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Calpains and proteasomes mediate degradation of ryanodine receptors in a model of cardiac ischemic reperfusion

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ABSTRACT

Type-2 ryanodine receptors (RyR2) – the calcium release channels of cardiac sarcoplasmic reticulum – have a central role in cardiac excitation–contraction coupling. In the heart, ischemia/reperfusion causes a rapid and significant decrease in RyR2 content but the mechanisms responsible for this effect are not fully understood. We have studied the involvement of three proteolytic systems – calpains, the proteasome and autophagy – on the degradation of RyR2 in rat neonatal cardiomyocyte cultures subjected to simulated ischemia/reperfusion (sI/R). We found that 8 h of ischemia followed by 16 h of reperfusion decreased RyR2 content by 50% without any changes in RyR2 mRNA. Specific inhibitors of calpains and the proteasome prevented the decrease of RyR2 caused by sI/R, implicating both pathways in its degradation. Proteasome inhibitors also prevented the degradation of calpastatin, the endogenous calpain inhibitor, hindering the activation of calpain induced by calpastatin degradation. Autophagy was activated during sI/R as evidenced by the increase in LC3-II and beclin-1, two proteins involved in autophagosome generation, and in the emergence of GFP-LC3 containing vacuoles in adenovirus GFP-LC3 transduced cardiomyocytes. Selective autophagy inhibition, however, induced even further RyR2 degradation, making unlikely the participation of autophagy in sI/R-induced RyR2 degradation. Our results suggest that calpain activation as a result of proteasome-induced degradation of calpastatin initiates RyR2 proteolysis, which is followed by proteasome-dependent degradation of the resulting RyR2 fragments. The decrease in RyR2 content during ischemia/reperfusion may be relevant to the decrease of heart contractility after ischemia.

1. Introduction

Ischemia/reperfusion (I/R) injury is a major cause of cell death in the heart [1]. The generation of reactive oxygen species and the increase in intracellular calcium concentration during ischemia and the subsequent reperfusion produce oxidative modification and proteolysis of several cardiac proteins [2–4], including sarcoplasmic reticulum (SR) calcium handling proteins such as ryanodine receptors (RyR2), sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) and phospholamban [2,3,5–7]. The decrease in the content of these SR proteins may exacerbate the characteristic calcium mishandling observed after I/R [8]. RyR2, the calcium release channels of cardiac sarcoplasmic reticulum, are particularly susceptible to ischemic injury. The tissue content of RyR2 decreases (50 to 70%) after ischemia [9] or after I/R [3,5]. RyR2 plays a crucial role in cardiac excitation–contraction coupling and its rapid down regulation during I/R may adversely affect cardiac cell recovery and functionality [7,10]. Greater understanding of the pathways involved in RyR2 degradation is therefore relevant for our ability to counteract the deleterious consequences of ischemia/reperfusion.

Calpains are calcium-dependent cysteine proteases activated during I/R, and are therefore potential contributors to RyR2 degradation [3]. Calpains, however, produce only limited proteolysis of their substrates and other proteolytic pathways further degrade the resulting fragments [11].

There are two other systems involved in cellular protein degradation in cardiac myocytes—macroautophagy and the ubiquitin–proteasome complex. Macroautophagy, from now on referred to simply as autophagy, is a degradative process whereby damaged proteins or organelles are sequestered in double membrane autophagosomes that fuse with lysosomes, where they are fully degraded and their aminoacids are recycled [12–14]. The proteasome is a barrel-

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shaped proteolytic complex that degrades ubiquitinated or oxidized proteins [15,16].

The aim of this work was to investigate the contribution of calpains, the proteasome and autophagy to RyR2 degradation following simulated I/R (sI/R) in a neonatal rat cardiomyocyte culture model.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), M199 medium, 2-deoxy-D-glucose, pancreatin, 3-methyladenine, E64d and clasto-lactacystin-β-lactone were purchased from Sigma-Aldrich Corp. Fetal bovine serum (FBS), penicillin and streptomycin were supplied by Biological Industries. Collagenase and newborn calf serum were obtained from Invitrogen (Paisley, Scotland, UK). Plastic Petri dishes were purchased from Falcon (BD Biosciences, Oxford, UK). MG-132 was acquired from Calbiochem and all other chemicals were of analytical grade and purchased from Merck Ltd. (Poole, Dorset, UK).

