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Ablation of junctin or triadin is associated with increased cardiac injury following ischaemia/reperfusion

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Aims Junctin and triadin are calsequestrin-binding proteins that regulate sarcoplasmic reticulum (SR) Ca^{2+} release by interacting with the ryanodine receptor. The levels of these proteins are significantly down-regulated in failing human hearts. However, the significance of such decreases is currently unknown. Here, we addressed the functional role of these accessory proteins in the heart's responses to ischaemia/reperfusion (I/R) injury.

Methods and results Isolated mouse hearts were subjected to global I/R, and contractile parameters were assessed in wild-type (WT), junctin-knockout (JKO), and triadin-knockout (TKO) hearts. Both JKO and TKO were associated with significantly depressed post-I/R contractile recovery. However, ablation of triadin resulted in the most severe post-I/R phenotype. The additional contractile impairment of TKO hearts was not related to a mitochondrial death pathway, but attributed to endoplasmic reticulum (ER) stress-mediated apoptosis. Activation of the X-box-binding protein-1 and transcriptional up-regulation of C/EBP-homologous protein (CHOP) provided a molecular mechanism of caspase-12-dependent apoptosis in myocytes. In addition, elevation of cytosolic Ca^{2+} during reperfusion was associated with the activation of calpain proteases and troponin I breakdown. Accordingly, treatment with the calpain inhibitor MDL-28170 significantly ameliorated post-I/R impairment of contractile recovery in intact hearts.

Conclusion These findings indicate that deficiency of either junctin or triadin impairs the contractile recovery in post-ischaemic hearts, which appears to be primarily attributed to increased ER stress and activation of calpain.

Keywords Junctin • Triadin • Ischaemia/reperfusion • ER stress • Calpain

1. Introduction

Ischaemia/reperfusion (I/R) injury, accompanied by contractile dysfunction and cellular damage contributes to cardiovascular disease and mortality.[1] Although due to multiple causes, defective Ca^{2+} cycling is a prominent mechanism contributing to I/R-related cardiac injury. The cytosolic Ca^{2+} overload that occurs during reperfusion, coupled with the subsequent futile cycles of Ca^{2+} release and re-uptake by the sarcoplasmic reticulum (SR), can exhaust intracellular ATP and consequently result in cardiomyocyte hyper-contracture.[2] Furthermore, the accumulation of Ca^{2+} in mitochondria, from either the cytoplasm or the SR, leads to opening of the mitochondrial permeability transition pore and apoptosis.[2] At the SR level, the activities of the SR Ca^{2+}-pump (SERCA) and the ryanodine receptor (RyR) are significantly depressed and enhanced, respectively, due to increased oxidative modification of these proteins upon reperfusion.[3] Thus, improving SR Ca^{2+} handling has been proposed to be an important target for reducing I/R injury. Indeed, adenoviral-mediated over-expression of SERCA or inducible expression of protein phosphatase-1 inhibitor-1 is associated with increased PLN phosphorylation and enhanced SERCA Ca^{2+} affinity was shown to limit infarct size and improve contractility after I/R.[4,5]

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Calsequestrin and the two accessory proteins junctin and triadin appear to play a significant role in the intraluminal control of RyR2 Ca^{2+} release. Junctin and triadin exhibit 60–70% amino acid homology in their transmembrane domains, including repeated KEKE motifs that are important for protein–protein interactions within their SR luminal tails.[6] The expression levels of both proteins are down-regulated in heart failure.[7] Ablation of triadin in the mouse resulted in significant decreases of cardiac SR proteins located in the junctional sarcoplasmic reticulum (jSR) cisternae (RyR2, calsequestrin, junctin, and junctophilin 1 and 2) and altered the architecture of cardiac Ca^{2+} release units.[8] None of these junctional proteins was altered in junctin knock-out (JKO) hearts.[6] Nevertheless, ablation of either junctin or triadin impaired SR Ca^{2+} release and overall EC coupling, rendering the hearts susceptible to ventricular arrhythmias and premature death.[6,8] However, there is no evidence of the role of junctin and triadin, in the heart's functional recovery and cell death following I/R injury.

During I/R, hypoxia/re-oxygenation (HR)-induced stress leads to accumulation of unfolded or mis-folded proteins, namely the unfolded protein response (UPR) or ER stress. In the early phase of reperfusion, cardiac function can be restored by correcting or eliminating the mis-folded proteins.[9] Meanwhile, sustained ER stress can activate cell apoptosis signalling cascades and drive SR Ca^{2+} into the cyotoplasm.[10] Intracellular Ca^{2+} release through RyR contributes to cytosolic Ca^{2+} overload, mitochondrial dysfunction, ER stress, and cell death.[11] In the present study, we found that the calsequestrin-binding proteins in the SR junctional complex, junctin and triadin, are important in the recovery of cardiac function and survival during I/R injury. Ablation of either protein exacerbated ER stress, promoting proteolysis of cardiac troponin I (cTnI), as well as activation of death-associated signal cascades.

2. Methods

For details regarding methods, refer to the Supplementary material online, Methods.

2.1 Animal models

The generation and characterization of the JKO[6] and triadin knock-out (TKO) mice[8] were previously described. The isogenic WT C57/BL6 mice were used as controls. The present study was in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and the National Research Council Guide for the Care and Use of Laboratory Animals: 8th Edition published by The National Academies Press, 2011, Washington, DC. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Cincinnati (Protocol No.04-04-19-02).

2.2 Global ischaemia/reperfusion *ex vivo*

Mice were anaesthetized by ip injection of pentobarbital (50 mg/kg body weight) and the adequacy of anaesthesia was evaluated by monitoring hind limb reflexes. The heart was excised, mounted on a Langendorff apparatus,[12] and perfused with Krebs–Henseleit (KH) physiological solution. Left ventricular end-diastolic pressure (LVEDP) was adjusted to 5–10 mmHg using a water-filled balloon inserted in the left ventricle. After 30 min stabilization period, the hearts were subjected to 40 min of no-flow, global ischaemia, followed by 60 min reperfusion. The heart's contractile parameters such as maximal rates of contraction (+d*P*/d*t* max) and relaxation (−d*P*/d*t* max), left ventricular developed pressure (LVDP), and LVEDP were monitored before ischaemia as well as during reperfusion.

2.3 Treatment of mouse hearts with calpain inhibitor

WT, JKO, or TKO mice were treated with a calpain inhibitor, MDL-28170 (Sigma-Aldrich, St Louis, MO, Cat.M6690, Cat.M6690) dissolved in KH buffer. Calpain inhibitor administration was carried out by a slightly modified method, compared with that described previously.[13] MDL-28170 (10 μM) was injected with constant speed into the perfusion buffer, 5 min prior to the induction of ischaemia, and continued from 0 to 5 min after reperfusion. The control group was treated with solvent following a similar protocol.

2.4 Assessment of cardiac injury and myocardial infarction

After I/R, the heart was stained with 2,3,5-triphenyltetrazolium chloride (TTC) (1%) (SigmaAldrich, T8877) and fixed in 10% formalin solution. As described earlier,[14] TTC-stained the viable tissue red, whereas the necrotic tissue remained pale. TTC stained hearts were sliced into five transverse sections and photographed. The infarct index is presented as a percentage of TTC unstained area relative to the area at risk (total section area).

