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ORIGINAL CONTRIBUTION

Studies on the role of apoptosis after transient myocardial ischemia: genetic deletion of the executioner caspases-3 and -7 does not limit infarct size and ventricular remodeling

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Abstract Although it is widely accepted that apoptosis may contribute to cell death in myocardial infarction, experimental evidence suggests that adult cardiomyocytes repress the expression of the caspase-dependent apoptotic pathway. The aim of this study was to analyze the contribution of caspase-mediated apoptosis to myocardial ischemia-reperfusion injury. Cardiac-specific caspase-3 deficient/full caspase-7-deficient mice (Casp3/7DKO) and wild type control mice (WT) were subjected to in situ ischemia by left anterior coronary artery ligation for 45 min followed by 24 h or 28 days of reperfusion. Heart function was assessed using M-mode echocardiography. Deletion of caspases did not modify neither infarct size determined by triphenyltetrazolium staining after 24 h of reperfusion (40.0 ± 5.1 % in WT vs. 36.2 ± 3.6 % in Casp3/7DKO), nor the scar area measured by pricosirius red staining after 28 days of reperfusion (41.1 ± 5.4 % in WT vs. 44.6 ± 8.7 % in Casp3/7DKO). Morphometric and echocardiographic studies performed 28 days after the ischemic insult revealed left ventricular dilation and severe cardiac dysfunction without statistically significant differences between WT and Casp3/7DKO groups. These data demonstrate that the executioner caspases-3 and -7 do not significantly contribute to cardiomyocyte death induced by transient coronary occlusion and provide the first evidence obtained in an in vivo model that argues against a relevant role of apoptosis through the canonical caspase pathway in this context.

Keywords Myocardium · Ischemia · Reperfusion · Apoptosis · Caspases

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Introduction

The extent of myocardial cell death, infarct size, is the most important predictor of survival and long-term outcome in patients with an acute myocardial infarction [25]. Although prompt restoration of myocardial perfusion is imperative to limit infarct size [34], myocardial salvage achieved by reperfusion is reduced by the occurrence of cardiomyocyte death during the reperfusion period, a phenomenon known as reperfusion injury [44].

There is solid evidence suggesting that a large fraction of cardiomyocyte death occurring during reperfusion takes place during the first minutes after restoration of blood flow, and involves sarcolemmal rupture, a major feature of necrotic cell death [17]. However, it is widely accepted that apoptosis contributes importantly to final infarct size [24,

31, 32, 52, 54]. A large number of studies suggests that activation of the apoptotic signaling during reperfusion results in the death of cardiomyocytes during both, the acute [23, 24, 31, 32], and late phase of reperfusion [2, 30, 33, 43], contributing to the loss of viable myocardium and progression to adverse ventricular remodeling.

Apoptosis is a regulated form of cell death that has been differentiated into two distinct pathways, the extrinsic pathway initiated by the activation of a death receptor, and the intrinsic or mitochondrial pathway induced by a variety of extra and intracellular stress stimuli. In both cases, the apoptotic signaling cascade triggers the cleavage and activation of the effector caspases-3 and -7, cysteinyl aspartate proteinases considered the executioners of the apoptotic cell death and extensively used as hallmarks of apoptosis [7, 28]. Moreover, apoptosis and, in particular, activation of caspases has been suggested as potential pharmacological target for the attenuation of reperfusion injury.

Although it has been suggested that reperfusion activates the mitochondrial apoptotic pathway [11], experimental data indicate that caspase-dependent signaling is important during heart morphogenesis [9] but is repressed in cardiomyocytes during terminal differentiation suggesting that apoptosis through the canonical caspase pathway is not involved in post-mitotic cardiomyocyte death [4, 9]. Although this evidence strongly argues against an important role of cardiomyocyte apoptosis in the reperfused myocardium, conclusive assessment of the role of caspase-mediated apoptosis in a clinically relevant experimental model is needed.

In the present study, we aimed to examine the contribution of the executioner caspases to acute myocardial reperfusion injury, post-infarct remodeling and heart failure by using a newly developed cardiac-specific caspase-3/caspase-7 null mouse model.

Methods

The experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, 8th Edition, published in 2011, and were reviewed and approved by the Research Commission on Ethics of the Hospital Vall d'Hebron and the Experimental Animal Ethic Committee of the University of Lleida (codes CEEA06-01/10, 07-01/10, 08-01/09 and 09-01/09).

