Tachycardia increases NADPH oxidase activity and RyR2 *S*-glutathionylation in ventricular muscle

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Abstract

We have shown previously that electrically induced tachycardia effectively produces myocardial preconditioning. Among other effects, tachycardia increases calcium release rates in microsomal fractions enriched in sarcoplasmic reticulum (SR) isolated from dog cardiac ventricular muscle. Here, we report that preconditioning tachycardia increased twofold the NADPH oxidase activity of isolated SR-enriched microsomal fractions, measured as NADPH-dependent generation of superoxide anion and hydrogen peroxide. Tachycardia also augmented the association of rac1 and the NADPH oxidase cytosolic subunit p47[phox] to the microsomal fraction, without modifying the content of the membrane integral subunit gp91[phox]. Microsomes from control animals displayed endogenous *S*-glutathionylation of cardiac ryanodine receptors (RyR2); in microsomal fractions isolated after tachycardia RyR2 *S*-glutathionylation levels were 1.7-fold higher than in controls. Parallel in vitro experiments showed that NADPH produced a transient increase in calcium release rates and enhanced 1.6-fold RyR2 *S*-glutathionylation in control microsomes but had marginal or no effects on microsomes isolated after tachycardia. Catalase plus superoxide dismutase, and the NADPH oxidase inhibitors apocynin and diphenyleneiodonium prevented the in vitro stimulation of calcium release rates and RyR2 *S*-glutathionylation induced by NADPH, suggesting NADPH oxidase involvement. Conversely, addition of reducing agents to vesicles incubated with NADPH markedly inhibited calcium release and prevented RyR2 *S*-glutathionylation. We propose that tachycardia stimulates NADPH oxidase activity, which by enhancing RyR2 redox modifications such as *S*-glutathionylation, would contribute to sustain faster calcium release rates during conditions of increased cardiac activity. This response may be an important component of tachycardia-induced preconditioning.

Keywords: Calcium release channels; Ryanodine receptor; Redox modulation; NOX activity; Reactive oxygen species; Cardiac muscle

1. Introduction

Massive generation of reactive oxygen species (ROS)—such as superoxide anion, hydrogen peroxide and hydroxyl radical—is an important factor in the damage and cell death induced by reperfusion of the ischemic myocardium [1]. Ischemic preconditioning, an adaptive response of the heart to short episodes of ischemia, decreases the damage produced by a prolonged subsequent ischemic episode [2]. Exposure to a low dose of oxygen radicals in the absence of ischemia reproduces the beneficial effects of ischemic preconditioning on infarct size and post-ischemic recovery of left ventricular function [3]. In contrast, antioxidants elimi-

nate the protective effect of ischemic preconditioning [4]. Thus, controlled ROS generation during these short episodes of ischemia and reperfusion is believed to play a role in ischemic preconditioning.

Preconditioning of the myocardium can also be achieved by five short episodes of electrically induced tachycardia [5]. Tachycardia-induced preconditioning increases both the density and the activity of two SR proteins involved in Ca^{2+} handling in heart muscle: the ryanodine receptor/Ca^{2+} release channel (RyR2) and the SERCA2a cardiac isoform of the Ca^{2+}-ATPase [6]. The resulting enhancement of both SR Ca^{2+} release and uptake may be determinant for successful preconditioning.

The primary signals responsible for increased activity and enhanced expression of RyR2 and SERCA2a in response to

tachycardia-induced preconditioning have not been identified. Periods of increased cardiac activity —including those induced by tachycardia—are likely to increase ROS generation. Through covalent redox modifications, ROS can alter the activity of key proteins for cardiac muscle function, such as the RyR2 channels that are particularly sensitive to redox modulation [7–9]. The cardiac NADPH oxidase may be a potential source of ROS generation during tachycardia. The activity of this enzyme has been shown to increase in human cardiomyocytes after acute myocardial infarction [10,11] and during the development of experimental cardiac hypertrophy [12–14].

Thus, the available evidence suggests that the NADPH oxidase plays a role in physiological and pathological conditions in the heart. Accordingly, the aims of this work were 1) to investigate the potential stimulatory effect of electrically induced tachycardia on the cardiac NADPH oxidase activity of isolated SR vesicles and 2) to explore whether tachycardia induces RyR2 redox modifications that may contribute to the enhanced Ca^{2+} release produced by preconditioning tachycardia.

2. Material and methods

2.1. Experimental design

Mongrel dogs of either sex, weighing on average 25 kg, were used to study the preconditioning effect of tachycardia, following previously described protocols in [5,6]. All experiments were done with approval from the Animal Care Committee, Facultad de Medicina, University of Chile, and in accordance with the "Position of the American Heart Association on Research and Animal Use". Dogs were anesthetized with sodium pentobarbital (30 mg kg^{-1}, i.v.); arterial blood pH, pO_2 and pCO_2 were stabilized by the administration of bicarbonate or adjusting the frequency and volume of the ventilator. Tachycardia was induced with five periods of stimulation at 216 ± 29 beats min^{-1} for 5 min, followed by intervening periods of 5 min of stimulation at 91 ± 9 beats min^{-1}; electrocardiograms indicated that this stimulation protocol did not produce ischemia. Controls were stimulated at a rate of 88 ± 5 beats min^{-1} for 50 min. Blood gases and pH were monitored periodically and no changes were observed during tachycardia. The number of animals used in the different determinations is stated in every case.

2.2. Isolation of sarcoplasmic reticulum (SR) vesicles

A microsomal fraction enriched in SR vesicles was isolated as previously described in [6,15], snap frozen in liquid nitrogen and kept at –80 °C. Fractions were used within 7 days after isolation.

2.3. Determination of NADPH oxidase activity

To determine the activity of this enzyme, we measured NADPH oxidation as well as generation of superoxide anion and H_2O_2. NADPH oxidation was measured spectroscopically at 25 °C using the molar extinction coefficient of 6250 l $(mol\ cm)^{-1}$ at 340 nm. Superoxide anion generation was measured by the lucigenin derived chemiluminiscence method in a Berthold FB 12 luminometer. To this aim, SR vesicles (0.2 mg ml^{-1}) were incubated at 25 °C with 100 mmol l^{-1} MOPS-Tris, pH 7.0, plus 100 μmol l^{-1} NADPH and 5 μmol l^{-1} lucigenin. Chemiluminiscence was expressed as nmoles of superoxide anion generated per mg protein per minute; calibration was done using hypoxanthine and xanthine oxidase as described in [16]. Hydrogen peroxide generation was measured at 37 °C using Amplex Red as described in [17]. The effect of diphenyleneiodonium (DPI, 10 μmol l^{-1}), apocynin (4 mmol l^{-1}), rotenone (200 nmol l^{-1}), N^G-nitro-L-arginine methyl ester (L-NAME, 1 mmol l^{-1}), allopurinol (250 μmol l^{-1}) and of the enzymes superoxide dismutase (SOD, 300 U ml^{-1}) or catalase (300 U ml^{-1}) was tested in some experiments.

