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Original article

CaMKII-dependent phosphorylation of cardiac ryanodine receptors regulates cell death in cardiac ischemia/reperfusion injury

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ABSTRACT

Ca^{2+}-calmodulin kinase II (CaMKII) activation is deleterious in cardiac ischemia/reperfusion (I/R). Moreover, inhibition of CaMKII-dependent phosphorylations at the sarcoplasmic reticulum (SR) prevents CaMKII-induced I/R damage. However, the downstream targets of CaMKII at the SR level, responsible for this detrimental effect, remain unclear. In the present study we aimed to dissect the role of the two main substrates of CaMKII at the SR level, phospholamban (PLN) and ryanodine receptors (RyR2), in CaMKII-dependent I/R injury. In mouse hearts subjected to global I/R (45/120 min), phosphorylation of the primary CaMKII sites, S2814 on cardiac RyR2 and of T17 on PLN, significantly increased at the onset of reperfusion whereas PKA-dependent phosphorylation of RyR2 and PLN did not change. Similar results were obtained in vivo, in mice subjected to regional myocardial I/R (1/24 h). Knock-in mice with an inactivated serine 2814 phosphorylation site on RyR2 (S2814A) significantly improved post-ischemic mechanical recovery, reduced infarct size and decreased apoptosis. Conversely, knock-in mice, in which CaMKII site of RyR2 is constitutively activated (S2814D), significantly increased infarct size and exacerbated apoptosis. In S2814A and S2814D mice subjected to regional myocardial ischemia, infarct size was also decreased and increased respectively. Transgenic mice with double-mutant non-phosphorylatable PLN (S16A/T17A) in the PLN knockout background (PLNDM) also showed significantly increased post-ischemic cardiac damage. This effect cannot be attributed to PKA-dependent PLN phosphorylation and was not due to the enhanced L-type Ca^{2+} current, present in these mice. Our results reveal a major role for the phosphorylation of S2814 site on RyR2 in CaMKII-dependent I/R cardiac damage. In contrast, they showed that CaMKII-dependent increase in PLN phosphorylation during reperfusion opposes rather than contributes to I/R damage.

1. Introduction

Myocardial infarction (MI) is the leading cause of morbidity and mortality in the Western world with >700,000 heart attacks diagnosed each year only in the United States [1]. Ischemic injury results from severe impairment of coronary blood supply and oxygen to the myocardium and prompt restoration of coronary flow can limit the infarct size and reduce mortality. Currently, primary reperfusion therapies, including percutaneous coronary intervention and thrombolysis, are the standard treatments for patients suffering from MI. However, the strategy of rapid reperfusion of ischemic myocardium is usually associated with additional cell damage, cardiac dysfunction and ventricular arrhythmias as a result of reperfusion injury [2–4] The causes of these deleterious effects are multifactorial, but altered Ca^{2+} handling has emerged as a major contributor to post-ischemic dysfunction and injury [3,5–7].

In the heart, Ca^{2+} handling during the excitation-contraction coupling process is mediated by a mechanism known as Ca^{2+}-induced

Abbreviations: AC3-I, Ca^{2+}-calmodulin kinase II-inhibitory peptide; Ca^{2+}, Calcium; CaMKII, Ca^{2+}-calmodulin kinase II; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; I/R, Ischemia/reperfusion; LAD, Left anterior descending; LDH, Lactodehydrogenase; MI, Myocardial infarction; PLN, Phospholamban; PKA, Protein kinase A; PLNDM, Double-mutant non-phosphorylatable PLN; SR, Sarcoplasmic reticulum; SR-AIP mice, Inhibitory CaMKII peptide at SR level; RyR2, Isoform 2 of ryanodine receptors; S16A/T17A mice, Serine16 mutated to Ala/threonine 17 mutated to Ala; S2814A mice, Serine 2814 mutated to Ala (RyR2 constitutively inactivated); S2814D mice, Serine 2814 mutated to Asp (RyR2 constitutively activated); SERCA2a, Isoform 2a of sarco/endoplasmic reticulum Ca^{2+}-ATPase; S, Serine; T, Threonine; TTC, Triphenyltetrazolium chloride; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; WT, Wild-type.

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Ca^{2+} release [8], by which a small influx of external Ca^{2+} through the L-type Ca^{2+} channel binds to and opens the cardiac Ca^{2+} release channel/ryanodine receptor (RyR2), producing a large release of Ca^{2+} from the sarcoplasmic reticulum (SR). Cytosolic Ca^{2+} is then re-sequestered into the SR by the SR Ca^{2+}-ATPase (SERCA2a). The function of this Ca^{2+} pump is regulated by the phosphorylation level of a regulatory protein, also associated with the SR, named phospholamban (PLN). PLN in turn, is subject of additional regulation by interacting partners like HAX-1 (an anti-apoptotic protein) and Gm or the anchoring subunit of protein phosphatase 1, which affect the overall SERCA-mediated Ca^{2+}-transport [9,10].

Recent studies strongly suggested that the phosphorylation of SR proteins by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is involved in reperfusion damage (necrosis, apoptosis, functional impairment) following prolonged ischemia. Conversely, inhibition of CaMKII activity at the SR level protects from the injury caused by ischemia/reperfusion (I/R) [11]. However, the downstream targets of CaMKII responsible for the detrimental effects of I/R remain unclear. For instance, it has been shown that CaMKII-dependent phosphorylation of the threonine 17 site of PLN (T17) occurs at the onset of reperfusion [11,12], yet the functional consequences of this phosphorylation events are uncertain. Indeed, the effects of enhanced SR Ca^{2+} uptake in the face of an ischemic insult are controversial. On one hand, experimental evidence indicated that gene transfer of SERCA2a alleviated post-ischemic cardiac injury in rat and porcine animal models [13,14], suggesting a beneficial effect of increasing SR Ca^{2+} uptake in I/R. On the other hand, several studies indicated that inhibiting SR Ca^{2+} uptake is cardioprotective whereas increasing it is harmful [15–18]. In line with these previous findings, more recent studies have shown that deletion of PLN eliminated the protective effects of a CaMKII-inhibitory peptide in mice (AC3-I) subjected to myocardial infarction. PLN deletion also exacerbated the deleterious cardiac effects of CaMKII transgenic mice [19]. Interestingly, the recently described protective effect of HAX-1/Hsp90 complex on I/R damage [10] can also be related to the inhibitory effect of the combined action of these compounds on SR Ca^{2+} uptake [10,20].

Referent to RyR2, it is known that the increased SR Ca^{2+} leak resulting from CaMKII-dependent phosphorylation of RyR2 is a main contributor to heart failure and reperfusion arrhythmias [4,21–23].

The main goal of the present study was to dissect the role of the two primary targets of CaMKII at the SR level, PLN and RyR2 in CaMKII-dependent I/R injury. We demonstrate here that RyR2 channels are phosphorylated by CaMKII during I/R and play a crucial role in CaMKII-dependent cardiac damage. Knock-in mice with a genetically inactivated CaMKII-phosphorylation site on RyR2 (S2814A) [22] are protected from necrosis, apoptosis and impaired cardiac function, following irreversible I/R injury. Conversely, knock-in mice with a phosphomimetic mutation on RyR2 (S2814D) [21] are more susceptible to cardiac damage following I/R. In contrast, PLN phosphorylation during reperfusion opposes rather than contributes to I/R damage.

Thus, CaMKII-dependent phosphorylation of RyR2 during reperfusion is crucial in determining cardiac damage and the main factor at the SR level responsible for the deleterious effect of CaMKII in I/R injury.

2. Materials and methods

An expanded Methods section is available in the online Supplementary material.