2.2. Primary culture

All animal experiments were approved by the Animal Care and Use Committee of the University of Chile and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, 1996). Cardiomyocytes were isolated from hearts from one- to three-day-old Sprague Dawley rats by enzymatic digestion using pancreatin (1.2 mg/ml) and collagenase (0.2 mg/ml) as described previously [17]. Cells were pre-plated to discard non-myocyte cells and the myocyte-enriched fraction was plated at 1.0×10^6 cells/mm^2 on gelatin-precoated 35-mm dishes and grown in DMEM/M199 (4:1) medium with 10% (w/v) FBS for 24 h before the experiments. Cardiomyocyte cultures were at least 95% pure as evaluated either morphologically or with anti-β-myosin heavy chain antibody (Vector Laboratories, Burlingame, CA, US) as described [18].

2.3. Simulated ischemia/reperfusion

Cells were incubated in ischemia-mimicking solutions containing (in mM) HEPES (5), 2-deoxy-D-glucose (10), NaCl (139), KCl (12), $MgCl_2$ (0.5), $CaCl_2$ (1.3), and lactic acid (20), pH 6.2, under 100% nitrogen ($O_2<1\%$) at 37 °C for 8 h. The lack of glucose or other nutrients together with the low pH and high potassium and lactate concentrations, mimics the changes occurring in the myocardium *in vivo* during no-flow ischemia [19]. Reperfusion was initiated by changing the ischemia-mimicking solution to DMEM/M199 (4:1) supplemented with 10% (w/v) FBS; incubation was continued for 16 h in 95% air, 5% CO_2. Parallel controls were similarly incubated but in buffer containing (in mM) HEPES (5), D-glucose (23), NaCl (139), KCl (4.7), $MgCl_2$ (0.5), $CaCl_2$ (1.3), pH 7.4 in 95% air, 5% CO_2 and were "reperfused" with the same solutions as cells subjected to sI/R.

2.4. Incubation of cardiomyocytes with inhibitors during simulated ischemia/reperfusion

To evaluate the involvement of calpains, proteasomes and autophagy on the degradation of RyR2, we performed sI/R in the presence of specific inhibitors for each different degradation pathway. To inhibit calpains we used 29 μM E64d, a concentration that completely prevented calpain activation during sI/R. Proteasomes were inhibited by 25 μM clasto-lactacystin-β-lactone (Lac) or 4.2 μM MG 132. These concentrations effectively blocked proteasomal activity, as judged by the accumulation of ubiquitinated proteins detected in Western blots and were similar to those used in other works [20,21]. To inhibit macroautophagy we used 10 mM 3-methyladenine [22].

2.5. Preparation of whole cell lysates and Western blots

Cardiomyocytes, rinsed twice with PBS to eliminate dead cells, were homogenized in cold lysis buffer containing (in mM) Tris–HCl (75), NaCl (225), EDTA (1.5), Nonidet P-40 (4.5%, w/v), sodium vanadate (5), sodium fluoride (40), sodium pyrophosphate (10), N-ethylmaleimide (10) and a protease inhibitor cocktail (Complete, Roche Diagnostics, Mannheim, Germany); final pH 7.4. The lysate was centrifuged at 4 °C (10 min at 10,000 ×*g*) and the supernatant was collected. Proteins were separated by SDS-PAGE (3.5–8% gradient gels