2.4. Detection of NADPH oxidase subunits in SR membrane fractions

Polyclonal antibodies against p47^{phox} and rac1 were a kind gift from Dr. Frans B. Wientjes (Division of Molecular Medicine, Department of Medicine, University College London, UK). Monoclonal antibodies against gp91^{phox} and p67^{phox} were a kind gift from Dr. Mark Quinn (Veterinary Molecular Biology, Montana State University, USA). A commercial antibody against gp91^{phox} (BD Biosciences, San Diego, CA) was also used in some experiments. To detect gp91^{phox}, p67^{phox} and p47^{phox}, SR fractions (10 μg) were loaded in 8% polyacrylamide gels; for detection of rac1, 10% gels were used. After SDS-PAGE under reducing conditions, proteins were transferred to PVDF membranes and probed with the above antibodies (dilution 1/1500). The antigen–antibody reaction was detected by ECL (Amersham, Biosciences).

2.5. Ca^{2+} release kinetics

SR vesicles (1 mg ml^{-1}) were actively loaded with calcium at 37 °C and calcium release was induced in a SX.18 MV stopped-flow fluorescence spectrometer (Applied Photophysics Ltd., Leatherhead, U.K.) as detailed elsewhere [6,15]. Changes in extravesicular calcium concentration with time were measured with the fluorescent indicator Calcium Green-5 N (Molecular Probes, Eugene, OR). Calcium release time courses followed double exponential kinetics; the rate constants (k) of the faster exponential were 5–10-fold higher than the k values of the slower exponential but both exponentials displayed similar amplitudes. The initial rate of calcium release was calculated from the amplitude and the k value of the faster exponential component. To investigate the effect of NADPH on release kinetics, 100 μmol l^{-1} of NADPH was added to the vesicles during the last 10 min of active calcium loading. When tested, 10 μmol l^{-1} of hydrogen peroxide was added 1 min before inducing Ca^{2+} release.

2.6. Detection of RyR2 S-glutathionylation

To detect endogenous *S*-glutathionylation, SR vesicles (30 μg) were incubated in non-reducing loading buffer plus 5 mmol l^{-1} N-ethylmaleimide at 60 °C for 20 min and separated by SDS-PAGE in 3.5–8% gradient gels under non-reducing conditions. After electrophoresis and transfer to PDF membranes, proteins were probed with anti-glutathione (anti-GSH) antibody (1:10000) from Virogen (Watertown, MA). After ECL detection of the antigen–antibody reaction, membranes were stripped and probed with anti-RyR antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were quantified by densitometric analysis using the Quantity One software (BioRad, Hercules, CA). Results were expressed as the ratio of anti-GSH/anti-RyR band densities. To detect NADPH-promoted *S*-glutathionylation, SR vesicles (1 mg ml^{-1}) were incubated at 37 °C for 10 min in a solution containing (mmol l^{-1}) 0.1 NADPH, 100 MOPS Tris pH 7.0.

2.7. Labeling of SR proteins with [^{35}S]-GSH

Vesicles were incubated for 10 min at 37 °C in a solution containing 100 mmol l^{-1} MOPS-Tris, pH 7.2, 100 μmol l^{-1} NADPH plus [^{35}S]-GSH as tracer. The reaction was terminated by addition of non-reducing sample buffer plus 5 mmol l^{-1} N-ethylmaleimide. SR proteins (30 μg) were separated in 3.5–8% non-reducing gels as above. Gels were stained with Coomassie Blue and the ^{35}S radioactivity incorporated into proteins was determined in a Molecular Imager FX system (BioRad) using a Phosphor Screen CP (Kodak, Rochester, NY).

2.8. Other procedures

The total glutathione content of the SR fraction was determined according to Griffith [18]. Protein concentration was determined according to Hartree [19] using commercial bovine serum albumin as standard. All reagents were of analytical grade. Ryanodine and protease inhibitors were obtained from Sigma Chemical Company (St Louis, MO).

2.9. Statistical analysis

Results are expressed as Mean ± S.E.M. For statistical analysis we used Student's *t*-test or one-way ANOVA followed by Tukey's Multiple comparison test. Differences were considered significant at *P* < 0.05.

3. Results

3.1. NADPH oxidase activity

We measured initial rates of NADPH oxidation and NADPH-dependent superoxide anion and hydrogen peroxide generation in SR-enriched microsomal fractions isolated from dog cardiac ventricular muscle of control animals or after inducing preconditioning tachycardia. We found that NADPH oxidation rates increased from 10.0 ± 2.9 nmol mg^{-1} min^{-1} in controls to 26.3 ± 6.6 nmol mg^{-1} min^{-1} after tachycardia (Fig. 1A, *P* < 0.05, *N* = 6 per group). Likewise, tachycardia doubled the initial rate of NADPH-dependent superoxide anion generation, from 10.1 ± 1.4 to 20.0 ± 2.2 nmol mg^{-1} min^{-1} (Fig. 1B, *P* < 0.05, *N* = 6 per group). Similarly, the rate of NADPH-dependent H$_2$O$_2$ generation measured at 37 °C increased from 15.2 ± 2.2 to 24.9 ± 0.4 nmol mg^{-1} min^{-1} after tachycardia (Fig. 1C, *P* < 0.05, *N* = 6 per group). When 100 μmol l^{-1} NADH was used as a substrate the initial rate of superoxide anion generation in controls was only 10–20% of the rate obtained with 100 μmol l^{-1} NADPH, indicating that in vitro NADPH is a better substrate of the cardiac NADPH oxidase than NADH.

In order to verify the source of superoxide anion and hydrogen peroxide generation, we tested the effect of different inhibitors. We found that L-NAME (a nitric oxide synthase inhibitor), allopurinol (a xanthine oxidase inhibitor), or rotenone (a mitocondrial inhibitor) did not inhibit the production of superoxide anion (Fig. 1D). In contrast, DPI, a non-specific flavoprotein inhibitor, decreased the production of superoxide anion by 85%, whereas the more specific NADPH oxidase inhibitor apocynin produced 52% inhibition (Fig. 1D). Apocynin also produced a similar degree of inhibition of hydrogen peroxide generation (not shown).

3.2. Detection of NADPH oxidase subunits in cardiac SR vesicles

In addition to membrane integral subunits, most NADPH oxidase isoforms possess cytosolic subunits that are recruited to the membrane upon activation. We found (Fig. 2A) that the isolated microsomal SR-enriched fraction used in this work contained the integral subunit gp91^{phox} and two of the cytosolic subunits, p67^{phox} and p47^{phox}, as well as of the small G protein rac1 that is required for the activation of some NADPH oxidase isoforms [20]. Equal amounts of the membrane integral subunit gp91^{phox} were present in SR vesicles from controls and after tachycardia (Fig. 2B, *N* = 6). In contrast, rac1 and the cytosolic subunit p47^{phox} increased over twofold in SR vesicles isolated after tachycardia (Fig. 2B, *P* < 0.05, *N* = 6). The cytosolic subunit p67^{phox} increased somewhat after tachycardia, but the increase was not statistically significant (Fig. 2B, *N* = 6).

