

# Reversible redox modifications of ryanodine receptor ameliorate ventricular arrhythmias in the ischemic-reperfused heart

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**Becerra R, Román B, Di Carlo MN, Mariangelo JI, Salas M, Sanchez G, Donoso P, Schinella GR, Vittone L, Wehrens XH, Mundiña-Weilenmann C, Said M.** Reversible redox modifications of ryanodine receptor ameliorate ventricular arrhythmias in the ischemic-reperfused heart. *Am J Physiol Heart Circ Physiol* 311: H713–H724, 2016. First published July 15, 2016; doi:10.1152/ajpheart.00142.2016.—Previous results from our laboratory showed that phosphorylation of ryanodine receptor 2 (RyR2) by Ca<sup>2+</sup> calmodulin-dependent kinase II (CaMKII) was a critical but not the unique event responsible for the production of reperfusion-induced arrhythmogenesis, suggesting the existence of other mechanisms cooperating in an additive way to produce these rhythm alterations. Oxidative stress is a prominent feature of ischemia/reperfusion injury. Both CaMKII and RyR2 are proteins susceptible to alteration by redox modifications. This study was designed to elucidate whether CaMKII and RyR2 redox changes occur during reperfusion and whether these changes are involved in the genesis of arrhythmias. Langendorff-perfused hearts from rats or transgenic mice with genetic ablation of CaMKII phosphorylation site on RyR2 (S2814A) were subjected to ischemia-reperfusion in the presence or absence of a free radical scavenger (mercaptopyronylglycine, MPG) or inhibitors of NADPH oxidase and nitric oxide synthase. Left ventricular contractile parameters and monophasic action potentials were recorded. Oxidation and phosphorylation of CaMKII and RyR2 were assessed. Increased oxidation of CaMKII during reperfusion had no consequences on the level of RyR2 phosphorylation. Avoiding the reperfusion-induced thiol oxidation of RyR2 with MPG produced a reduction in the number of arrhythmias and did not modify the contractile recovery. Conversely, selective prevention of S-nitrosylation and S-glutathionylation of RyR2 was associated with higher numbers of arrhythmias and impaired contractility. In S2814A mice, treatment with MPG further reduced the incidence of arrhythmias. Taken together, the results suggest that redox modification of RyR2 synergistically with CaMKII phosphorylation modulates reperfusion arrhythmias.

arrhythmias; ischemia/reperfusion; ryanodine receptor type 2; redox modifications

## NEW & NOTEWORTHY

*This study demonstrates that redox changes of ryanodine receptor type 2 occur together with Ca<sup>2+</sup> calmodulin-dependent kinase II phosphorylation of the sarcoplasmic reticulum Ca<sup>2+</sup> channel in hearts subjected to ischemia/reperfusion. Both post-translational modifications seem to act synergistically in determining reperfusion arrhythmogenesis.*

RESTORATION OF CORONARY FLOW after a myocardial ischemic event rescues the heart from further damage. However, reperfusion creates a pathophysiological scenario of its own. A contractile dysfunction and myocyte cell death are pathological manifestations of the reperfusion injury, which depend on the duration of ischemia. Even after a brief period of ischemia, which causes no or little tissue damage, life-threatening ventricular arrhythmias can occur (54). Several clinical reports have referred to the presence of arrhythmias during the relief of coronary artery spasm, exercise-induced ischemia, and silent ischemia or after thrombolysis subsequent to acute myocardial infarction (30, 37, 41).

During reperfusion, a great part of the arrhythmic events is thought to be related to an imbalance in ionic currents favoring a depolarizing net inward current (triggered arrhythmias) (3). Early afterdepolarizations occur during the action potential (AP), usually in the setting of prolonged repolarization, and are classically attributed to reactivation of L-type Ca<sup>2+</sup> current (*I<sub>Ca</sub>*) (20, 32). Delayed afterdepolarizations (DADs) occur after completion of AP repolarization and are a consequence of spontaneous sarcoplasmic reticulum (SR) Ca<sup>2+</sup> release and activation of a transient inward current (*I<sub>ti</sub>*), primarily mediated by the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (36). This initial stimulus of nonreentrant nature may give rise to episodes of ventricular tachycardia or fibrillation. Previous results from our laboratory showed that, during the early reperfusion of stunned heart, most of the ventricular premature beats (VPBs) detected were triggered by DADs and, as expected, decreased when the SR function was disabled (40). These reperfusion-induced arrhythmias were diminished by 70% in the presence of a Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) inhibitor and were reduced by 50% in mice lacking the CaMKII phosphorylation site on the ryanodine receptor (RyR2) (40). These results provided compelling evidence for a critical role of CaMKII target proteins, particularly RyR2, in reperfusion

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arrhythmogenesis. They also suggest that other mechanisms cooperate in an additive way to these rhythm alterations. Reperfusion after ischemia is a condition of oxidative stress in which reactive oxygen and nitrogen species (ROS/RNS) are generated by several different cellular sources, which include the mitochondria, NADPH oxidase (NOX), xanthine oxidase (XO), and nitric oxide synthase (NOS). Redox modifications of both CaMKII and RyR2 have been described in different cardiac diseases, such as heart failure and diabetes (9, 13, 18, 23). However, these changes have been poorly studied in the ischemic heart injury (2).

Oxidation and autophosphorylation of CaMKII are post-translational modifications resulting in autonomous activation of the enzyme (10). Redox-dependent CaMKII activation has been found to be related to angiotensin II and aldosterone-induced apoptosis (10, 17, 49), electrical remodeling (51), sinus node dysfunction (46), and cardiac glycoside treatment (19), all situations in the course of which life-threatening arrhythmias can occur. Moreover, oxidized CaMKII has been reported to be enhanced in atria from patients with atrial fibrillation (38). Recently, an NO-dependent mechanism for myocardial CaMKII activation has been elucidated. Gutierrez et al. (15) showed that CaMKII activity was enhanced in the presence of NO donors, and Curran et al. (5) extended these findings showing *S*-nitrosylation of CaMKII in response to  $\beta$ -adrenergic stimulation. These studies reported that the increased NO-dependent CaMKII activity was associated with an increased SR  $\text{Ca}^{2+}$  leak. Both oxidation and nitrosylation of CaMKII might occur during ischemia/reperfusion. It is still unknown whether these mechanisms are involved in the activation of CaMKII, the consequent phosphorylation of RyR2, and the generation of arrhythmias observed under this pathological condition.

Extensive information in the literature shows that RyR2 possesses cysteine residues highly susceptible to redox modifications, which have remarkable effects on RyR2 function (18). RyR2 is endogenously *S*-glutathionylated (43) and *S*-nitrosylated (55), being both reversible redox modifications. Whereas increases in *S*-glutathionylation enhanced RyR2-mediated  $\text{Ca}^{2+}$  release and decreased RyR2-mediated  $\text{Ca}^{2+}$  leak (7, 42), the consequences of *S*-nitrosylation of RyR2 remain controversial. It has been reported that decreased *S*-nitrosylation of RyR2 through genetic deletion or pharmacological inhibition of neuronal NOS is able to induce either an increase or a decrease in spontaneous SR  $\text{Ca}^{2+}$  release in isolated cardiomyocytes (6, 12, 52). The conflicting results in these and other reports (25, 56) suggest that the impact of *S*-nitrosylation on RyR2 activity is critically dependent on the underlying cell conditions. Surprisingly, no evidence has been reported about the changes in the redox state of RyR2 during myocardial reperfusion and their possible functional implications. The present study was designed to elucidate the occurrence of CaMKII and RyR2 oxidation in isolated rat hearts subjected to ischemia/reperfusion and their involvement in the genesis of reperfusion arrhythmias.

## METHODS

The experiments were performed on male Wistar rats (200–300 g body wt) and transgenic mice (25–30 g) with genetic ablation of the CaMKII site on RyR2 (S2814A) (4), approved by the Institutional Animal Care and Use Committee (IACUC) of the School of Medicine,

National University of La Plata, Argentina (Nro T05022014) conforming to the *Guide for the Care and Use of Laboratory Animals* (NIH, 2011).

