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Exercise and tachycardia increase NADPH oxidase and ryanodine receptor-2 activity: possible role in cardioprotection

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Aim Our objective was to investigate in cardiac muscle the contribution of NADPH oxidase to (a) ryanodine receptor-2 (RyR2) *S*-glutathionylation and (b) the preconditioning effects of exercise and tachycardia on infarct size following coronary artery occlusion.
Methods and results We measured NADPH oxidase activity, RyR2 *S*-glutathionylation, and calcium release kinetics in sarcoplasmic reticulum (SR) vesicles isolated from dog ventricular muscle after exercise and tachycardia, plus or minus prior administration of the NADPH oxidase inhibitor apocynin. In ventricular muscle sections, we studied the colocalization of NADPH oxidase and RyR2 by confocal microscopy using fluorescent antibodies. We determined the effect of apocynin on the infarct size produced by occlusion of the descendent anterior coronary artery in animals preconditioned by exercise or tachycardia. Exercise and tachycardia increased NADPH oxidase activity, RyR2 *S*-glutathionylation, and calcium release rates in isolated SR vesicles. Cardiac muscle sections displayed significant colocalization of NADPH oxidase and RyR2, suggesting direct and specific effects of reactive oxygen species (ROS) produced by NADPH oxidase on RyR2 activation. The NADPH oxidase inhibitor apocynin prevented the increase in RyR2 *S*-glutathionylation, reduced calcium release activity, and completely prevented the protective effects of exercise and tachycardia on infarct size.
Conclusions The loss of cardioprotection induced by the NADPH oxidase inhibitor suggests that ROS generated by this enzyme are important mediators of the preconditioning response, which presumably involves NADPH oxidase-induced RyR2 *S*-glutathionylation.

1. Introduction

Cardiac preconditioning is a powerful strategy to protect the myocardium against ischaemia–reperfusion damage. Different interventions are effective in conferring resistance against the damage produced by prolonged coronary artery occlusion, but the underlying mechanisms are not well understood.[1] Reactive oxygen species (ROS) produced during preconditioning[2,3] have been proposed to act as second messengers in the development of cardioprotection, which can be triggered by oxidants[2] and blocked by antioxidants.[4] Mitochondria have been suggested as the primary source of ROS during preconditioning.[5,6] Likewise, the NADPH oxidase, which is a major ROS source in cardiac tissue,[7–9] has a crucial role in preconditioning induced by ischaemia[10] or angiotensin II.[11,12] Yet the *in vivo* ROS source responsible for cardiac preconditioning has not been identified.

Brief episodes of tachycardia[13] and exercise[14] also reduce infarct size in dogs following coronary artery occlusion. Preconditioning by tachycardia increases both the activity of NADPH oxidase and the activity of cardiac ryanodine receptors (RyR2) in isolated sarcoplasmic reticulum (SR) vesicles.[15]

Oxidative stress increases protein *S*-glutathionylation, i.e. the formation of a mixed disulphide between a protein cysteine residue and glutathione, which is a protein post-translational modification present under physiological conditions.[16] Cardiac RyR2, which are endogenously

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S-glutathionylated, increase their S-glutathionylation levels after tachycardia-induced preconditioning.[15] Moreover, *in vitro* activation of NADPH oxidase increases both RyR2 S-glutathionylation and the calcium release activity of SR-enriched cardiac vesicles, suggesting that these changes are produced by NADPH oxidase-dependent ROS generation.[15]

It is not known if preconditioning exercise mimics the effects that preconditioning tachycardia exerts on RyR2 and NADPH oxidase activities. The aims of this work were to investigate if brief episodes of exercise increase NADPH oxidase activity and enhance RyR2 S-glutathionylation. In addition, we investigated if *in vivo* incubation with an NADPH oxidase inhibitor affected the cardioprotective effects of exercise or tachycardia.

2. Methods

2.1 Experimental protocols

All experiments reported in this work were done in adult mongrel dogs of either sex, 20–32 kg of body weight with the approval of the Animal Care Committee of the School of Medicine, Universidad de Chile. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996).

Detailed protocols for preconditioning exercise and tachycardia have been described previously.[13,14] The SR-enriched fractions used in this work were obtained as described below from the following nine groups:

Group 1. Control; dogs remained at rest in the lab for 60 min or they were anaesthetized (pentobarbital 30 mg/kg, i.v.) and stimulated at basal rate for 60 min before heart removal. There were no differences between these two control groups; therefore data were pooled together in a single group.

Group 2. Exercise; dogs run on a treadmill for five periods of 5 min each at 6 km/h with intervening periods of rest of 5 min. After the last exercise period, the animals rested for 10 min to allow the heart rate and the aortic pressure to return to basal values and the heart was removed under anaesthesia.