These results, which are consistent with the increased NADPH oxidation and superoxide anion/hydrogen peroxide production displayed by SR-enriched fraction isolated after tachycardia, strongly suggest that tachycardia increases the generation of ROS through NADPH oxidase activation.

The microsomal SR-enriched fraction used in this work contained attached sarcolemmal/transverse tubule membranes, as evidenced by the binding densities of [^{3}H]-nitrendipine (2.4 nmol mg^{-1} protein) and [^{3}H]-ouabain (3.5 nmol mg^{-1} protein). Furthermore, these plasma mem-
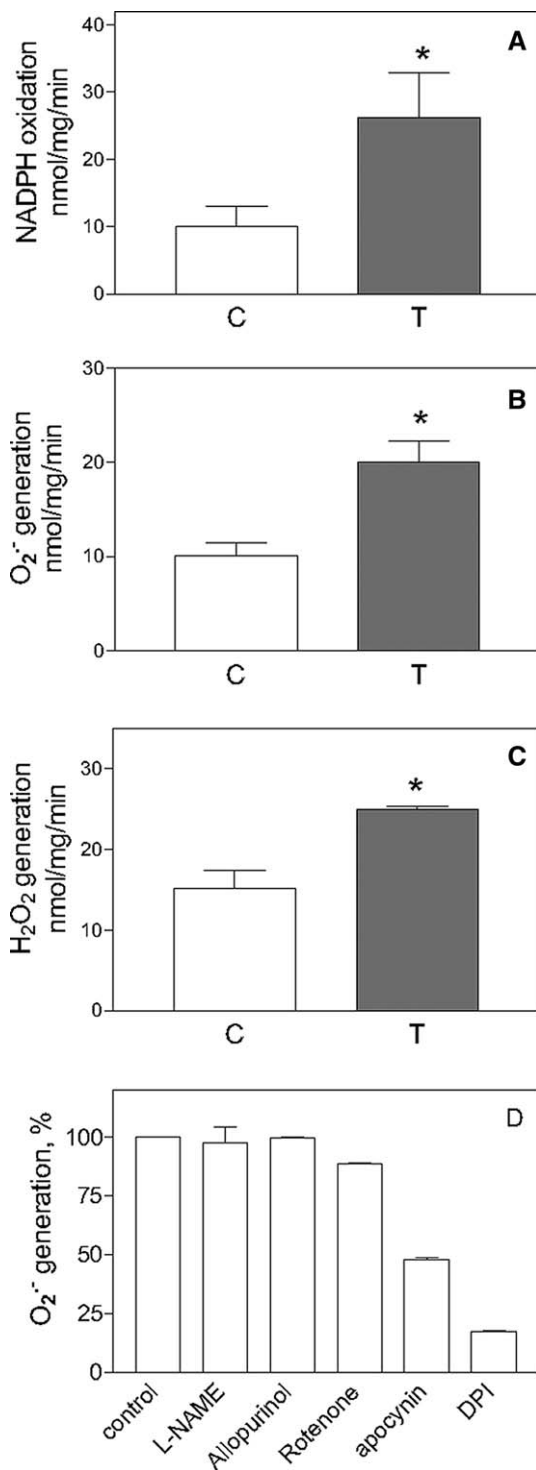
Fig. 1. Tachycardia increases NADPH oxidase activity. Initial rates of NADPH oxidation (**panel A**), superoxide anion generation (**panel B**) and hydrogen peroxide generation (**panel C**) were determined in SR vesicles isolated from control (open bars) and after electrically induced tachycardia (solid bars) * $P < 0.05$, $N = 6$. **Panel D**: percentage of the initial rate of superoxide anion generation after addition of the indicated inhibitors ($N = 3$ in each case). For further details, see text.

brane components remained associated to SR after centrifugation in sucrose density gradients. Therefore, although we cannot discriminate whether the measured NADPH oxidase activity is associated to SR or plasma membranes, in either case the NADPH oxidase generates ROS in close proximity to RyR2 channels.

3.3. Endogenous S-glutathionylation of RyR2 from controls and after tachycardia

Protein *S*-glutathionylation, i.e. the formation of mixed disulfides between GSH and cysteine SH residues, has been shown to increase after oxidative stress in isolated cardiomyocytes and hearts [21,22]. RyR channels contain highly reactive cysteine residues that can undergo redox modifications in vitro [8,9]. Therefore we investigated if tachycardia induced *S*-glutathionylation of cysteine residues of cardiac RyR in vivo. Using anti-GSH antibodies, we found that RyR and several other proteins present in the isolated SR fraction were endogenously *S*-glutathionylated both in control (Fig. 3A, lane 1) and after electrically induced tachycardia (Fig. 3A, lane 3). *S*-glutathionylation was completely abolished by incubation of vesicles with DTT (Fig. 3A, lanes 2 and 4, control and tachycardia, respectively), indicating that this redox modification can be readily reversed. A single high MW band was recognized by anti-RyR antibody after stripping the membrane (Fig. 3A, lanes 1′, 2′, 3′ and 4′). Incubation with DTT increased the amount of RyR detected in the Western blot (lanes 2′ and 4′), probably because DTT disrupted disulfide bridges between RyR subunits or with other proteins that prevented RyR entry into the gel. To correct for any difference in protein content, *S*-glutathionylation was normalized by the RyR content of the same band determined with anti-RyR. As shown in Fig. 3B, tachycardia increased *S*-glutathionylation of RyR by 60% compared to controls ($P < 0.05$, $N = 6$).

3.4. In vitro S-glutathionylation of RyR2

It has been reported that GSH acts as a cofactor for the stimulation of calcium release mediated by H_2O_2 in cardiac myocytes [23]. Noteworthy, protein *S*-glutathionylation, including RyR1 *S*-glutathionylation, can be induced in vitro by incubation with GSH plus H_2O_2 [24,25]. The SR fraction used in this work contained 0.48 ± 0.3 nmol mg^{-1} protein of total GSH (Mean $\pm$ S.D., $N = 3$). Therefore we tested if H_2O_2—NADPH oxidase generated—plus endogenous GSH supported *S*-glutathionylation of RyR2 in vitro. Fig. 3C shows that RyR2 *S*-glutathionylation increased 70%, on average, after incubation of control vesicles with 100 µmol l^{-1} NADPH. SR vesicles incubated with NADPH in the presence of DPI (not shown) or apocynin (Fig. 3C) did not present increased RyR2 *S*-glutathionylation relative to the controls; likewise, apocynin alone was without effect on the basal level of *S*-glutathionylation (Fig. 3C).