2.5 Evaluation of cardiomyocte apoptosis

Heart sections were processed for TUNEL assays using the DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI, USA Cat.G3250), following the manufacturer's instructions. TUNEL-positive nuclei were counted on each heart section from 10 randomly chosen fields per section. The TUNEL-positive nuclei were expressed as a fraction of total 10 000 nuclei counted per section. In addition, DNA fragmentation was analysed in cardiac lysates using a Cell Death Detection ELISA plus kit (Roche, Indianapolis, IN, USA, Cat.11774425001), which quantifies the cytoplasmic histone-associated DNA fragments.

2.6 Cytosolic Ca^{2+} measurements following hypoxia/re-oxygenation

Isolated cells[6] were exposed to hypoxic conditions of 1% O_2 and 20% CO_2, while the culture media was changed with a hypoxic buffer for 40 min, followed by 1 h of re-oxygenation. After HR treatment, myocytes were loaded with 4 μM fluo-4 AM (Invitrogen, Grand Island, NY, USA, F14201) for 30 min at 37°, washed three times and harvested as a cell suspension for diastolic Ca^{2+} measurements using flow cytometry.[15,16] Following cell loading with fluo-4AM, propidium iodide was added to the suspension to exclude non-viable cells from measurements. Fluorescence signals from fluo-4 AM loaded cells excited at 488 nm were collected at emission of >510 nm. The total fluorescence intensity was analysed from ~10 000 viable myocytes per sample. FlowJo software (Tree Star, Inc., Ashland, OR, USA) was used to generate histograms of cell distribution according to fluorescence intensity, and to calculate the arithmetic mean of fluo-4AM intensity as an indication of cytosolic Ca^{2+} concentration.

2.7 Analysis of spliced XBP-1 by PstI digestion and real-time PCR

Total RNA was extracted from frozen heart tissue using an mRNA extraction kit (Qiagen, Valencia, CA, USA) and cDNA was prepared with the SuperScript II first-strand synthesis system (Invitrogen, Cat.11904-018), as described previously.[17]

2.8 Assessment of calpain, caspase-9, and caspase-12 activities

The activities of calpain and caspases 9 and 12 were detected by a fluorometric assay, according to the manufacturer's instructions (Biovision, Mountain View, CA, USA, Cat NO: K118 and K139). Calpain,

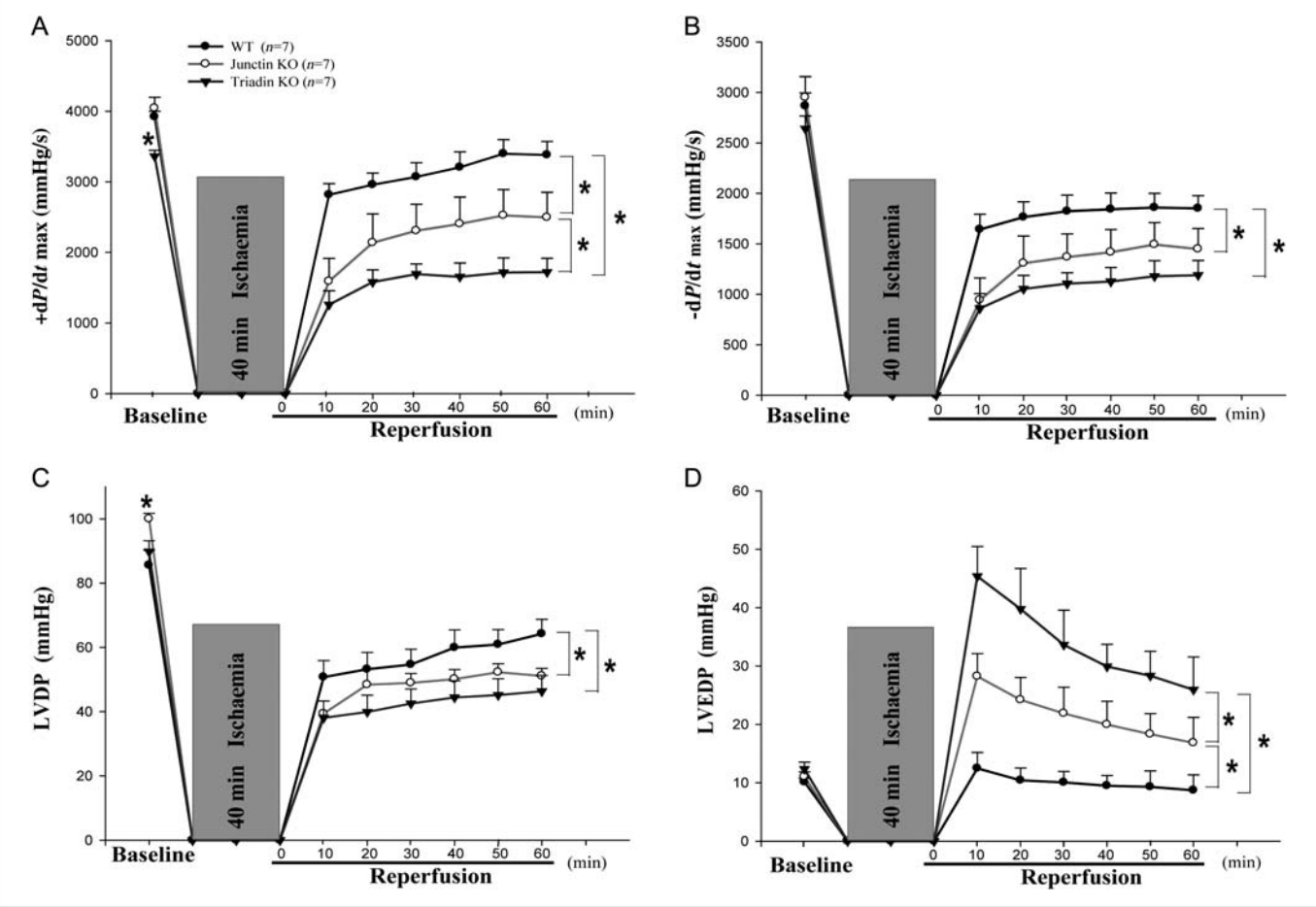

Figure 1 Ablation of either junctin or triadin impairs the contractile recovery in isolated perfused hearts after I/R. Cardiac performance was evaluated by: (*A*) maximal rates of contraction (+d*P*/d*t* max); (*B*) maximal rates of relaxation (−d*P*/d*t* max); (*C*) left ventricular developed pressure (LVDP); and (*D*) left ventricular end diastolic pressure (LVEDP). The data indicate that the recovery of post-ischaemic contractility was significantly reduced in JKO and TKO hearts compared with WT hearts (*$P < 0.05$, $n = 7$).

caspase-9, and caspase-12 activities were quantified using a fluorescence plate reader (Model:GENios, TECAN system, Inc., San Jose, CA, USA) (excitation: 400 nm, emission: 505 nm).