2.1. Animals

Experiments were performed in knock-in male mice (4 months old) in which the S2814 site of RyR2 was either replaced by alanine (S2814A mice), to genetically inhibit RyR2 phosphorylation by CaMKII [22] or aspartic acid (S2814D mice), to mimic constitutive phosphorylation of RyR2 by CaMKII [21]. An additional experimental group was performed in transgenic male mice, expressing a mutant PLN in which both phosphorylatable residues (S16 and T17) were replaced by Ala (PLNDM) [24] (obtained from MMRRC, University of Missouri/Harlan, Mouse Regional Resource Center, NCRR, NIH) [24]. The corresponding wild type (WT) littermates were utilized as controls for the S2814A/D mice [21]. C57BL/6 mice were used as controls for the PLNDM mice. Finally, a small group of global CaMKIIδ-knockout mice [25] was also used in the in vivo experiments (see Results). Animals were inbreeded and maintained in our animal facilities in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). The protocols were approved by the Ethics Committee of the Cardiovascular Research Center, National Research Council (CONICET).

2.2. Preparations

a. *Ex-vivo experiments: Langendorff perfusion and experimental protocol*
Isolated hearts were perfused according to the Langendorff technique [26]. After a 10 min stabilization period, hearts were subjected to 45 min of global ischemia followed by 120 min of reperfusion [11].
b. *Isolated myocytes*
Enzymatic isolation of ventricular myocytes and fluorescence measurements were performed as previously described [27,28]. The standard whole-cell configuration of the patch-clamp technique was used for voltage-clamp recordings with a patch-clamp amplifier [29].
c. *In-vivo experiments*
Mice were anesthetized by sodium pentobarbital (45 mg/kg, i.p.) and then subjected to myocardial ischemia induced by the left anterior descending (LAD) coronary artery occlusion for 1 h followed by reperfusion for 24 h. Sham operated mice were subjected to the same surgical procedures without LAD ligation [25].

2.3. Infarct size

Infarct size was assessed by the triphenyltetrazolium chloride (TTC) technique [12,25].

2.4. LDH determinations

Cardiac injury was evaluated by LDH released in the perfusion effluent. LDH samples were taken every minute during the first 10 min of reperfusion [11,12].

2.5. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end (TUNEL) labeling

TUNEL assay was performed on myocardial slices fixed in buffered formalin and processed for histological examination [11,12]

2.6. Electrophoresis and Western blot analysis

Cardiac homogenates were prepared as previously described [11,30]. Proteins were probed with antibodies raised against S16 and T17-phosphorylated PLN, total PLN, S2814 and S2808-phosphorylated RyR2, total RyR2, Bcl-2 and Bax [3,11]. GAPDH was used as control loading.

2.7. Statistical analysis

Data are expressed as mean ± SEM. Unpaired and paired Student *t*-test or ANOVA followed by Tukey post hoc test were used for statistical comparisons when appropriate. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Increased CaMKII, but not PKA-dependent phosphorylation of RyR2 and PLN during I/R

To define the cardioprotective mechanisms associated with CaMKII-inhibition at the SR level, we performed ex-vivo experiments in Langendorff perfused mouse hearts subjected to a protocol of 45 min ischemia followed by reperfusion. At different times during reperfusion (3 and 15 min), hearts were freeze-clamped to measure the phosphorylation status of RyR2 at S2814 and S2808 and PLN at T17 and S16, using phospho-epitope specific antibodies. Selection of these reperfusion times for phosphorylation assessment was based on previous results that showed that maximal CaMKII-dependent phosphorylation of PLN occurred at the onset of reperfusion and then declined [11,26,31], consistent with the abrupt increase in diastolic Ca^{2+} observed at the onset of reperfusion [32]. Western blotting of ventricular lysates revealed a significant increase in S2814 phosphorylation (CaMKII site) in perfused hearts subjected to myocardial infarction followed by 3 min of reperfusion, compared to pre-ischemia (Fig. 1A). After 15 min of reperfusion, phosphorylation of S2814 site was still higher than pre-ischemic values. In contrast, there were no significant changes in S2808 phosphorylation (PKA site) of RyR2 in either group. Moreover, we also observed a decrease in RyR2 abundance at the onset of reperfusion, similar to previous findings [11,33]. Phosphorylation of PLN at T17 (CaMKII site) also significantly increased in hearts subjected to 3 and 15 min of reperfusion, whereas phosphorylation of PLN at S16 (PKA site) remained unchanged in reperfused hearts versus pre-ischemic controls (Fig. 1B), consistent with previous reports [11,12,25,26,31]. Control experiments in which hearts were continuously perfused for a period of time identical to that of hearts submitted to I/R protocol did not show any significant change in either the expression or phosphorylation of RyR2 or PLN (results not shown).

To further confirm the role of RyR2 phosphorylation in I/R *in vivo* we performed an additional series of experiments in WT mice submitted to regional myocardial ischemia followed by reperfusion (1/24 h). Supplementary Fig. 1 shows immunoblots and averaged results of these experiments. No changes in total expression/degradation of RyR2 were apparent after 24 h of reperfusion in the in vivo experiments, which suggests that RyR2 alterations during global I/R might be rescued during reperfusion. Moreover, and similar to the results in the intact heart, no significant changes in S2808 phosphorylation were observed in WT mice after the 1/24 hI/R protocol. However, WT mice showed significantly increased RyR2 phosphorylation at S2814 site, whereas there were no significant changes in the phosphorylation of this site in parallel I/R experiments performed in CaMKIIδ-KO mice [25].

3.2. Inhibition of CaMKII-dependent Ser2814 phosphorylation on RyR2 protects against necrosis and apoptosis following I/R injury

To determine whether genetic inhibition of CaMKII phosphorylation of RyR2 prevented cardiac damage following I/R, we studied RyR2 knock-in mice [22] in which the S2814 phosphorylation site was mutated to alanine (S2814A). CaMKII-phosphorylation patterns of PLN in these mice were similar to those in WT mice (Supplementary Fig. 2). At baseline, twitch shortening and Ca^{2+} transient amplitude during electric field stimulation (0.5 Hz) and SR Ca^{2+} load (assessed by rapid application of 25 mmol/L caffeine) in isolated myocytes were similar to those of WT mice (Supplementary Fig. 3). Basal contractility was also similar in WT vs. S2814A mice in the intact heart (Fig. 2). At the onset of global ischemia, the isovolumetric left ventricular developed pressure (LVDP) dropped essentially to 0 in both groups. However, on reperfusion, S2814A mice exhibited better post-ischemic mechanical recovery, *i.e.* LVDP was higher in S2814A mice compared to WT (Fig. 2A). The vast contractile depression observed in both groups of mice after 45 min ischemia may not only be attributed to the infarct