We also verified RyR2 *S*-glutathionylation through the incorporation of ^{35}S from [^{35}S]-GSH into RyR2. Control vesicles were incubated with 100 µmol l^{-1} NADPH in the presence of tracer amounts of [^{35}S]-GSH and ^{35}S-label incorporation was analyzed in a phosphorimager system. Fig. 4A
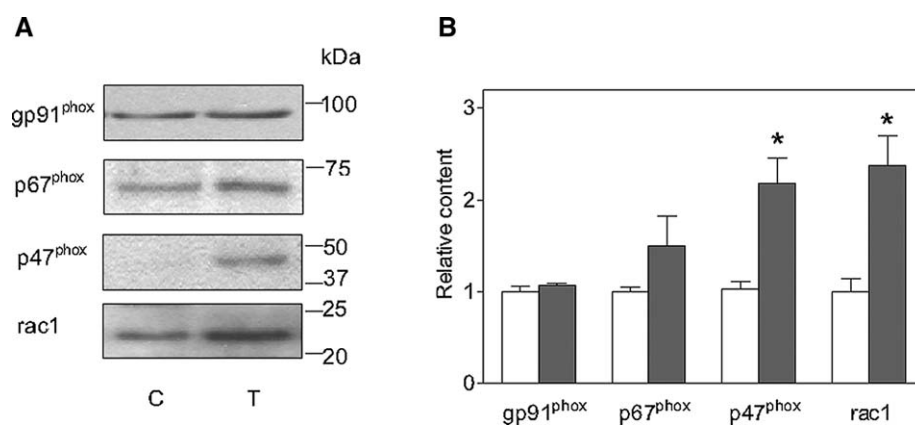
Fig. 2. Tachycardia increases the association of the cytosolic subunits of NADPH oxidase to the SR-enriched membrane fraction. **Panel A**: Representative Western blots of cardiac SR vesicles isolated from control (C) and after tachycardia (T). The amount of protein loaded per lane was 10 μg in each case. **Panel B**: Densitometric analysis of Western blots like those shown in Panel A. * $P < 0.05$, $N = 6$ for control (open bars) and tachycardia (solid bars).

illustrates a Coomassie blue-stained gel of SR vesicles incubated without NADPH (lane 1) with NADPH (lane 2) or with NADPH plus DTT (lane 3). The corresponding radioactive image of the same gel shows that ^{35}S incorporation into RyR2 occurred only when the incubation solution contained NADPH (Fig. 4A, lane 2′). Almost no radioactivity was incorporated into RyR2 in the absence of NADPH (Fig. 4A, lane 1′) or when DTT was included during the incubation with NADPH (Fig. 4A, lane 3′). The role played by ROS in the radioactive labeling of RyR2 is confirmed by the results shown in Fig. 4B. The presence of SOD and catalase during the incubation with NADPH decreased the incorporation of [^{35}S]-glutathionyl residues into RyR2 (lane 5′, Fig. 4B) when compared to the incorporation produced solely by NADPH (lane 4′, Fig. 4B). On average less that 10% of this RyR2 radioactive labeling remained in the presence of SOD plus catalase (Fig. 4C).

3.5. Effect of NADPH on Ca^{2+} release kinetics

We have shown before that microsomal SR-enriched fractions isolated after tachycardia display faster calcium release kinetics than controls [6], denoting higher activity, higher content of RyR2, or both. Representative release records obtained for both types of vesicles are shown in Fig. 5A. Calcium release was transient and after < 0.5 s SR vesicles reverted from calcium release to calcium uptake, as shown in Fig. 5B. These results show that despite the higher initial rate of calcium release observed after tachycardia, the calcium release process ceased as rapidly in vesicles isolated after tachycardia as in controls, allowing SR refilling. Thus, the stimulation of RyR2 activity produced by tachycardia does not seem to affect the process whereby RyR2 rapidly close after opening [15].

Addition of thapsigargin eliminated calcium uptake [6] and preincubation with 100 μM Ryanodine abolished calcium release (not shown). These results, which indicate that all the calcium accumulated by the SR membrane fraction is taken up by the SERCA and that calcium is released solely through RyR2, rule out eventual transverse tubule contribution to uptake and release.

Phosphorylation of RyR2 has been shown to increase the activity of single channels in lipid bilayers [26] as well as calcium release kinetics in isolated cardiac myocytes [27]. To investigate if a higher degree of RyR2 phosphorylation contributed to the faster release rates observed after tachycardia, we measured the incorporation of ^{32}P from [γ-^{32}P]-ATP into RyR2 during or after active calcium loading. Both RyR2 in tachycardic or control SR had similar ^{32}P incorporation during active loading. Addition of [γ-^{32}P]-ATP after 20 min of active calcium loading did not result in incorporation of ^{32}P into RyR2 from controls or after tachycardia. We interpret these results as an indication that in both types of vesicles RyR2 became equally and maximally phosphorylated by endogenous kinases during active calcium loading. Therefore, the higher release rate observed in vesicles obtained after tachycardia cannot be attributed to a different degree of RyR2 phosphorylation. Different extents of calcium loading can also be discarded; both types of vesicles removed all the extravesicular calcium from the loading solution and thus attained the same calcium load (45 nmol mg^{-1}) before mixing with the release solution.