Cardiac-specific caspase-3/caspase-7 double knockout mice

Full caspase-7-deficient cardiac-specific caspase-3-deficient mouse strain (casp3/7DKO) was generated by crossing of caspase-7-deficient [28] and caspase-3 floxed mice [45] with the Nkx2.5::Cre transgenic mouse strain, a kind gift of Dr. Eric N. Olson (UT Southwestern Medical Center, Dallas, TX, USA) [36]. Genotypes were analyzed by polymerase chain reaction (PCR) using tail DNA as a template (primer sequences can be found in Supplemental material S1) and the expression of both caspases was determined by western blot and immunohistochemistry. The breeding program followed to obtain single and double mutant mice as well as control wild type (WT) mice is specified in Cardona et al. (Suppl. Fig. A) [9]. All genotypes were obtained following Mendelian ratios. In order to reduce to a minimum the possibility of differential phenotype due to changes in genetic background, the control (wild type) animal group was composed of $Cre^{+/?}Casp3^{+/+}Casp7^{+/+}$; $Cre^{-/-}Casp3^{+/+}Casp7^{+/+}$; $Cre^{-/-}Casp3loxP^{/+}Casp7^{+/+}$ and $Cre^{-/-}Casp3loxP/loxPCasp7^{+/+}$ mice born at the same time than double KO mice. Therefore control mice had virtually the same genetic background than knockout mice.

In vivo ischemia/reperfusion protocol

All mouse experiments were performed on 24–28 g and 8–12 week old mice of both genders. Mice were anaesthetized with isoflurane (5 % induction, 2–3 % maintenance) and mechanically ventilated (Inspira ASV, Harvard Apparatus). Anesthesia was maintained with 1–2 % isoflurane. The heart was exposed through the fourth intercostal space and the left anterior descending coronary artery (LAD) ligated approximately 1 mm below the edge of the left atrial appendage with an 8–0 silk suture. Regional ischemia was confirmed by visual inspection of a pale color in the occluded distal myocardium and ST-segment. After occlusion for 45 min, the suture was loosened to start reperfusion. The thorax and the skin incision were closed with 6–0 silk sutures (Lab Arago, Spain) and buprenorphine (0.01 mg/kg, Buprex, Merck & Co. Inc) was given for pain relief. Mice with lack of ST-elevation during ischemia or lack of ST-recovery at reperfusion were excluded from further evaluation.

To determine the contribution of caspases-3 and -7 to the extent of infarct size, hearts ($n = 18$; nine WT, nine Casp3/7DKO, four females and five males in each group) were excised after 24 h of reperfusion, mounted in a Langendorff apparatus, and perfused with saline for blood removal. The LAD was re-occluded and 2 % Evans blue injected into the beating left ventricle (LV) to delineate the area at risk. Mice with unclear delineation of the area at risk were excluded from analysis. Hearts were cut into five transverse sections that were incubated at 37 °C in 1 % triphenyltetrazolium chloride (Sigma Chemical) for 15 min, and imaged. The area at risk and the area of

necrosis were measured semi-automatically (Image Pro-Plus software, Media Cybernetics) in the digitalized images. Infarct size was calculated as a percentage of necrosis at the region at risk. The area of contraction band necrosis was analyzed in sections stained with Masson's trichrome and compared with the infarct area as previously described by our group ($n = 3$ per group) [5].

To analyze genotype-based differences in the cardiac tolerance to ischemia/reperfusion, WT ($n = 5$) and Casp3/7DKO ($n = 5$) male mice were subjected to an ischemic preconditioning protocol consisting in three cycles of 5 min ischemia and 5 min of reperfusion.

The effect of caspase-3 and -7 deletion on post-infarct remodeling and heart function was analyzed in mice reperfused for 28 days ($n = 19$; eight WT and nine Casp3/7DKO; four males in the WT group and five males in the Casp3/7DKO group). After reperfusion hearts were rapidly excised, weighed, fixed in buffered 4 % paraformaldehyde and embedded in paraffin for histological evaluation. Sections at the papillary muscles level were cut at 5 µm and stained with picrosirius red to visualize the area of scar and interstitial and peri-vascular fibrosis. The percentage of the left ventricle stained for collagen was calculated as the ratio of picrosirius-red positively stained area over total tissue area using Image Pro-Plus analysis software.

Echocardiographic and morphometric analysis

Mice underwent transthoracic echocardiography at baseline (before surgery) and at 28 days after surgery. Echocardiography was performed using a Vivid portable ultrasound system with a i12L-RS 12 MHz transducer (GE Healthcare) as described earlier [21]. The left ventricular end-systolic (LVESd) and diastolic (LVEDs) internal diameters were measured in M-mode recordings. Left ventricular ejection fraction (LVEF) and fractional shortening (FS) were calculated according to standard formulas.