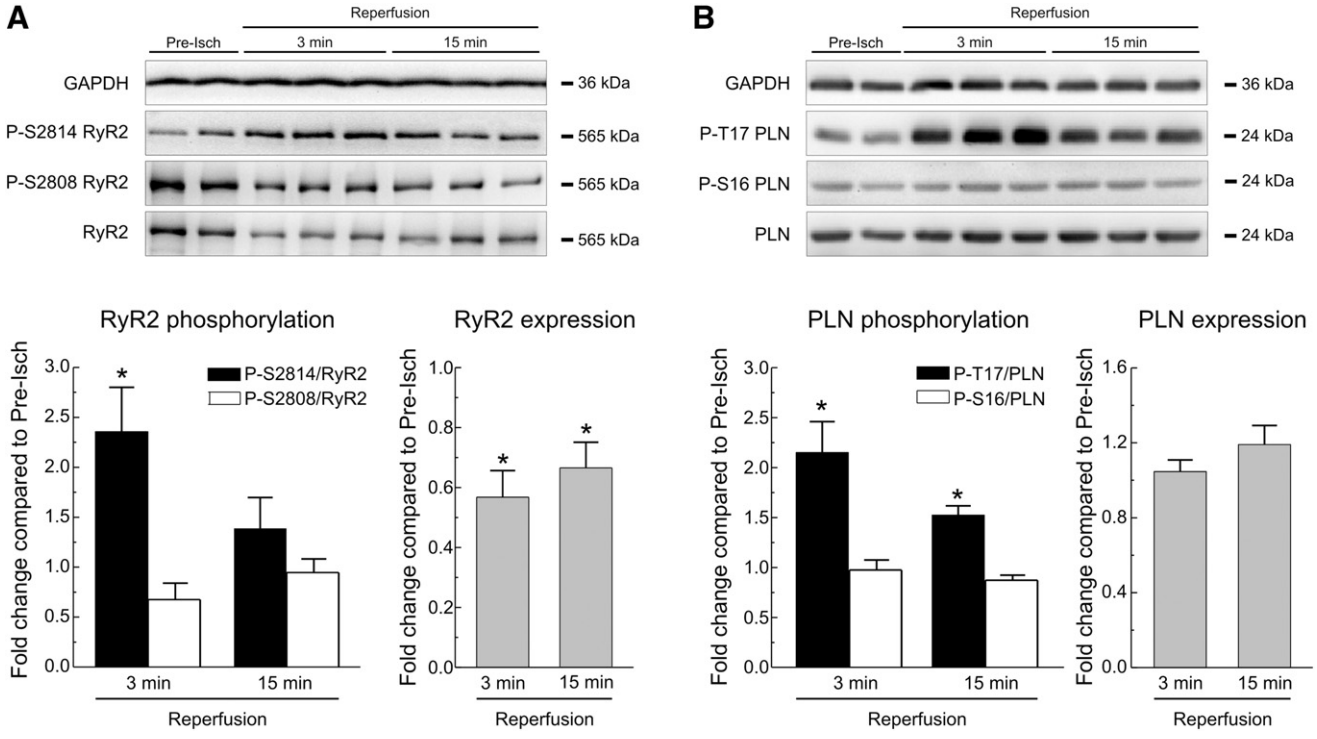

Fig. 1. Reperfusion significantly increases CaMKII dependent phosphorylation of RyR2 and PLN. A. Western blots and summary results of CaMKII and PKA-dependent phosphorylation of RyR2 at S2814 and S2808 respectively, and of RyR2 expression, in Langendorff perfused WT mouse hearts subjected to 45 min ischemia followed by various reperfusion times. Comparing with pre-ischemic values (Pre-Isch) there was a significant increase in S2814 phosphorylation at 3 min of reperfusion, without significant changes in the phosphorylation of S2808. A decrease in the expression of RyR2 was present at 3 and 15 min of reperfusion. B. Results of PLN phosphorylation sites during reperfusion. A significant increase in the phosphorylation of the CaMKII-dependent site, T17, was evident at 3 and 15 min of reperfusion with no significant changes in the PKA dependent site, S16. PLN expression did not change relative to pre-ischemic values. GAPDH was used as a loading control. Data represent the average ± SEM of values from 4 to 9 hearts per group.*$P < 0.05$ vs. the corresponding pre-ischemic values.

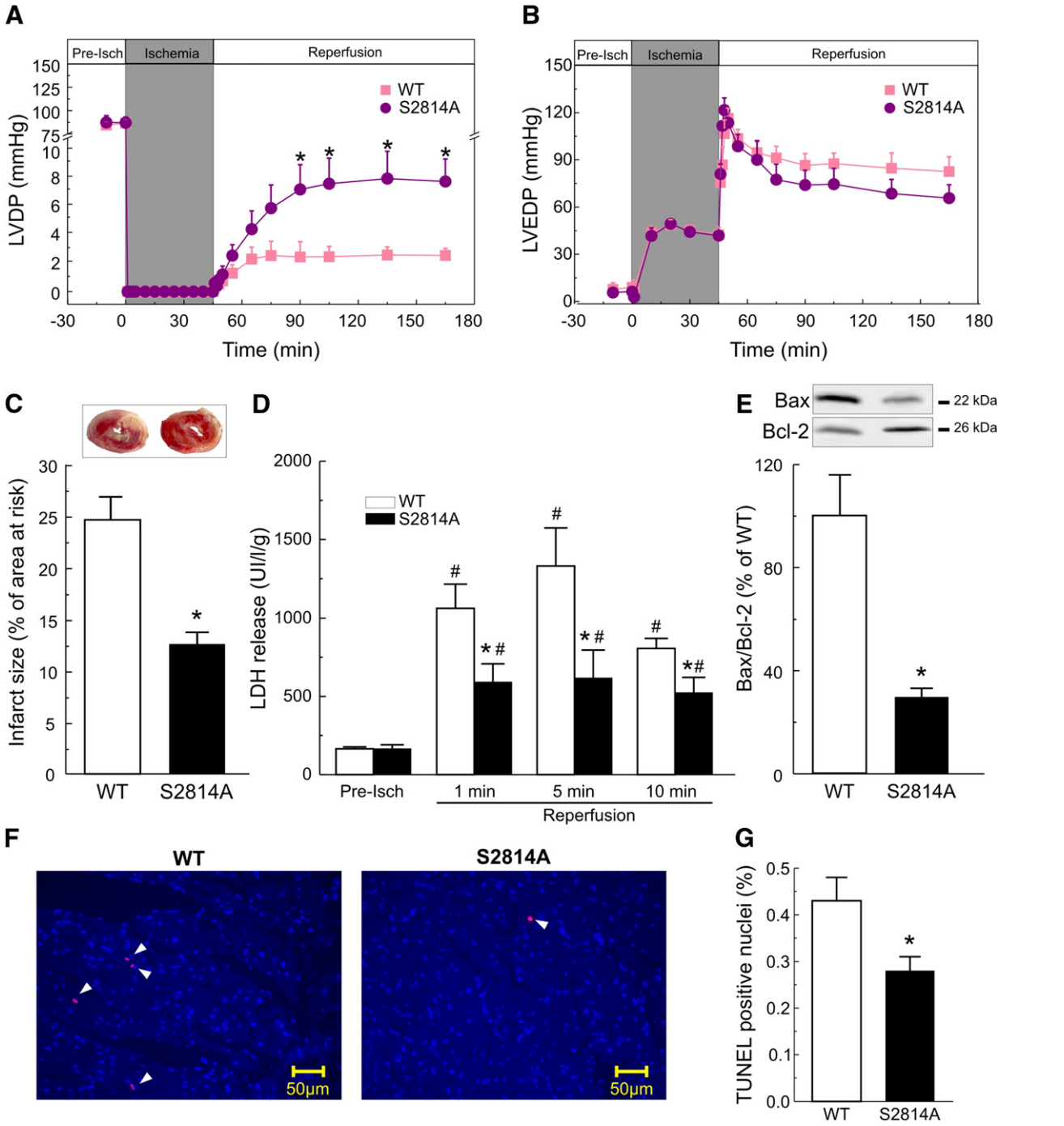

Fig. 2. The lack of CaMKII-phosphorylation of RyR2 protects against I/R injury. Perfused hearts from S2814A mice subjected to I/R (45/120 min) showed a significant improvement of contractile parameters during reperfusion when compared to WT mice, *i.e.* the left ventricular developed pressure (LVDP), was increased (A). The left ventricular end diastolic pressure (LVEDP) showed a trend to be lower in S2814A vs. WT mice, although without reaching significant levels (B). Myocardial viability was enhanced in S2814A vs. WT mice, as reflected by a decrease in the infarct size at the end of reperfusion (C) and in LDH released during the first 10 min of reperfusion (D). Apoptosis was also diminished in S2814A vs. WT mice: Typical immunoblots and overall results after 120 min of reperfusion, depicted in panel (E), showed a decrease in the ratio between the proapoptotic protein Bax and the antiapoptotic protein Bcl2 (Bax/Bcl2). TUNEL assay confirmed the apoptotic death decrease in S2814A mice hearts relative to WT. Examples of TUNEL stained cardiac tissue are shown in (F). Total nuclei were stained with DAPI (light blue). Apoptotic nuclei, in red, are marked with arrow heads. Original magnification is 40×. Overall results of this assay are shown in (G). Data represent the average ± SEM of n = 5–12 hearts per group.*P < 0.05 vs. WT mice. #P < 0.05 vs. the corresponding pre-ischemic values.

areas (see below) but also to the presence of myocardial stunning areas peripheral to the infarct size zone. Postischemic dysfunction (stunned myocardium) has been shown to revert after approximately 48/72 h of reperfusion [34]. This would greatly influence contractility depression observed in acute experiments after prolonged ischemic periods, as used herein. By comparing Figs. 2A and B, it is clear than in this model of I/R with a prolonged ischemic period, the alteration of contractile (systolic) activity is trivial contrasted with the marked elevation of diastolic pressure. However, the increase in LVEDP was not significantly different between the WT and S2814A animals. In spite of that, it was possible to detect a significant difference in LVDP. To better study the mechanical recovery, an additional series of experiments was performed in which ischemia was reduced to 20 min. This ischemic period also produced infarct damage in mouse hearts (results not shown).