The present results show that RyR2 exhibited increased *S*-glutathionylation after tachycardia. Thus, we investigated if the stimulation of RyR2 *S*-glutathionylation promoted in vitro by NADPH caused an increased rate of calcium release in SR-enriched microsomal fractions isolated from controls. To this purpose vesicles were incubated with 100 μmol l^{-1} NADPH after active calcium loading and before inducing release. In the experiment shown in Fig. 5C, incubation of control vesicles with NADPH increased by 40% the magnitude of the faster component of calcium release and increased its rate constant from 113 to 200 s^{-1}. These values imply that NADPH produced a 2.5-fold increase in the initial rate of calcium release (calculated as the magnitude of release multiplied by the rate constant value). In contrast, NADPH had no effect after tachycardia (Fig. 5D), since NADPH did not affect the magnitude of release and on average, k values in
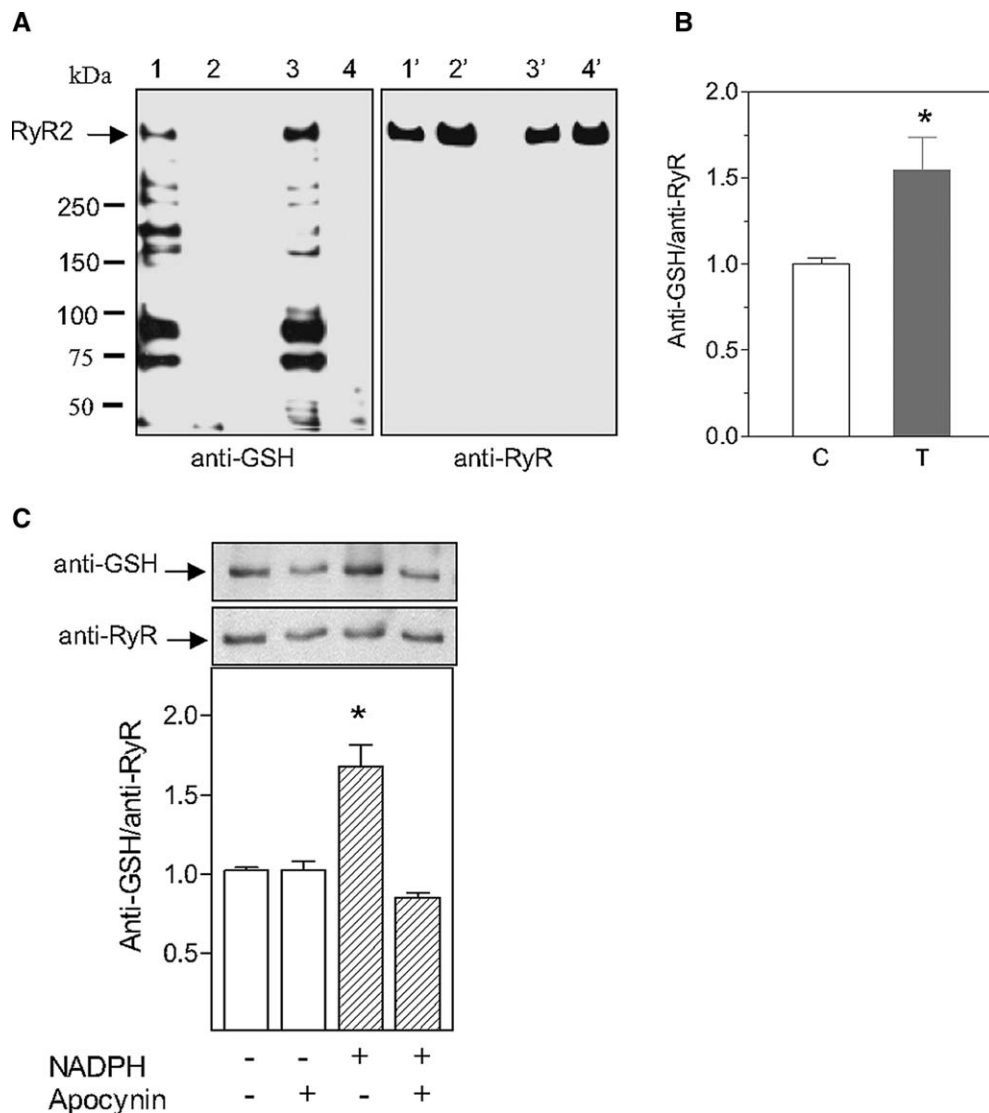
Fig. 3. *S*-glutathionylation of RyR2. **Panel A**: Representative Western blots obtained in SR vesicles from controls probed with anti-GSH antibody (lanes 1, 2) or anti-RyR antibody (lanes 1′, 2′), or from SR vesicles after tachycardia, probed with anti-GSH (lanes 3, 4) or anti-RyR (lanes 3′, 4′). Lanes 2, 2′ and 4, 4′ were obtained after addition of DDT (100 mmol l⁻¹) to the protein samples. **Panel B**: The ratio anti-GSH/anti-RyR was calculated from the analysis by densitometry of Western blots like those shown in A; values were normalized with respect to controls. $P < 0.05$, $N = 6$ for control (C) and tachycardia (T). **Panel C**: RyR2 *S*-glutathionylation in controls (open bars) in the absence ($N = 5$) or in the presence of apocynin (4 mmol l⁻¹; $N = 3$), and with NADPH (100 µmol l⁻¹, hatched bars) in the absence ($N = 6$) or in the presence of apocynin (4 mmol l⁻¹; $N = 3$) *$P < 0.05$.

tachycardia were (in s⁻¹) 165 ± 9 ($N = 18$) before and 163 ± 19 ($N = 5$) after addition of NADPH. Addition of DTT (250 µmol l⁻¹) during the incubation with NADPH greatly inhibited calcium release in vesicles isolated from both controls and after tachycardia (Fig. 5C, D).

In contrast to NADPH, addition of 100 µmol l⁻¹ NADH, which as described above is a poor substrate of the cardiac NADPH oxidase, did not modify calcium release rates in control SR; yet, as reported [28] increasing NADH to 1 mM decreased calcium release rates (not shown). Incubation of control SR vesicles with 1 mM glutathione (GSH), a reducing agent present in millimolar concentrations in cardiac cells, inhibited rather than activated calcium release in the absence of NADPH (Fig. 5E). The inhibitory effect of GSH is consistent with its ability to maintain RyR2 SH residues in a reduced state, a condition that favors RyR2 closing [7–9]. In contrast,

as reported previously [29] incubation of SR vesicles with H_2O_2 (10 µmol l⁻¹) increased considerably calcium release rates (Fig. 5F).

The effects of NADPH on the rates of calcium release from control SR vesicles are summarized in Fig. 6. On average, and considering all experiments, incubation with NADPH increased the magnitude of the faster component of calcium release by 30% and its rate constant (k) from 113 ± 6.4 s⁻¹ ($N = 19$) to 190 ± 16 s⁻¹ ($N = 5$), resulting in an average two-fold increase in the initial rate of calcium release ($P < 0.05$). The stimulation by NADPH was prevented by SOD and catalase, implying involvement of superoxide anion and hydrogen peroxide. In contrast, addition of SOD and catalase in the absence of NADPH did not modify the initial rates of calcium release. Similarly, apocynin (4 mmol l⁻¹) abolished the observed enhancement in calcium release produced by
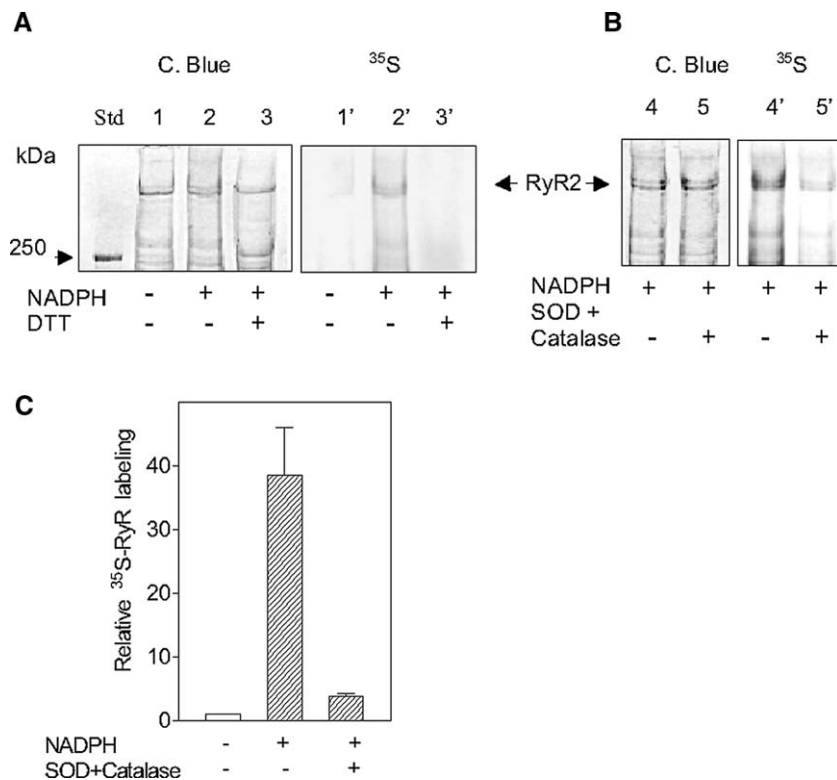
Fig. 4. Incorporation of the [35S]-glutathionyl residue into RyR2. **Panel A, Left**: Coomassie blue stained non-reducing SDS-containing gel of control SR proteins incubated with [35S]-GSH. Lane 1: control; Lane 2: vesicles incubated with 100 µmol l^{-1} NADPH; Lane 3: vesicles incubated with 100 µmol l^{-1} NADPH plus 100 mM l^{-1} DTT. **Panel A, Right**: 35S-radioactivity obtained with a phosphorimager of the same gel shown in Panel A, left. **Panel B, Left**: Coomassie blue stained non-reducing SDS-containing gel of control SR proteins incubated with [35S]-GSH and 100 µmol l^{-1} NADPH in the absence (lane 4) or in the presence of SOD and catalase (lane 5). **Panel B, Right:** 35S-radioactivity obtained with a phosphorimager of the same gel shown in Panel B, Left. **Panel C**: Quantification of the data shown in A and B. Values were normalized with respect to controls. *$P < 0.05$.