After completion of functional measurements, the heart was excised and mean LV wall thickness (LVW) and intraventricular septum thickness (IVS) were measured in transverse sections fixed in diastole and stained with Masson's trichrome. The largest endocardial circumference from a single LV section was used as an index for the extent of LV dilation [15].

Western blot and immunohistochemistry

Protein expression was analyzed in total protein extracts from tissues by SDS-PAGE as described previously [4]. Primary antibodies: caspase-3 cell signalling (9662), 1/3000; caspase-6 cell signalling (9762), 1/1000; caspase-7 Enzo Life Sciences (ADI-AMM-127) 1/1000; Gapdh Abcam (ab8245) 1/10,000. Densitometric quantification of the bands was performed with the ImageJ software from scanner images of film exposures in which bands were not saturated. Values were expressed as arbitrary units (AU) corresponding to the signal numerical value given by ImageJ.

For immunohistochemistry analysis, P2 pups were killed by decapitation; the heart was excised, fixed in 4 % paraformaldehyde for 24 h at 4 °C and included into paraffin. Slices (3 µm) were deparaffinized, endogenous peroxydase was chemically inhibited with 3 % H_2O_2 for 30 min and antigens were unmasked with citrate buffer at pH 6. Tissue was incubated with the rabbit anti-caspase-3 primary antibody (cell signalling, 1:250 in PBS, 2 % goat serum, overnight, 4 °C) and after three washes in PBS, slides were incubated with a biotinylated goat anti-rabbit antibody (DAKO, 1:200 in PBS, 2 % goat serum, 1 h, RT). Samples were rinsed and incubated with avidin–biotin-HRP complex (Cultek, 1:100 in PBS, 1 h, RT), developed with diaminobenzidine and H_2O_2, contrasted with Meyer's hematoxylin, dehydrated and mounted.

TUNEL assay

TUNEL-positive cardiomyocytes were measured in hearts obtained from WT, Casp3/7DKO and endonuclease G null (endoG$^{-/-}$) mice after 24 h of reperfusion. Generation and characterization of endoG$^{-/-}$ null mice has been described by Irvine RA [22], and used by our group to define the role of endonuclease G in maladaptive cardiac hypertrophy [35]. The endoG$^{-/-}$ group was included in the study to demonstrate that the presence of TUNEL+ nuclei in a sample is not a definitive proof of the involvement of caspases in the cell death process. The TUNEL assay was performed with the ApoTag Peroxidase kit (S7100, Millipore) in deparaffinized transverse ventricular slices following manufacturer's instructions. Sections were counterstained by hematoxylin-eosin and myocyte nuclei were identified by position and morphology. The results were expressed as the ratio of the number of TUNEL-positive myocytes to the total number of myocytes after examination of 20 fields per each slice obtained at 400× magnification ($n = 3$ per group).

Statistics

All data were analyzed by a single observer blinded to mouse genotypes. Results are expressed as mean ± standard error. Statistical significance of difference among groups were evaluated by either an unpaired Student's t test or one-way ANOVA followed by Tukey's multiple-group comparisons test. Statistical significance was assumed at $P < 0.05$. Survival was compared using the log-rank test.

Results

Generation of cardiac-specific caspase-3 deficient/full caspase-7 deficient mice

We have designed a conditional knockout mouse in which caspase-3 gene deletion depends on loxP recombination driven by Cre recombinase expressed under the control of the Nkx2.5 basal promoter-cardiac enhancer. Nkx2.5 promoter directs gene expression from the onset of cardiac commitment [36]. Caspase-7 was deleted ubiquitously because the lack of phenotype observed previously [28]. Caspase-3 and -7 double mutant mice were obtained by intercrossing caspase-3lox/lox, caspase-7$^{-/-}$ and Nkx2.5::Cre mice. Genotyping reaction (Table S1) was designed to distinguish between caspase-3 floxed (e.g. tail) and knockout (myocardium) alleles, showing the presence of both in the heart due to floxed caspase-3 in non-myocytes (Fig. 1a). Western blot confirmed lack of caspase-7 expression in knockout mice and remnant caspase-3 expression in the neonatal heart (Fig. 1b), which was absent in myocytes as shown by immunohistochemistry (Fig. 1c). The phenotypic description of casp3/7DKO has been recently published by our group [9].