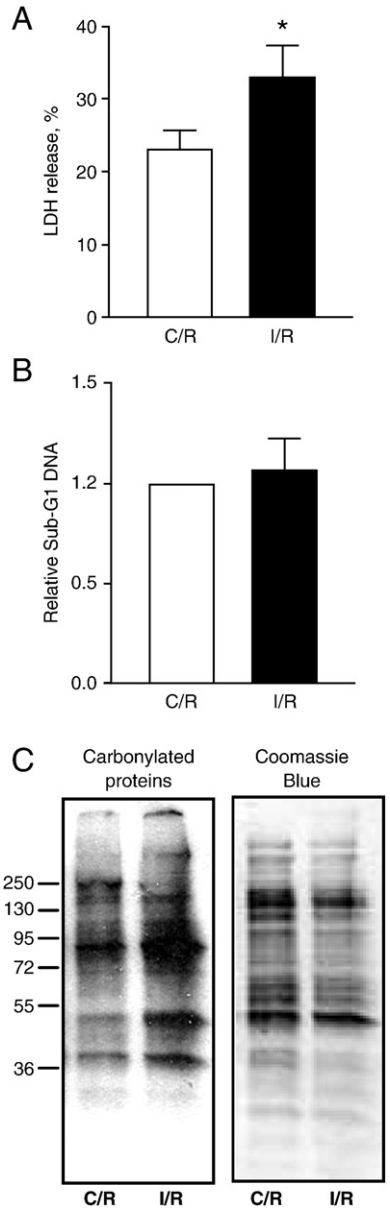

Fig. 1. Effect of simulated ischemia–reperfusion on cardiomyocyte death. (A) Release of LDH to the culture medium was determined as an index of necrosis and was expressed as % of total activity ($n=7$) (B) DNA fragmentation was detected by measuring sub-G1 DNA with propidium iodide labelling followed by flow cytometry in permeabilized cells ($n=3$). Results are expressed as relative sub-G1 DNA respect to C/R. (C) A representative Western blot for carbonylated protein content of total cell lysate (left) and Coomassie Blue staining of the same PVDF membrane as load control (right). Values are given as mean ± SEM. $^*P<0.05$.

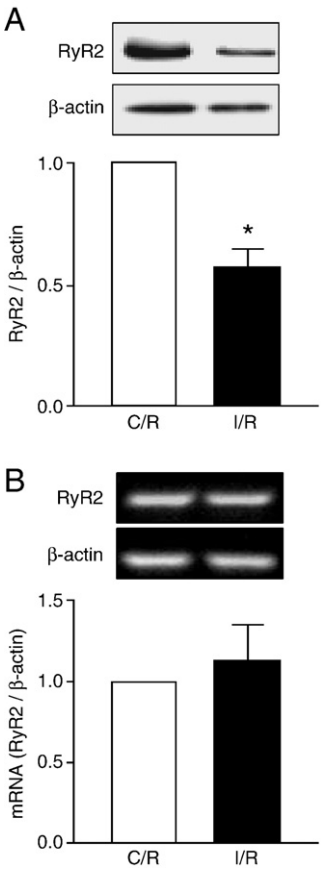

Fig. 2. RyR2 protein and mRNA content following simulated ischemia–reperfusion. (A) Representative Western blots obtained in total cell lysates reacted with anti-RyR2 or anti-β-actin. The bar graphs show the ratio of RyR2/β-actin calculated from densitometric analysis of Western blots like those shown in the figure ($n=9$). (B) Semi-quantitative RT-PCR for RyR2 and β-actin mRNAs ($n=3$). Values are given as mean ± SEM. $^{*}P<0.05$

for RyR2, 15% gels for microtubule associated protein light chain 3 (LC3-II), or 8% gels for beclin-1, calpastatin and ubiquitin) and transferred onto PVDF membranes (Millipore Corp., Bedford, MA). Membranes were probed with the following primary antibodies: anti-RyR2 (Affinity Bioreagents), anti-LC3B for LC3-I and LC3-II (Cell Signaling), anti-beclin-1 (Santa Cruz Biotechnology), anti-calpastatin (Santa Cruz Biotechnology) or anti-β-actin (Sigma). After incubation with the appropriate secondary antibody, antigen–antibody reaction was detected by ECL (Amersham, Biosciences). Blots were quantified by densitometric analysis using the software Quantity One (BioRad, Hercules, CA); results were normalized with respect to β-actin, a protein that did not change in this sI/R model. Blots were only visually compared when testing with anti-ubiquitin antibodies (Cell Signaling, 1: 200,000 dilution).