NADPH without changing calcium release rates in the absence of NADPH, whereas the nitric oxide synthase inhibitor L-NAME did not modify calcium release before or after incubation with NADPH (Fig. 6).

4. Discussion

The present results show that a microsomal cardiac fraction enriched in SR isolated after preconditioning tachycardia displayed increased NADPH oxidase activity, suggesting that stimulation of this enzyme may be a key feature of preconditioning tachycardia. Noteworthy, a recent preliminary report [30] shows that ischemic preconditioning does not occur in NADPH oxidase gp91phox subunit-knockout mice, suggesting that this enzyme has a crucial role in cardiac ischemic preconditioning.

Previous studies performed in isolated hearts have shown that interventions that modify the cellular redox status, such as simulated ischemia, metabolic inhibition or treatment with low concentrations of hydrogen peroxide, increase disulfide bonding and *S*-glutathionylation of cellular proteins [21,22]. Yet, isolated organs and cells are devoid of normal circulation and experiments with isolated systems are usually performed under higher oxygen tension than present in the tissue. For this reason, it was relevant to study whether redox

modifications such as *S*-glutathionylation can also occur in vivo under stressful heart conditions such as preconditioning tachycardia.

We found that RyR2 channels from dog cardiac ventricular muscle are *S*-glutathionylated in vivo, and that RyR2 in vesicles isolated after tachycardia had increased *S*-glutathionylation levels. To our knowledge, this is the first study to report endogenous *S*-glutathionylation of cardiac RyRs and the increase in *S*-glutathionylation produced by electrically induced tachycardia in vivo. This redox modification of RyR2 release channels might be responsible, at least in part, for the stimulation of calcium release rates displayed by SR vesicles after tachycardia [6]. Thus, enhanced RyR2 *S*-glutathionylation may represent an important mechanism to sustain faster rates of calcium release in vivo in response to increased cardiac activity. Yet, it is important to point out that both RyR2 from controls or after tachycardia closed equally fast following activation. Thus, the stimulation of calcium release rates induced by tachycardia should not affect calcium re-uptake into the SR during diastole.

Preconditioning tachycardia produced a parallel increase in NADPH oxidase activity and RyR2 *S*-glutathionylation. These results suggest that increased ROS production by this enzyme was responsible for the increase in RyR2 *S*-glutathionylation, with the consequent stimulation of RyR2-mediated calcium release reported earlier [6] and confirmed
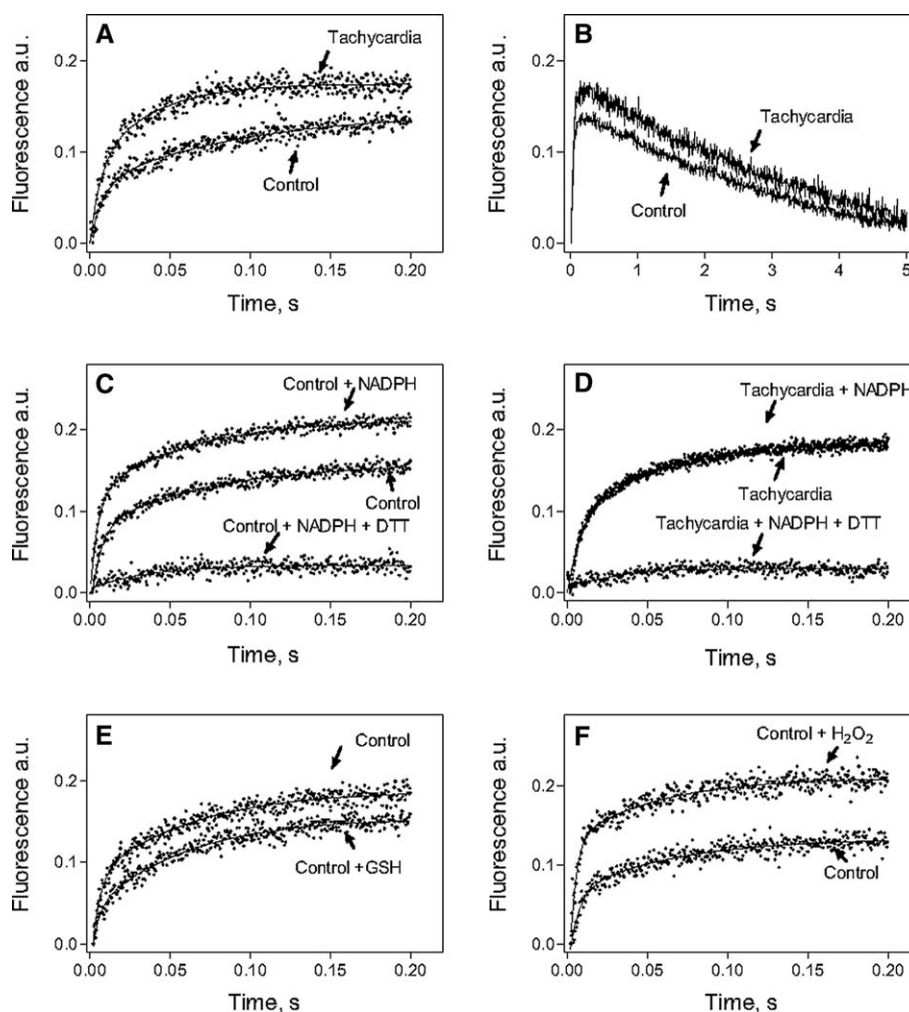
Fig. 5. Calcium release kinetics in control and after tachycardia. SR vesicles (1 mg ml^{-1}) actively loaded with Ca^{2+} were mixed (1:10) in a stopped flow fluorescence spectrometer with a solution that produced upon mixing pCa 6 and 1.2 mmol l^{-1} free ATP. Release kinetics was measured following the change in fluorescence of Calcium Green 5 N and were fitted to the double exponential function: A$_1$exp (–k$_1$t) + A$_2$exp (–k$_2$t). **Panels A and B**: Representative fluorescent records illustrating calcium release kinetics in control vesicles and after tachycardia in two different time frames. The parameters for the faster exponential release were A$_1$ = 0.06 units; k$_1$ = 122 s^{-1} for control and A$_1$ = 0.08 units; k$_1$ = 157 s^{-1} for tachycardia. **Panel C**: Calcium release kinetics in SR vesicles from control before and after incubation with 100 μmol l^{-1} NADPH or with 100 μmol l^{-1} NADPH plus 250 μmol l^{-1} DTT. The parameters for the faster exponential release were A$_1$ = 0.085 units; k$_1$ = 113 s^{-1} in the absence of NADPH and A$_1$ = 0.120 units; k$_1$ = 200 s^{-1} after incubation with NADPH. **Panel D**: Calcium release kinetics in SR vesicles after tachycardia before and after incubation with 100 μmol l^{-1} NADPH or 100 μmol l^{-1} NADPH plus 250 μmol l^{-1} DTT. **Panel E**: Effect on calcium release kinetics of incubation of control SR vesicles with 1 mM GSH, before calcium loading. **Panel F**: Stimulation of calcium release kinetics from control SR vesicles by 10 μmol l^{-1} H$_2$O$_2$.