Cardiac-specific deletion of caspase-3 and -7 does not modify infarct size

To determine whether caspase-3 and -7 contribute to reperfusion-induced cardiomyocyte death, infarct size was determined in casp3/7DKO and WT mice subjected to 45 min of LAD occlusion followed by 24 h of reperfusion. Mortality rate during the surgical procedure was 22 % (two WT and two casp3/7DKO mice). One mouse was excluded for no clear confirmation of ST-elevation during ischemia and an additional mouse was excluded for unsuccessful delimitation of the area at risk. The results showed no differences between WT and casp3/7DKO mice in the mass of myocardium at risk as assessed by Evans Blue staining, nor in the infarct size, expressed as percentage of area at risk developing necrosis, (40.0 ± 4.2 % in reperfused WT group vs. 36.2 ± 3.3 % in casp3/7DKO group; P = ns, Fig. 2). We calculated the probability of having obtained by chance (beta error) these results despite a significant contribution of caspase-dependent apoptosis to infarct size that is, a reduction of 30 % in the casp3/7DKO group, assuming variances in each group to be equal to those observed. This probability was 0.079.

The area of cell death detected by triphenyltetrazolium was composed of contraction band necrosis in both WT and casp3/7DKO mice (Fig. 2d shows an area of contraction band necrosis measured in control and transgenic mice).

The percentage of TUNEL+ myocyte nuclei in WT and Casp3/7DKO hearts was similar (28.22 ± 2.31 and 24.96 ± 2.68 %, respectively, P = ns) while it was significantly reduced in the endoG$^{-/-}$ group (11.05 ± 2.02, $P < 0.001$) (Fig. 3). These results are in agreement with a previous study from our group demonstrating that primary cultured cardiomyocytes subjected to experimental

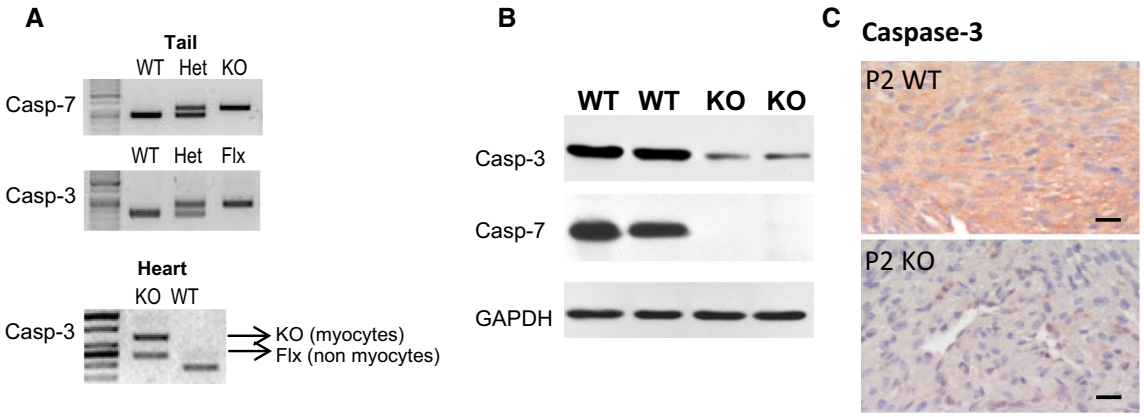

Fig. 1 Characterization of cardiomyocyte-specific caspase-3 deficient/full caspase-7-deficient mice. **a** Genotyping by genomic PCR of tail and heart DNA. Primer set includes three primers for detecting caspase-3 and four for detecting caspase-7 (see Table S1 for sequences and fragment lengths). *WT* wild type allele, *Het* heterozygous, *KO* deleted knockout allele (for caspase-7), *Flx* floxed allele. *Lower panel* shows caspase-3 genotyping products from cardiac DNA. *KO* deleted knockout allele (cardiomyocytes), *Flx* floxed (non-myocytes). **b** Immunodetection of caspase-3 and caspase-7 in P2 neonatal hearts of wild type (WT) and knockout (KO) mice. Remnant expression of caspase-3 is detected in KO hearts due to non-myocytes. Caspase-7 ubiquitous deletion assures total absence in heart extracts. **c** Immunohistochemistry of caspase-3 (*brown staining*) in WT and KO heart slices confirms deletion in cardiomyocytes. *Bar* 20 μm