### *Ex Vivo Experiments: Langendorff Perfusion and Experimental Protocol*

Animals were anaesthetized by intraperitoneal injection of ketamine and xylazine (70 and 5 mg/kg, respectively). The dose was sufficient to produce a surgical level of anesthesia (loss of pedal withdrawal reflex) without profoundly affecting the cardiovascular function. Central thoracotomy and heart excision were performed immediately after phase III of anesthesia was reached. Isolated rat hearts were perfused at a constant temperature (37°C), heart rate (4 Hz), and flow (14 ml/min) with bicarbonate buffer solution (BBS) composed of (in mM): 128.30 NaCl, 4.70 KCl, 1.35  $\text{CaCl}_2$ , 20.20  $\text{NaHCO}_3$ , 0.40  $\text{NaH}_2\text{PO}_4$ , 1.10  $\text{MgSO}_4$ , 11.10 glucose, and 0.04  $\text{Na}_2\text{EDTA}$ . This solution was equilibrated with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  to give a pH of 7.4, as described (50). Perfusion of the isolated mouse hearts was modified as follows: heart rate 6 Hz, flow 4 ml/min, and 2.5 mM  $\text{CaCl}_2$  in the perfusate. The mechanical activity of the hearts was assessed by introducing into the left ventricle (LV) a latex balloon connected to a pressure transducer (ADInstruments) and filled with aqueous solution to achieve an LV end-diastolic pressure (LVEDP) of ~5–10 mmHg. LV contractility was evaluated measuring the developed pressure (LVDP) and the maximal rate of pressure development (+dP/dt) (50).

*Epicardial monophasic APs.* Monophasic APs (MAPs) were obtained by using a silver/silver chloride Ag/AgCl electrode opposed to the epicardial surface of the free LV wall as previously described (39). MAP recordings obtained satisfied previously documented criteria of a stable baseline and triangular MAP morphology, rapid upstroke phase, and consistent amplitude (21, 39). Although MAP measurements are local, they were always associated with global changes in contractility. This makes it possible to correlate these electrical events with biochemical changes measured in the whole ventricle. Quantification of VPBs was accomplished by counting the number of extra MAPs that do not follow the basal heart rhythm. VPBs and ventricular tachycardia events (VT) were studied during the first 3 min of reperfusion.

*Experimental protocol.* After a 10-min stabilization period, rat/mouse hearts were subjected to 20/15 min of global normothermic ischemia followed by 30 min of reperfusion with BBS. In previous experiments, we have shown that this protocol did not produce irreversible tissue damage (27). When drugs were used, they were perfused 10 min before the onset of ischemia and during the reperfusion period [10  $\mu\text{M}$  apocynin (APO), 100  $\mu\text{M}$  *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), 2 mM mercaptopropionylglycine (MPG), 1 mM 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), and 10  $\mu\text{M}$  *S*-methyl-L-thiocitrulline (SMTC)]. APO, L-NAME, MPG, and DMSO, used for the dilution of drugs, did not affect basal contractility and MAP duration at 90% of repolarization (MAPD90) (Table 1). A group of hearts perfused but not submitted to ischemia/reperfusion protocol was used as control (Ctrl). For biochemical assays, hearts were freeze clamped at 1 min of reperfusion (28).

### *Electrophoresis and Western Blot*

Pulverized frozen ventricles were homogenized in four volumes of the following (in mM): 25 NaF, 300 sucrose, 1 EDTA, 30  $\text{KH}_2\text{PO}_4$  (pH 7.0) plus protease inhibitor cocktail. The homogenate was sedimented at 13,000 g for 10 min, and the supernatant was fractionated in small aliquots, frozen in liquid  $\text{N}_2$ , and stored at  $-80^\circ\text{C}$ . Protein concentration was measured using the Bradford method with bovine serum albumin as the standard. For immunological detection of phosphorylated CaMKII (pCaMKII), phospholamban (PLN), phosphorylated PLN (pThr17-PLN), and GAPDH, 20–50  $\mu\text{g}$  of homogenate protein was electrophoresed per gel lane in 10% acrylamide gels

Table 1. Effects of MPG, APO, and L-NAME on basal contractility, relaxation, and MAP duration

|                | LVDP,<br>mmHg | +dP/dt, mmHg/s | $t_{1/2}$ , ms | MAPD <sub>90</sub> ,<br>ms |
|----------------|---------------|----------------|----------------|----------------------------|
| Control        | 84.6 ± 7.4    | 2761.3 ± 238.5 | 48.8 ± 1.9     | 51.8 ± 5.8                 |
| MPG (n = 8)    | 92.7 ± 12.3   | 2560.0 ± 144.8 | 54.6 ± 2.8     | 52.5 ± 2.8                 |
| Control        | 88.8 ± 3.3    | 2696.6 ± 128.3 | 48.4 ± 0.8     | 50.6 ± 6.9                 |
| APO (n = 6)    | 90.1 ± 5.6    | 2686.7 ± 155.9 | 53.9 ± 2.2     | 55.8 ± 0.7                 |
| Control        | 96.2 ± 8.0    | 3340.3 ± 243.5 | 49.8 ± 3.9     | 56.0 ± 1.5                 |
| L-NAME (n = 9) | 97.5 ± 6.0    | 3389.7 ± 203.4 | 55.8 ± 5.3     | 57.0 ± 0.6                 |

Values are means ± SE and were obtained at the end of the stabilization (Control) and at the end of the 10-min period of drug perfusion, prior to ischemia. LVDP, left ventricular developed pressure; +dP/dt, maximal rate of pressure development;  $t_{1/2}$ , half relaxation time; MAPD<sub>90</sub>, monophasic action potential duration at 90% repolarization; MPG, mercaptopropionylglycine; APO, apocynin; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester.

according to Porzio and Pearson (11). For immunological detection of RyR2 and phosphorylated RyR2 (pS2814-RyR2), 100 µg of homogenate protein was electrophoresed per gel lane in 3.5–8.0% gradient acrylamide gels according to Laemmli (11). For immunological detection of the redox state of CaMKII (oxidized CaMKII, ox-CaMKII) and RyR2 (S-glutathionylation and S-nitrosylation), 50 µg of homogenate protein was resuspended in nonreducing loading solution containing 35 mM N-ethylmaleimide (NEM) and separated in 10% acrylamide (ox-CaMKII) and 3.5–8.0% gradient Tris-acetate (RyR2) gels under nonreducing conditions (42). Proteins were transferred to PVDF membranes (Immobilon-P, Millipore). Blots were blocked with Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4) with or without the addition of 0.1% Tween (TBST) and 5% skim milk or albumin, as indicated for each antibody, and then incubated with the following primary antibodies: ox-CaMKII (Millipore, 1:1,000 in 5% milk TBST) and Thr286-phosphorylated CaMKII (Abcam, 1:1,000 in 1% albumin TBST), RyR2 (Santa Cruz Biotechnology, 1:5,000 5% milk TBST), Ser2814-phosphorylated RyR2 (Badrilla, 1:2,500 in 5% milk TBST), Thr17-phosphorylated PLN (Badrilla, 1:2,500 in 5% milk TBST), PLN (Thermo Fisher Scientific, 1:5,000 in 1% milk TBST), GAPDH (Millipore, 1:10,000 in 1% milk TBST), anti-glutathione (GSH) (Virogen, 1:1,000 in 5% albumin TBST), and anti-nitrosocysteine (AG-Scientific, 1:1,000 in 5% albumin TBST). The membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, 1:10,000 in 1% milk TBST) and developed using an enhanced chemiluminescence reagent (Millipore). The signals emitted for chemiluminescence were detected using Chemidoc Imaging system (Bio-Rad) and analyzed with ImageJ software (NIH). The results were normalized to corresponding densitometry signal of unphosphorylated protein or GAPDH.

#### Determination of NOX Activity

Superoxide production was measured by lucigenin chemiluminescence in SR vesicles. Microsomal fractions enriched in SR vesicles were obtained from frozen hearts homogenized in four volumes of 20 mM MOPS-Tris (pH 6.8) and 300 mM sucrose with protease inhibitors (4 µg/ml leupeptin, 4 µg/ml pepstatin A, 1 mM benzamidine, and 1 mM PMSF). The homogenate was sedimented at 3,800 g for 15 min, and the resulting pellet was rehomogenized 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 was resuspended at 10 mg protein/ml in homogenization buffer, fractionated in small aliquots, frozen in liquid N<sub>2</sub>, and stored at –80°C. SR vesicles (0.2 mg/ml) were incubated in 100 mM MOPS-Tris (pH 7.0), 100 µM NADPH, and 5 µM lucigenin at 25°C. Chemiluminescence was measured in a Berthold FB 12 luminometer

and expressed as nanomoles of superoxide anion per milligram of protein per minute (42).