Supplementary Fig. 4 indicates that the mechanical recovery was significantly more important in S2814A than in WT mice, similar to the results obtained with prolonged ischemia.

To further delineate the effects of inhibition of RyR2 phosphorylation in post-ischemic damage, the extent of I/R-induced myocardial infarction was assessed. The infarct size post-I/R (45/120 min) was 24.6 ± 2.2% in WT hearts, whereas it was significantly attenuated in S2814A mice (12.6 ± 1.2%; $P < 0.05$) (Fig. 2C). Moreover the extent of necrotic cell death examined by lactate dehydrogenase (LDH) efflux during the first 10 min of reperfusion was decreased by approximately 40% in S2814A mice relative to WT (Fig. 2D). Finally, the extent of apoptotic cell death examined by the ratio between the proapoptotic protein Bax and the antiapoptotic protein Bcl2 (Bax/Bcl2) and TUNEL assay was also significantly lower in S2814A than in WT mice after reperfusion (Figs. 2E–G).

Taken together these results indicate that prevention of S2814 phosphorylation diminished I/R injury, *i.e.* necrotic and apoptotic death, and improved functional recovery in the intact heart.

3.3. Constitutive phosphorylation of S2814 does not affect contractile recovery but enhances I/R damage

To further assess the role of RyR2 in the deleterious effect of CaMKII in I/R, we performed experiments in mice, with a S2814D mutation in RyR2 to mimic constitutive phosphorylation [21]. CaMKII-phosphorylation patterns of PLN in these mice were similar to those in WT mice (Supplementary Fig. 2). Supplementary Figs. 3A and B show that twitch shortening and Ca^{2+} transient amplitude were similar in S2814D compared to WT and S2814A mice, although SR Ca^{2+} load was lower than in the other two strains. The decrease in SR Ca^{2+} load in S2814D is presumably the consequence of the higher diastolic SR Ca^{2+} leak observed in these mice [21]. Thus, S2814D mice exhibited enhanced fractional SR Ca^{2+} release (ratio of twitch/caffeine-induced Ca^{2+} transient), compared to WT or S2814A mice, supporting the notion that RyR2 phosphorylation at S2814 activates both diastolic and systolic RyR2 Ca^{2+} release [21,35]. Basal contractility in the intact heart was also similar in S2814D and WT mice and post-ischemic mechanical recovery (LVDP) was not significantly altered (Figs. 3A and B). Similar results were obtained when the ischemic period was reduced to 20 min (Supplementary Fig. 4). In contrast to the contractile behavior, infarct size measured at the end of 45/120 min I/R was significantly greater in S2814D than in WT mice, as shown in the typical examples and overall results of Fig. 3C. LDH release was slightly higher in S2814D compared to WT mice at baseline and significantly increased in the first minutes of reperfusion in S2814D mice compared to WT mice. This difference disappeared however in samples obtained at the end of 10 min of reperfusion (Fig. 3D). These results suggest that necrosis was primarily enhanced at the onset of reperfusion in S2814D hearts. Apoptotic cell death was also increased in S2814D mice. Figs. 3E to G shows show a significantly higher Bax/Bcl2 ratio and more TUNEL positive nuclei after reperfusion in S2814D mice than in WT animals.

Interestingly, Ser2814D mice showed a dissociation between infarct size and mechanical recovery, *i.e.* whereas the mechanical recovery was not significantly different relative to WT, the infarct size was significantly increased. A possible explanation for these somewhat discordant results may lie on the increased fractional release observed in these mice (see above and Supplementary Fig. 3), which would be able to counteract the deleterious mechanical effect of I/R.

To further test the impact of CaMKII-dependent phosphorylation of RyR2, we performed an additional series of in vivo experiments in S2814A, S2814D and WT mice subjected to regional myocardial ischemia induced by the left anterior descending (LAD) coronary artery occlusion for 1 h followed by 24 h of reperfusion. Supplementary Fig. 5 shows that the infarct size relative to the area at risk was significantly decreased in S2814A mice and increased in S2814D compared to WT mice. Taken together, the data in S2814A and S2814D mice demonstrate that CaMKII-dependent phosphorylation of RyR2 is an essential signaling event that promotes cardiac damage and contributes to irreversible I/R injury.

3.4. CaMKII-dependent PLN phosphorylation does not participate in the deleterious effect of CaMKII in I/R

To determine whether CaMKII-dependent phosphorylation of PLN also contributes to I/R damage, we performed experiments in transgenic mice in which the PKA and CaMKII-dependent phosphorylation sites of PLN (T17 and S16) were both mutated to Ala, and thereby rendered non-phosphorylatable. Supplemental Fig. 6A shows that the ability of isoproterenol to increase PLN phosphorylation at T17 was blocked in these PLN double mutant (PLNDM) mice. The relaxant effect of isoproterenol was likewise blunted although the positive inotropic effect of the β-agonist was similar in both groups (Supplementary Figs. 6B and C). The similar inotropic action of isoproterenol in PLNDM and WT mice was attributed to enhanced L-type Ca^{2+} current in these animals [24].

Fig. 4A shows that LVDP was significantly impaired in PLNDM mice during reperfusion after 45 min of ischemia whereas LVEDP did not significantly change (B), consistent with previous findings in which perfused hearts were submitted to a shorter ischemic period [6]. Infarct size and LDH release were not reduced but, on the contrary they were significantly enhanced in PLNDM compared to WT mice (Figs. 4C and D). The finding that preventing PLN phosphorylation exacerbates the functional and structural heart damage after MI suggests that the CaMKII-dependent phosphorylation of PLN observed during reperfusion (Fig. 1B) favors post-ischemic recovery and protects from I/R cardiac damage.

An important limitation of PLNDM mice is that they develop an increase in L-type Ca^{2+} current [24] which was interpreted to be a compensatory mechanism since basal contractility was not affected (Fig. 4 and Supplementary Fig. 6). To further explore whether this increase in L-type Ca^{2+} current contributes to the increased infarct size observed in PLNDM mice, additional experiments were performed to determine the effect of returning this current to basal levels. In patch-clamp experiments performed in isolated myocytes from PLNDM and WT mice, we observed an increase in L-type Ca^{2+} current of approximately 20%, in agreement with previous findings [24]. This increase was inhibited in the presence of 10 nmol/L of the Ca^{2+} channel blocker nifedipine. Figs. 5A and B show typical records and overall results of these experiments. Figs. 5C to F show that contractile recovery, infarct size and LDH release were not significantly changed in the presence of a nifedipine concentration sufficient to block the increase in L-type Ca^{2+} current in PLNDM myocytes. Of note, this nifedipine concentration slightly decreased LVDP under basal conditions although without reaching significant levels. These findings indicate that the increase in L-type Ca^{2+} current does not significantly contribute to the detrimental effect of I/R on PLNDM mice. Taken together the above results reveal that phosphorylation of T17 of PLN at the beginning of reperfusion is not detrimental. On the contrary, this response serves to oppose to the deleterious action of RyR2 phosphorylation.