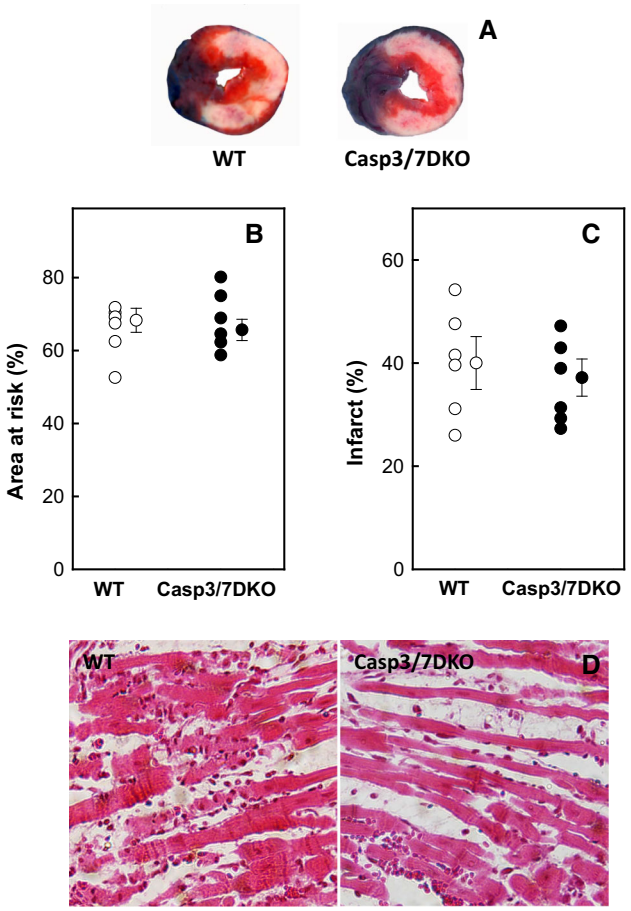

Fig. 2 Deletion of caspase-3 and caspase-7 does not modify infarct size. **a** Images of representative transverse heart section after *Evans blue* and triphenyltetrazolium staining. **b** Quantification of area at risk and **c** infarct size, expressed as percentage of area at risk, in WT and Casp3/7KO hearts submitted to 45 min of ischemia and 24 h of reperfusion. **d** Photomicrographs of infarcted myocardial section stained with Masson's trichrome showing the presence of contraction band necrosis. Values are mean ± SEM. $n = 6$ animals per group

ischemia undergo TUNEL+ DNA damage that cannot be prevented by pan-caspase inhibitors but is blocked by endonuclease G or Bnip3 gene silencing [58]. Therefore, our data suggest that the presence of TUNEL+ nuclei in a sample is not a definitive proof of the involvement of caspases in the cell death process.

To discard that the lack of differences in infarct size were consequence of genotype-based differences in the tolerance to ischemia/reperfusion, infarct size was analyzed in casp3/7DKO and WT mice subjected to a preconditioning protocol. Ischemic preconditioning reduced infarct size in WT and casp3/7DKO mice with respect their control groups (24.3 ± 3.9 and 20.2 ± 2.9 %, respectively). No significant differences between both groups with respect the magnitude of protection were observed (39 % in WT and 44 % in casp3/7DKO mice).

Lack of executioner caspases does not affect post-infarct remodeling and ventricular function

To test whether deletion of caspases-3 and -7 modifies post-infarct remodeling and ventricular performance, casp3/7DKO and WT mice were subjected to a long-term follow-up ischemia/reperfusion protocol consisting in 45 min of LAD occlusion followed by 4 weeks of reperfusion.

Mortality was 26 %: two WT mice (one mouse during surgery and one mouse at 24 h of reperfusion) and three transgenic mice (two mice during surgery and one mouse after 3 days of reperfusion). One animal of each group was excluded for no confirmation of ST-elevation during ischemia.

To assess postischemic left ventricular remodeling and function, echocardiographic measurements were obtained before occlusion and at 4 weeks of reperfusion. M-mode echocardiographic analysis revealed no differences on LV dimensions and function between WT and casp3/7DKO mice at baseline. Myocardial infarction resulted in a significant increase in LVEDD and LVESD and a decrease in FS and LVEF in both groups. However, there were no significant differences in cardiac dilation and dysfunction between WT and casp3/7DKO mice (Fig. 4).

Morphometric measurements were made in hearts obtained after completion of the echocardiographic analysis. No significant differences in mean LV wall thickness (0.56 ± 0.09 mm in WT mice vs. 0.54 ± 0.13 mm in casp3/7DKO mice), septum thickness (0.89 ± 0.12 mm in WT mice vs. 0.84 ± 0.09 mm in casp3/7DKO mice) and in the LV endocardial circumference (11.64 ± 1.58 mm in WT mice vs. 12.55 ± 1.85 mm in casp3/7DKO mice) were observed.