#### Assessment of Reduced GSH Levels

Total reduced GSH content was determined according to Ellman's method (44). Briefly, pulverized frozen ventricles were treated with four volumes of 10% tricarboxylic acid, placed in an ice water bath for at least 20 min, and then sedimented at 8,500 g for 15 min. The determination of GSH levels in the supernatant was based on the reaction of nonprotein sulfhydryl groups with 5,5'-dithiobis 2-nitrobenzoic acid (0.01 M) in 0.4 M Tris buffer pH 8.9 to give a compound that absorbs at 412 nm. GSH levels were expressed as micrograms per gram of tissue.

#### Detection of ROS Production

ROS generation was determined using dihydroethidium (DHE, Sigma) fluorescence method. DHE produces red fluorescence when oxidized to ethidium bromide, which is then intercalated into DNA. Fresh heart sections were frozen in Tissue Tec optimal cutting temperature compound (Sakura Finetek Europe). With the use of a Leica cryostat, 20-µm transverse sections were cut, mounted on slides (Pearl), and incubated with 10 µM DHE at 37°C for 30 min in a light-protected and humidified chamber. Sections were washed twice with BBS and stored in the dark. Images were acquired using an epifluorescence microscope (Nikon Eclipse E200) with rhodamine filter. The fluorescence intensity per image was quantified using ImageJ analysis software (NIH). Five frozen cardiac sections per animal were analyzed, and 20 photographs were randomly taken for each section with a ×40 objective (26).

#### RyR2 Free Thiol Content

The content of free thiols in RyR2 was determined using the monobromobimane (mBB, Calbiochem) fluorescence method. Briefly, frozen heart tissue was homogenized in five volumes HEN buffer containing 250 mM Hepes (pH 7.4), 1 mM EDTA, 50 mM neocuproine, 300 mM sucrose, and protease inhibitors (0.5 µg/ml leupeptin, 1.0 µg/ml pepstatin, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM benzamidine, and protease inhibitor cocktail). Homogenates were centrifuged at 12,000 g for 10 min, and supernatants were incubated with 4 mM NEM, 10 mM dithiothreitol (DTT) or untreated for 30 min in the dark and at room temperature. Samples were then centrifuged at 40,000 g for 1 h, and pellets were washed and resuspended in HEN buffer. Incubation with 5 mM mBB was performed for 1 h in the dark and at room temperature. To remove unbound mBB, samples were centrifuged at 40,000 g for 1 h, and the pellets were washed, resuspended in HEN buffer, and combined with nonreducing loading buffer with a final concentration of 2 mM NEM. Samples were heated for 20 min at 50°C, and proteins were separated in 3.5–8.0% Tris-acetate gels under nonreducing conditions. Images were acquired using Chemidoc Imaging system (Bio-Rad) and analyzed with ImageJ software (NIH). mBB fluorescence was normalized to RyR2 amount determined using Coomassie blue staining of the gels (12, 47). Treatment with reducing (DTT) and oxidizing (NEM) agents was used to obtain maximum and minimum mBB fluorescence values, respectively, for each intervention.

#### Statistics

Data are expressed as means ± SE. Statistical significance was determined by Student's *t*-test for paired or unpaired observations as appropriate and ANOVA when different groups were compared. The Newman-Keuls test was used to examine statistical differences observed with the ANOVA. Numbers of arrhythmias between two different groups were analyzed using the Mann-Whitney test. Values of *P* < 0.05 were considered significant.

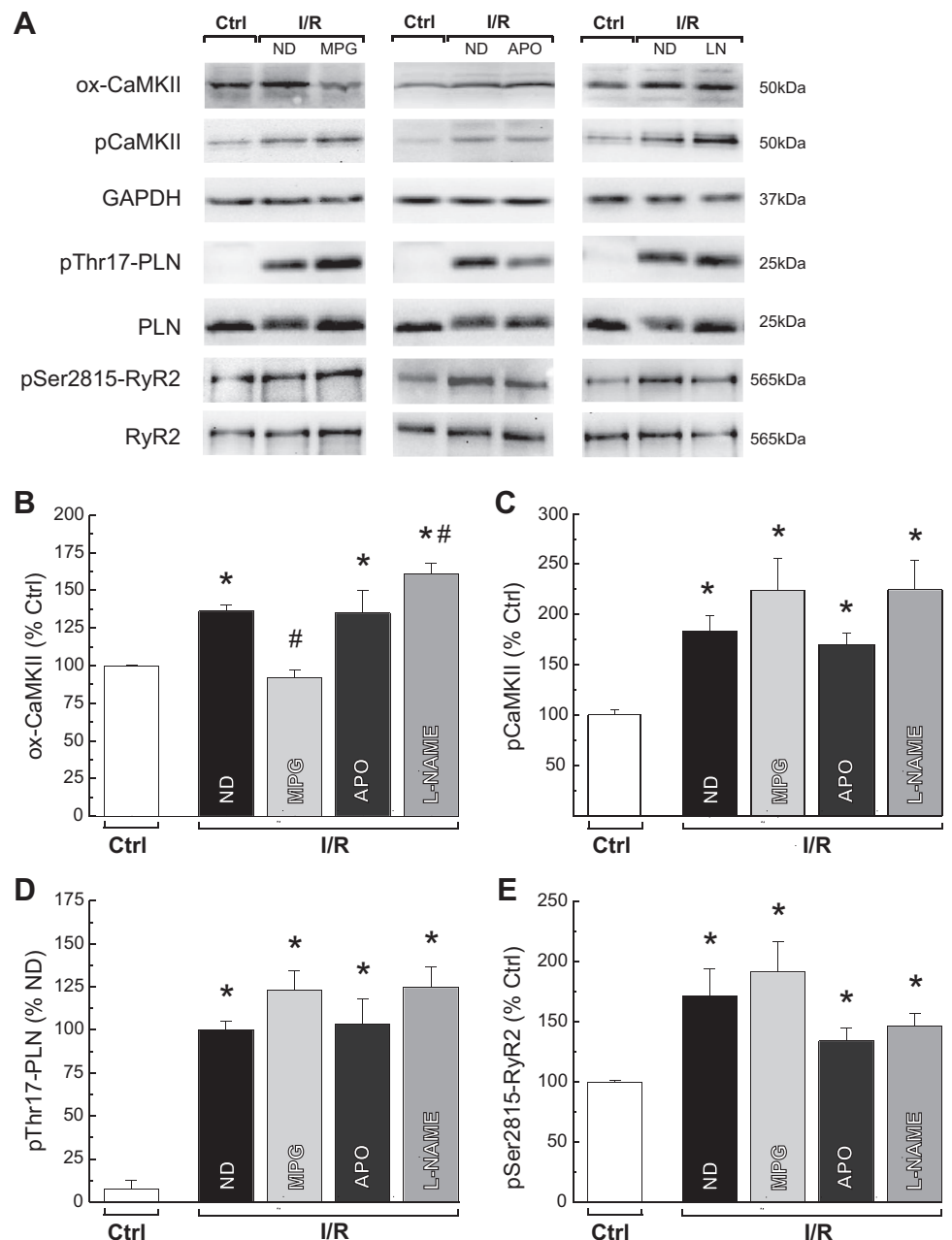
## RESULTS

*Oxidation of CaMKII During Ischemia/Reperfusion*

Oxidative stress is a hallmark feature of reperfusion injury, and CaMKII is directly modified by ROS. Therefore, we examined the possible involvement of oxidation-dependent activation of CaMKII in rat hearts subjected to ex vivo ischemia/reperfusion. Oxidation of CaMKII (ox-CaMKII) increased by 36% at the beginning of reperfusion. This enhancement was only abrogated by the free radical scavenger MPG. Neither the NOX inhibitor, APO, nor the general NOS inhibitor (L-NAME) was able to reduce the reperfusion-induced increase in ox-CaMKII (Fig. 1, A and B). To determine the effect of ox-CaMKII, we studied the phosphorylation of three CaMKII targets: the kinase itself, PLN, and RyR2 in the

absence and the presence of the different antioxidant treatments (Fig. 1, A–E). The reduction in ox-CaMKII induced by MPG was not associated with a diminished phosphorylation level of CaMKII, Thr17 of PLN, and Ser2815 of RyR2. As expected, the inhibition of NOX and NOS by APO and L-NAME, respectively, did not modify the phosphorylation of CaMKII targets. The use of another free radical scavenger, Tiron, mimicked the effects of MPG on reperfusion-induced ox-CaMKII levels and autophosphorylation of CaMKII. Whereas Tiron significantly reduced ox-CaMKII from  $130.9 \pm 6.0\%$  to  $114.3 \pm 2.9\%$  control, no changes in pCaMKII were observed ( $260.0 \pm 25.7\%$  vs.  $356.2 \pm 66.3\%$  control),  $n = 5-13$ . The overall findings suggest that, although ox-CaMKII increases during early reperfusion, it seems not to be necessary to sustain CaMKII activity at least toward its SR substrates.