Group 3. Tachycardia; dogs were anaesthetized and after a complete atrioventricular blockade, the heart was stimulated at 215 $\pm$ 22 cycles per min for five periods, 5 min each, with intervening periods of 5 min of stimulation at basal rate; the heart was removed after the last stimulation period. Three additional dogs were stimulated as above but at a lower frequency of 150 cycles per min.

Group 4. Control plus apocynin; animals were treated as group 1, but apocynin (10 mg/kg body weight) was administered either intravenously or intracoronary. There was no difference between these two forms of administration; therefore data were pooled together in a single group.

Group 5. Exercise plus apocynin; animals were treated as group 2, but apocynin (10 mg/kg body weight) was administered intravenously just before exercise.

Group 6. Tachycardia plus apocynin; animals were treated as group 3, but apocynin (10 mg/kg body weight) was administered intracoronary during the basal rate stimulation periods.

In the following groups, we measured the infarct area produced by occlusion of the descendent coronary artery for 50 min followed by 4.5 h of reperfusion in anaesthetized dogs (abbreviated as IR).

Group 7. Ischaemia in non-preconditioned dogs plus apocynin; as group 4, followed by IR.

Group 8. Ischaemia in dogs preconditioned with exercise plus apocynin; as group 5, followed by IR.

Group 9. Ischaemia in dogs preconditioned with tachycardia plus apocynin; as group 6, followed by IR.

The electrocardiogram and the haemodynamic conditions of the anaesthetized animals were continuously monitored as in previous work.[13,14] At the end of the reperfusion period, the heart was extracted and the size of the infarction relative to the risk region was measured by the triphenyltetrazolium staining technique as described.[13]

2.2 Isolation of SR vesicles

A microsomal fraction enriched in SR vesicles was isolated by differential centrifugation as described.[17,18] Briefly, the left ventricle was finely minced and homogenized (*Heidolph Diax 600* homogenizer, three 15 s bursts at 24 000 rpm) in four volumes of 300 mM sucrose and 20 mM MOPS–Tris, pH 6.8, with protease inhibitors (4 μg/mL leupeptin, 4 μg/mL pepstatin A, 1 mM benzamidine, and 1 mM PMSF). The homogenate was sedimented at 3800 *g* for 15 min and the resulting pellet was re-homogenized as above. The combined supernatants were sedimented at 28 000 *g* for 15 min. The resulting supernatant was sedimented at 120 000 *g* for 1 h after addition of solid KCl to a final concentration of 0.65 M. The pellet, enriched in SR vesicles, was resuspended at 10 mg of protein per mL in homogenization buffer, fractionated in small aliquots, frozen in liquid N_2, and stored at -80°C.

2.3 Determination of NADPH oxidase activity

Superoxide production was measured by lucigenin chemiluminiscence by incubating SR vesicles (0.2 mg/mL) in 100 mM MOPS–Tris, pH 7.0, 100 μM NADPH, and 5 μM lucigenin at 25°C. Chemiluminiscence was measured in a Berthold FB 12 luminometer and expressed as nmoles of superoxide anion per mg protein per min.[15]

2.4 Detection of NADPH oxidase subunits in SR membrane fractions

SR-enriched fractions (10 μg of protein) were mixed with SDS–PAGE sample buffer, boiled for 3 min, subjected to SDS–PAGE, transferred to PVDF membranes, and probed with antibodies against NADPH oxidase subunits. Polyclonal antibodies against $p47^{phox}$ and rac1 were a kind gift from Dr Frans B. Wientjes (University College London, UK). The antibody against $gp91^{phox}$ was from BD Biosciences (San Diego, CA, USA). Antigen–antibody reaction was detected by ECL (Amersham, Biosciences).

2.5 Detection of RyR2 S-glutathionylation

S-Glutathionylation of RyR2 was detected after SDS–PAGE under non-reducing conditions[15] utilizing anti-GSH antibodies from Virogen (Watertown, MA, USA). After ECL detection of the antigen–antibody reaction, membranes were stripped and probed with anti-RyR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Results were obtained by densitometric analysis of the blots and expressed as the ratio of anti-GSH/anti-RyR band densities.

2.6 Ca^{2+} release kinetics

Calcium release kinetics was determined in an SX.18MV stopped-flow fluorescence spectrometer (Applied Photophysics Ltd, Leatherhead, UK) as detailed elsewhere.[15,17,18]

2.7 Calcium leak

SR vesicles (0.2 mg/mL) were incubated at 30°C in a solution containing (in mM): 0.01 $CaCl_2$, 100 KCl, 10 phosphocreatine, and 20 imidazole–MOPS, pH 7.2, plus 15 U/mL creatine kinase and 0.1 μM Calcium Green-2. Calcium uptake was initiated by adding a small volume of Mg–ATP (final 2 mM ATP and 3 mM $MgCl_2$). Extravesicular free calcium ($[Ca^{2+}]e$) was monitored in a fluorescence spectrophotometer (JOBIN YVON-SPEX FluoroMax-2). When $[Ca^{2+}]e$ stabilized at <0.3 μM, thapsigargin (2 μM) was added to inhibit the

Ca^{2+}-ATPase. The resulting release flux, mostly due to spontaneous RyR2 activity, increased $[Ca^{2+}]e$ with time. The initial rate of this non-stimulated Ca^{2+} flux represents the magnitude of calcium leak.