After 28 days of reperfusion, the scar area, measured as the area of fibrosis in the infarcted heart, was similar in both groups (41.2 ± 6.0 % in WT mice and 44.7 ± 6.6 % in casp3/7DKO mice, P = ns, Fig. 5) and not statistically different than those of infarct size obtained after 24 h of reperfusion. Like in the case of infarct size at 24 h, the probability of having obtained this results despite a 30 % reduction in scar in the casp3/7DKO group due to a beta error was very low (0.044).

Discussion

In this manuscript we have tested the hypothesis that apoptosis is an important contributor to cell death in cardiomyocytes after transient ischemia in a way that it had never been tested before, and the results are clearly against the validity of that hypothesis. This study demonstrates, by using a new developed cardiac-specific caspase double

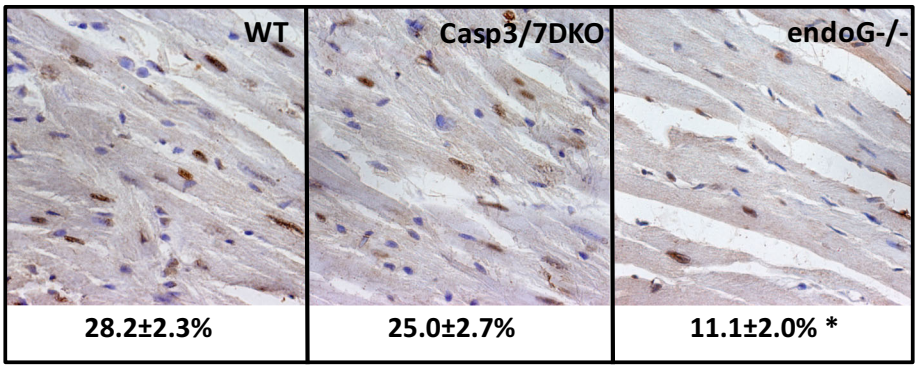

Fig. 3 TUNEL-positive DNA damage in cardiomyocytes depends on endonuclease G as is independent of caspase-3 and caspase-7. Representative images of TUNEL stained myocardial section at 24 h of reperfusion and percentage of TUNEL-positive cardiomyocytes (*brown*) in WT, Casp3/7KO and endonuclease knockout (endoG$^{-/-}$) mice. Values are mean ± SEM. $n = 3$ animals per group. *$P < 0.05$ vs. WT group

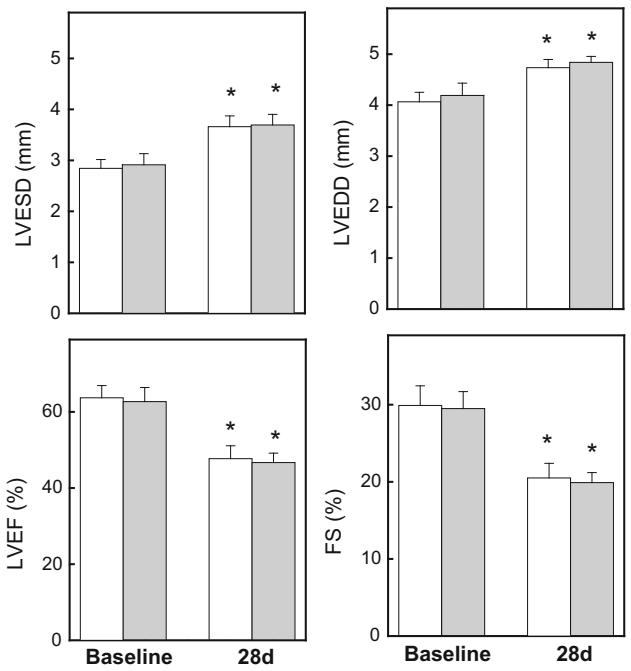

Fig. 4 Caspase-3 and caspase-7 deletions does not alter postinfarct echocardiographic parameters. Body weight (BW), heart rate (HR) and echocardiographic data in WT and Casp3/7KO mice before ischemia (baseline) and at 28 days of reperfusion. *FS* fractional shortening, *LVEF* left ventricular ejection fraction, *LVEDD* left ventricular end-diastolic diameter, *LVESD* left ventricular end-systolic diameter. Values are mean ± SEM. $n = 6$ animals per group. *$P < 0.05$ vs. baseline data