Fig. 1. Reperfusion-induced increase in  $\text{Ca}^{2+}$  calmodulin-dependent kinase II (CaMKII) activity is independent of its oxidation state. Typical blots (A) and overall results of experiments showing oxidation (ox-CaMKII, B) and autophosphorylation of CaMKII (pCaMKII, C) and phosphorylation of its 2 substrates, Thr17 site of phospholamban (PLN) (D) and Ser2815 of ryanodine receptor 2 (RyR2) (E), at the onset of ischemia/reperfusion (I/R) in the absence (ND) or presence of different antioxidants are shown. Reperfusion-induced increase in ox-CaMKII was only abolished by mercaptopropionylglycine (MPG) treatment. MPG, apocynin (APO), or  $N^G$ -nitro-L-arginine methyl ester (L-NAME) (LN) were not able to prevent the CaMKII-dependent increase in protein phosphorylations. The data represent means  $\pm$  SE ( $n = 5-13$  hearts). \* $P < 0.05$  with respect to control values (Ctrl). # $P < 0.05$  with respect to reperfusion with no drug ND.



Moreover, the lack of effect of L-NAME on the phosphorylation of CaMKII and its targets indirectly indicates that the described activation by S-nitrosylation of CaMKII (5) is not occurring in our experimental conditions.

*Oxidative/Nitrosative Modifications of RyR2 and Their Impact on Reperfusion Arrhythmias*

ROS/RNS signaling plays a role in the regulation of RyR2 channel gating by promoting redox posttranslational modifications. It has been shown that oxidative stress developed during the course of different cardiac diseases induces RyR2 SR Ca<sup>2+</sup> leak, leading to the appearance of arrhythmias. In these experiments, we particularly examined the S-glutathionylation and S-nitrosylation of the channel in the stunned heart and its correlation with the presence of arrhythmias. The level of these two RyR2 posttranslational modifications increased during early reperfusion, and these changes were prevented when the ischemia/reperfusion protocol was performed in the presence of MPG (Fig. 2, A and B). In addition, MPG decreased the number of VPBs (Fig. 3, A and B) and diminished the average duration of VT, a more severe type of arrhythmia (Fig. 3, C and D), with a tendency to decrease the incidence of these events (Fig. 3E). Similar results were obtained when MPG was given only at reperfusion. Overall, these findings are in line with the widely accepted concept that oxidized RyR2 is prone to arrhythmias.

To determine the relative contribution of S-glutathionylation and S-nitrosylation to the generation of arrhythmias, the number of VPBs and the duration and incidence of VTs were evaluated in the presence of APO and L-NAME. The reperfusion-induced increase in NOX activity (from 2.6 ± 0.1 to 4.1 ± 0.4 nmol O<sub>2</sub>·mg protein<sup>-1</sup>·min<sup>-1</sup>, P < 0.05) was diminished to control values by APO (2.1 ± 0.1 nmol O<sub>2</sub>·mg protein<sup>-1</sup>·min<sup>-1</sup>). This treatment selectively suppressed the S-glutathionylation of RyR2 without affecting the level of S-nitrosylation (Fig. 4, A and B). In addition, L-NAME abolished the S-nitrosylation of RyR2 in the absence of changes in

S-glutathionylation of RyR2 (Fig. 4, A and B). Neither APO nor L-NAME treatment alleviated arrhythmogenesis. Conversely, the number of VPBs significantly increased (Fig. 4C), whereas VTs episodes showed a trend toward a longer duration and higher incidence with respect to ischemia/reperfusion hearts with no intervention (Fig. 4, D and E). Similarly to L-NAME, treating the hearts with a NOS-1-specific inhibitor, SMTc, increased the number of VPBs at early reperfusion (SMTc: 63 ± 3; L-NAME: 65 ± 6 vs. ND: 45 ± 4, n = 3–8).

*Oxidative/Nitrosative Modifications of RyR2 and Their Impact on Contractile Recovery*

Because arrhythmogenic activity during reperfusion has been linked to SR Ca<sup>2+</sup> leak, changes in SR Ca<sup>2+</sup> load and therefore in contractility could be expected. The contractile recovery after ischemia was not modified by MPG; +dP/dt and LVEDP attained levels similar to control hearts perfused in the absence of drugs (Fig. 5, A and B). However, both mechanical parameters were drastically impaired by APO and L-NAME; +dP/dt was significantly reduced, whereas LVEDP was enhanced (Fig. 5, C and D). Therefore, removing S-glutathionylation and/or S-nitrosylation of RyR2 by a reducing agent (MPG) or through selective enzyme inhibitors (APO, L-NAME) seemed to be associated with different effects on contractile recovery after ischemia.

*Redox Balance During Ischemia/Reperfusion*

Depending on the pharmacological tool used to prevent the S-glutathionylation and S-nitrosylation, beneficial or deleterious effects on electrical and mechanical activities during reperfusion were observed. To assess whether this discrepancy originates from a different oxidative environment derived from the treatment used, reduced GSH levels were measured in hearts subjected to ischemia/reperfusion in the presence or absence of MPG, APO, or L-NAME. The level of GSH was reduced in ischemia/reperfusion hearts, confirming that oxida-

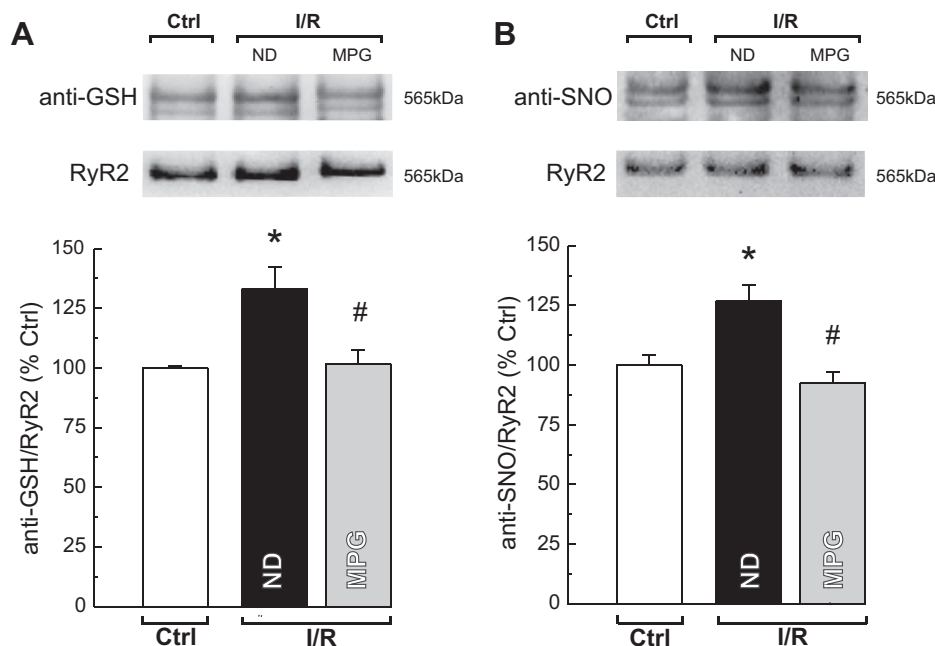


Fig. 2. Reperfusion increases the S-glutathionylation and S-nitrosylation of RyR2. Typical blots and overall results of experiments showing the S-glutathionylation (A) and S-nitrosylation (B) of RyR2 in hearts subjected to I/R in the absence (ND) or presence of MPG are shown. Reperfusion-induced increase in S-glutathionylation and S-nitrosylation of RyR2 was prevented by MPG. The data represent means ± SE (n = 7–13 hearts). \*P < 0.05 with respect to Ctrl. #P < 0.05 with respect to reperfusion with ND. GSH, glutathione; SNO, nitrosocysteine.