2.8 Immunolocalization and colocalization of gp91^{phox} and RyR2 in cardiac tissue sections by confocal microscopy

Ventricular tissue was frozen in isopentane cooled by liquid nitrogen; 6 μm sections were obtained in a cryostat and fixed following standard protocols. After 15 min of permeabilization (PBS, 0.1% Triton X-100), sections were blocked with PBS plus 1% BSA for 90 min followed by incubation with anti-gp91^{phox} (1:100, BD Biosciences) and anti-RyR2 (1:200, ABR) at 4°C overnight. After incubation with Alexa Fluor® 488- or 543-labelled secondary antibodies (Molecular Probes), two-channel fluorescent images were recorded by confocal microscopy (LSM 5-Pascal, Axiovert 200, Zeiss): intensity $I \in [0, 255]$, ×63 oil, NA 1.4, excitation/emission were 488/505–530 nm for Alexa 488 and 543/560–580 nm for Alexa 543. Confocal raw-images were deconvolved by Huygens Scripting (SVI, Hilversum, Netherlands). All subsequent image-processing routines for visualization, for the segmentation of regions of interest (ROIs), and for the calculation of Manders colocalization coefficients ($M1$ and $M2$) were developed in our laboratory on the basis of IDL (ITT, Boulder, USA). Following Manders *et al.*[19] $M1$ and $M2$, calculate the contribution of fluorescence intensities in the colocalizing region (ROI1 ∩ ROI2, yellow ROIs in *Figure 5E*) with respect to the fluorescence intensities in the segmented region in each channel (ROI1 or ROI2, red and green ROIs in *Figure 5E*).

$$M1 = \frac{\sum_i I_{ROI1 \cap ROI2}}{\sum_i I_{ROI1}}, M2 = \frac{\sum_i I_{ROI1 \cap ROI2}}{\sum_i I_{ROI2}} \quad (1)$$

ROI1 and ROI2 were defined by threshold values in the intensity histograms of the corresponding fluorescent channels. For better segmentation, fluorescent structure borders were enhanced by gradient filters. The quality of the segmentation was optimized interactively by overlaying the original fluorescent images with the ROIs in each channel. The statistical significance of the colocalization of the segmented fluorescent pattern was determined by probability density functions (PDFs) according to Costes *et al.*[20] In contrast to Costes *et al.*, we did not use pixel blocks to scramble the images, but introduced radial displacements to shift one fluorescent channel with respect to the other. $M1/M2$ were calculated for each displacement (*Figure 5F*) and constituted the data for the generation of the corresponding PDFs (*Figure 5G*). The statistical significance of colocalizing structures was evaluated by P-values ($P < 0.05$); which test if $M1/M2$ values calculated at the original channel positions (no displacement) are significantly greater than $M1/M2$-values calculated for successive displacements. All fluorescent images in *Figure 5* were displayed in the opacity colour mode [4, dimx, dimy]. Here, the first three channels represent the red/green/blue colour channels, and the fourth channel represents the opacity-channel. An α-value of 0 corresponds to blend factor of 0 (no opacity or full transparency) and an α-value of 255 corresponds to a blend factor of 1.0.

2.9 Other procedures

Protein concentration was determined according to Hartree.[21]

Apocynin was obtained from Sigma-Aldrich, Milwaukee, WI, USA. All other reagents were of analytical grade.

2.10 Statistical analysis

Results, expressed as mean ± SEM, except otherwise specified, were analyzed by one-way ANOVA followed by Dunnett's test. Differences were considered significant at $P < 0.05$.

3. Results

The haemodynamic effects of exercise and tachycardia in the absence and in the presence of apocynin are shown in *Table 1*. Exercise significantly increased heart rate and mean aortic pressure by 58 and 15%, respectively. Heart rate stimulation during tachycardia was 2.4-fold higher than basal rate and the mean aortic pressure was damped to control values by an external blood reservoir as described.[13] Apocynin by itself did not modify heart rate or mean aortic pressure. In the presence of apocynin, exercise increased heart rate and mean aortic pressure by 61 and 20%, respectively. ECG repolarization changes were not observed during tachycardia and exercise in any experimental condition.