here. This proposed role for the NADPH oxidase in cardiac signal transduction in vivo is further supported by the present in vitro experiments showing an increase in RyR2 *S*-glutathionylation and calcium release rates in SR vesicles from control animals upon incubation with NADPH. The fact that NADPH did not increase in vitro calcium release rates in SR vesicles obtained from animals subjected to tachycardia (Fig. 5, panel D) suggests that tachycardia increases in vivo RyR2 *S*-glutathionylation to its maximal extent, at least in terms of its effects on calcium release rates.

We found that RyR2 *S*-glutathionylation increased in vitro upon incubation of control SR vesicles with NADPH. This result suggests that ROS generated by the NADPH oxidase, together with the endogenous glutathione present in the vesicles, promote RyR2 *S*-glutathionylation. Further supporting this proposal is the fact that RyR2 *S*-glutathionylation

induced by NADPH in vitro was inhibited by apocynin, a specific inhibitor of NADPH oxidase. Apocynin also blocked the increase in calcium release rates induced by NADPH. In contrast, an inhibitor of nitric oxide synthase did not affect the stimulation of calcium release produced by NADPH, making highly unlikely the participation of nitric oxide synthase in this response. The lack of effect on superoxide anion generation of rotenone, a mitochondrial electron transport blocker, and of a xanthine oxidase inhibitor also suggests that NADPH enhances ROS formation via the NADPH oxidase and not through stimulation of other ROS-producing enzymes. In addition, the lack of effect of rotenone confirms our previous results showing negligible contamination of our isolated cardiac SR fraction with mitochondria [6].

A number of studies have shown that RyRs are modulated by their redox state [7–9,31]; this property makes RyR poten-
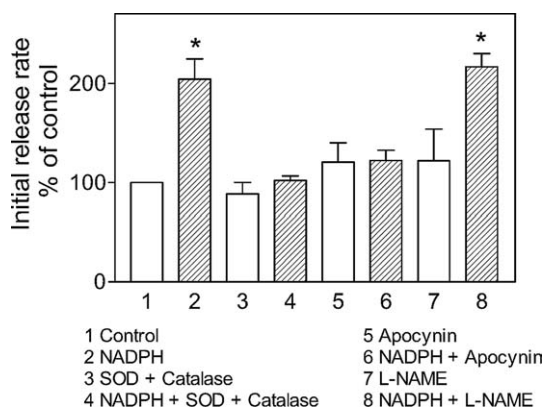
Fig. 6. Effect of various inhibitors on the initial rates of calcium release. The initial rate of calcium release was enhanced following incubation of control SR vesicles with NADPH (100 μmol l^{-1}; $N = 5$). The rates of release were also determined after addition of SOD (300 U ml^{-1}) plus catalase (300 U ml^{-1}; $N = 4$); apocynin (4 mmol l^{-1}; $N = 4$) or L-NAME (1 mmol l^{-1}; $N = 3$) in the absence (open bars) or the presence of 100 μmol l^{-1} NADPH (hatched bars). The results are expressed as percent of the control. *$P < 0.05$.

tial redox sensors, as recently discussed [32]. In particular *S*-glutathionylation of skeletal RyR1 causes significant stimulation of channel activity [25,33]. In addition to being endogenously *S*-glutathionylated, as reported here, RyR2 channels are also endogenously *S*-nitrosylated and this modification activates single channel activity in lipid bilayers [34]. It remains to be studied whether the stimulatory effect of *S*-glutathionylation on calcium release is due to activation of the RyR2 protein itself or to modifications of inhibitory interactions with RyR2-associated proteins, as shown for RyR1 [25]. *S*-glutathionylation of critical cysteine residues could constitute a protective mechanism against the irreversible oxidation of the RyR2 protein, as it does for a number of other proteins [35], since extensive oxidation of RyR2 cysteine residues produces in vitro uncontrolled calcium release and eventually inactivates this protein [31]. Furthermore, the increased calcium release caused by *S*-glutathionylation of RyR2 may enhance calcium-dependent gene expression [36]; it is interesting to recall in this regard that tachycardia enhances the expression of RyR2 and SERCA2a [6]. All these mechanisms could possibly operate in the heart and may serve a protective role during preconditioning [37,38].

The mechanisms responsible for tachycardia-induced activation of NADPH oxidase in the heart remain to be investigated. In leukocytes—the best-known system—the NADPH oxidase is activated via PKC dependent phosphorylation of the cytosolic subunit p47phox and the prenylation of Rac that occurs downstream of phosphatidylinositol-3-kinase (PI3K) activation [39]. In vascular cells, Angiotensin II activates the NADPH oxidase via AT1 receptors, which trigger a signaling cascade involving PI3K and PKC [40]. The stimulation protocol used in this work may activate similar signal transduction pathways but more work is needed to establish the particular pathway involved in the NADPH oxidase activation induced by tachycardia.

References

[1] Becker LB. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. Cardiovasc Res 2004;61:461–70.

[2] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124–36.

[3] Tritto I, D'Andrea D, Eramo N, Scognamiglio A, De Simona C, Violante A, et al. Oxygen radicals can induce preconditioning in rabbit hearts. Circ Res 1997;80:743–8.