4. Discussion

Previous works from our laboratory has shown that CaMKII-dependent phosphorylation of Ca^{2+} handling proteins at the SR level is responsible for the detrimental effects of CaMKII during irreversible I/R injury [11,12]. The salutary effects of CaMKII-inhibition at the SR level resulted from the normalization of SR Ca^{2+} homeostasis that contributes to post-ischemic cell death and mechanical dysfunction. We have further shown that I/R-induced activation of CaMKII and phosphorylation of SR Ca^{2+} handling proteins contribute to mitochondrial dysfunction and subsequent necrotic and apoptotic cell deaths [11,12]. However the specific targets phosphorylated by CaMKII at the SR level and responsible for this effect were not dissected in those previous

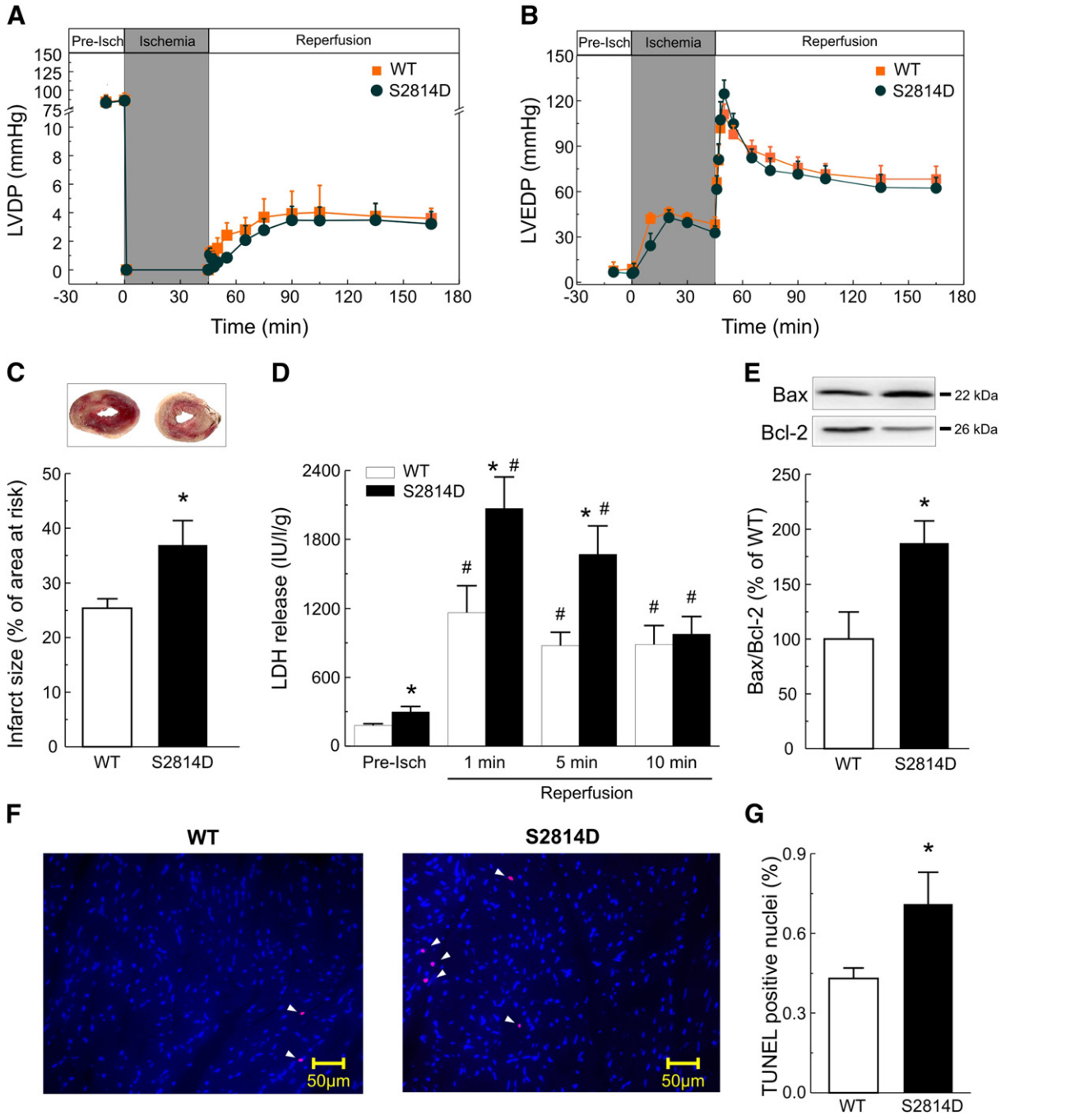

Fig. 3. Constitutive activation of CaMKII-dependent S2814 site of RyR2 does not change post-ischemic mechanical recovery but enhances I/R cardiac damage. Perfused hearts from mice with constitutive phosphorylation of S2814 on RyR2 (S2814D mice) showed a similar post-ischemic mechanical recovery (LVDP and LVEDP) compared with WT mice (A, B). In contrast, constitutive activation of S2814 increased infarct size (C) and LDH release at 1 and 5 min of reperfusion (D). The apoptotic death was also enhanced at the end of reperfusion in S2814D mice relative to WT, when evaluated by the increase in either Bax/Bcl2 ratio (E) or TUNEL staining. Typical examples and overall results of the TUNEL assay are shown in panels (F) and (G) respectively. Apoptotic nuclei, in red, are marked with arrowheads. Data represent the average ± SEM of n = 7–12 hearts per group.*P < 0.05 vs. WT mice. #P < 0.05 vs. the corresponding pre-ischemic values.

papers. In the present work, we explored two main candidates for this action: RyR2 and PLN, based on the facts that they are the two main proteins involved in SR Ca^{2+} handling and that both are targets of CaMKII.

Here we provide evidence that CaMKII-dependent phosphorylation of RyR2 during reperfusion is a critical determinant of the severity of detrimental effects of CaMKII (necrosis, apoptosis and contractile depression) following I/R injury. We used knock-in mice which express either the CaMKII-phosphomimetic S2814D or the non-phosphorylatable S2814A mutants on RyR2, which enabled us to evaluate the importance of CaMKII phosphorylation of RyR2 [21,22]. Our results provide direct evidence showing that CaMKII-dependent phosphorylation of RyR2 is the main target responsible for I/R damage at the SR level. The in vivo experiments, in which I/R was produced by the left coronary artery ligation, the gold standard model of I/R, also revealed the critical role of CaMKII-dependent RyR2 phosphorylation in I/R injury, giving further support to the above conclusion. Furthermore, CaMKII-dependent PLN phosphorylation at the onset of reperfusion did not contribute to the deleterious effect of I/R. To the best of our knowledge, this is the first report that shows that RyR2 phosphorylation is a major mediator of CaMKII-induced injury in I/R.