3.1 NADPH oxidase activity

The initial rate of superoxide anion generation in SR vesicles isolated from hearts obtained after exercise was 2.8-fold higher than control and similar to the increase produced by tachycardia (*Figure 1A*). Prior administration of 10 mg per kg body weight of apocynin abolished the increase in superoxide anion produced by exercise or tachycardia (*Figure 1A*), suggesting direct NADPH oxidase involvement in these responses. Heart rate during exercise attained lower values than those induced by electrical stimulation during tachycardia, yet the activation of NADPH oxidase was similar. To assess the contribution of heart rate *per se* to the increase in enzyme activity, we stimulated hearts at the frequency attained during exercise (150 cycles per min). Stimulation at this frequency did not increase NADPH oxidase activity (*Figure 1A*), suggesting that exercise stimulates the enzyme by pathways not involving increased heart rate.

Increased membrane association of NADPH oxidase cytoplasmic regulatory subunits is another index of its activation.[22] *Figure 1B* shows that exercise increased significantly the association of the two cytoplasmic regulatory subunits p47^{phox} and rac1 to SR-enriched vesicles,

Table 1 Effect of apocynin on haemodynamics

			Heart rate (cycles/min)	MAP (mmHg)
Group 1 ($n = 16$)	C	Basal	94 ± 8	95 ± 5
Group 2 ($n = 16$)	E	Basal	100 ± 6	98 ± 4
		Exercise	158 ± 4*	113 ± 5*
Group 3 ($n = 7$)	T	Basal	88 ± 11	91 ± 6
		Tachycardia	215 ± 12*	80 ± 4
Group 4 ($n = 10$)	C + A	Basal	85 ± 8	90 ± 8
		Apocynin	92 ± 2	85 ± 5
Group 5 ($n = 9$)	E + A	Basal	96 ± 5	87 ± 8
		Apocynin	95 ± 7	90 ± 5
		Exercise	153 ± 10*	108 ± 4*
Group 6 ($n = 8$)	T + A	Basal	83 ± 3	92 ± 5
		Apocynin	80 ± 6	94 ± 5
		Tachycardia	212 ± 3*	90 ± 4

MAP, mean aortic pressure; C, control; E, exercise; T, tachycardia; A, apocynin.
*$P < 0.05$.

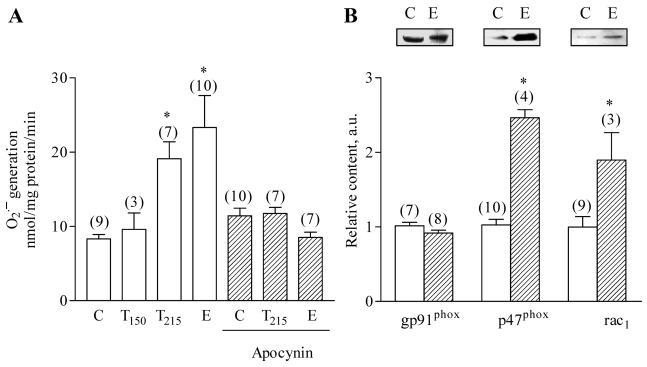

Figure 1 NADPH oxidase activity. (*A*) Initial rates of superoxide anion generation in SR vesicles isolated from control (C), exercise (E), tachycardia at 150 cycles per min (T_{150}), and tachycardia at 215 cycles per min (T_{215}) without (open bars) and with (hatched bars) previous administration of apocynin at 10 mg/kg body weight. The number of animals is shown on top of each bar. (*B*) The relative contents of NADPH oxidase subunits gp91^{phox}, p47^{phox}, and rac1 after exercise were quantified in Western blots. Representative Western blots, and the number of animals used, are shown on top of each bar. $^*P < 0.05$.

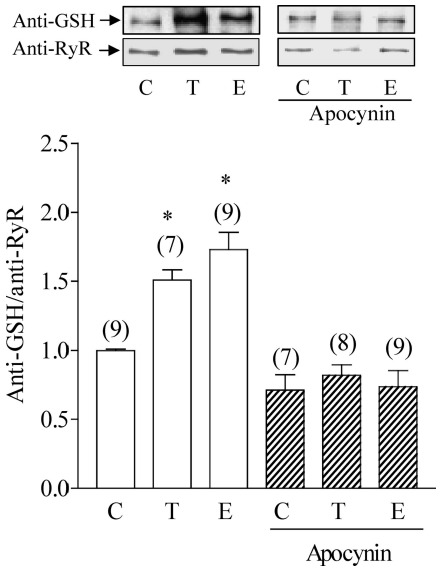

Figure 2 Effect of increased heart rates on RyR2 *S*-glutathionylation; inhibition by apocynin: Representative Western blots obtained with SR vesicles from controls (C), exercise (E) and tachycardia (T) probed with anti-GSH antibody (upper) and with anti-RyR antibody after stripping (lower), plus or minus prior apocynin administration. The graph bar shows the ratio anti-GSH/anti-RyR calculated from densitometric analysis of Western blots like those shown on top. The numbers over the bars represent the number of animals analyzed. $^*P < 0.05$.

without changing the relative content of the membrane-bound catalytic subunit gp91^{phox}. These results support NADPH oxidase activation by exercise.