[4] Chen W, Gabel S, Steenbergen C, Murphy E. A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. Circ Res 1995;77:424–9.

[5] Domenech RJ, Macho P, Vélez D, Sánchez G, Liu X, Dhalla N. Tachycardia preconditions infarct size in dogs. Role of adenosine and protein kinase C. Circulation 1998;97:786–94.

[6] Domenech RJ, Sánchez G, Donoso P, Parra V, Macho P. Effect of tachycardia on myocardial sarcoplasmic reticulum and Ca^{2+} dynamics: a mechanism for preconditioning? J Mol Cell Cardiol 2003;35:1429–37.

[7] Morad M, Suzuki YJ. Redox regulation of cardiac muscle calcium signaling. Antioxid Redox Signal 2000;2:65–71.

[8] Meissner G. Molecular regulation of cardiac ryanodine receptor. Cell Calcium 2004;35:621–8.

[9] Hidalgo C, Bull R, Behrens MI, Donoso P. Redox regulation of RyR-mediated Ca^{2+} release in muscle and neurons. Biol Res 2004;37: 539–52.

[10] Krijnen PA, Meischl C, Hack CE, Meijer CJ, Visser CA, Roos D, et al. Increased Nox2 expression in human cardiomyocytes after acute myocardial infarction. J Clin Pathol 2003;56:194–9.

[11] Fukui T, Yoshiyama M, Hanatani A, Omura T, Yoshikawa J, Abe Y. Expression of p22-phox and gp91-phox, essential components of NADPH oxidase, increases after myocardial infarction. Biochem Biophys Res Commun 2001;281:1200–6.

[12] Byrne JA, Grieve DJ, Bendall JK, Li JM, Gove C, Lambeth JD, et al. Contrasting roles of NADPH oxidase isoforms in pressure-overload versus angiotensin II-induced cardiac hypertrophy. Circ Res 2003;93: 802–5.

[13] Bendall JK, Cave AC, Heymes C, Gall N, Shah AM. Pivotal role of a gp91^{phox}-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. Circulation 2002;105:293–6.

[14] Li JM, Gall NP, Grieve DJ, Chen M, Shah AM. Activation of NADPH oxidase during progression of cardiac hypertrophy to failure. Hypertension 2002;40:477–84.

[15] Sanchez G, Hidalgo C, Donoso P. Kinetic studies of calcium-induced calcium release in cardiac sarcoplasmic reticulum vesicles. Biophys J 2003;84:2319–30.

[16] Javesghani D, Magder SA, Barreiro E, Quinn MT, Hussain SN. Molecular characterization of a superoxide-generating NAD(P)H oxidase in the ventilatory muscles. Am J Respir Crit Care Med 2002;165: 412–8.

[17] Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal Biochem 1997;253:162–8.

[18] Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 1980;106:207–12.

[19] Hartree EF. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal Biochem 1972;48:422–7.

[20] Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase. Role in cardiovascular biology and disease. Circ Res 2000;86:494–501.

[21] Eaton P, Byers HL, Leeds N, Ward MA, Shattock MJ. Detection, quantitation, purification, and identification of cardiac proteins *S*-thiolated during ischemia and reperfusion. J Biol Chem 2002;277:9806–11.

[22] Brennan JP, Wait R, Begum S, Bell JR, Dunn MJ, Eaton P. Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. J Biol Chem 2004;279:41352–60.

[23] Suzuki YJ, Cleemann L, Abernethy DR, Morad M. Glutathione is a cofactor for H_2O_2-mediated stimulation of Ca^{2+}-induced Ca^{2+} release in cardiac myocytes. Free Radic Biol Med 1998;24:318–25.

[24] Li J, Huang FL, Huang K-P. Glutathiolation of proteins by glutathione disulfide *S*-oxide derived from *S*-nitrosoglutathione. Modifications of rat brain neurogranin/Rc3 and neuromodulin/Gap-43. J Biol Chem 2001;276:3098–105.

[25] Aracena P, Tang W, Hamilton SL, Hidalgo C. Effects of *S*-glutathionylation and *S*-nitrosylation on calmodulin binding to triads and FKBP-12 binding to type 1 calcium release channels. Antiox Redox Signal 2005;7:870–81.

[26] Valdivia HH, Kaplan JH, Ellis-Davies GC, Lederer WJ. Rapid adaptation of cardiac ryanodine receptors: modulation by Mg^{2+} and phosphorylation. Science 1995;267:1997–2000.

[27] Ginsburg KS, Bers DM. Modulation of excitation–contraction coupling by isoproterenol in cardiomyocytes with controlled SR Ca^{2+} load and Ca^{2+} current trigger. J Physiol 2004;556:463–80.

[28] Cherednichenko G, Zima AV, Feng W, Schaefer S, Blatter LA, Pessah IN. NADH oxidase activity of rat cardiac sarcoplasmic reticulum regulates calcium-induced calcium release. Circ Res 2004;94:478–86.

[29] Goldhaber JL, Liu E. Excitation–contraction coupling in single guinea-pig ventricular myocytes exposed to hydrogen peroxide. J Physiol 1994;477:135–47.

[30] Bell RM, Cave AC, Johar S, Shah AM, Shattock MJ. The role of a gp91[phox]-containing NADPH oxidase in early ischemic preconditioning. Abstract. Circulation 2003;108 (IV-46).

[31] Marengo JJ, Hidalgo C, Bull R. Sulfhydryl oxidation modifies the calcium dependence of ryanodine-sensitive calcium channels of excitable cells. Biophys J 1998;74:1263–77.

[32] Hidalgo C, Donoso P, Carrasco MA. The ryanodine receptors Ca^{2+} release channels: cellular redox sensors? IUBMB Life 2005;57:315–22.

[33] Aracena P, Sánchez G, Donoso P, Hamilton SL, Hidalgo C. *S*-glutathionylation decreases Mg^{2+} inhibition and *S*-nitrosylation enhances Ca^{2+} activation of RyR1 channels. J Biol Chem 2003;278:42927–35.

[34] Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. Science 1998;279:234–7.

[35] Giustarini D, Rossi R, Milzani A, Colombo R, Dalle-Donne I. *S*-glutathionylation: from redox regulation of protein functions to human diseases. J Cell Mol Med 2004;8:201–12.

[36] Berridge MJ, Bootman MD, Roderick HL. Calcium signaling; dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 2003;4:517–29.

[37] Das DK, Maulik N. Preconditioning potentiates redox signaling and converts death signal into survival signal. Arch Biochem Biophys 2003;420:305–11.

[38] Eaton P, Bell RM, Cave AC, Shattock MJ. Ischemic preconditioning: a potential role for protein S-thiolation? Antiox Redox Sig 2005;7:882–8.

[39] Quinn MT, Gauss KA. Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. J Leukoc Biol 2004;76:760–81.

[40] Cai H, Griendling KK, Harrison DG. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. Trends Pharmacol Sci 2003;24:471–8.