2.9 Western blot analysis

Proteins were analysed by Western blots as previously described.[12]

2.10 Statistical analysis

Data were expressed as mean ± SEM. Multiple comparisons among three or more groups were performed using ANOVA, and the Bonferroni exact test was conducted for *post hoc* analyses (SPSS 13.0. IBM Co., Armonk, NY, USA). A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Recovery of contractile function in junctin and triadin-deficient hearts after ischaemia/reperfusion

Previous studies showed that ablation of triadin impaired SR Ca^{2+} storage and release, resulting in depressed Ca^{2+} handling in cardiomyocytes,[8] while ablation of junction enhanced Ca^{2+} cycling and contractile parameters in isolated cardiomyocytes.[6] Using the Langendorff perfusion system, we first examined the basal contractile parameters of JKO and TKO hearts in an unloaded mode and under these conditions, there were no significant differences in contractile parameters among WT, JKO, and TKO hearts, with the exceptions of a depressed maximal rate of contraction (+d*P*/d*t* max) in TKO (*Figure 1A*) and enhanced LVDP in JKO hearts (*Figure 1C*). To determine the role of junctin and triadin in I/R injury, the hearts were subjected to 40 min of global no-flow ischaemia, followed by 60 min of reperfusion. As shown in *Figure 1*, compared with WT hearts, the maximal rates of contraction (+d*P*/d*t* max) (*Figure 1A*) and relaxation (−d*P*/d*t* max) (*Figure 1B*) and LVDP (*Figure 1C*) were significantly decreased, while LVEDP was significantly increased (*Figure 1D*) in JKO and TKO hearts. These findings indicate that post-ischaemic contractile function was impaired in JKOs, and even further impaired in TKO hearts.

3.2 Myocardial injury after ischaemia/reperfusion

Myocyte necrosis resulting from infarction constitutes an irreversible injury to the myocardium that can weaken contractile function. Here, the myocardial infarct size was determined by TTC staining after I/R, as described earlier. The average infarct sizes were 14, 35, and 43% of area at risk in WT, JKO, and TKO hearts,

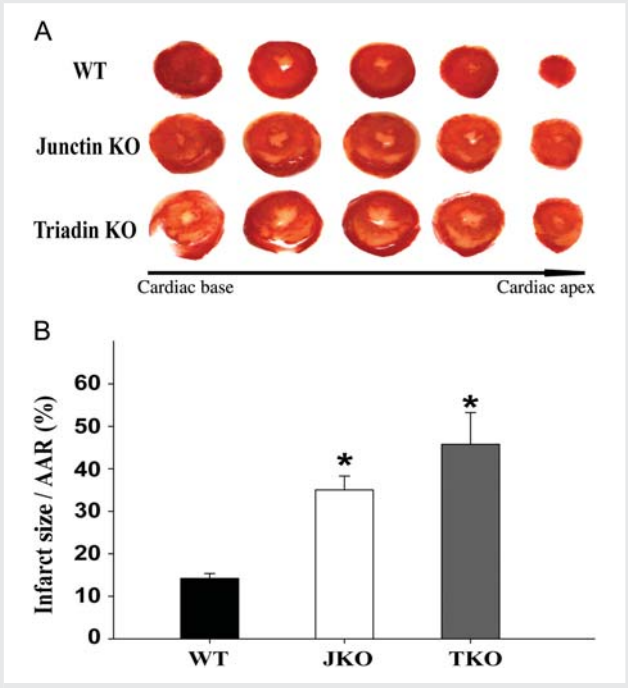

Figure 2 Ablation of junctin or triadin exacerbates myocardial injury upon I/R. (*A*) Representative cross-sectional images of WT, JKO, and TKO hearts stained with TTC following *ex vivo* I/R injury. (*B*) The myocardial infarct size was normalized to the area at risk after 40 min global-ischaemia and subsequent 60 min of reperfusion. ($n = 6$, $^*P < 0.05$ vs. WT I/R hearts).

respectively (*Figure 2A* and *B*). The difference in the infarct area between JKO and TKO hearts was not significant, suggesting that besides necrosis, other mechanisms such as apoptosis, autophagy, and degradation of troponin I (see later text) must contribute to lower recovery of contractile function in TKO hearts.

3.3 Apoptosis in junctin and triadin-knockout hearts

Cardiomyocyte apoptosis is also an important factor contributing to the loss of contractile function in I/R injury and heart failure. At basal conditions, out of 10 000 nuclei randomly selected on sections of paraffin-embedded hearts, we detected ~37 in WT, ~42 in JKO, and ~57 in TKO TUNEL positive nuclei. These numbers were increased to 69 (approximately two-fold), 181 (approximately four-fold), and 257 (approximately five-fold) in post-I/R WT, JKO, and TKO hearts, respectively. Thus, in post-reperfusion myocardium, the number of TUNEL-positive nuclei as well as the degree of DNA fragmentation increased in all three groups and this increase was significantly higher in KOs, when compared with WT hearts (*Figure 3A–C*).

To determine the mechanisms underlying apoptosis, we assessed the level of BAX and Bcl-2 in these hearts. BAX is a prominent pro-apoptotic molecule that can facilitate the release of cytochrome *c* from the mitochondrial inner-membrane to the cytosol, whereas Bcl-2 has anti-apoptotic effects that can promote cell survival by interfering with activation of the cytochrome c/Apaf-1 pathway through stabilization of the mitochondria. Thus, the ratio of BAX to Bcl-2 expression (BAX/Bcl-2) reflects mitochondrial-dependent apoptosis under stress conditions. Western blot analysis demonstrated that