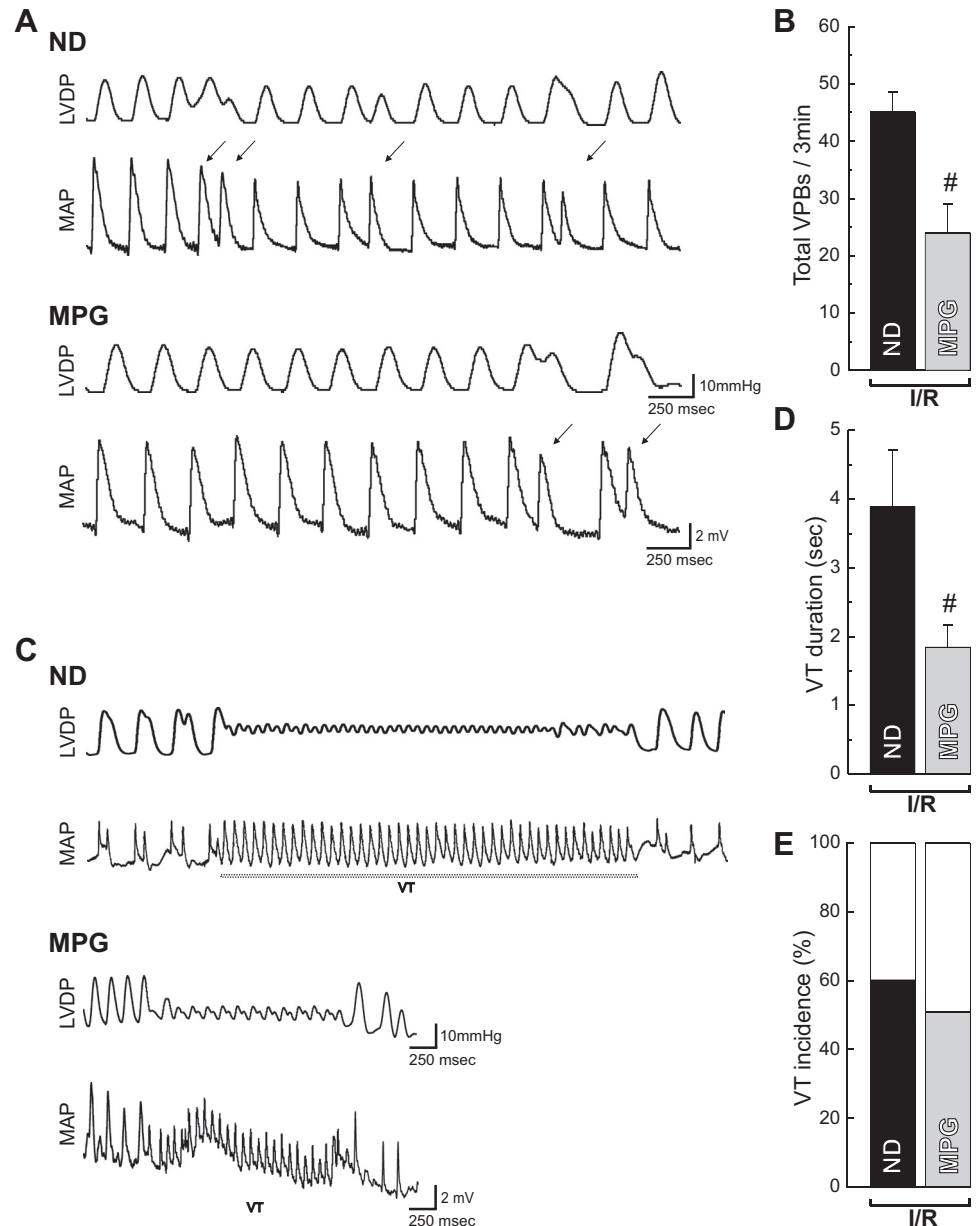


Fig. 3. MPG reduces the severity of reperfusion arrhythmias. Representative simultaneous recordings of mechanical (left ventricular developed pressure, LVDP) and electrical activity (monophasic action potential, MAP) showing ventricular premature beats (VPBs) (A) and ventricular tachycardia (VT) (C) during the early reperfusion in the absence (ND) or the presence of MPG are shown. Overall results of total VPBs (B) and VT, duration, and incidence (D and E) in the first 3 min of reperfusion are shown. The antioxidant MPG decreased the number of total VPBs (Mann-Whitney test) and the duration of VT episodes. The data represent means  $\pm$  SE ( $n = 5-14$  hearts).  $\#P < 0.05$  with respect to reperfusion with ND.

tive stress is increased by the injury. Treatment with MPG restored GSH to control levels; however, APO or L-NAME did not (Fig. 6A), indicating that only MPG was able to prevent the oxidative stress induced by ischemia/reperfusion. The antioxidant effect of MPG was confirmed by quantifying ROS production using DHE staining. The increased ethidium fluorescence intensity observed in hearts subjected to ischemia/reperfusion was reduced by MPG treatment (Fig. 6, B and C). These changes in ROS production were paralleled by changes in the redox state of RyR2, as determined by the mBB fluorescence method. Ischemia/reperfusion decreased the level of free thiol content on RyR2, consistent with increased oxidation of the SR  $\text{Ca}^{2+}$  channel, and the presence of MPG precluded this effect (Fig. 6, D and E). Thus the apparent beneficial or deleterious effects of S-glutathionylation and S-nitrosylation of RyR2 on the electrical and mechanical activity during reper-

fusion seem to depend on the cellular redox milieu, which is differentially affected by the three antioxidants used.

#### Impact of CaMKII-Dependent Phosphorylation and Oxidation of RyR2 on the Propensity for Reperfusion Arrhythmias

As we previously described, knockin mice lacking the principal CaMKII phosphorylation site in RyR2 (S2814A) showed a marked decrease in reperfusion arrhythmogenesis (40). To test whether antioxidant treatment further reduced the appearance of arrhythmias, S2814A mice were subjected to ischemia/reperfusion in the absence and presence of MPG (Fig. 7). Reduction of cellular oxidative stress by MPG, which avoids the oxidation of RyR2 during reperfusion (Fig. 6, D and E), further diminished the number of VPBs by 50%. These results give support to the concept that both phosphorylation and