3.2 Effect of NADPH oxidase activity on RyR2 *S*-glutathionylation

Oxidation of cysteine residues by cellular ROS generates mixed disulphides with glutathione. We showed previously that RyR2 *S*-glutathionylation increases after tachycardia,[15] but the relationship between this redox modification and *in vivo* activation of NADPH oxidase has not been demonstrated. Exercise increased RyR2 *S*-glutathionylation by 1.7-fold (*Figure 2*), similar to the increase produced by tachycardia. Administration of apocynin did not change RyR2 S-glutathionylation level in control animals but eliminated the increase produced by exercise or tachycardia (*Figure 2*), suggesting that ROS produced by NADPH oxidase are involved in this modification.

3.3 Effect of apocynin on RyR2-mediated calcium release activity

Tachycardia and RyR2 *S*-glutathionylation increase calcium release activity in isolated SR vesicles.[15,17] Therefore, we investigated the effects of exercise and apocynin on calcium release kinetics following the time-dependent changes in [Ca^{2+}]e with the calcium sensitive dye Calcium Green-5N as reported.[15,17,18] Representative calcium release records are shown in *Figure 3*. Apocynin did not modify calcium release kinetics in controls (*Figure 3A*) but prevented the stimulation of calcium release kinetics produced by exercise (*Figure 3B*) or tachycardia (*Figure 3C*). Calcium release records were fitted to double exponential functions; the values of the faster rate constant *k*1, an index of RyR2 activity in response to a sudden increase in [Ca^{2+}]e,[18] also indicate that apocynin prevented release stimulation by exercise and tachycardia (*Figure 3D*). Apocynin did not act by changing RyR2 density, since equilibrium [^{3}H]ryanodine binding density was the same in SR vesicles from controls or after apocynin administration (data not shown). Likewise, apocynin administration did not change the total calcium load since in all cases SR vesicles reached the same load.

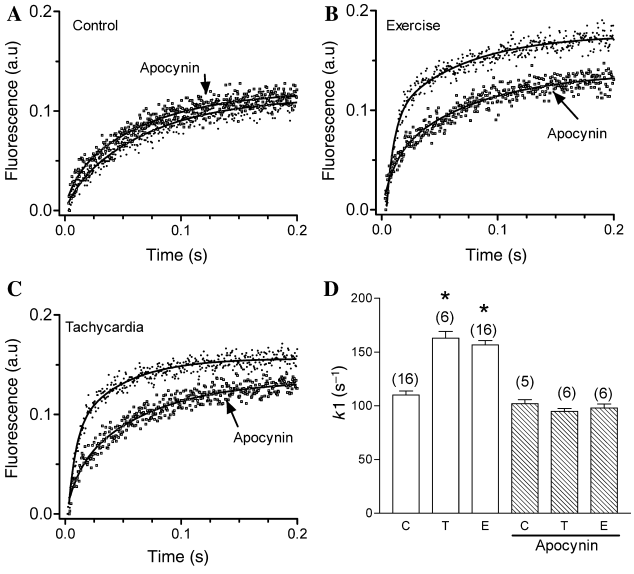

Figure 3 Calcium release kinetics. SR vesicles (1 mg/mL) actively loaded with Ca^{2+} were mixed (1:10) in a stopped-flow fluorescence spectrometer with a solution that produced upon mixing pCa 6 and 1.2 mM free ATP. Release kinetics was measured from the change in fluorescence of Calcium Green 5N fitted to a double exponential function: $A1 \exp(-k1t) + A2 \exp(-k2t)$. Representative fluorescent records illustrating calcium release kinetics in vesicles obtained in control (*A*), after exercise (*B*), and after tachycardia (*C*) with and without administration of apocynin. (*D*) Comparison of release rate constants obtained in controls and after exercise or tachycardia, and the effects of apocynin. The numbers over the bars represent the number of animals analyzed. $^*P < 0.05$.

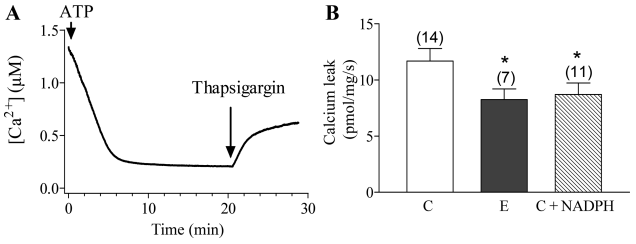

Figure 4 Effect of RyR2 *S*-glutathionylation on calcium leak. (*A*) Calcium uptake upon addition of Mg–ATP and calcium leak following addition of thapsigargin (2 μM). (*B*) Calcium leak was expressed as the initial rate of extravesicular calcium concentration increase after thapsigargin in control vesicles (C), after exercise (E) or control vesicles plus 100 μM NADPH (C + NADPH). For further details, see Section 2.