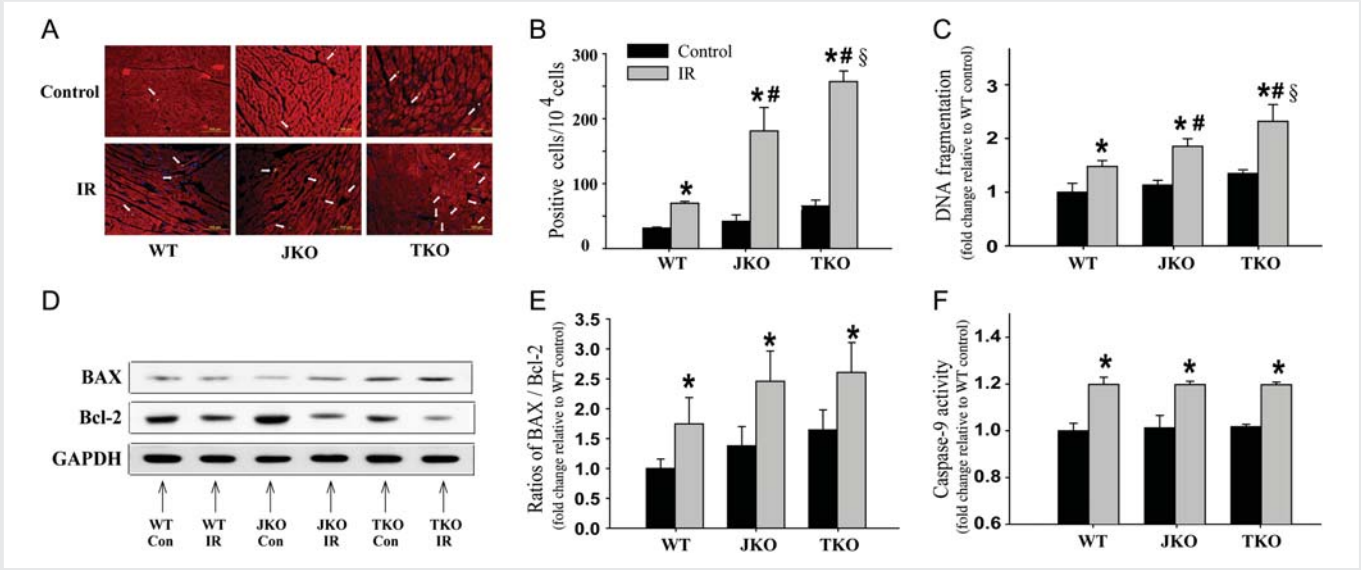

Figure 3 Ablation of junctin or triadin increases I/R-induced cardiomyocte apoptosis. (*A*) Representative TUNEL staining in WT, JKO, and TKO cardiac sections before and after I/R injury. (*B*) Junctin and triadin knock-out hearts, subjected to 40 min of no-flow ischaemia followed by 60 min reperfusion, exhibited increased TUNEL-positive nuclei compared with WT hearts. (*C*) DNA fragmentation (mono- and oligo-nucleosomes contents) in JKO and TKO hearts were detected by ELISA in the presence and absence of I/R injury. (*D*) Representative Western blots illustrating expression levels of BAX, Bcl-2, and GAPDH. (*E*) Quantitative analysis of the ratios of BAX to Bcl-2 obtained from the immunoblots. (*F*) caspase-9 activity was evaluated in heart homogenates and it was expressed as fold change relative to the WT control group ($n = 6$, $^*P < 0.05$ vs. control hearts of the same genotype; $\#P < 0.05$ vs. WT I/R hearts; $\S P < 0.05$ vs. JKO I/R hearts).

there were no differences in the BAX/Bcl-2 ratios among WT, JKO, and TKO hearts under basal conditions or in response to I/R injury (*Figure 3D–E*). Next, we assessed caspase 9, which becomes activated after cytochrome *c* release and is considered to be a critical regulator of mitochondrial-mediated apoptosis. Fluorometric assays revealed that caspase 9 activity increased to the same extent in WT, JKO, and TKO post-ischaemic hearts (*Figure 3F*), suggesting that the mitochondria-dependent pathway is not the main mechanism causing the observed enhanced apoptosis in the JKO or TKO hearts.

3.4 Cytosolic Ca^{2+} following ischaemia/reperfusion

Since cytosolic Ca^{2+} overload plays an important role in the pathophysiological mechanism of I/R, we used fluo-4 AM, a fluorescent Ca^{2+} indicator, to detect diastolic Ca^{2+} in isolated myocytes subjected to simulated I/R (HR). Under basal conditions (*Figure 4A*), there was no difference in the fluorescence signal among the three groups. Upon HR, diastolic Ca^{2+} increased by 147% in WT, and it was further increased to 167 and 199% in JKO and TKO myocytes, respectively. Analysis of DNA fragmentation indicated that ablation of junctin was associated with increased apoptosis, and this was even higher in the absence of triadin (Supplementary material online, *Figure S1*A). There were no alterations in caspase-9 activity among WT, JKO, and TKO myocytes after HR (Supplementary material online, *Figure S1*B), but the activity of caspase-12, an important player in apoptosis, was increased by 1.4-, 1.7-, and 2.1-fold in WT, JKO, and TKO myocytes (*Figure 4B*). Additionally, Western blot analysis indicated that the SERCA2a expression levels were decreased to 68 and 74% in JKO and TKO myocytes, respectively, whereas no change was observed in post-hypoxic WTs (*Figure 4C*).

3.5 ER stress and autophagy

It has been reported that disturbed Ca^{2+} handling is associated with the ER stress response and sustained ER stress can lead to apoptosis.[18,19] Thus, studies were performed to detect the effect of junctin or triadin ablation on activation of the three ER-associated stress pathways: ATF6 (activation of transcription factor 6), PERK (protein kinase RNA-like endoplasmic reticulum kinase), and IRE-1α (inositol-requiring enzyme 1α).[20] Our data show that the ATF6 signal cascade was not altered among the three groups, as evidenced by the lack of differences in the X-box-binding protein-1 (XBP-1) mRNA levels, a downstream indicator of ATF6 activation (Supplementary material online, *Figure S2A*). Similarly, the PERK signal cascade was not altered among the three groups, as indicated by the phosphorylation levels of its eIF2a substrate, under basal or I/R conditions (Supplementary material online, *Figures S2B* and *S2C*). However, the IRE-1α pathway was activated to a greater extent in both JKO and TKO hearts upon I/R, as demonstrated by the quantitative analysis of the mRNA levels of spliced XBP-1 (XBP-1) using RT–PCR. This spliced form was similar among the three groups under basal conditions, but it increased by 1.6-, 2.3-, and 2.8-fold in WT, JKO, and TKO mouse hearts after I/R injury (*Figure 5B*). Furthermore, activation of XBP-1, reflected by its spliced mRNA transcript, was associated with 1.2-, 1.6-, and 2.0-fold increases in the expression of C/EBP-homologous protein (CHOP), which is a transcription factor that can up-regulate pro-apoptotic gene expression and down-