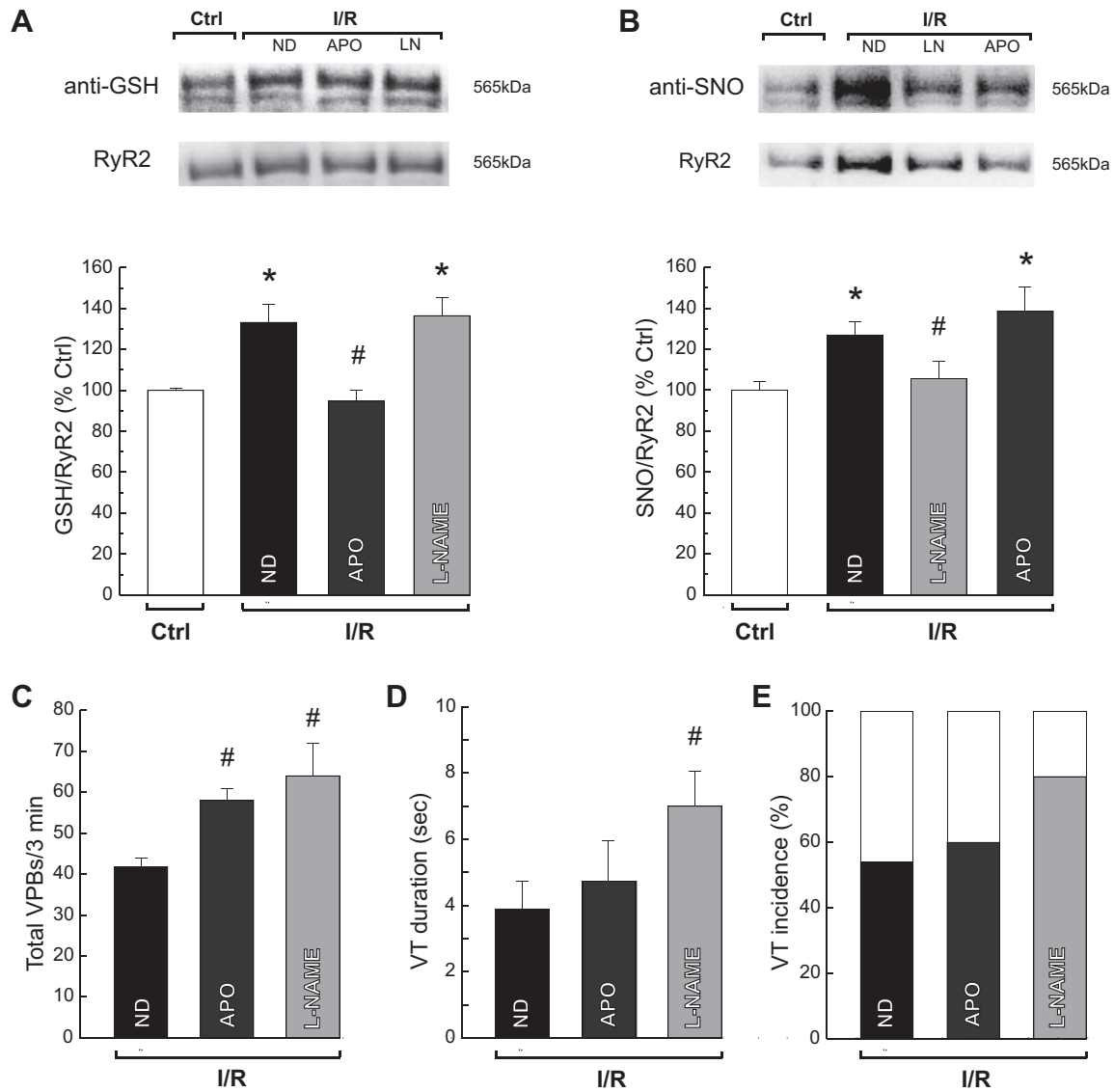


Fig. 4. Inhibition of specific oxidative/nitrosative changes of RyR2 increases reperfusion arrhythmias. Typical blots and overall results of experiments show the reperfusion-induced increase in *S*-glutathionylation (A), *S*-nitrosylation of RyR2 (B), total VPBs (C), VT, and duration and incidence (D and E) in the first 3 min of reperfusion in the absence (ND) or the presence of APO and L-NAME. APO selectively abolished the *S*-glutathionylation of RyR2, and L-NAME did the same with the *S*-nitrosylation of RyR2. Both drugs augmented the number of VPBs (Mann-Whitney test) and showed a tendency to increase the duration and incidence of VT. The data represent means  $\pm$  SE ( $n = 4-11$  hearts). \* $P < 0.05$  with respect to I/R. # $P < 0.05$  with respect to reperfusion with ND.

oxidation of RyR2 are involved in the generation of reperfusion arrhythmias. Furthermore, the results suggest that oxidation of RyR2 contributes to reperfusion arrhythmias, even in the absence of the CaMKII-dependent phosphorylation of RyR2.

**DISCUSSION**

Oxidative stress is a prominent feature in the onset and progression of a number of heart diseases, including ischemia/reperfusion injury. It is well known that CaMKII and RyR2 are functionally influenced by redox modifications (9, 13, 18, 23). Our results indicate that both proteins become oxidized following ischemia/reperfusion in the intact heart, but the impact of such alterations on the genesis of arrhythmias and the recovery of contractility are apparently different. Although oxidation of CaMKII seems to have no consequences in terms

of the activity of the enzyme and the phosphorylation of two SR target proteins, the redox modifications of RyR2 appear to have both beneficial and detrimental effects. In the present work, we particularly studied the *S*-glutathionylation and *S*-nitrosylation of RyR2, two reversible posttranslational modifications of the channel that seem to play a protective role in the arrhythmogenesis and the contractile recovery of the stunned heart.

*CaMKII Mechanism of Activation During Ischemia/Reperfusion*

Redox-dependent CaMKII activation alters different mechanisms that may contribute to arrhythmia propensity (23). In postischemic reperfusion, a well-known situation of oxidative stress, experiments from our laboratory previously demonstrated that CaMKII-dependent phosphorylation of RyR2 is a

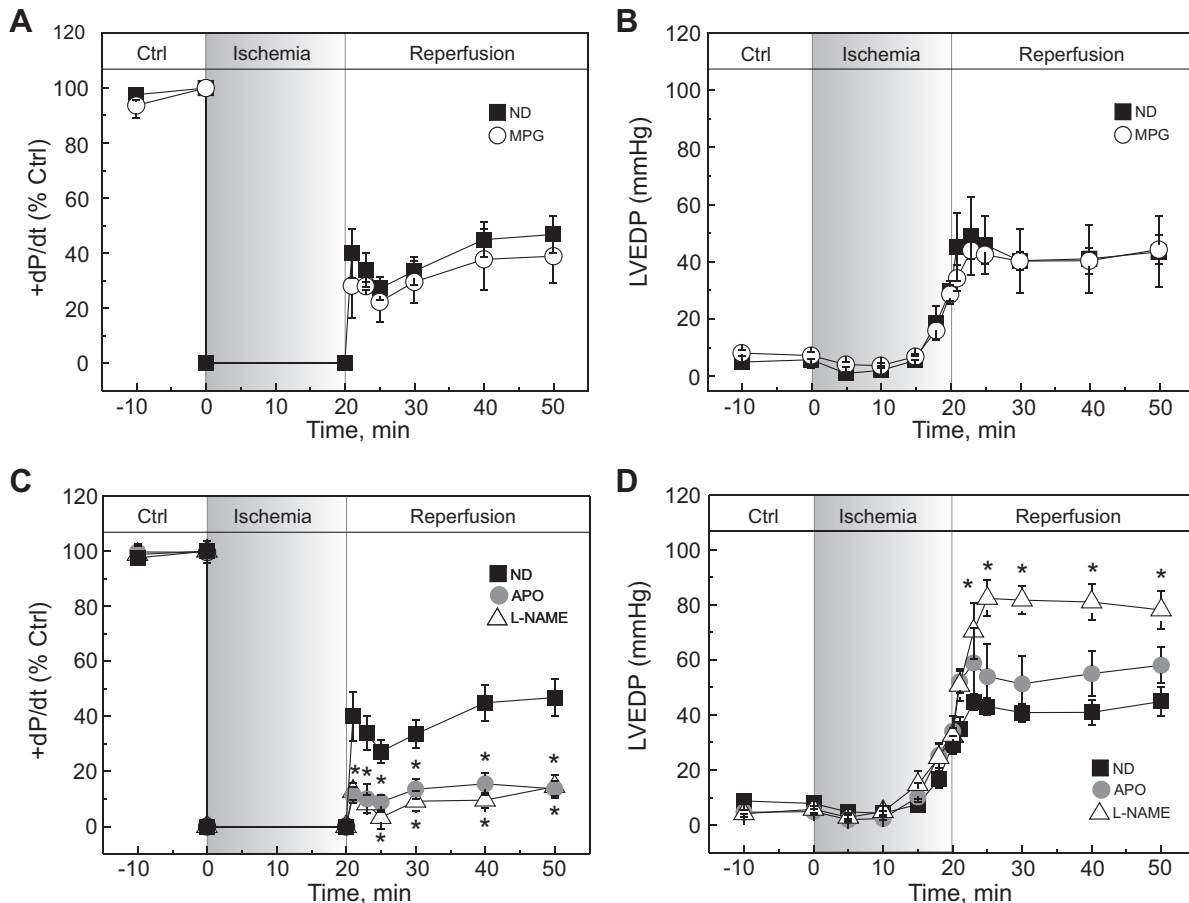


Fig. 5. Differential effects of the antioxidants on the contractile recovery during reperfusion. Time courses are shown of maximal rate of pressure development ( $+dP/dt$ , A and C) and left ventricular end-diastolic pressure (LVEDP) (B and D) of rat hearts submitted to I/R protocols in the absence (ND) or presence of MPG (A and B), APO, and L-NAME (C and D). Hearts treated with MPG showed a similar recovery pattern to ND. The presence of APO or L-NAME deteriorated the contractility with respect to ND and further increased LVEDP. The data represent means  $\pm$  SE ( $n = 4-11$  hearts). \* $P < 0.05$  with respect to ND.

crucial factor for triggering arrhythmias (40). The present findings further showed that ox-CaMKII increased during reperfusion by a NOX-uncoupled/NOS-independent mechanism. In this scenario, mitochondria appear as a good candidate to provide the ROS involved in CaMKII oxidation. However, redox-dependent activation of this enzyme did not influence the degree of RyR2 phosphorylation. The lack of relationship between oxidation and activation of CaMKII has also been reported by Bell et al. (2), who showed similar levels of ox-CaMKII in stunned male and female rat hearts associated with different phosphorylation extent of CaMKII and PLN. Taken together, the overall results suggest that ox-CaMKII is not essential to maintain the kinase activity in ischemia/reperfusion at least toward its SR substrates. It is likely that the massive  $Ca^{2+}$  overload at early reperfusion produced by both an uncontrolled  $Ca^{2+}$  influx (34) and SR  $Ca^{2+}$  depletion (48) provides the necessary intracellular  $Ca^{2+}$  to activate and sustain CaMKII activity independently of its oxidation state.