3.4 Effect of RyR2 *S*-glutathionylation on SR calcium leak

We investigated if the increase in RyR2 activity produced by *S*-glutathionylation enhanced calcium leak from SR vesicles by activating the spontaneous activity of RyR2 channels at near resting [Ca^{2+}]. We actively loaded SR vesicles with calcium, while constantly monitoring the decrease in [Ca^{2+}]e with the fluorescent indicator Calcium Green 2 (*Figure 4A*). When calcium uptake was completed, [Ca^{2+}]e approached 0.2 μM; addition at this point of 2 μM thapsigargin to block the SERCA pump increased [Ca^{2+}]e. This increase was due to spontaneous RyR2-mediated calcium release events (*Figure 4A*), since preincubation with 100 μM ryanodine blocked the increase in [Ca^{2+}]e induced by thapsigargin (data not shown). Calcium leak, determined as the initial rate of [Ca^{2+}]e increase upon thapsigargin addition, was decreased by exercise from 11.7 ± 1.1 ($n = 14$) to 8.3 ± 1.0 ($n = 7$) pmoles per mg per second ($P < 0.05$; *Figure 4B*). Incubation of calcium loaded SR vesicles from controls with NADPH, which increases RyR2 *S*-glutathionylation,[15] produced a 26% decrease in calcium leak compared with the basal condition (*Figure 4B*). These results indicate that conditions that increase RyR2 *S*-glutathionylation, such as exercise or incubation with NADPH, have an inhibitory effect on RyR2-mediated calcium leak.

3.5 Localization of NADPH oxidase in cardiac myocytes

A longitudinal section of ventricular muscle stained with an antibody against $gp91^{phox}$ (*Figure 5A*) shows that this antibody binds in a regular pattern across the cardiac myocytes and at the cell boundaries and the intercalated discs. At higher magnification (*Figures 5B* and *5C*), regularly spaced bands for $gp91^{phox}$ (red) and RyR2 (green) are apparent. Colocalization between $gp91^{phox}$ and RyR2 can be observed in *Figures 5D* and *5E*. *Figure 5D* shows a two-channel image that results from the merging of the color tables of *Figures 5B* and *5C*. *Figure 5E* presents the segmented regions for $gp91^{phox}$ (ROI1, red), RyR2 (ROI2, green), and the colocalization region (ROI1 ∩ ROI2, yellow). Colocalization coefficients, calculated for six different samples, yielded $M1 = 0.32 \pm 0.04$ and $M2 = 0.30 \pm 0.04$. As *Figures 5F* and *5G* demonstrate, the calculated degrees of colocalization are not the result of an arbitrary distribution of the corresponding fluorescence signals. *Figure 5F* shows a representative behaviour of *M*1 calculated for

