (the difference between bovine and human XOR activity, for example, spans three orders of magnitude[55]). Experiments involving non-human mammals may therefore be at risk of overestimating the importance of XOR as a source of O_2^- if extrapolated to humans.

In mice, Khan *et al.*[34] showed that XOR and nNOS co-localized in the SR, and furthermore that they co-immunoprecipitated (*Figure 5A*), suggesting a protein–protein interaction between the two enzymes. O_2^- production by XOR was ~60% higher in $nNOS^{-/-}$ mice compared with wild-type controls suggesting that nNOS deficiency (but *not* eNOS deficiency) may stimulate myocardial O_2^- production by XOR. An alternative interpretation is that nNOS gene deletion reduces the availability of NO to scavenge XOR-derived O_2^-. It was also found that aspects of the myocardial positive force-frequency response (FFR) attenuation previously observed in $nNOS^{-/-}$ mice at high pacing frequencies[56] could be restored following incubation with allopurinol[34] (*Figure 5B* and *C*). The authors originally proposed that the absence of nNOS from the SR caused diastolic Ca^{2+} leak through RyR2. This could be an effect of uninhibited or unscavenged O_2^- production by XOR causing increased

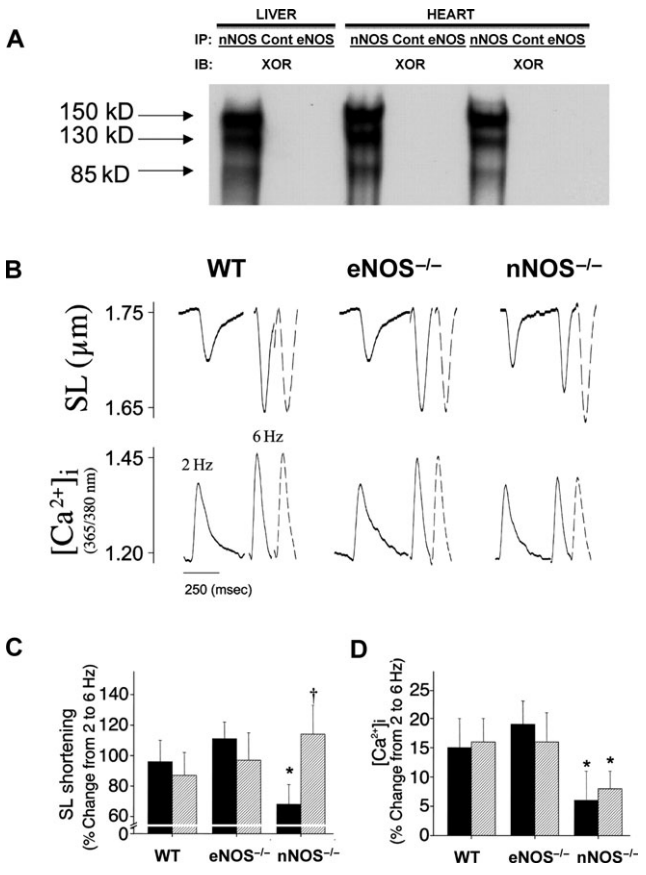

Figure 5 The products of xanthine oxidoreductase (xanthine dehydrogenase, XDH: 150 kDa; and xanthine oxidase, XO: 130 and 85 kDa) coimmunoprecipitate with nNOS, but not with eNOS, in heart and liver (*A*). Control represents total protein from tissue lysate and reveals little XOR protein. The force frequency response (FFR) is depressed in $nNOS^{-/-}$ compared with WT and $eNOS^{-/-}$ (*B* and *C*, black bars; $^*P < 0.05$). Cell shortening is restored by XOR inhibition with allopurinol (100 μM) (C, grey bars; † $P < 0.05$ vs. $nNOS^{-/-}$ without allopurinol), but the frequency-induced increase in the Ca^{2+} transient amplitude is not (*D*; $^*P < 0.05$ $nNOS^{-/-}$ with and without allopurinol vs. WT and $eNOS^{-/-}$). These data suggest that increased O_2^- production by XOR results in decreased myofilament Ca^{2+} sensitivity at 6 Hz. Modified from reference.[34]