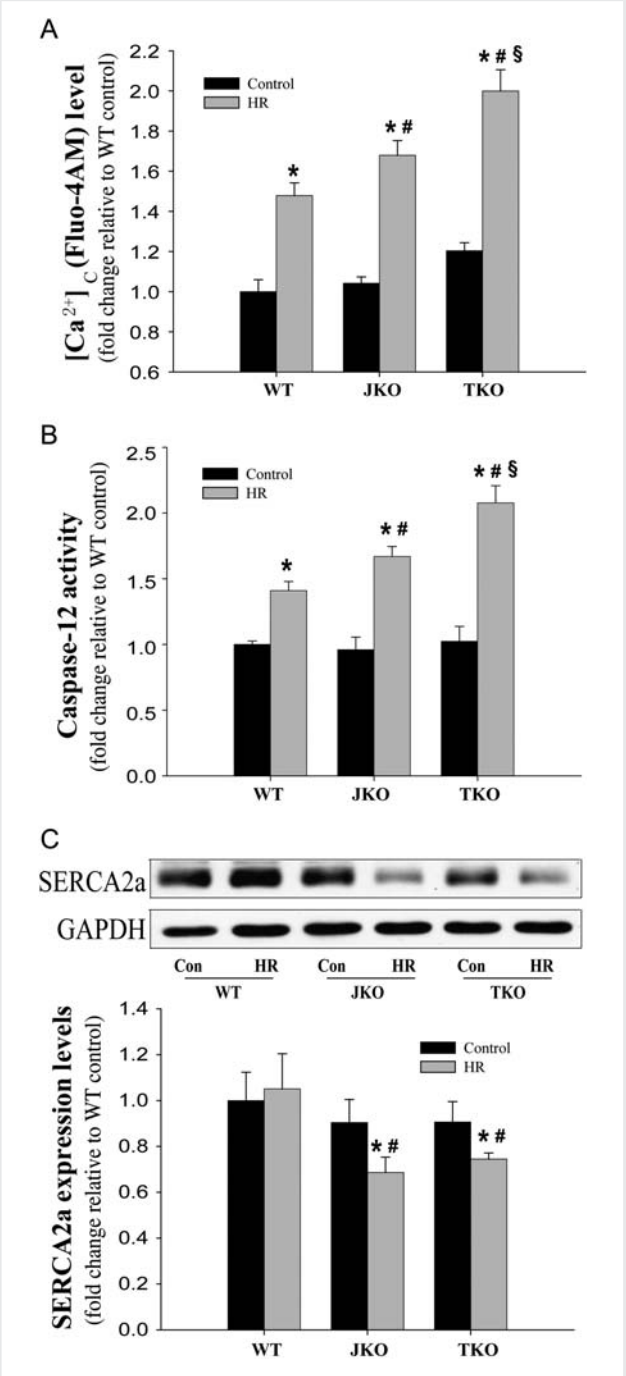

Figure 4 Cytosolic Ca^{2+} overload increased in junctin or triadin-deficient myocytes in response to hypoxia/re-oxygenation. (*A*) Quantitative analysis of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) based on fluo-4AM mean fluorescence intensities, which are expressed as fold change relative to the WT control group. (*B*) caspase-12 activities in the presence and absence of hypoxia/re-oxygenation. (*C*) Representative Western blots illustrating SERCA2a and GAPDH and the quantitative analysis of SERCA2a expression levels relative to the WT control group after normalization to GAPDH levels (cell lysates from $n = 4–5$ hearts/group, $^*P < 0.05$ vs. control hearts of the same genotype; $\#P < 0.05$ vs. WT I/R hearts; $\S P < 0.05$ vs. JKO I/R hearts).

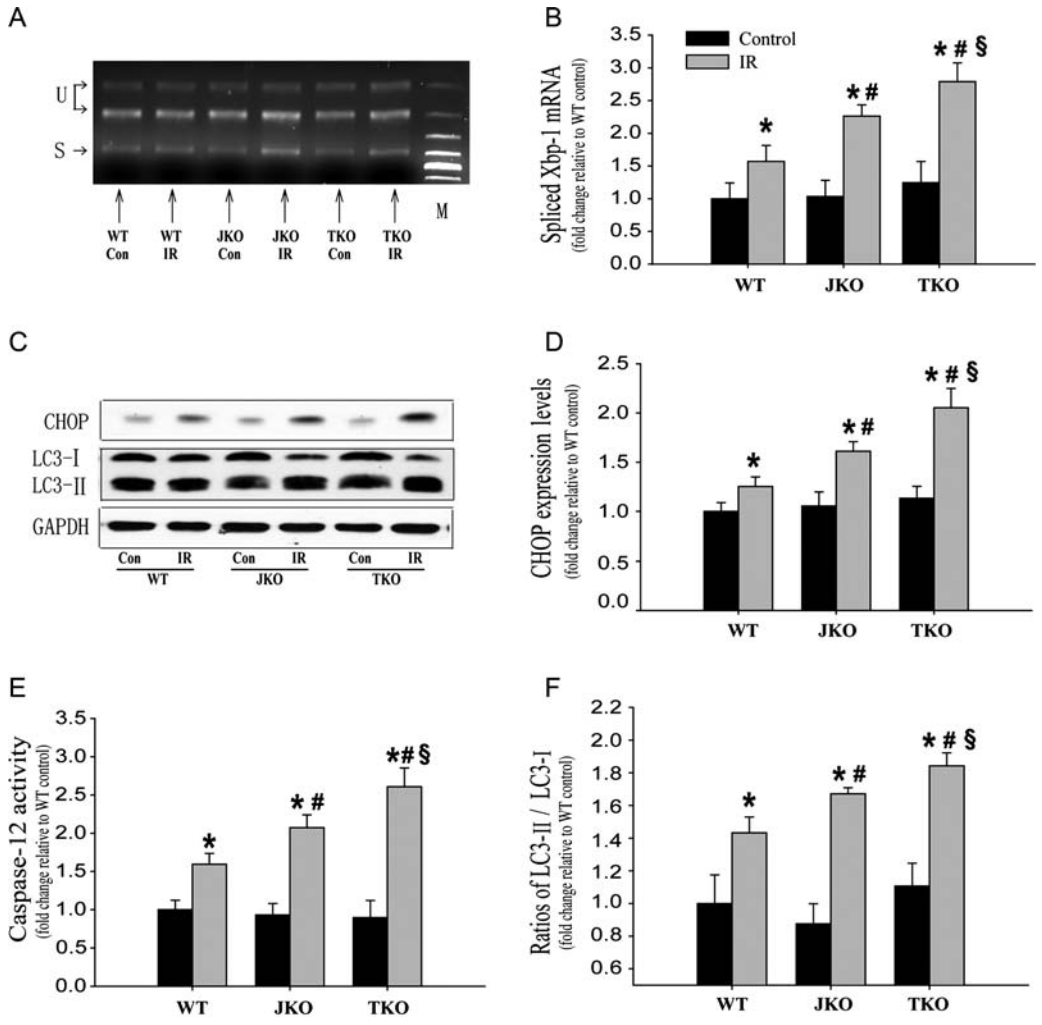

Figure 5 Deficiency of junctin or triadin exacerbates the ER stress response to I/R. (*A*) Total XBP-1 was amplified and digested with *Pst I*. Identification of XBP-1 unspliced *(U)* and spliced *(S)* forms using agarose gel electrophoresis. (*B*) Quantification of mRNA expression levels of spliced XBP-1 using real-time-PCR. (*C*) Representative Western blots illustrating CHOP, LC3, and GAPDH. (*D*) Quantitative analysis of CHOP expression levels relative to the WT control group, after normalization to GAPDH levels. (*E*) Quantification of caspase-12 activity in heart homogenates, expressed as fold change relative to the WT control group. (*F*) Quantitative analysis of the LC3-II/LC3-I ratio, expressed as fold change relative to the WT control group. ($n = 6–8$, $^*P < 0.05$ vs. control hearts of the same genotype; $\#P < 0.05$ vs. WT I/R hearts; §$P < 0.05$ vs. JKO I/R hearts).

regulate anti-apoptotic gene expression, in post-ischaemic WT, JKO, and TKO mouse hearts, compared with basal conditions (*Figure 5C* and *D*). In addition caspase-12, a cysteine protease that promotes cell apoptosis after sustained ER stress was increased by 1.6-, 2.0-, and 2.6-fold in post-ischaemic WT, JKO, and TKO hearts, respectively (*Figure 5E*).

Emerging evidence indicates that ER stress is also a potent inducer of autophagy, a process whereby eukaryotic cells recycle their macromolecules and organelles.[21] An important component in autophagy production is the glycine exposed form of microtubule light chain (LC3), also called LC3-I, which can exist as a lipid form (LC3-II) after combining with phosphatidylethanolamine. Thus, the ratio of LC3-II to LC3-I is a parameter for evaluating the formation of autophagosomes. As shown in *Figure 5C* and *F*, there were no differences in the ratios of LC3-II/LC3-I among all groups under basal conditions. However, after 1 h reperfusion, the ratios of LC3-II/LC3-I significantly increased in all three groups but the increases were higher in the JKO and TKO hearts. Together, these results indicate that the enhanced apoptosis in JKO and TKO hearts post I/R may be attributed to exacerbated ER stress, resulting in activation of caspase-12 via the IRE-pathway and autophagy. Additionally, triadin ablation can induce ER stress to a greater extent than junctin-deficiency upon I/R challenge.

