Journal of Molecular and Cellular Cardiology 52 (2012) 761–772

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Reduced cardiac CapZ protein protects hearts against acute ischemia–reperfusion injury and enhances preconditioning

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ARTICLE INFO

Article history:
Received 24 October 2011
Received in revised form 17 November 2011
Accepted 21 November 2011
Available online 2 December 2011

Keywords:
CapZ
Ischemia–reperfusion
Preconditioning
Protein kinase C
Myofilaments

ABSTRACT

The Z-disc protein CapZ has historically been classified as a structural element, anchoring sarcomeric actin. Our previous work expanded its role to include signal transduction, as CapZ transgenic myofilaments are less sensitive to protein kinase C (PKC). Myocardial PKC has paradoxical effects, mediating both preconditioning and ischemia–reperfusion (IR) injury. Our objective was to determine how decreased CapZ affects IR injury and cardiac preconditioning. Mouse hearts were subjected to 20 min global ischemia and 60 min reperfusion. Some hearts were preconditioned with intermittent IR (IPC). Left ventricular function was assessed and myocardial tissue collected post-IR for molecular analysis and tissue staining. Post-ischemic function was significantly better and infarct size smaller in CapZ transgenic hearts, as compared to wildtype. IPC decreased IR damage in both wildtype and CapZ transgenic hearts, although CapZ transgenic hearts performed significantly better than wildtype. Immunoblotting revealed increased myofilament-associated PKC-α and -ε following IR in wildtype hearts, but no change in PKC-δ or -ζ. By contrast, post-IR myofilament-associated PKC-α was significantly higher in CapZ transgenic mice but the rise in PKC-ε was attenuated. Both PKC-δ and PKC-ζ decreased in CapZ transgenic myofilaments following IR. IPC increased myofilament-associated PKC-α and -ε, while decreasing PKC-δ in wildtype hearts. Preconditioned CapZ IPC hearts showed attenuated increases in myofilament PKC-α and -ε, but also a significant decrease in myofilament PKC-δ and -ζ. These data demonstrate significant differences in post-IR myofilament PKC in untreated and preconditioned CapZ transgenic mice. CapZ reduction did not dramatically affect post-IR myofilament function, nor did preconditioning. These results demonstrate that CapZ deficiency decreases IR injury, while providing enhanced cardioprotection with IPC. The cardioprotected phenotype of CapZ transgenic mice is associated with an altered translocation of PKC-isoforms to cardiac myofilaments.

1. Introduction

Cardiac ischemia is commonly caused by coronary artery disease, arterial thrombosis, and stenosis, and is a significant cause of heart failure (reviewed in [1]). Although the re-establishment of blood flow and oxygen delivery following ischemia is the paramount goal of clinicians, post-ischemic reperfusion is associated with impaired cardiac function, arrhythmias, and cardiomyocyte death (reviewed in [2–3]). There is an increasing interest in understanding the molecular mechanisms that cause the dysfunctional changes associated with ischemia–reperfusion (IR) injury, as this information could be used to identify novel therapeutic targets and design more effective interventions.

Myocardial preconditioning was first described by Murry and colleagues [4] who reported that short, repeating episodes of ischemia protect canine hearts from damage caused by a subsequent and prolonged ischemic insult. The adaptive phenomenon of "ischemic preconditioning" can be mimicked by a number of pharmacological compounds including adenosine receptor agonists [5], stimulators of α-adrenergic receptors [6], and opioids [7–9]. While preconditioning may be induced by a number of agents, one common element of many of these events is the activation of protein kinase C (PKC) [10]. Paradoxically, PKC activation is also a powerful mediator of post-ischemic dysfunction [11–12]. The discrepant effects of PKC activation in IR are not fully explained, although there is strong evidence to suggest that specific PKC-isoforms are responsible for affecting the cardioprotective phenotype of preconditioning, while other isoforms produce post-ischemic damage and dysfunction [11–13]. More recently it has been suggested that the subcellular location of PKC may be fundamental in determining post-IR recovery [14].

The molecular basis for preconditioning has not been definitively determined, although several models have been advanced and are

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doi:10.1016/j.yjmcc.2011.11.013

supported by experimental evidence. Many studies have identified the cardiac myocyte mitochondrion as one of the end-effectors in preconditioning, with the overriding hypothesis that the protection of mitochondrial energy production is crucial for post-ischemic recovery (reviewed in [15–16]). It has also been noted that the slowed depletion of metabolic resources during IR offers significant protection against IR [17]. Given that cardiac myofilaments consume the majority of the ATP supply within the cardiac myocyte, we previously tested the hypothesis that acute inhibition of cardiac myofilament ATP consumption could provide a cardioprotective advantage against IR injury. We found that preconditioning was associated with a PKC-dependent suppression of myofilament activation, and that a PKC-independent slowing of myofilament ATP use was sufficient to induce a cardioprotective phenotype [18–19]. Together these findings suggest that acute PKC-dependent inhibition of cardiac myofilaments may play a role in the beneficial outcome of preconditioning and post-IR recovery.