oxidative stress and irreversible activation of RyR2.[46] However, this theory does not explain why FFR attenuation in $nNOS^{-/-}$ should increase at higher stimulation frequencies when diastolic interval (and therefore the opportunity for diastolic Ca^{2+} leak) is shorter. Ziolo and Bers[57] suggested that a decrease in SR Ca^{2+} load at higher stimulation frequencies would be more likely to result from decreased Ca^{2+} reuptake through SERCA2a, probably secondary to a reduction in phospholamban phosphorylation in the $nNOS^{-/-}$ myocardium.[58] Moreover, while the application of allopurinol returned sarcolemma length shortening in $nNOS^{-/-}$ myocytes to wild-type levels, it did not restore the Ca^{2+} transient amplitude or SR Ca^{2+} load (*Figure 5D*).[34] Increased cell shortening without a change in systolic Ca^{2+} suggests that the allopurinol is correcting a XOR-derived, O_2^--mediated reduction in myofilament Ca^{2+} sensitivity. This is in agreement with earlier work in which allopurinol and oxypurinol were shown to improve the force generated by stunned cardiac myofilaments, while at the same time reducing systolic Ca^{2+} transients, thereby enhancing myofilament Ca^{2+} sensitivity.[59] But again, this does not explain why the FFR attenuation in $nNOS^{-/-}$ mice occurred only at high stimulation frequencies.

Other major sources of redox stress are the nicotinamide-adenine dinucleotides NAD^+, NADH, and NADPH. The regulatory effects of these molecules on cardiac and skeletal isoforms of RyR incorporated into planar lipid bilayers were investigated by Zima *et al*.[60] Interestingly, they found that NADH decreased cardiac RyR2 P_{open} and that this was counteracted by NAD^+, but conversely that both NADH and NAD^+ increased the skeletal RyR1 P_{open}. No effect was found for NADPH on either RyR isoform.[60] In a subsequent paper, they confirmed the finding that NADH significantly lowered the Ca^{2+} spark frequency in rat permeabilized cardiomyocytes.[61] Interestingly, they also found that application of just 5 μM of spermine NONOate at low P_{O2} produced 93% inhibition of NADH oxidase activity. Therefore, both RyR2 and NADH oxidase (which co-purifies with SR membrane) appear to be regulated by NO. The direct activating effect of NO on RyR2[46] is augmented by simultaneous inhibition of NADH oxidase, which relieves the negative modulation of NADH on RyR2.[61] Furthermore, the authors have shown that in addition to NADH directly causing an 84% reduction in RyR2 activity, there also appears to be an indirect (O_2^--mediated) NADH inhibition of SERCA2a activity.[62] Finally, it has recently been shown that local production of O_2^- by NAD(P)H oxidase on the SR of coronary artery smooth muscle cells is involved in regulating CICR from the RyR.[63] It should be noted that the mitochondrion, being an important source of both NO and ROS, is also a key modulator of the myocardial redox state (for a review, see Turrens[64]).

7. Indirect, cGMP-dependent modulation of RyR2

So far, direct (poly-)*S*-nitrosylation has been the main mechanism by which NO has been considered to modulate RyR2 P_{open}. *S*-nitrosylation of the skeletal RyR1 isoform is thought to occur at cysteine-3635.[65] This residue is localized to the calmodulin-binding domain of RyR1, and the covalent binding of NO to cysteine-3635 reverses the inhibitory effect of calmodulin on the channel.[27] However, the classical second messenger pathway involving soluble guanylate cyclase—cGMP—protein kinase G (PKG) should not be discounted. PKG has been shown to be capable of phosphorylating RyR *in vitro*,[66] but whether this forms part of a physiological pathway *in vivo* is unknown and likely to be underestimated by experiments in isolated RyR preparations.