3.6 Calpain activity

Calpain is a type of non-lysosomal cysteine protease, whose activation depends on intracellular Ca^{2+}, and has been implicated as an essential component in ER-stress-induced cell death pathways.[22] As shown in *Figure 6A*, there was no change in calpain expression levels among WT, JKO, and TKO hearts under basal or I/R conditions. However, during I/R, calpain activity was increased by 146, 162, and 183% in WT, JKO, and TKO hearts, respectively (*Figure 6B*). Similar findings were obtained in cardiomyocytes subjected to HR treatment (Supplementary material online, *Figure S1C*). Accordingly, cTnI, a characteristic proteolytic substrate of calpain, was degraded to a greater extent

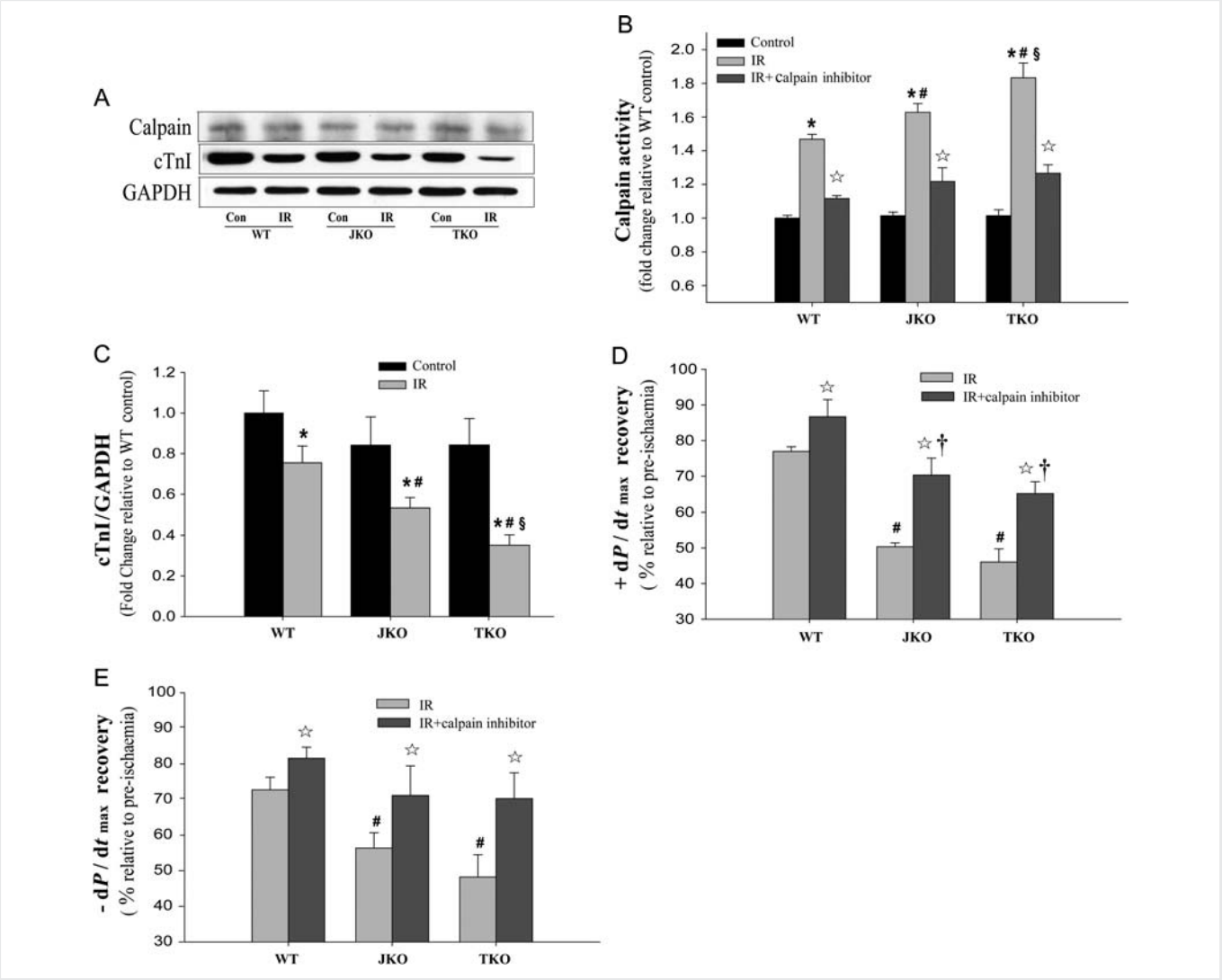

Figure 6 Ablation of junctin or triadin-enhanced calpain activation in response to I/R. (*A*) Representative Western blot illustrating calpain, troponin I (cTnI), and GAPDH levels. (*B*) Assessment of calpain activity in heart homogenates using the fluorometric assay. (*C*) Quantitative analysis of cTnI, expressed relative to the WT control group after normalizing to GAPDH levels. (*D*) Maximal rates of contraction (+d*P*/d*t* max) and (*E*) maximal rates of relaxation (−d*P*/d*t* max) under MDL28170 treatment to evaluate the recovery of the cardiac performance ($n = 6$ hearts/group, $^{*}P < 0.05$ vs. control hearts of the same genotype; $\#P < 0.05$ vs. WT I/R hearts; §$P < 0.05$ vs. JKO I/R hearts; ☆$P < 0.05$ vs. the I/R hearts of the same genotype; †$P < 0.05$ vs. WT I/R hearts treated with calpain inhibitor).

in JKO and TKO hearts than in WT (*Figure 6A* and *C*). Together, these data suggest that I/R induced a higher Ca^{2+} overload in JKO and TKO myocytes than in WT, and this was associated with a concordant increase in calpain activation and troponin I proteolysis.

3.7 Recovery of contractile function after treatment with a calpain inhibitor

To determine the degree to which calpain and consequent cell death in JKO and TKO myocytes played a role in their decreased recovery after I/R, we added MDL28170, a calpain inhibitor,[13] to the perfusate 5 min prior to I/R to determine whether this could partially rescue contractility and cell death. MDL28170 had no effect on basal contractile parameters. Its addition to the perfusate suppressed I/R-induced calpain activation (*Figure 6B*) and equalized active calpain levels and apoptosis, quantified by the number of TUNEL positive nuclei, in WT, JKO, and TKO hearts (*Figure 6B*, Supplementary material online, *Figures S3A* and *S3B*). Furthermore, MDL28170 reduced the infarct area and the degree of cTnI degradation after I/R in all three groups although these detrimental effects remained greater in KO hearts, compared with WTs (Supplementary material online, *Figures S3C* and *S4*). As a result, contractile recovery in all three groups was significantly improved. However, while the rates of relaxation (−d*P*/d*t* max) were similar among the three groups, the rates of contraction (+d*P*/d*t* max) remained slightly depressed in the JKO and TKO hearts, compared with WTs (*Figure 6D* and *E*).