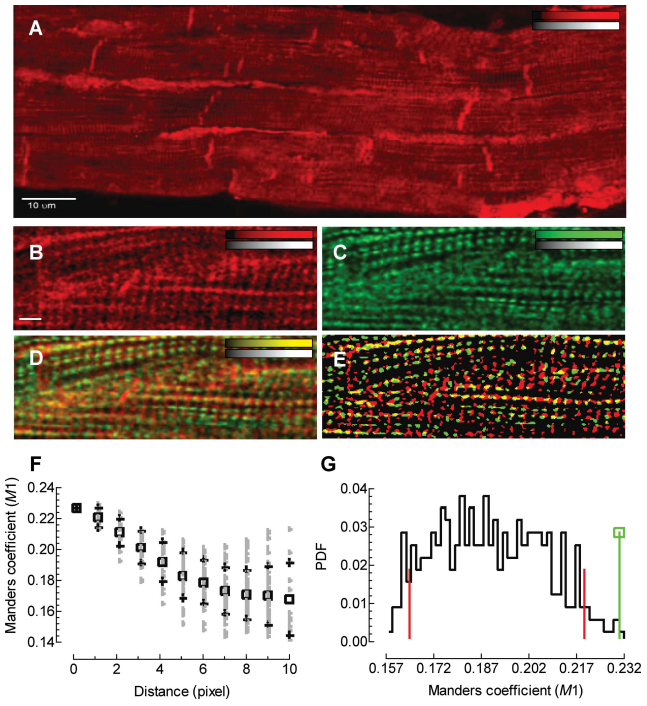

Figure 5 Immunolocalization and colocalization of $gp91^{phox}$ and RyR2 in cardiac tissue sections. (*A–D*) Representative fluorescent images of anti-$gp91^{phox}$ (A and B, red), anti-RyR (C, green), and superposition of anti-$gp91^{phox}$/anti-RyR (D, yellow). Images were displayed as described in Section 2. The black-grey bars represent the α-channel or opacity-values. (*E*) Segmented ROIs for $gp91^{phox}$ (red), anti-RyR2 (green), and colocalization of ROIs for $gp91^{phox}$/anti-RyR2 (yellow). Scale bar in (A) represents 10 μm, scale bar for (B–E) represents 2 μm. Manders colocalization coefficients for $gp91^{phox}$ (*M*1) and anti-RyR2 (*M*2) were calculated from the intensity values in the segmented ROIs [Eq. (1)]. (*F*) Manders colocalization coefficient for $gp91^{phox}$ (*M*1) as a function of the radial displacement from the original image position in pixels. Mean values (black squares) were derived from the individual Manders coefficients (grey plus signs) calculated for each displacement at the indicated distance (pixel). Standard deviations are plotted as black plus signs. (*G*) Probability density function (PDF) plotted as a histogram of the Manders coefficient *M*1 calculated in (F). Red bars represent the $P < 0.05$ confidence values. The green bar marks the Manders coefficient *M*1 calculated at the original image position (displacement = 0).

successive radial displacements (see Section 2). *M*1 drops sharply from $M1 \sim 0.23$ (no displacement) to $M1 \sim 0.18$ at a radial displacement of more than 6 pixel. This result indicates that the degree of colocalization for a random distribution of the observed proteins is 18%. Evidence that $M1 \sim$ 23% actually represents a statistically significant colocalization in the context of the PDF is shown in *Figure 5G*: the $M1 \sim 23\%$ value (green bar) is located outside the $P < 0.05$ confidence limits (red bars). *M*1 and *M*2 values obtained in controls or after exercise were not significantly different indicating that exercise did not change the degree of colocalization of these proteins. The localization of NADPH oxidase in the proximity of RyR2 would allow the controlled redox modification of this protein, since ROS are short-lived species and their action is confined to molecules located within their immediate vicinity.

3.6 Effect of apocynin on the preconditioning induced by exercise or tachycardia

Exercise and tachycardia reduce the infarct size produced by the prolonged occlusion of a coronary artery.[13,14] In this work, we investigated the effect of apocynin on the

Table 2 Effect of apocynin on haemodynamics during ischaemia and reperfusion

			Heart rate (cycles/min)	MAP (mmHg)
Group 7 ($n = 4$)	C + A	Basal	90 ± 5	87 ± 8
		Apocynin	92 ± 3	85 ± 5
		I 30 min	98 ± 7	80 ± 7
		R 180 min	105 ± 6	85 ± 6
Group 8 ($n = 5$)	E + A	Basal	90 ± 6	84 ± 5
		Apocynin	92 ± 4	85 ± 8
		Exercise	150 ± 8*	107 ± 6*
		I 30 min	95 ± 9	83 ± 7
		R 180 min	100 ± 10	79 ± 8
Group 9 ($n = 6$)	T + A	Basal	85 ± 6	87 ± 5
		Apocynin	88 ± 7	90 ± 6
		Tachycardia	200 ± 8*	88 ± 4
		I 30 min	93 ± 3	82 ± 5
		R 180 min	101 ± 4	81 ± 6

MAP, mean aortic pressure; C, control; E, exercise; T, tachycardia; A, apocynin.

I 30 min: values obtained after 30 min of ischaemia.

R 180 min: values obtained after 180 min of reperfusion following ischaemia.

*$P < 0.05$.

preconditioning effect of tachycardia and exercise. The administration of apocynin did not modify significantly heart rate or mean arterial pressure (*Table 2*); both variables were similar to previously reported values obtained in controls and preconditioned animals during ischaemia and reperfusion.[13,14] Zones at risk, expressed as percentage of risk zone volume/left ventricular wall volume, in dogs treated with apocynin were (mean ± SD) control: 53 ± 10 ($n = 4$), tachycardia: 51 ± 6 ($n = 6$), and exercise: 52 ± 4 ($n = 5$), similar to previously published values.[13,14] Administration of apocynin did not modify the infarct size in control animals but abolished the protective effects of exercise and tachycardia (*Figure 6*). The blockade of the preconditioning effects of exercise and tachycardia produced by apocynin is likely related to NADPH oxidase inhibition, since apocynin did not modify collateral blood flow (data not shown).

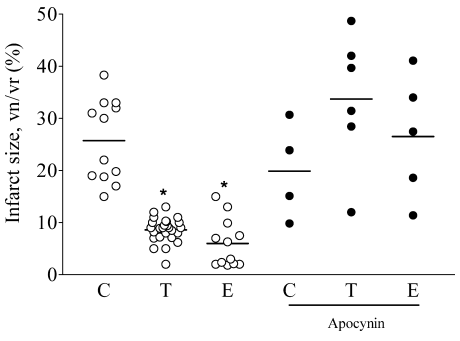

Figure 6 Effect of *in vivo* NADPH oxidase inhibition with apocynin on infarct size. Infarct size, expressed in terms of necrotic region volume as percent of the risk region volume (vn/vr, %), was measured after occlusion of the anterior descendent coronary artery in controls and preconditioned animals treated with apocynin as described in Section 2 (filled circles). Data obtained in animals without apocynin were taken from ref. 13 (T) and ref. 14 (E), and are shown for comparison purposes (open circles). $P < 0.05$.