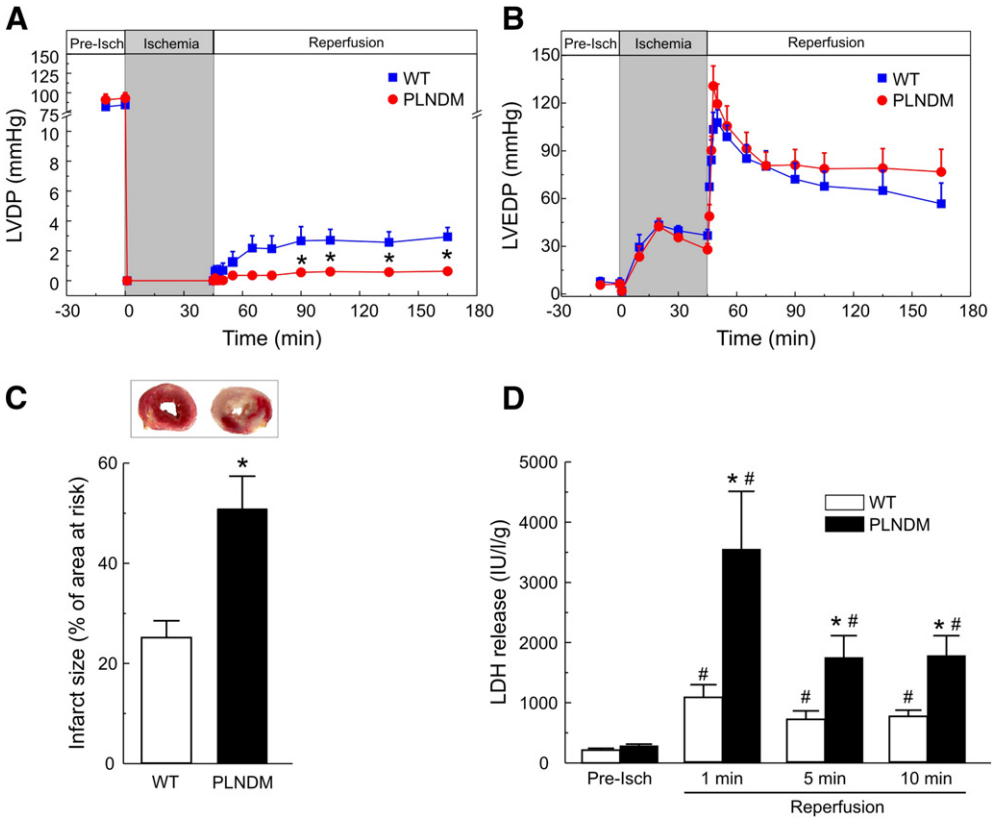

Fig. 4. The lack of PLN phosphorylation increases I/R injury. Double PLN mutant mice (PLNDM) exhibited a significant decrease in contractile recovery vs. WT mice (A), without significant changes in LVEDP (B). Infarct size at the end of reperfusion (C) and LDH released during the first 10 min of reperfusion (D) were also significantly increased when compared with WT mice. Data represent the average ± SEM of n = 7–14 per group. *P < 0.05 vs. WT mice. #P < 0.05 vs. the corresponding pre-ischemic values.

4.1. The RyR2 is a major downstream target of CaMKII, mediating its detrimental effects during I/R

A major conclusion from the present work is that when RyR2 cannot be phosphorylated by CaMKII, the irreversible damage associated with I/R (necrosis, apoptosis) as well as the associated mechanical dysfunction, are largely prevented. A second major conclusion is that constitutive CaMKII-phosphorylation of RyR2 increases susceptibility to post-ischemic myocardial damage, in both ex-vivo and in-vivo models. An important corollary of these two findings is that the increase in CaMKII-dependent phosphorylation of RyR2 that occurs during reperfusion (Fig. 1A and Supplementary Fig. 1) is a primary determinant of CaMKII-induced necrosis and apoptosis following I/R injury. Although we did not assess either diastolic Ca^{2+} or SR Ca^{2+} leak upon reperfusion in these mice, it is possible to speculate that the increase in SR Ca^{2+} leak produced by RyR2 phosphorylation may favor mitochondria Ca^{2+} overload and cardiac damage. Thus, preventing this phosphorylation event could ameliorate cardiac reperfusion injury. In previous experiments performed in the rat heart [11], we demonstrated that CaMKII-dependent cardiac damage during irreversible I/R was reliant on a CaMKII-phosphorylation at the SR level. In these experiments there was a trend to increase in the ratio of phosphorylated S2814/RyR2. However, this increase failed to reach statistical significance. The reason for the discrepancy between these previous findings and the present results is not apparent to us. Although we cannot discard that species differences may underlie this inconsistency, it is also possible that the simultaneous changes in RyR2 expression and Ser2814 phosphorylation that occur at the onset of reperfusion enhance data spreading, making the detection of a significant increase in the Ser2814/RyR2 ratio more difficult. The present experiments in mouse hearts, showing a significant increase in the ratio Ser2814/RyR2 and in knock-in mice revealing a significant decrease and increase of I/R injury in Ser2814A and Ser2814D mice, respectively, together with the fact that T17 phosphorylation seems not to contribute to cardiac damage, clearly show however that the damaging effect of CaMKII in I/R is chiefly dependent on the phosphorylation of S2814 on RyR2. The previously described diminished expression levels of RyR2 at the onset of reperfusion [33] were also found in the present experiments (Fig. 1A). Although this alteration seems to be reversible, since it was not apparent in the in vivo experiments (Supplementary Fig. 2), a possible contribution of RyR2 degradation to I/R damage cannot not be discarded from the present results. Current experiments in our laboratory are exploring this possibility.

4.2. Beneficial effects of CaMKII-dependent PLN phosphorylation in I/R injury

The present experiments also indicate that preventing CaMKII-dependent phosphorylation of PLN at T17 site at the onset of reperfusion, as assessed in mice in which PLN phosphorylation sites S16 and T17 are ablated, increased cardiac injury. This finding cannot be attributed to the inhibition of S16 phosphorylation, since phosphorylation of this site did not increase during reperfusion (Fig. 1B). Neither can it be due to the 20% increase in L-type Ca^{2+} current, the typical compensation observed in these transgenic mice [24] since similar results were obtained in the presence of a nifedipine concentration that blocked the Ca^{2+} current increase observed in isolated myocytes from PLNDM mice (Fig. 5). These experiments therefore imply that there is a beneficial effect of CaMKII-dependent PLN phosphorylation in I/R injury.

The role of PLN phosphorylation in I/R has been difficult to ascertain and indeed previous results support either a beneficial or a detrimental effect of PLN phosphorylation [11,36,37]. These contradictory observations may reflect the unique effect of PLN phosphorylation on accelerating SR Ca^{2+} uptake, which could result in two opposite consequences. On the one hand, it would counteract the diastolic Ca^{2+} elevation