2.6. Semi-quantitative RT-PCR

We measured the amounts of RyR2 mRNA during sI/R using the following oligonucleotides: RyR2 (forward, 5′-CTACTCAGGATGAGGTGCGA-3′; reverse, 5′-CTCTCTTCAGATCCAAGCCA-3′) and β-actin (forward, 5′-TACATGGCTGGGGTCTTGAA-3′; reverse, 5′-TCTACAATGAGCTGCGTGTG-3′). After DNA denaturation (3 min, 94 °C), the following thermal profile was performed: 30 cycles of 30 s at 94 °C, 30 s at 52 °C and 1 min at 72 °C plus a final 10 min elongation step at 72 °C. Expression of mRNA was evaluated from ethidium bromide-stained agarose gel electrophoresis. Expression levels were calculated as the ratio of the respective RyR2 and β-actin bands and normalized to the control.

2.7. Immunocytochemistry of LC3-II

Cardiomyocytes were transduced with adenovirus GFP-tagged LC3 at a multiplicity of infection of 100 for 4 h before sI/R. Autophagosomes were quantified in cardiomyocytes via fluorescence imaging of GFP-LC3 as described [22].

2.8. Other procedures

Lactate dehydrogenase (LDH) activity in the culture medium, determined by measuring the decrease in NADH spectrophotometrically at 340 nm, was normalized to total LDH activity. DNA fragmentation was determined in methanol permeabilized cardiomyocytes labeled with propidium iodide; the sub-G1 population was quantified by flow cytometry [23]. Calpain activity and protein carbonylation were determined with commercial kits according to the manufacturer's instructions (Calbiochem and Chemicol International, respectively). Protein was determined according to Hartree [24].

2.9. Statistical analysis

Data are shown as mean ± S.E.M. of the number (n) of independent experiments. Data were analyzed by Student's t-test or one-way ANOVA followed by Tukey's test. Differences were considered significant at $P<0.05$.

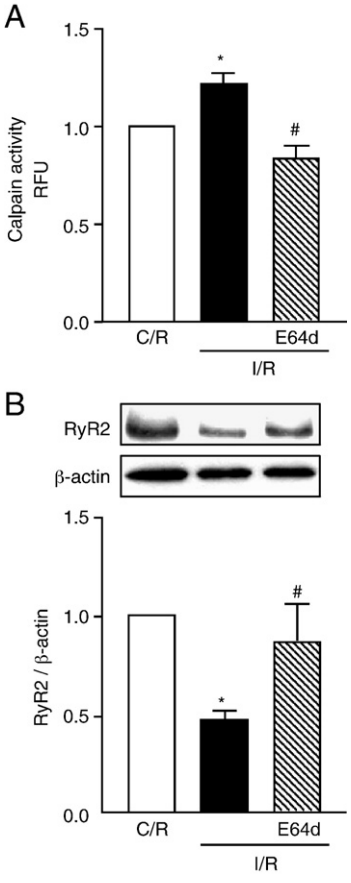

Fig. 3. Activation of calpains during simulated ischemia–reperfusion results in RyR2 degradation. (A) Effect of E64d (29 μM) during sI/R on calpain activity measured *in vitro* in whole cell lysates ($n=4$). (B) Representative Western blots obtained from total cell lysates with anti-RyR2 and anti-β-actin. The bar graphs show the ratio of RyR2/β-actin calculated from densitometric analysis of Western blots like those shown in the figure. Values are expressed as mean ± SEM. $^{*}P<0.05$ C/R vs I/R, $^{\#}P<0.05$ I/R vs I/R + E64d.

3. Results

3.1. Characterization of the simulated ischemia/reperfusion model

First, we evaluated the effects of simulated ischemia/reperfusion on the viability of neonatal cardiomyocytes. Our protocol (ischemia for 8 h, reperfusion for 16 h) resulted in a diminution in viability characterized by changes in morphology with appearance of rounded cells (not shown) and increased LDH leakage (Fig. 1A), suggestive of necrotic cell death. In contrast, apoptosis, as evaluated by flow cytometry of propidium iodide-stained cells, was not significantly different after sI/R when compared with time-matched controls (Fig. 1B). These results indicate that sI/R produces necrotic but not apoptotic cell death. Our sI/R model was also associated with increased oxidative stress [2,4,25]. Enhanced protein carbonylation, a marker of severe oxidative damage, was clearly visibly in several proteins present in cells extracts obtained after sI/R (Fig. 1C).