4. Discussion

Our results in cardiac SR-enriched fractions isolated from animals preconditioned by exercise show an enhancement in the activity of NADPH oxidase and in RyR2 *S*-glutathionylation, as shown before for tachycardia.[15] Administration of apocynin *in vivo*, prevented the enhancement of both NADPH oxidase activity and RyR2 *S*-glutathionylation, suggesting that this redox modification is dependent on NADPH oxidase activity. The decrease of RyR2 *S*-glutathionylation to basal levels induced by apocynin paralleled the reduction in calcium release activity to control values when exercise and tachycardia were done in the presence of apocynin.

Increases in NADPH oxidase activity have been related to pathological conditions such as cardiac hypertrophy and remodelling.[23] Our results provide evidence that short periods of physiological stimulation by exercise also increase NADPH oxidase activity. Furthermore we have identified RyR2 as one of the targets for ROS generated by NADPH oxidase. RyR2 are sensitive to redox regulation through the modification of critical cysteine residues of the molecule.[24,25] Reducing thiol reagents like DTT greatly inhibit RyR2 channel activity *in vitro*[15,18] and calcium transients in isolated cardiomyocytes.[25]

The colocalization of $gp91^{phox}$ and RyR2 supports a role for NADPH oxidase as modulator of RyR2 activity. Thus, ROS generation in its vicinity may promote RyR2 *S*-glutathionylation as a possible physiological mechanism to increase channel activity in the overall reducing cytoplasmic environment. In macrophages, recruitment of the small G protein rac and $p47^{phox}$ is needed to form a catalytically active complex with $gp91^{phox}$. Yet the pathways leading to NADPH oxidase activation via recruitment of these regulatory proteins are not known in cardiac cells. In endothelial cells, NADPH oxidase is activated by membrane depolarization.[26] Our results, showing that stimulation at the cardiac frequency produced by exercise did not activate the enzyme, argue against depolarization-induced activation in exercise. Cyclic changes in stretch activate NADPH oxidase in endothelial cells[27] and may also underlie stimulation of this enzyme in cardiac muscle cells. The threshold for stretch activation of NADPH oxidase is very narrow,[27] raising the possibility that higher stimulation rates by tachycardia are needed to match the cellular stretch produced by exercise.

Other reports have shown increased ROS generation by increases in stimulation rate in isolated cardiac myocytes.[28] Increases in stimulation frequency of 10–20% significantly enhance NADPH oxidase activity in rat hearts independently of adrenergic activity.[29] Our results, however, suggest a role for adrenergic stimulation during exercise. Alpha-adrenergic stimulation of cardiac tissue promotes $p47^{phox}$ phosphorylation and increases in NADPH oxidase activity.[30] Adrenergic stimulation could produce higher increases in intracellular calcium than electrical stimulation alone. Although $gp91^{phox}$-containing NADPH oxidase is not calcium dependent, the activation of calcium-dependent pathways could contribute to enzyme activation. The prevention of the protective effects of exercise and tachycardia preconditioning by *in vivo* inhibition of NADPH oxidase with apocynin shown here is in agreement with previous data showing a critical role of this enzyme in ischaemic and pharmacological preconditioning.[10–12] Ischaemic preconditioning fails

in isolated hearts obtained from knock-out animals for NADPH oxidase,[10] whereas Angiotensin II preconditioning in conscious rats[11] and isolated hearts[12] depends on enhanced ROS generation by NADPH oxidase, since it is abolished by the ROS scavenger *N*-acetyl cysteine and by apocynin.

The redox modification of RyR2 shown in this work had two functional consequences *in vitro*: it increased RyR2-mediated calcium release activity and decreased RyR2-mediated calcium leak. These effects may be of marked physiological relevance, since enhanced RyR2 *S*-glutathionylation would increase *in vivo* the systolic calcium release rate during periods of increased cardiac activity but would decrease calcium release during diastole, decreasing the chances of occurrences of arrhythmias. Increasing RyR2 activity in systole and decreasing it during diastole would also increase SR fractional calcium release and decrease SR calcium content. Even small depletions of SR calcium load would increase the ability of the SR to cope with the calcium flooding that is bound to occur during ischaemia–reperfusion and that may constitute an important aspect of the cardioprotective effects produced by exercise and tachycardia. In addition to RyR2 modulation, ROS generated by NADPH oxidase may also act as triggers of ROS production by the mitochondria, which may contribute to the development of the preconditioning response as suggested for Angiotensin II mediated preconditioning.[11]

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