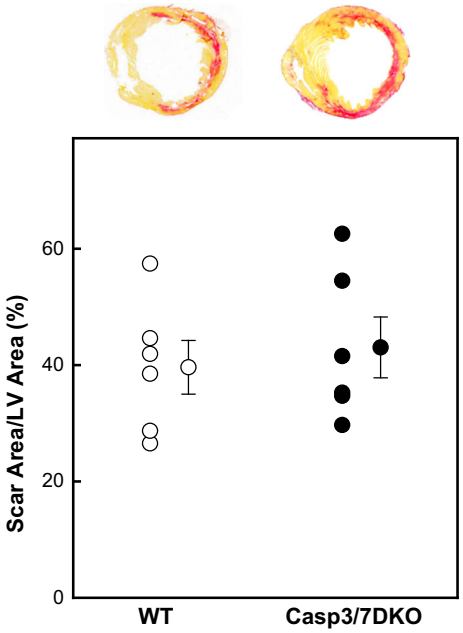

Fig. 5 Deletion of caspase-3 and caspase-7 does not modify postinfarct scar area. **a** Representative images showing pricosirius *red* staining of transverse cardiac sections. **b** Bar graphs shows infarct area quantification in WT and Casp3/7KO hearts submitted to 45 min of ischemia and 28 days of reperfusion and expressed as scar area per LV area. Values are mean ± SEM. $n = 6$ animals per group

knockout mice, that the executioner caspases-3 and -7 do not significantly contribute neither to the acute effects of myocardial ischemia/reperfusion injury nor to post-infarct adverse remodeling. These results obtained support the concept that cell death occurs mainly during the first hours of reperfusion and by a necrotic mechanism.

Cardiomyocyte cell death is the most detrimental consequence of myocardial reperfusion injury. The fact that the release of intracellular proteins accurately predicts the final infarct size [8, 16], and that reperfused infarcts are mainly composed of areas of contraction band necrosis corresponding to myocytes showing sarcolemmal rupture [5, 38], indicates that necrosis is the main form of cell death during reperfusion. Furthermore, the time course of markers of necrosis and the fact that most strategies against reperfusion injury loss their effectiveness when their administration is delayed only few minutes after restoration of flow, indicates that most of cell death occurs during the initial minutes of reperfusion [17].

Despite that, since Gottlieb et al. evaluated the occurrence of apoptosis in an animal model of transient ischemia [18], it has been widely accepted that cardiomyocytes could also die by apoptosis within the first hours of reperfusion [26, 40], and in lower magnitude as the process of remodeling progresses [23, 48]. However, the relative impact of apoptosis to the extent of cardiac damage still being debated due to the large differences in its magnitude reported by different investigators. In this regard it has been described that apoptosis represents the major form of acute cell death after 2 h of reperfusion after transient coronary occlusion in rats (86 vs. 14 % of necrosis) [23], but also that it is of minor importance after global ischemia and 6 h of reperfusion in isolated canine hearts [14]. This strong variability has been explained at least in part as consequence of the different temporal progression of each form of cell death. Zhao et al. observed that while the extent of necrotic cell death was maximal at 24 h of reperfusion, apoptosis progressively developed during late reperfusion in dogs subjected to transient coronary occlusion [59]. It is also proposed that necrosis occurs in the ischemic area exposed to the most severe ATP depletion, while apoptosis is predominantly found in the adjacent border areas [49], playing an important role in expanding the infarct border zone during the post-infarct remodeling process [13].

Contrary to these observations, experimental evidence suggests that apoptosis is not relevant for post-mitotic cardiomyocyte cell death. Apoptosis is mainly executed by caspases-3 and -7, which are highly regulated by the activation of signaling cascades in response to a diversity of both extrinsic and intrinsic signals [12]. According to this, the contribution of apoptosis to myocardial infarct size is based in the assumption that the caspase-dependent signaling is functional in differentiated cardiomyocytes. However, Bahí et al. demonstrated a global reduction of the whole caspase-dependent pathway expression in cardiomyocytes during cell differentiation and that these proteins are not up-regulated after a hypoxic stimulus in isolated cardiomyocytes [4]. In addition, more recently, Cardona et al. showed that while required for heart development, the executioner caspases-3, -6 and -7 are silenced in the terminally differentiated myocardium [9].

Therefore, in the present study we applied an in vivo protocol of ischemia/reperfusion to a newly developed transgenic mouse model with a simultaneous cardiac-specific deletion of the executioner caspases -3 and -7. The results herein reported prove that caspase mediated apoptosis is not causally involved in the death of cardiomyocytes during the acute phase of reperfusion. In addition, the lack of effect of caspase depletion on LV dimensions, scar formation and LV function after 28 days of reperfusion is also against a significant contribution of caspases to the process of postinfarct remodeling and heart failure.