4. Discussion

Junctin and triadin exhibit 60 to 70% amino acid homology in their transmembrane domain, and both of them are important calsequestrin-binding proteins that regulate SR Ca^{2+} release by interacting with RyR2.[6] It has been previously reported that ablation of

triadin in heart is accompanied by significant decreases in the expression levels of SR Ca^{2+}-cycling proteins, including junctin (~95% decrease), calsequestrin and RyR2, while ablation of junctin does not change the expression of these proteins.[6,8] Thus, the TKO model may be considered as a 'double-knockout'. Since the expression of both triadin and junctin are reduced in failing hearts, it is intriguing to ask whether ablation of junctin expression (JKO) or 'combined ablation of triadin and junctin expression' (TKO) affect myocardial survival through I/R injury and to what extent. Here, we addressed this question using an *ex vivo* perfusion model with 40-min ischaemia (no flow) followed by reperfusion and investigated functional recovery and cell-death events, including necrosis, apoptosis and autophagy. This study provides the first evidence that the absence of either junctin or triadin/junctin aggravates the detrimental effects of I/R injury by impairing the contractile recovery and increasing cell death in the myocardium. These effects in junctin and triadin/junctin-deficient hearts are associated with exacerbated ER stress, increased autophagy, and over-activation of calpain, while the mitochondria-dependent apoptotic pathways are not altered. Furthermore, blockade of calpain activation partially rescues the impaired function in both JKO and TKO hearts. Thus, the presence of junctin and triadin appears important for maintaining the heart's contractile function and for the survival of cardiomyocytes during I/R, since the loss of either can increase dysfunction and injury.

Augmentation of apoptosis has been recognized as a common cellular event involved in the pathogenesis of heart failure, and it is considered as an epiphenomenon in post-ischaemic hearts.[23] For example, transgenic mice overexpressing active caspase-8, which exhibit a 10-fold increase in apoptosis (apoptotic rate of 0.023%), develop severe dilated cardiomyopathy over 8 weeks and die within 2–6 months, compared with healthy wild-type mice that exhibit apoptotic rates of ~0.002%.[24] Our semi-quantitative analysis of apoptosis, based on TUNEL staining data collected post-I/R, showed that the degree of apoptosis was ~1% in WTs, ~2% in JKOs and ~3% in TKOs. However, the $+dP/dt$ max post-I/R was reduced by 20, 50, and 57% in WTs, JKOs, and TKOs, respectively, indicating that apoptosis could only partially contribute to the impaired contractile function.

Calcium overload plays a pivotal role in the mechanisms underlying cell death events, including necrosis, apoptosis, and autophagy. At the initial time of reperfusion, the increase in cytosolic Ca^{2+} is mirrored by a decrease in SR Ca^{2+} content,[25] and we observed that Ca^{2+} overload was increased in both junctin- or triadin-deficient myocytes, following HR. This may be partially due to down-regulation of SERCA2a expression levels, which can deter Ca^{2+} re-entry into the SR. Furthermore, previous studies indicated aberrant Ca^{2+}-release in junctin or triadin null cardiomyocytes,[6,8] which would also contribute to Ca^{2+}-overload post I/R. In addition, the activation of the reverse mode of NCX may exacerbate this condition.[26] The depletion of SR Ca^{2+} store leads to accumulation of mis- or unfolded proteins, called the UPR or ER stress,[20] for which ATF6, PERK, and IRE-1α are ER membrane sensors that monitor UPR.[20] In the current study, although both ATF6 and PERK-associated pathways were activated in response to I/R injury, these cascades were not tuned up to a greater extent in the junctin or triadin-deficient hearts. However, activation of the IRE-1α signalling pathway was increased in the absence of junctin or triadin post I/R. Actually, dissociation of IRE-1α from GRP78 leads to its dimerization and its autophosphorylation,[27] which subsequently splices XBP-1 mRNA by removing a 26nt sequence, resulting in translational frame shift and production of an active XBP-1 transcription factor.[20] Indeed, our data show the enhanced induction of spliced XBP-1 in post-I/R JKO hearts, which was even more pronounced in the absence of triadin.

CHOP is an ER stress-associated transcription factor, that can promote expression of pro-apoptotic factors while suppressing anti-apoptotic gene transcription,[28] resulting in activation of caspase-12 to execute apoptosis.[29] In the present study, the expression levels of CHOP as well as caspase-12 activity were more elevated in post-ischaemia reperfused KO than WT hearts. Importantly, these increases were more pronounced in the absence of triadin, corresponding to the increased apoptosis and depressed contractile function of the TKO hearts. These results suggest that I/R-induced heart injury, especially cardiomyocyte apoptosis, may be primarily dependent on deterioration of the ER stress in junctin-deficient or triadin-deficient hearts.

Besides increased myocyte apoptosis, is there any other molecular mechanism accounting for the diminished contractile recovery in KO hearts? Our study indicates that calpain was activated to a greater extent in the absence of these jSR adaptors during I/R, and this may be attributed to the higher observed diastolic Ca^{2+}, because calpain is a non-lysosomal cysteine protease whose activity is dependent on cytosolic Ca^{2+} concentration. Activation of calpain is involved not only in impairment of Ca^{2+} handling proteins[30] but also in proteolysis of the cardiac sarcomere components[31] during I/R. Cardiac TnI is part of the troponin complex that, in concert with tropomyosin, regulates myocyte contraction. Calpain activation has been shown to degrade the COOH-terminal cTnI in I/R and this correlates with I/R-induced contractile dysfunction.[32,33] Along these lines, we found that increased calpain activity was associated with reduced expression of cTnI in JKO hearts upon I/R, and ablation of triadin intensified this proteolytic effect, suggesting that increased cardiac TnI proteolysis may be partly responsible for the impaired cardiac function in post-ischaemic JKO and TKO hearts. In support of this mechanism, we found that administration of MDL28170, a calpain inhibitor, could partially rescue the recovery of contractile function in JKO and TKO hearts. This partial recovery may be due to other factors such as autophagy, energetics, and necrosis. Despite the fact that MDL28170 treatment significantly reduced the infarct area and the degree of cTnI degradation in JKO and TKO hearts, these detrimental effects remained greater in the KO models, suggesting a more pronounced injury than WTs (Supplementary material online, *Figures S3*C and *S4*). Notably, calpain inhibition resulted in similar contractile recovery of post-ischaemic JKO and TKO hearts, supporting the notion that this pathway contributes to the exacerbated dysfunction in TKOs.

