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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

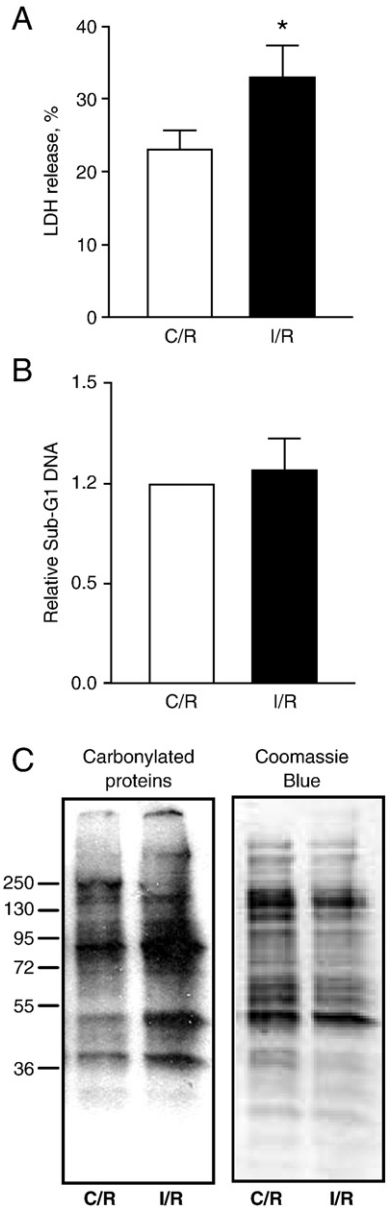

Fig. 1. Effect of simulated ischemia–reperfusion on cardiomyocyte death. (A) Release of LDH to the culture medium was determined as an index of necrosis and was expressed as % of total activity ($n=7$) (B) DNA fragmentation was detected by measuring sub-G1 DNA with propidium iodide labelling followed 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. $^*P<0.05$.