3.2. Decreased RyR2 content after sI/R in cultured cardiomyocytes

The content of RyR2 decreased significantly (50%) in cells subjected to sI/R (Fig. 2A) in agreement with previous reports [3,5,9]. The amount of RyR2 mRNA, did not change during sI/R (Fig. 2B), suggesting that the decrease in RyR2 protein content was caused by protein degradation.

3.3. Calpains are involved in RyR2 degradation

Calpains, which are activated during ischemia, contribute to the degradation of several structural proteins in cardiomyocytes [2,11,26,27]. The protective effect of leupeptin, a cysteine protease inhibitor, on RyR2 protein levels suggests that calpains degrade RyR2 during I/R [3]. We measured calpain activity and evaluated the effect of E64d (29 μM), a specific calpain inhibitor, on RyR2 degradation. There was a small but significant increase in calpain activity in whole cell lysates prepared from cardiomyocytes subjected to sI/R and this was prevented by E64d (Fig. 3A). This calpain inhibitor also prevented RyR2 degradation during sI/R (Fig. 3B), confirming that calpains contribute to this degradation. Addition of E64d to cardiomyocytes did not change RyR2 content under control conditions (not shown) and had no effect on the appearance of polyubiquitinated proteins in Western blots (not shown), indicating that proteasomal activity was insensitive to E64d.

3.4. Proteasomes are involved in RyR2 degradation

The proteasome is the primary site for the degradation of intracellular proteins [16]. Activation of calpain increases proteasomal protein degradation [28]. We investigated the effects of two proteasome inhibitors, clasto-lactacystin-β-lactone (Lac) and MG 132, on sI/R-induced RyR2 degradation. Lac inhibits chymotrypsin- and trypsin-like proteolytic activities while MG 132, a structurally