The phenotypic characterization of casp3/7DKO mice has been recently published by our group [9]. The fact that the tolerance to ischemia/reperfusion injury, based on the cardioprotection obtained in response to an ischemic preconditioning protocol, was similar in control and transgenic mice rule out the possibility that the results obtained were a mere consequence of phenotype-based differences.

Although our observations are in agreement with the described down-regulation of the whole caspase signaling in differentiated cardiomyocytes [4, 9], the present study does not rule out the possibility of some residual caspase-6 activity in the infarcted or peri-infarct myocardium. In addition, since caspase-7 deletion was total and cardiac-specific deletion of caspase-3 using the NKx2.5 promoter may induce caspase-3 deletion in cardiac cells other than cardiomyocytes, we cannot completely discard an indirect effect of apoptosis on cardiomyocyte survival and heart function as consequence of the presence of caspase activity in other cardiac cell types [41, 50]. However, the absence of any difference in infarct size after 24 h of reperfusion and in the magnitude of adverse post-infarction remodeling or functional recovery observed in mice with myocardial deletion of caspases-3 and -7 suggests that the potential contribution of apoptosis in non-cardiomyocyte cells to the death of cardiomyocytes is of minor relevance at best in the context of acute myocardial infarction and subsequent LV remodeling. Our study does not exclude other regulated forms of cell death independent of caspases, such as necroptosis. This process, which is activated by the stimulation of a death receptor and requires the kinase activity of RIP1, induces cell death with morphological features of necrosis [40].

There are several reasons that could explain why apoptosis of cardiomyocytes during reperfusion is so extensively documented. Almost all studies on apoptotic death use neonatal cardiomyocytes, which still expressing caspases or total heart homogenates, that include cell types other than cardiomyocytes [1, 13, 19, 39, 55, 57]. In this regard, Zidar et al. described that active caspase-3 staining in sections of human hearts after myocardial infarction was overwhelmingly higher in cells other than cardiomyocytes [60]. In other studies, the experimental models used retain the ability to proliferate and have a functional caspase system as it occurs in cardiomyocytes from zebra fish [29], or cell lines derived from atrial cardiomyocytes obtained from mouse AT-1 (HL-1) [51].

Furthermore, different groups have documented cardioprotection by using caspase inhibitors [20, 40]. However, the conclusions obtained from these studies are not conclusive for two main reasons. First, many studies have

failed to observe this beneficial effect [27, 37, 42]; second, it has been suggested that the mechanisms of the reported protection could be unrelated to apoptosis (i.e. caspase-dependent cleavage of contractile proteins) [42, 46, 56], or explained by inhibition of other proteases involved in necrosis as a consequence of limited drug selectivity [6]. Condorelli et al. described that forced cardiac-specific expression of caspase-3 in vivo in transgenic mice increases infarct size [10]. However, this study is completely compatible with the fact that in non-transgenic mice infarct size is independent of caspases-3 and -7.

Finally, many studies have inferred that caspase signaling is functional in differentiated cardiomyocytes from the experimental evidence showing mitochondrial damage and release of mitochondrial proteins as cytochrome c and AIF. These two proteins are to cause caspase activation and the cleavage of DNA rendering 3′-OH ends detectable by TUNEL assay [53]. However, opening of mitochondrial permeability transition pore (MPTP) during reperfusion results in mitochondrial membrane depolarization, uncoupling of oxidative phosphorylation and cardiomyocyte death in a caspase-independent manner [3, 47]. In addition, the detection of TUNEL-positive cardiomyocyte nuclei in a sample is not a definitive proof of the involvement of caspases in the DNA fragmentation. Different studies have shown that TUNEL-positive cardiomyocytes detected in heart slices, may also be necrotic [41]. Furthermore, MPTP favors the release of endonuclease-G which is proposed to be responsible for the caspase-independent cleavage of DNA in primary cultured cardiomyocytes subjected to hypoxia [58]. Our data provide further evidence to this previous study by demonstrating a reduction of TUNEL-positive cardiomyocytes in reperfused myocardium from endonuclease G null mice. Therefore, these data indicate that the presence of TUNEL+ nuclei in cardiomyocytes depends not only on caspase activation.

Conclusions

Our study provides the first evidence obtained in an in vivo model that argues against a relevant role of caspase-mediated cardiomyocyte cell death occurring in reperfused myocardium. These findings are in agreement with previous studies demonstrating the repression of the caspase signaling in post-mitotic cardiomyocytes, and may have important therapeutic implications. Our results show that activation of effector caspases is not a good target for prevention of lethal myocardial reperfusion injury and that therapeutic efforts should be aimed to prevent necrotic cell death occurring early during the reperfusion period.

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Compliance with ethical standards

Conflict of interest None.

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