In conclusion, the present findings indicate that ablation of junctin or that of both triadin and junctin is associated with increased cardiac I/R injury, through deteriorated ER stress and extensive calpain activation, suggesting that these junctional-SR calsequestrin-binding regulators play important roles in maintaining cardiac contractility and cell survival in response to I/R injury.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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References

1. Lowenstein CJ. Myocardial reperfusion injury. *N Engl J Med* 2007;**357**:2409–2410.
2. Ruiz-Meana M, Fernandez-Sanz C, Garcia-Dorado D. The SR-mitochondria interaction: a new player in cardiac pathophysiology. *Cardiovasc Res* 2010;**88**:30–39.
3. Tang WH, Kravtsov GM, Sauert M, Tong XY, Hou XY, Wong TM *et al*. Polyol pathway impairs the function of SERCA and RyR in ischemic-reperfused rat hearts by increasing oxidative modifications of these proteins. *J Mol Cell Cardiol* 2010;**49**:58–69.
4. Talukder MA, Kalyanasundaram A, Zhao X, Zuo L, Bhupathy P, Babu GJ *et al*. Expression of SERCA isoform with faster Ca2+ transport properties improves postischemic cardiac function and Ca^{2+} handling and decreases myocardial infarction. *Am J Physiol Heart Circ Physiol* 2007;**293**:H2418–2428.
5. 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**:1012–1020.
6. Yuan Q, Fan GC, Dong M, Altschafl B, Diwan A, Ren X *et al*. Sarcoplasmic reticulum Ca^{2+} overloading in junctin deficiency enhances cardiac contractility but increases ventricular automaticity. *Circulation* 2007;**115**:300–309.
7. Gergs U, Berndt T, Buskase J, Jones LR, Kirchhefer U, Muller FU *et al*. On the role of junctin in cardiac Ca^{2+} handling, contractility, and heart failure. *Am J Physiol Heart Circ Physiol* 2007;**293**:H728–734.
8. Chopra N, Yang T, Asghari P, Moore ED, Huke S, Akin B *et al*. Ablation of triadin causes loss of cardiac Ca^{2+} release units, impaired excitation-contraction coupling, and cardiac arrhythmias. *Proc Natl Acad Sci U S A* 2009;**106**:7636–7641.
9. Toldo S, Severino A, Abbate A, Baldi A. The role of PDI as a survival factor in cardiomyocyte ischemia. *Methods Enzymol* 2011;**489**:47–65.
10. Sammels E, Parys JB, Missiaen L, De Smedt H, Bultynck G. Intracellular Ca^{2+} storage in health and disease: a dynamic equilibrium. *Cell Calcium* 2010;**47**:297–314.
11. Ruiz A, Matute C, Alberdi E. Intracellular Ca^{2+} release through ryanodine receptors contributes to AMPA receptor-mediated mitochondrial dysfunction and ER stress in oligodendrocytes. *Cell Death Dis* 2010;**1**:e54.
12. Qian J, Ren X, Wang X, Zhang P, Jones WK, Molkentin JD *et al*. Blockade of Hsp20 phosphorylation exacerbates cardiac ischemia/reperfusion injury by suppressed autophagy and increased cell death. *Circ Res* 2009;**105**:1223–1231.
13. Hernando V, Inserte J, Sartorio CL, Parra VM, Poncelas-Nozal M, Garcia-Dorado D. Calpain translocation and activation as pharmacological targets during myocardial ischemia/reperfusion. *J Mol Cell Cardiol* 2010;**49**:271–279.
14. Zhou X, Fan GC, Ren X, Waggoner JR, Gregory KN, Chen G *et al*. Overexpression of histidine-rich Ca^{2+}-binding protein protects against ischemia reperfusion-induced cardiac injury. *Cardiovasc Res* 2007;**75**:487–497.
15. Li F, Suqishita K, Su Z, Ueda I, Barry WH. Activation of connexin-43 hemichannels can elevate $[Ca^{2+}]_i$ and $[Na^+]_i$ in rabbit ventricular myocytes during metabolic inhibition. *J Mol Cell Cardiol* 2001;**33**:2145–2155.
16. Boston DR, Koyama T, Rodriguez-Larrain J, Zou A, Su Z, Barry WH. Effects of Antiotensin II on intracellular calcium and contracture in metabolically inhibited cardiomyocytes. *J Pharmacol Exp Ther* 1998;**285**:716–723.
17. Tsuchiya M, Tye CE, Sharma R, Smith CE, Bartlett JD. XBP1 may determine the size of the ameloblast endoplasmic reticulum. *J Dent Res* 2008;**87**:1058–1062.
18. Wang X, Eno CO, Altman BJ, Zhu Y, Zhao G, Olberding KE *et al*. ER stress modulates cellular metabolism. *Biochem J* 2011;**435**:285–296.
19. Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* 2011;**13**:184–190.
20. Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005;**115**:2656–2664.
21. Hoyer-Hansen M, Jaattela M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ* 2007;**14**:1576–1582.
22. Martinez JA, Zhang Z, Svetlov SI, Hayes RL, Wang KK, Larner SF. Calpain and caspase processing of caspase-12 contribute to the ER stress-induced cell death pathway in differentiated PC12 cells. *Apoptosis* 2010;**15**:1480–1493.
23. Fan GC, Ren X, Qian J, Yuan Q, Nicolaou P, Wang Y *et al*. Novel cardioprotective role of a small heat-shock protein, Hsp20, against ischemia/reperfusion injury. *Circulation* 2005;**111**:1792–1799.
24. Whelan RS, Kaplinskiy V, Kitsis RN. Cell death in the pathogenesis of heart disease: mechanisms and significance. *Annu Rev Physiol* 2010;**72**:19–44.
25. Valverde CA, Kornyeyev D, Ferreiro M, Petrosky AD, Mattiazzi A, Escorbar AL. Transient Ca^{2+} depletion of the sarcoplasmic reticulum at the onset of reperfusion. *Cardiovasc Res* 2010;**85**:671–680.
26. Wei GZ, Zhou JJ, Wang B, Wu F, Bi H, Wang YM *et al*. Diastolic Ca^{2+} overload caused by Na^+/Ca^{2+} exchanger during the first minutes of reperfusion results in continued myocardial stunning. *Eur J Pharmacol* 2007;**572**:1–11.
27. Ali MM, Bagratuni T, Davenport EL, Nowak PR, Silva-Santisteban MC, Hardcastle A *et al*. Structure of the Ire1 autophosphorylation complex and implications for the unfolded protein response. *Embo J* 2011;**30**:894–905.
28. Chiribau CB, Gaccioli F, Huang CC, Yuan CL, Hatzoglou M. Molecular symbiosis of CHOP and C/EBP beta isoform LIP contributes to endoplasmic reticulum stress-induced apoptosis. *Mol Cell Biol* 2010;**30**:3722–3731.
29. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA *et al*. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000;**403**:98–103.
30. 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* 2010;**1802**:356–362.
31. Portbury AL, Willis MS, Patterson C. Tearin' up my heart: proteolysis in the cardiac sarcomere. *J Biol Chem* 2011;**286**:9929–9934.
32. McDonough JL, Arrell DK, Van Eyk JE. Troponin I degradation and covalent complex formation accompanies myocardial ischemia/reperfusion injury. *Circ Res* 1999;**84**:9–20.
33. Maekawa A, Lee JK, Nagaya T, Kamiya K, Yasui K, Horiba M *et al*. Overexpression of calpastatin by gene transfer prevents troponin I degradation and ameliorates contractile dysfunction in rat hearts subjected to ischemia/reperfusion. *J Mol Cell Cardiol* 2003;**35**:1277–1284.