#### Oxidative/Nitrosative Changes of RyR2 and Arrhythmogenesis During Ischemia/Reperfusion

RyR2 is highly sensitive to redox state attributable to the large number of cysteines that compose the homo-tetramer (18). Depending on the degree of oxidative stress, RyR2 free

thiols can be reversibly or irreversibly oxidized (31). Three types of reversible redox modifications have been described for RyR2: disulfide oxidation, S-nitrosylation, and S-glutathionylation. Whereas irreversible oxidation unambiguously increases RyR2 activity (31, 45, 53), the impact of reversible redox changes on the channel function remains controversial (6, 7, 12, 25, 42, 52, 56). It has been suggested that RyR2 intersubunit cross linking or alterations in RyR2 association with auxiliary proteins, as a result of oxidative stress, promote an increase in RyR2 activity and diastolic  $Ca^{2+}$  leak (24, 33). This  $Ca^{2+}$  leak could be the cause of the formation of  $Ca^{2+}$  waves sufficient to induce spontaneous APs and therefore cardiac arrhythmias during reperfusion. Among protein redox modifications, S-nitrosylation and S-glutathionylation have been proposed to conspire to the redox-induced deleterious effects by protecting the reactive thiol groups from further irreversible oxidation (14, 29). In the stunned heart, neither the occurrence of redox changes of RyR2 nor their functional consequences on arrhythmias and contractile dysfunction have been previously described.

In the present study, we found that S-nitrosylation and S-glutathionylation of RyR2 augmented during early reperfusion after ischemia. Prevention of these reversible redox modifications in the reperfusion-induced oxidative environment



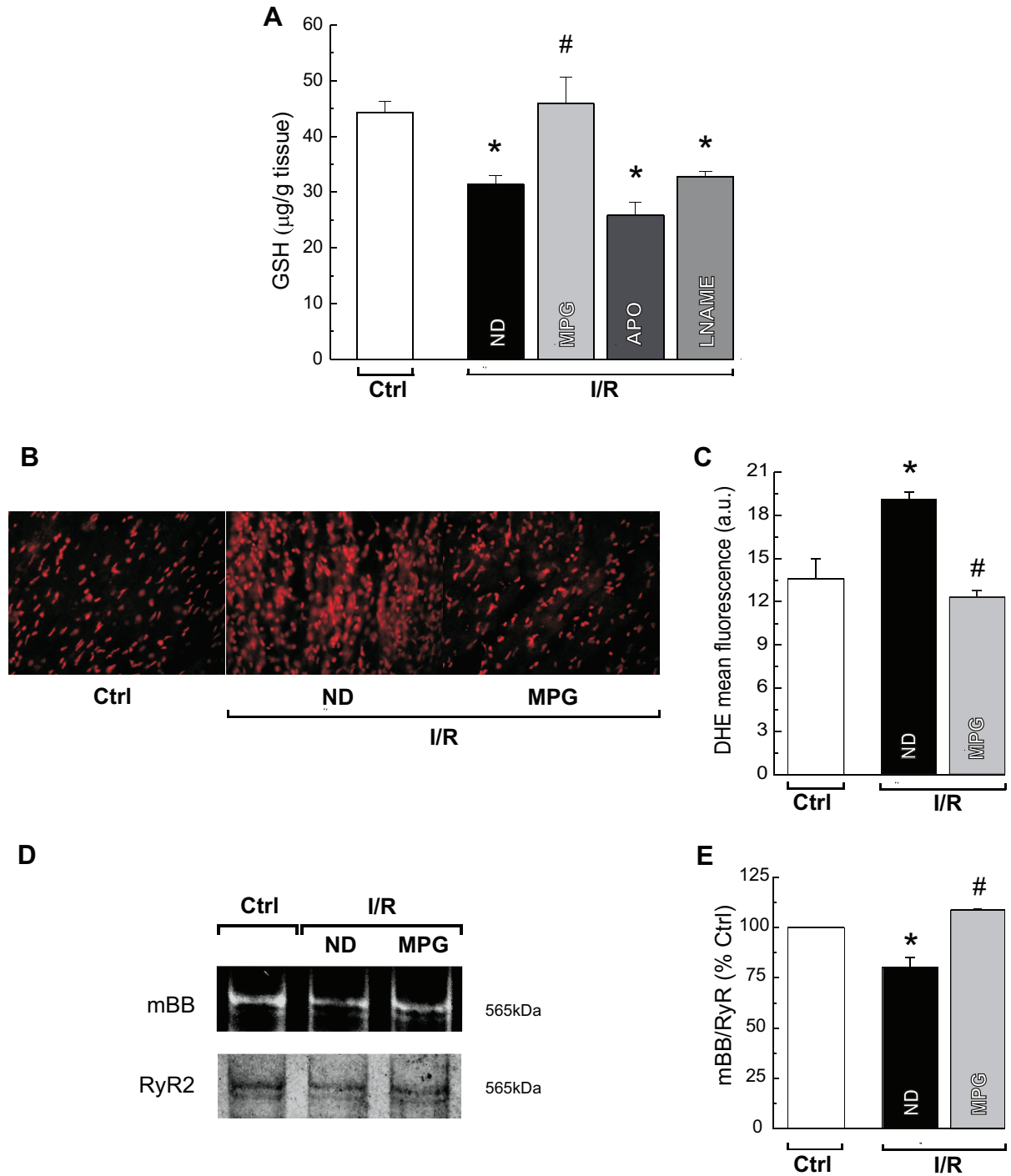


Fig. 6. Effects of the antioxidants on reduced GSH content, reactive oxygen species (ROS) formation, and RyR2 free thiol content during reperfusion. Average levels of reduced form of GSH measured in tissue homogenates from Ctrl or I/R rat hearts in the absence (ND) or presence of MPG, APO, or L-NAME are shown. Only MPG treatment restored GSH levels to Ctrl values (A) and overall results (C) of dihydroethidium (DHE) staining for ROS production in Ctrl and I/R hearts without (ND) or with MPG treatment are shown. MPG reduces ROS production to Ctrl levels. Representative (monobromobimane, mBB) fluorescence intensity and Coomassie blue-stained gels of RyR2 (D) and average results of free thiol content on RyR2 (E) in Ctrl and I/R hearts in the absence (ND) or presence of MPG are shown. Content of free thiols decreases in I/R, and it is restored by treatment with MPG. The data represent means  $\pm$  SE ( $n = 3-6$  hearts). \* $P < 0.05$  with respect to Ctrl. # $P < 0.05$  with respect to reperfusion with ND.

was associated with a higher number of arrhythmias and an impaired contractile recovery. It is likely that, in this context, S-nitrosylation and S-glutathionylation emerge as protective mechanisms. Our findings are consistent with Cuttler et al. (6), who found that the combination of decreased S-nitrosylation

and increased oxidation of RyR2 leads to a striking increase in spontaneous SR  $Ca^{2+}$  release and ventricular arrhythmias, in intact hearts exposed to elevated  $[Ca^{2+}]$  and oxidative stress, two prevailing conditions in ischemia/reperfusion. Similar results were described in the failing heart (1, 13).