CapZ is an actin capping protein that locates at cardiac Z-discs and anchors sarcomeric actin. Transgenic mice overexpressing CapZ in cardiac myocytes develop a lethal cardiac hypertrophy, while a large reduction in CapZ protein causes severe myofibrillar disarray and death [20]. However, a transgenic mouse model that contains modest reductions in cardiac CapZ protein levels is viable, and is associated with decreased PKC-dependent regulation of myofilament function [21]. In a complimentary model of CapZ deficiency in which CapZ protein levels are reduced by biochemical extraction, PKC-dependent regulation of cardiac myofilaments was also found to be reduced [22].

The dichotomous nature of PKC in IR injury and the role of CapZ in PKC signaling, has led us to investigate whether post-ischemic cardiac function is altered in CapZ hearts, and whether these hearts can be protected by preconditioning. Furthermore, we sought to investigate how myofilament-associated PKC and other signaling molecules activated by IR are altered in CapZ transgenic mice.

2. Methods

2.1. Animal care

All animals were cared for in accordance with the guidelines of the Animal Care and Use Committee of the University of Guelph, and the Canadian Council on Animal Care.

2.2. Transgenic mice

The transgenic mouse model in which CapZ protein is specifically decreased in the heart has been described previously [20–22]. Single actin capping protein subunits are unstable and non-functioning [23–25]. Overexpression of one isoform replaces the endogenous isoform [20]. To decrease cardiac CP-β1, we generated a mouse model in which CP-β2 was overexpressed, lowering the level of the Z-line-associated CP-β1 in the functional α/β heterodimer by approximately 7% [20]. Previous work has shown that this approach reduces the amount of CP-β1 associated with the Z-line without causing CP-β2 to localize to the Z-discs [20]. Transgenic mice that are deficient in the Z-line-associated CP-β1 are henceforth referred to as "CapZ transgenic mice." CapZ transgenic mice were homozygous for the transgene allele and were backcrossed to wildtype C57BL/6 mice between 3 and 8 generations. All studies used female mice that were 3–5 months of age. Wildtype mice were age, gender, and strain (C57BL/6) matched.

2.3. Langendorff perfusion

Myocardial function was determined in Langendorff perfused hearts using a pressure transducer (AdInstruments, Powerlab 4/30) to measure left ventricular function. Briefly, hearts were excised from CO_2 euthanized mice and placed in ice-cold Krebs–Henseleit solution containing heparin. Excess tissue was dissected from the aortas and hearts were cannulated using a 22-gauge needle for retrograde perfusion. Hearts were perfused with oxygenated (95% O_2/5% CO_2) Krebs–Henseleit solution maintained at 37 °C and paced at 7 Hz. A fluid-filled balloon attached to the pressure transducer was passed through the left atrium into the left ventricle. The balloon was inflated to give a left ventricular end diastolic pressure (LVEDP) of <5 mm Hg and developed pressure (LVDP) of greater than 70 mm Hg. Hearts were perfused for 20 min to ensure stable function before measuring the baseline.

2.4. IR and preconditioning protocols

All hearts were subjected to 20 min of global ischemia by stopping Langendorff perfusion and immersing hearts in nominally oxygen-free Krebs–Henseleit buffer contained in an organ bath to maintain temperature (37 °C) (Fig. 1A). After global ischemia, hearts were reperfused for 60 min. For ischemic preconditioning, hearts were subjected to two cycles of 2.5 min of ischemia and 2.5 min of reperfusion, after 20 min of baseline perfusion (Fig. 2A).

2.5. Infarct area measurement

After reperfusion, some hearts were cut through the root of aorta and snap-frozen in liquid nitrogen. Frozen hearts were subsequently sectioned and incubated in 1% triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, Oakville, ON) for 10 min to stain infarct areas. After fixing overnight, sections were photographed and the infarct area was analyzed by ImageJ (NIH, Bethesda, MD). Sections were weighed to determine percent of total heart, and infarct areas were added across all sections to produce values that are expressed as a percentage of the total heart.

2.6. Myofilament isolation

Cardiac myofilaments were isolated and prepared according to a modified protocol from Pyle et al. [21]. Briefly, hearts were homogenized in an ice-cold Standard Buffer and centrifuged at 12,000 *g* for 15 min at 4 °C. The pellets were resuspended in ice-cold Skinning Buffer and gently agitated on ice for 40 min. The suspension was centrifuged at 1100 *g* for 15 min at 4 °C and washed three times with ice-cold Standard Buffer. Protein concentration was determined with the Bio-Rad Bradford Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, ON).

2.7. Actomyosin MgATPase

Actomyosin MgATPase activity was determined using a modified Carter assay [26]. Purified myofilaments (50 μg) were incubated in reaction buffers made by mixing Activating and Relaxing buffers, creating buffers containing varying levels of free calcium. Free calcium was calculated using the program from Patton et al. [27]. Myofilaments were incubated in reaction buffers for 5 min at 32 °C, and reactions were quenched with 10% trichloroacetic acid. The amount of inorganic phosphate produced was measured by adding an equal volume of 0.5% $FeSO_4$ and 0.5% ammonium molybdate in 0.5 M H_2SO_4, and reading the absorbance at 630 nm.

2.8. Immunoblotting

Immunoblotting was performed using a modified protocol from Pyle et al. [18]. Myofilament proteins (75 μg for PKC and 150 μg for protein phosphatases) were resolved by SDS-PAGE using 10% (PKC) or 12% (protein phosphatases) separating gels. Proteins were transferred to nitrocellulose membranes and probed with primary