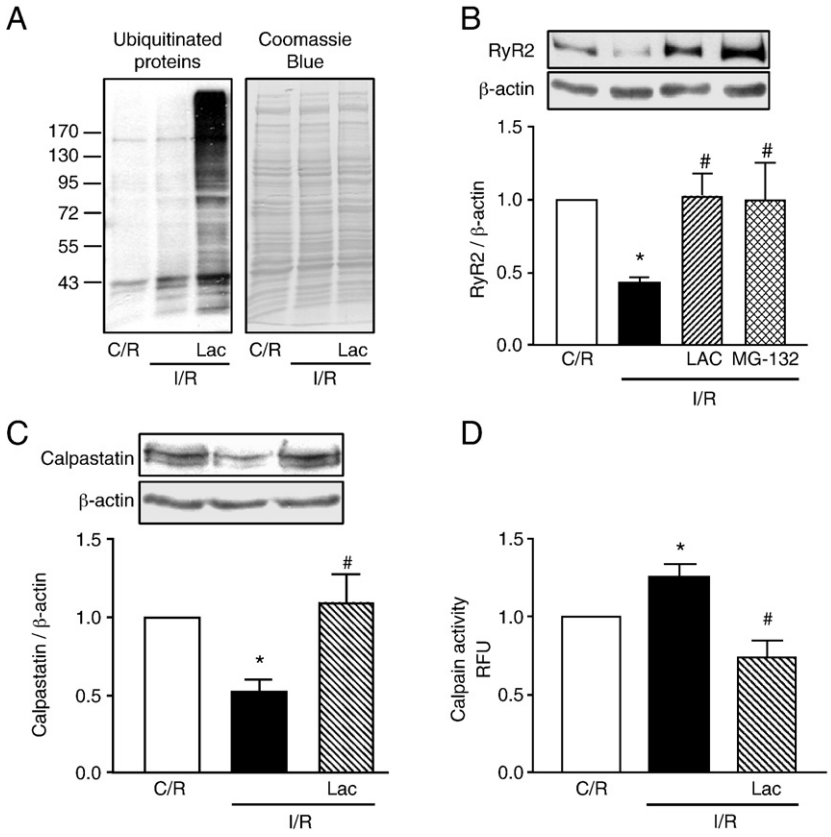

Fig. 4. Proteasomal degradation of RyR2 during simulated ischemia–reperfusion. (A) Representative Western blot, obtained with anti-ubiquitin antibody, of whole cell lysates obtained from controls or following sI/R in the presence and absence of the proteasome inhibitor Lac (25 μM); Coomassie blue staining of the same PVDF membrane is shown as load control (right). (B) Representative Western blots obtained in total cell lysates with anti-RyR2 or anti-β-actin in controls and after sI/R in the presence of the proteasome inhibitors Lac (25 μM) and MG-132 (4.2 μM). The bar graph shows the ratio of RyR2/β-actin calculated by densitometric analysis. (C) Representative Western blots obtained with anti-calpastatin or anti-β-actin in total cell lysates from cells in which sI/R was performed in the presence of the proteasome inhibitor Lac. The bar graphs show the ratio of calpastatin/β-actin calculated from densitometric analysis of Western blots like those shown in the figure. (D) Effect of Lac on calpain activity during sI/R. The activity was assessed *in vitro* using whole cell lysates ($n=4$). Values are expressed as mean ± SEM ($n=7$). $^{*}P<0.05$ C/R vs I/R; $^{\#}P<0.05$ I/R vs I/R + Lac or I/R + MG-132.

unrelated inhibitor, inhibits chymotrypsin- and caspase-like proteolytic activities of the proteasome. Lac (at 25 μM) increased markedly the amounts of ubiquitinated proteins (Fig. 4A); similar results were observed with MG 132 (at 4.2 μM, not shown), indicating that these agents effectively inhibited proteasomal activity at the concentrations used. Both inhibitors completely prevented the decrease in RyR2 content following sI/R (Fig. 4B). Neither inhibitor affected the amount of RyR2 in control cardiomyocytes (data not shown).

These results suggest that both calpains and the proteasome mediate RyR2 degradation during sI/R in cultured cardiomyocytes. The fact that specific inhibitors for each pathway can independently and effectively prevent RyR2 degradation suggests a relationship between both degradation systems.

The degradation of calpastatin, a highly specific endogenous inhibitor of calpain [29], is necessary for calpain activation during I/R [30]. We measured the content of calpastatin following sI/R and investigated the effect of proteasome inhibition on calpastatin levels. The amount of calpastatin was greatly diminished (by 50%) after sI/R and this decrease was totally prevented by Lac (Fig. 4C). Lac also prevented the increase in calpain activity observed after sI/R (Fig. 4D). We conclude that proteasomal activity promotes calpain activation by inducing the breakdown of calpastatin.

3.5. Autophagy is activated by sI/R but is not implicated in RyR2 degradation

Beclin-1 and LC3-II, two proteins involved in the formation of autophagosomes, are specific markers for the activation of autophagy [12–14,31]. As found in other models of I/R [31,32] there was a significant increase in both LC3-II and beclin-1 after sI/R (Fig. 5A–B), suggesting that autophagy increased under these conditions. We confirmed this increase by transducing cells with a GFP-LC3 tagged adenovirus. Activation of autophagy is seen as a punctuate fluorescent pattern arising from the formation of autophagic vacuoles [22]. Images obtained by immunofluorescence microscopy of GFP-LC3 transfected cardiomyocytes showed that the number of cells with vacuoles increased after sI/R (Fig. 5C). These results indicate that autophagy is activated during sI/R. Inhibition of autophagy by 3-methyladenine (3-MA; 10 mM) did not prevent, however, the RyR2 decrease induced by sI/R but rather stimulated a further decrease