Cyclic adenosine diphosphate ribose (cADPR) is thought to be an endogenous modulator of RyR2, and has been shown to increase P_{open}, and the gain of the CICR process in rat and guinea-pig ventricular myocytes.[67] Furthermore, PKG phosphorylates and activates ADP-ribosyl cyclase (the enzyme that synthesises cADPR from β-NAD^+ *in vivo*) or a regulator of the enzyme, in a NO- and cGMP-dependent manner.[68,69] cGMP can also inhibit or activate PDEs that control the concentration of cAMP[70] and thus PKA-mediated phosphorylation. Marx *et al*. suggested that RyR2 phosphorylation by PKA occurs in response to β-adrenergic stimulation and increases RyR2 P_{open}. They also proposed that excessive phosphorylation of RyR2 (hyperphosphorylation) contributed to RyR2 dysregulation and diastolic SR Ca^{2+} leak in HF.[17,71–73]

However, other groups have disputed these findings. Li *et al.* showed that changes in Ca^{2+} spark frequency and amplitude observed after β-adrenergic stimulation were due to an increase in SR Ca^{2+} content and not to direct stimulation of RyR2.[74] Two other groups have failed to detect hyperphosphorylation of RyR2 by PKA in the failing heart.[75] In summary, it is unclear whether PKA phosphorylation of RyR2 plays a role in the modulation of RyR2 function and whether this can be affected by cGMP.

8. Changes in NO modulation of RyR2 in the failing heart

In the failing heart, there is evidence that the physical association between nNOS and RyR2 may decrease. This was first shown in an experimental model of myocardial infarction (MI) in senescent rats.[76] Extracts from non-infarcted hearts showed no immunoreactivity between nNOS and caveolin-3, the scaffolding protein localized to caveolae in the sarcolemma and usually associated with eNOS.[2] After MI, however, the amount of nNOS/cav-3 complexes was dramatically increased. Migration of nNOS from its association with RyR2 on the SR in the healthy myocardium to a localization with caveolin-3 on the sarcolemma post-MI has subsequently been seen in a rat model of MI-induced HF[29] (*Figure 3*) and in the human failing myocardium.[30] Translocation of nNOS from the SR to caveolae may have dual effects on myocardial contractility. First, increased NO production in caveolae may augment feedback inhibition on LTCC,[33] whereas secondly, decreased local NO at the SR may disrupt normal regulation of RyR2 or SERCA2A. Loss of nNOS-derived NO from the SR may not only cause decreased reversible activation of RyR2 by *S*-nitrosylation;[46] it may also remove inhibition of XOR, allowing increased O_2^- production. Furthermore, it has been shown that XOR expression is upregulated in the failing heart,[77] and the resulting oxidative stress may enhance the risk of unregulated and irreversible RyR2 activation by O_2^-, diastolic Ca^{2+} leak, and depletion of the SR Ca^{2+} stores. All these changes are likely to depress cardiac contractility and contribute to the phenotype of HF.

9. Conclusions

In summary, there is increasing evidence suggesting that RyR2 P_{open} may be regulated by NO derived from both eNOS (under conditions of myocardial stretch)[23] and nNOS. RyR2 and nNOS co-immunoprecipitate,[29] RyR2 is constitutively *S*-nitrosylated,[46] and NO has been shown to modulate RyR2 P_{open} (in a PKA activity- and concentration-dependent manner).[47,48] It is also increasingly likely that RNS and ROS interact. For a long time NO has been known to scavenge O_2^- and produce $ONOO^-$. More recently, data have emerged that nNOS and XOR (a major source of O_2^-) physically interact in the murine LV myocardium[34] and that nNOS-derived NO has an inhibitory, and therefore cardioprotective, effect on O_2^- generation by XOR.[52] This may be significant in the failing heart, where it has been shown that nNOS translocates from the SR to the sarcolemma,[29] thus potentially removing the inhibition on XOR and allowing irreversible activation of RyR2 by O_2^-.[46] Of course, XOR is not the only relevant source of redox stress in the heart. NO has also been shown to inhibit NADH oxidase and thereby reduce the inhibitory effect of NADH on RyR2.[61] The interplay between these proteins is important in order to understand the potential therapeutic benefits of, for example, administering NO donors, HNO donors, or XOR inhibitors in HF.

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