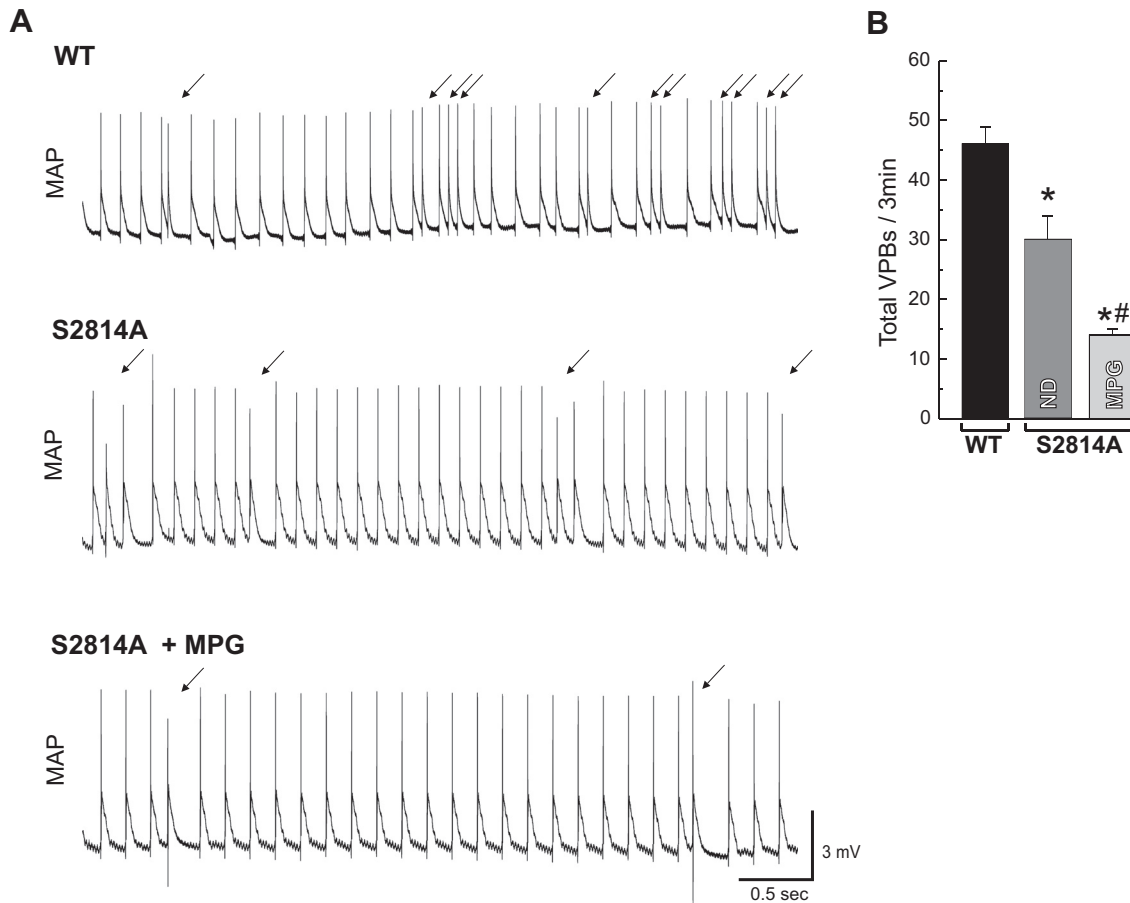


Fig. 7. CaMKII-dependent phosphorylation of RyR2-Ser2814 site and redox changes are critical for generation of reperfusion arrhythmias. Representative recordings of electrical activity (MAP) showing VPBs (arrows) during early reperfusion in WT mice or mice with genetic mutation of Ser2814 site of RyR2 (S2814A) in the absence or presence of MPG (A) and overall results of total VPBs in the first 3 min of reperfusion (B) are shown. There was a significant decrease in VPBs in RyR2-S2814A mice relative to WT, but the treatment with MPG declined them further. The data represent means  $\pm$  SE ( $n = 3-8$  hearts). \* $P < 0.05$  with respect to WT. # $P < 0.05$  with respect to S2814A without MPG.

A different situation occurred when the reperfusion-induced oxidative stress was precluded by the presence of the free radical scavenger, MPG. Although the *S*-nitrosylation and *S*-glutathionylation of RyR2 were blunted, the number of VPBs and the duration of VTs decreased. In this environment, both the reversible and the irreversible oxidations of RyR2 might be prevented, turning RyR2 into a less leaky channel and decreasing the severity of arrhythmias. The fact that the content of free thiols on RyR2 in those hearts subjected to ischemia/reperfusion in the presence of MPG was similar to control conditions supports this possibility. The antiarrhythmic effects of MPG treatment were not associated with an enhancement of the contractile recovery after ischemia. This behavior might reflect the capacity of myocyte  $Ca^{2+}$  handling for autoregulation (8). Although the SR  $Ca^{2+}$  content might be transiently higher in the presence of MPG, the compensatory changes in  $Ca^{2+}$  fluxes would restore  $Ca^{2+}$  cycling and steady-state contractility after ischemia. However, this mechanism might be overwhelmed when the severity of reperfusion arrhythmias increased, as in the case of APO and L-NAME. The lack of reversible oxidations, which turns RyR2 hyperactive as a result of irreversible oxidation, could lead to a drastic SR  $Ca^{2+}$  leak, which would deplete the SR, decrease  $Ca^{2+}$  transient, and impair contractility.

Several experiments studying the impact of redox changes on RyR2 activity have been performed in artificial planar bilayer systems or isolated myocytes (1, 16, 47). The novelty of the present findings is that we were able to detect reperfusion-induced redox modifications of RyR2 in the intact ex vivo heart, a model in which the general nitroso/redox balance is better preserved.

#### Contribution of Nitroso/Redox Imbalance to Ischemia/Reperfusion Arrhythmias in S2814A Mice

Numerous studies have shown that either CaMKII-dependent phosphorylation or oxidation of RyR2 favors the generation of arrhythmias (1, 4). Recently, a potential interaction of the two posttranslational modifications of RyR2 has been described in different animal models (6, 22, 53). In the mdx mouse model of Duchenne muscular dystrophy (22, 53), the oxidation state and the activity of RyR2 were markedly reduced when RyR2 phosphorylation was genetically inhibited. In contrast, the reduced *S*-nitrosylation of RyR2 caused by inhibition of neuronal NOS increased the oxidation of RyR2 and  $Ca^{2+}$ -triggered arrhythmias in intact guinea pig hearts, together with a decrease in the phosphorylation of Ser2814 site of RyR2. A cross talk between ROS/RNS modifications and

phosphorylation of RyR2 was not evident in the present experiments of hearts subjected to ischemia/reperfusion. Antioxidant treatments, which modified the redox state of RyR2, altered the propensity to arrhythmias, without affecting the reperfusion-induced increase in CaMKII phosphorylation of RyR2. Moreover, in mice lacking the CaMKII phosphorylation site of RyR2, which showed a decreased number of VPBs compared with wild-type, MPG further reduced the propensity to arrhythmias. Thus, in our experimental conditions, oxidation and phosphorylation seem to act as independent and additive pathways of regulation of RyR2 activity.

In conclusion, our present results provide new insights into the mechanisms underlying the reperfusion arrhythmias. They suggest that, whereas S-nitrosylation and S-glutathionylation of RyR2 restrain the propensity for arrhythmias, other oxidations of RyR2 amplify the arrhythmogenic effect of the CaMKII phosphorylation of the channel. These mechanisms, working in concert, provide the molecular basis for reperfusion arrhythmias, and they must be considered as therapeutic targets in the ischemia/reperfusion injury.

#### Limitations of This Study

The present data show that nitroso/redox imbalance is an important mechanism that contributes to the triggering of VPBs and VT during the early reperfusion phase and suggest that RyR2 is a putative player in this scenario. We are aware of the fact that a causal relationship between RyR2 redox modification and arrhythmias is difficult to ascertain in the intact heart in which pharmacological interventions are unlikely to have effects limited only to RyR2. Alteration of other redox-sensitive proteins cannot be excluded. In this context, it was recently reported that SERCA2a oxidation plays a significant role in repolarization alternans associated with myocardial infarction (35). Future work is needed to validate the contribution of selective RyR2 redox modifications to reperfusion arrhythmogenesis.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

R.B., B.S.R., M.N.D.C., J.I.M., M. Salas, G.S., G.R.S., C.M.-W., and M. Said performed experiments; R.B., B.S.R., M.N.D.C., J.I.M., M. Salas, G.S., G.R.S., C.M.-W., and M. Said analyzed data; R.B., M. Salas, G.R.S., L.V., C.M.-W., and M. Said interpreted results of experiments; R.B., B.S.R., C.M.-W., and M. Said prepared figures; M. Salas, L.V., C.M.-W., and M. Said conception and design of research; M. Salas, G.S., P.D., L.V., X.H.W., C.M.-W., and M. Said edited and revised manuscript; L.V., C.M.-W., and M.

Said drafted manuscript; L.V., X.H.W., C.M.-W., and M. Said approved final version of manuscript.

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