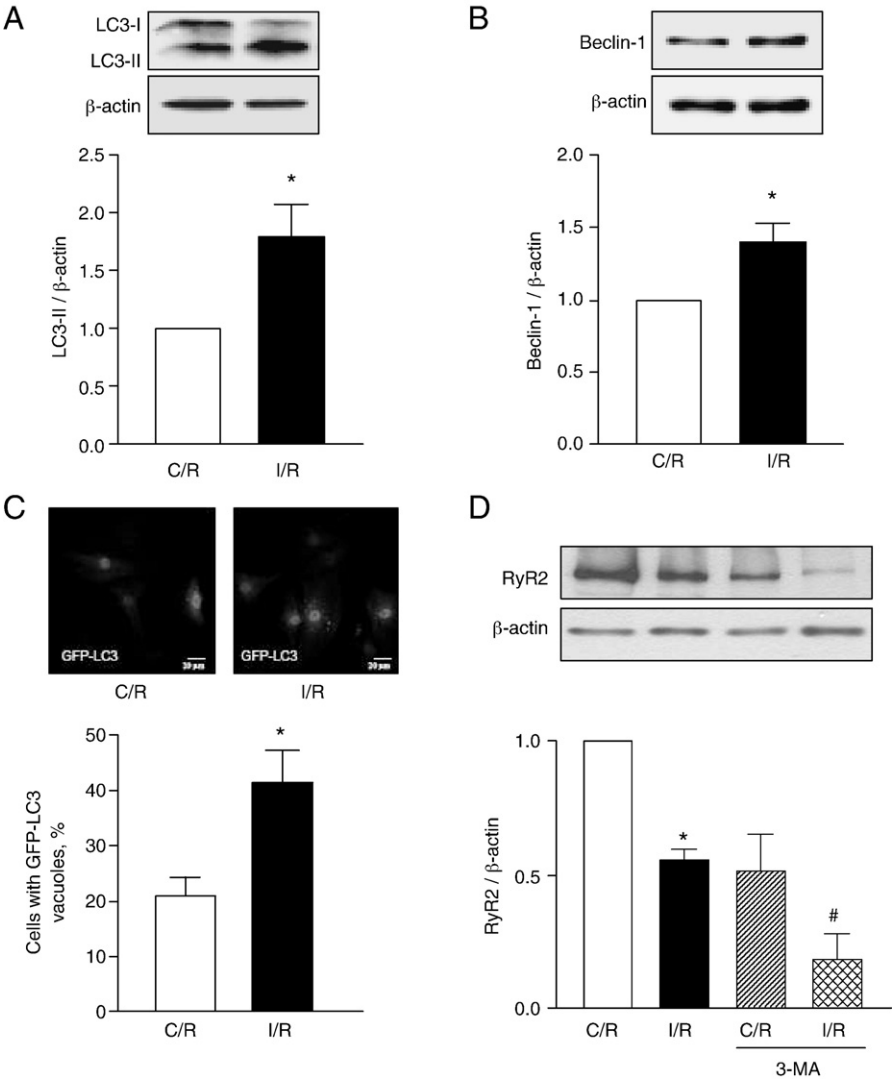

Fig. 5. Inhibition of macroautophagy increased RyR2 degradation during simulated ischemia–reperfusion. Representative Western blot, obtained with anti-LC3B or anti-β-actin, of whole cell lysates obtained from controls or following sI/R. Blots were assayed with anti-LC3B (A) or anti-Beclin-1 (B). The bar graphs show the ratios of LC3-II/β-actin (A, $n=7$) and beclin-1/β-actin (B, $n=6$) calculated from densitometric analysis of Western blots like those shown in the figure. (C) Fluorescent images of GFP-LC3 transduced cardiomyocytes in control conditions or following sI/R. Bar graphs represent the percentage of cells exhibiting more than 5 vacuoles, a sign of macroautophagy ($n=3$). (D) Representative Western blot, obtained with anti-RyR2 or anti-β-actin of whole cell lysates obtained from controls or following sI/R with and without the macroautophagy inhibitor 3-MA (10 mM). The bar graphs show the ratios of RyR2/β-actin calculated by densitometric analysis. Values are expressed as mean ± SEM ($n=6$). $^{*}P<0.05$ C/R vs I/R, $^{\#}P<0.05$ I/R vs I/R + 3-MA.

(Fig. 5D). Additionally, RyR2 content was significantly diminished by 3-MA in control cells not subjected to sI/R. (Fig. 5D). Autophagy thus seems not to be involved in the degradation of RyR2 induced by sI/R.

4. Discussion

Our present results indicate that RyR2 is degraded during sI/R by the concerted actions of calpains and the proteasome. Autophagy, in contrast, has no role in this process. We have also shown that the proteasome contributed to calpain activation after sI/R by promoting breakdown of calpastatin.

In our model system we observed increased oxidative stress and necrotic cell death but saw no evidence of increased apoptosis, in agreement with other reports showing that total deprivation of oxidizable substrates causes cardiomyocyte death exclusively by necrosis due to the rapid loss of ATP [33]. The extent of LDH leakage during sI/R represented only 35% of the total LDH, suggesting that this protocol produced rather mild ischemia (65% of the cells surviving). Nevertheless, surviving cells exhibited a 50% decrease in RyR2 content. The amounts of RyR2 mRNA were unaffected by sI/R and the amount of RyR2 protein was sustained when sI/R was accompanied by calpain or proteasome inhibitors. We assume therefore that RyR2 was degraded by both calpains and the proteasome during sI/R.