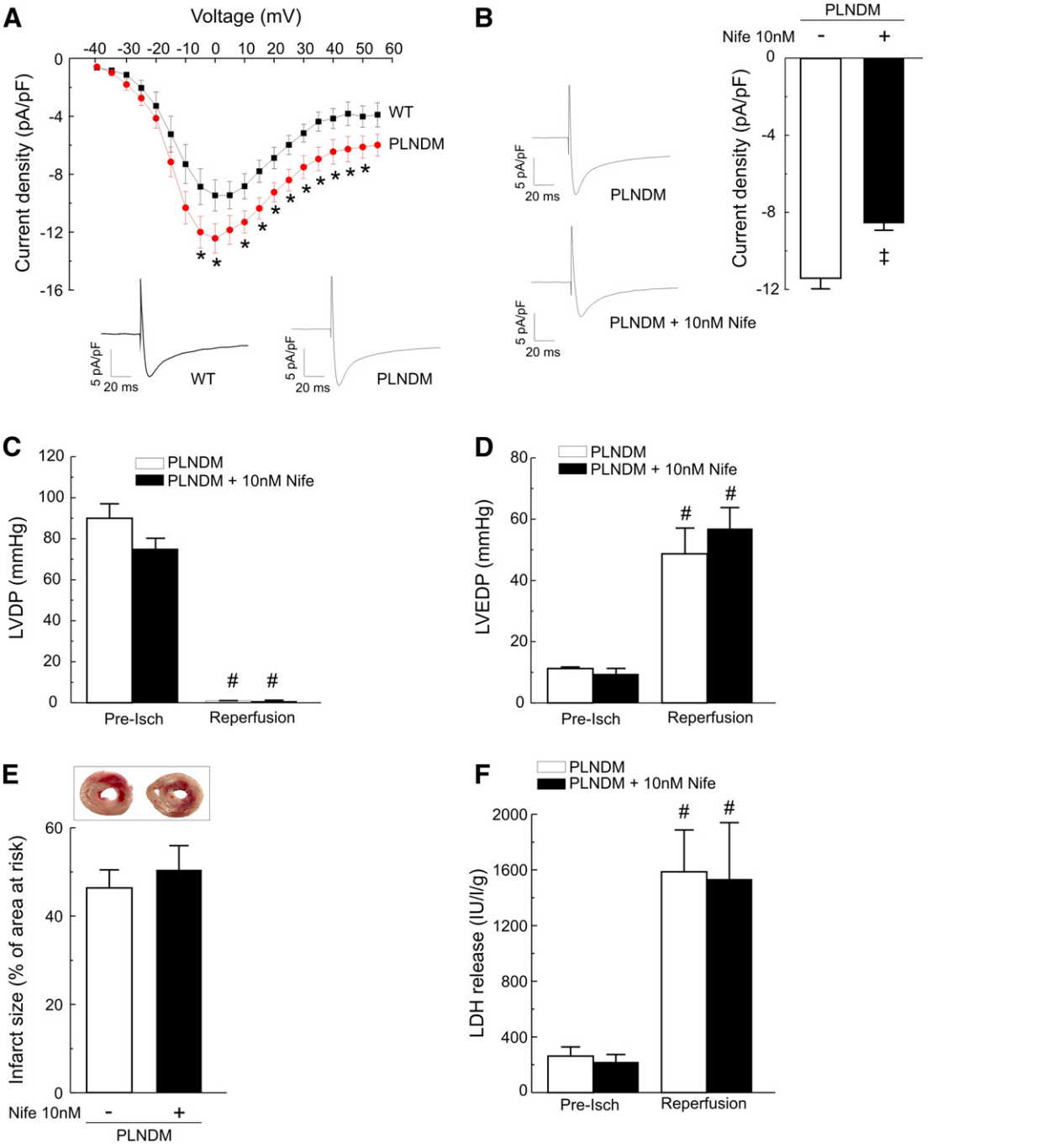

Fig. 5. Enhanced I/R injury in PLNDM mice is not due to the increased L-type Ca^{2+} current. A. Current density–voltage relations for average data of peak current density collected from WT mice (n = 8) and from PLNDM mice (n = 11). Representative traces of ICa, compensated for cell capacitance evoked by the voltage-clamp depolarizing step to 0 mV, recorded in WT and PLNDM myocytes, are shown. B. Representative traces of ICa, compensated for cell capacitance, recorded in a PLNDM myocyte before and after application of nifedipine (Nife) to the bath solution (left) and average data of peak ICa density of 6 cells recorded at 0 mV before and after nifedipine (right), are displayed. The currents were evoked by a voltage-clamp depolarizing step to 0 mV (250 ms) from a prepulse potential of −40 mV. C. There were not significant differences in the left ventricular developed pressure, (LVDP, C), left ventricular end-diastolic pressure, (LVEDP, D), infarct size at the end of reperfusion (E) and LDH released during the first 10 min of reperfusion (F) between PLNDM with or without nifedipine. *P < 0.05 vs. WT; ‡P < 0.05 PLNDM vs. PLNDM + Nife; #P < 0.05 vs. the corresponding pre-ischemic values.

resulting from SR Ca^{2+} leak, thus avoiding an increase in mitochondrial Ca^{2+} and favoring contractile recovery. Indeed, previous studies in PLNDM mice submitted to I/R revealed an increase in diastolic Ca^{2+} during reperfusion relative to WT mice [6]. On the other hand, by increasing SR Ca^{2+} load, it may favor SR Ca^{2+} leak due to the regulatory effect of intra-SR Ca^{2+} on RyR2 function. This would eventually produce a futile circle in which the continuous SR Ca^{2+} leak would evoke mitochondrial Ca^{2+} overload and cardiac injury. This latter sequence may underlie the detrimental effect of maximally increasing SR Ca^{2+} uptake in I/R, when interbreeding AC3-I mice (which were protected against I/R) with PLNKO mice [38] and may also explain the cardioprotective effect of inhibiting SR Ca^{2+} uptake, like the one produced by HAX-1/hsp90 complex [10]. The role of PLN phosphorylation in I/R would therefore depend on several factors among which are the status of the different PLN protein partners, the degree and time course of PLN phosphorylation, the Ca^{2+} threshold for SR Ca^{2+} leak and the status (integrity, phosphorylation, redox alterations) of RyR2. This latter factor appears to be crucial, since it is clear that in the hearts of PLNKO mice, which mimic

a maximum degree of PLN phosphorylation but conserve intact RyR2 under resting conditions, there is no evident necrosis or apoptosis [38].

Previous experiments from our laboratory using perfused mouse hearts that express an inhibitory CaMKII peptide at the SR level (SR-AIP mice) reported that infarct size was reduced by approximately 60% [11], not different from the reduction observed in S2814A mice (present results). Notably in SR-AIP mice, CaMKII-dependent phosphorylation of PLN and of RyR2 are both inhibited while in the S2814A mice only the CaMKII-dependent phosphorylation of RyR2 was prevented. This finding indicates that when CaMKII-dependent RyR2 phosphorylation is precluded, prevention of PLN phosphorylation failed to increase cardiac injury as should be expected from the results in PLNDM mice. Thus, CaMKII-dependent inhibition of RyR2 phosphorylation seems to be necessary and sufficient to prevent the CaMKII-dependent cardiac damage that originates at the SR level in I/R.

In summary, we demonstrate for the first time that phosphorylation of S2814 of RyR2 plays a major role in the detrimental effect of CaMKII in I/R. Our studies using knock-in mice which express either the CaMKII-phosphomimetic S2814D or the non-phosphorylatable S2814A mutants of RyR2 provide direct evidence showing that RyR2 are the main CaMKII targets at the SR responsible for I/R damage. Our previous results indicated that CaMKII promotes mitochondrial dysfunction and subsequent necrotic and apoptotic cell deaths during I/R through a phosphorylation occurring at the SR level. The present findings revealed that CaMKII-dependent phosphorylation of RyR2 by enhancing SR Ca^{2+} leak is the main responsible for this post-ischemic cardiac damage. In contrast, CaMKII-dependent PLN phosphorylation does not contribute to this injury but rather opposes to it. Our experiments further indicate that inhibition of RyR2 phosphorylation may be necessary and sufficient to prevent the CaMKII-dependent cardiac damage that originates at the SR level in I/R. Given the critical role of RyR2 in I/R damage, the strategy of impairing CaMKII phosphorylation at these Ca^{2+} release sites would represent a promising therapeutic strategy for the prevention/treatment of I/R damage.

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Disclosure statement

None.

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Appendix A. Supplementary data

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References

[1] Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. Heart disease and stroke statistics—2014 update: a report from the American Heart Association. Circulation 2014;129:e28-292.
[2] Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? J Clin Invest 1985;76:1713–9.
[3] Garcia-Dorado D, Ruiz-Meana M, Inserte J, Rodriguez-Sinovas A, Piper HM. Calcium-mediated cell death during myocardial reperfusion. Cardiovasc Res 2012;94:168–80.
[4] Said M, Becerra R, Valverde CA, Kaetzel MA, Dedman JR, Mundina-Weilenmann C, et al. Calcium–calmodulin dependent protein kinase II (CaMKII): a main signal responsible for early reperfusion arrhythmias. J Mol Cell Cardiol 2011;51:936–44.
[5] Piper HM, Kasseckert S, Abdallah Y. The sarcoplasmic reticulum as the primary target of reperfusion protection. Cardiovasc Res 2006;70:170–3.
[6] Valverde CA, Mundina-Weilenmann C, Reyes M, Kranias EG, Escobar AL, Mattiazzi A. Phospholamban phosphorylation sites enhance the recovery of intracellular Ca^{2+} after perfusion arrest in isolated, perfused mouse heart. Cardiovasc Res 2006;70:335–45.
[7] Steenbergen C, Murphy E, Watts JA, London RE. Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. Circ Res 1990;66:135–46.
[8] Fabiato A, Fabiato F. Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rabbit, rat, and frog hearts and from fetal and new-born rat ventricles. Ann N Y Acad Sci 1978;307:491–522.
[9] Kranias EG, Hajjar RJ. Modulation of cardiac contractility by the phospholamban/SERCA2a regulatome. Circ Res 2012;110:1646–60.
[10] Lam CK, Zhao W, Cai W, Vafiadaki E, Florea SM, Ren X, et al. Novel role of HAX-1 in ischemic injury protection involvement of heat shock protein 90. Circ Res 2013;112:79–89.
[11] Salas MA, Valverde CA, Sanchez G, Said M, Rodriguez JS, Portiansky EL, et al. The signalling pathway of CaMKII-mediated apoptosis and necrosis in the ischemia/reperfusion injury. J Mol Cell Cardiol 2010;48:1298–306.
[12] Vila-Petroff M, Salas MA, Said M, Valverde CA, Sapia L, Portiansky E, et al. CaMKII inhibition protects against necrosis and apoptosis in irreversible ischemia–reperfusion injury. Cardiovasc Res 2007;73:689–98.
[13] del Monte F, Lebeche D, Guerrero JL, Tsuji T, Doye AA, Gwathmey JK, et al. Abrogation of ventricular arrhythmias in a model of ischemia and reperfusion by targeting myocardial calcium cycling. Proc Natl Acad Sci U S A 2004;101:5622–7.
[14] Prunier F, Kawase Y, Gianni D, Scapin C, Danik SB, Ellinor PT, et al. Prevention of ventricular arrhythmias with sarcoplasmic reticulum Ca^{2+} ATPase pump overexpression in a porcine model of ischemia reperfusion. Circulation 2008;118:614–24.
[15] Kumada Y, Yamamoto F, Yamamoto H, Ishikawa T, Kagisaki K, Hirose H. Decreasing sarcoplasmic reticular calcium gives rise to myocardial protection—the effect of thapsigargin for myocardial protection under conditions of normothermia. Jpn J Thorac Cardiovasc Surg 1998;46:368–74.
[16] Zucchi R, Ronca F, Ronca-Testoni S. Modulation of sarcoplasmic reticulum function: a new strategy in cardioprotection? Pharmacol Ther 2001;89:47–65.
[17] Avellanal M, Rodriguez P, Barrigon S. Protective effects of cyclopiazonic acid on ischemia–reperfusion injury in rabbit hearts. J Cardiovasc Pharmacol 1998;32:845–51.
[18] Cross HR, Kranias EG, Murphy E, Steenbergen C. Ablation of PLB exacerbates ischemic injury to a lesser extent in female than male mice: protective role of NO. Am J Physiol 2003;284:H683–90.
[19] Zhang T, Guo T, Mishra S, Dalton ND, Kranias EG, Peterson KL, et al. Phospholamban ablation rescues sarcoplasmic reticulum Ca^{2+} handling but exacerbates cardiac dysfunction in CaMKIIdelta(C) transgenic mice. Circ Res 2010;106:354–62.
[20] Zhao W, Waggoner JR, Zhang ZG, Lam CK, Han P, Qian J, et al. The anti-apoptotic protein HAX-1 is a regulator of cardiac function. Proc Natl Acad Sci U S A 2009;106:20776–81.
[21] van Oort RJ, McCauley MD, Dixit SS, Pereira L, Yang Y, Respress JL, et al. Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure. Circulation 2010;122:2669–79.
[22] Chelu MG, Sarma S, Sood S, Wang S, van Oort RJ, Skapura DG, et al. Calmodulin kinase II-mediated sarcoplasmic reticulum Ca^{2+} leak promotes atrial fibrillation in mice. J Clin Invest 2009;119:1940–51.
[23] Ling H, Zhang T, Pereira L, Means CK, Cheng H, Gu Y, et al. Requirement for Ca^{2+}/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. J Clin Invest 2009;119:1230–40.
[24] Brittsan AG, Kranias EG. Phospholamban and cardiac contractile function. J Mol Cell Cardiol 2000;32:2131–9.
[25] Ling H, Gray CB, Zambon AC, Grimm M, Gu Y, Dalton N, et al. Ca^{2+}/calmodulin-dependent protein kinase II delta mediates myocardial ischemia/reperfusion injury through nuclear factor-kappaB. Circ Res 2013;112:935–44.
[26] Said M, Vittone L, Mundina-Weilenmann C, Ferrero P, Kranias EG, Mattiazzi A. Role of dual-site phospholamban phosphorylation in the stunned heart: insights from phospholamban site-specific mutants. Am J Physiol 2003;285:H1198–205.
[27] Palomeque J, Petroff MV, Sapia L, Gende OA, Mundina-Weilenmann C, Mattiazzi A. Multiple alterations in Ca^{2+} handling determine the negative staircase in a cellular heart failure model. J Card Fail 2007;13:143–54.
[28] Palomeque J, Rueda OV, Sapia L, Valverde CA, Salas M, Petroff MV, et al. Angiotensin II-induced oxidative stress resets the Ca^{2+} dependence of Ca^{2+}-calmodulin protein kinase II and promotes a death pathway conserved across different species. Circ Res 2009;105:1204–12.
[29] Aiello EA, Cingolani HE. Angiotensin II stimulates cardiac L-type Ca^{2+} current by a Ca^{2+}- and protein kinase C-dependent mechanism. Am J Physiol 2001;280:H1528–36.

[30] Zhang T, Johnson EN, Gu Y, Morissette MR, Sah VP, Gigena MS, et al. The cardiac-specific nuclear delta(B) isoform of Ca^{2+}/calmodulin-dependent protein kinase II induces hypertrophy and dilated cardiomyopathy associated with increased protein phosphatase 2A activity. J Biol Chem 2002;277:1261–7.
[31] Vittone L, Mundina-Weilenmann C, Said M, Ferrero P, Mattiazzi A. Time course and mechanisms of phosphorylation of phospholamban residues in ischemia-reperfused rat hearts. Dissociation of phospholamban phosphorylation pathways. J Mol Cell Cardiol 2002;34:39–50.
[32] Valverde CA, Kornyeyev D, Ferreiro M, Petrosky AD, Mattiazzi A, Escobar AL. Transient Ca^{2+} depletion of the sarcoplasmic reticulum at the onset of reperfusion. Cardiovasc Res 2010;85:671–80.
[33] Pedrozo Z, Sanchez G, Torrealba N, Valenzuela R, Fernandez C, Hidalgo C, et al. Calpains and proteasomes mediate degradation of ryanodine receptors in a model of cardiac ischemic reperfusion. Biochim Biophys Acta 1802;2010:356–62.
[34] Cohen MV, Yang XM, Downey JM. Smaller infarct after preconditioning does not predict extent of early functional improvement of reperfused heart. Am J Physiol 1999;277:H1754–61.
[35] Ferrero P, Said M, Sanchez G, Vittone L, Valverde C, Donoso P, et al. Ca^{2+}/calmodulin kinase II increases ryanodine binding and Ca^{2+}-induced sarcoplasmic reticulum Ca^{2+} release kinetics during beta-adrenergic stimulation. J Mol Cell Cardiol 2007;43:281–91.
[36] Nicolaou P, Rodriguez P, Ren X, Zhou X, Qian J, Sadayappan S, et al. Inducible expression of active protein phosphatase-1 inhibitor-1 enhances basal cardiac function and protects against ischemia/reperfusion injury. Circ Res 2009;104:1012–20.
[37] Yang Y, Zhu WZ, Joiner ML, Zhang R, Oddis CV, Hou Y, et al. Calmodulin kinase II inhibition protects against myocardial cell apoptosis in vivo. Am J Physiol 2006;291:H3065–75.
[38] Luo W, Grupp IL, Harrer J, Ponniah S, Grupp G, Duffy JJ, et al. Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of beta-agonist stimulation. Circ Res 1994;75:401–9.