Our observation of the role of calpain in RyR2 supports previous results showing that leupeptin prevents the decrease in RyR2 content in isolated hearts subjected to I/R [3]. Experiments in vitro have shown, however, that exogenously added calpain II degrades RyR2 in isolated cardiac SR vesicles with production of two proteolytic fragments of 150 and 350 KDa. There was no further degradation of the 150 KDa fragment even when proteolysis was allowed to proceed for 30 min at a relatively high concentration of calpain [34]. If calpain displays similar activity in whole cells, there must be other pathways for degrading the resulting large RyR2 fragments.

Proteasomes are reportedly inhibited by I/R, but this inhibition is not complete and depends on the extent and duration of ischemia [4,35,36]. We did not find an increase in ubiquitinated proteins after sI/R in neonatal cardiomyocytes, suggesting that in our model, proteasomes were either not inhibited or their remaining activity was enough to degrade the proteolytic fragments produced by calpain.

Polyubiquitination is a general signal for proteasomal degradation. Some forms of polyubiquitination and monoubiquitination, however, have roles in protein localization, protein function and cell signalling [37,38]; moreover, oxidized proteins can be degraded by the proteasome without previous ubiquitination [39]. Accordingly, we did not investigate the ubiquitination of RyR2 during sI/R because, as stated above, ubiquitination of one specific protein cannot be taken as equivalent to proteasomal degradation. The specific proteasome inhibitors, Lac and MG 132, completely prevented RyR2 degradation; this result, together with the observation that proteasomal inhibition did not lead to large fragments recognized by the antibody to RyR2, suggested that calpains were also inhibited in the presence of Lac.

The regulation of calpain *in vivo* is not fully understood. The calpain/calpastatin ratio may be a better indicator of calpain potential activity than calpain activity alone. In isolated hearts subjected to I/R, the calpain/calpastatin ratio increases shortly after I/R; this increase occurs mainly through increased calpastatin degradation, which is not prevented by calpain inhibitors [40]. Although breakdown of calpastatin by the proteasome has been demonstrated *in vitro* [41], this is the first evidence for a role of the proteasome in calpastatin degradation, which in turn promotes calpain activation in cell cultures during sI/R.

Autophagy, which in the heart, is normally responsible for limited degradation of long-lived proteins and organelles, is increased following I/R [31,32]. Changes in specific marker proteins indicate that autophagy was also increased in our model of sI/R. RyR2 is a long-lived protein [42]; this feature plus its location in the SR, suggested that autophagy might be the primary degradation mechanism. Our results have not supported this hypothesis. The enhanced degradation of RyR2 produced as a consequence of the inhibition of autophagy may be attributable to a compensatory up-regulation of proteasomal activities or other proteolytic pathways.

Neonatal cardiomyocytes are phenotypically different from their adult counterparts but posses functional RyR2 [43,44]. On the assumption that proteolytic processing is similar in adult and neonatal cells, we feel that our model is appropriate for investigation of RyR2 degradation after sI/R.

In summary our results suggests that proteasomal degradation of calpastatin during sI/R enhances calpain activation, which in turn proteolyses RyR2. The resulting fragments are then fully degraded by the proteasome.

RyR2 has a key role in cardiac excitation–contraction coupling [45]. Down regulation of RyR2 may be responsible, at least in part, for the reversible contractile dysfunction, or stunning, that follows IR [46,47]. RyR2 down regulation will also alter the relative contribution of SR calcium release and L-type calcium channel-mediated calcium entry to calcium increase during systole [48]. This change may alter calcium signalling in the heart and contribute to the adverse cardiac remodelling produced by myocardial ischemia [49]. The attenuation of RyR2 degradation by calpain or proteasome inhibitors may serve to maintain contractile function. This effect could be the basis of the protective effects of calpastatin overexpression [50] and of inhibitors of the proteasome [51] or calpain [30] observed in experimental models of I/R.

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