Biochemical and Biophysical Research Communications xxx (2017) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

CaMKII inhibition mitigates ischemia/reperfusion-elicited calpain activation and the damage to membrane skeleton proteins in isolated rat hearts

Ling-Heng Kong [a, b, 1], Xiao-Ming Gu [a, 1], Feng Wu [c], Zhen-Xiao Jin [d], Jing-Jun Zhou [a, *]

[a] Department of Physiology, Fourth Military Medical University, Xi'an, China
[b] Institute of Basic Medical Science, Xi'an Medical College, Xi'an, China
[c] Department of Cardiology, Xi'an, China
[d] Department of Cardiovascular Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an, China

ARTICLE INFO

Article history:
Received 4 July 2017
Accepted 22 July 2017
Available online xxx

Keywords:
Ischemia/reperfusion injury
CaMKII
Calpain
Heart

ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been implicated in myocardial ischemia/reperfusion (IR) injury. The aim of this study was to determine the effect of CaMKII on the damage to membrane skeleton proteins, which is an important cause of IR injury. Isolated rat hearts were subjected to 45-min global ischemia/2-h reperfusion. Both KN-62 and KN-93 were used to inhibit CaMKII. Compared with controls, the hearts in the IR group exhibited remarkable myocardial injury area, LDH release, cell apoptosis and contractile dysfunction, along with an increase in the phosphorylation of CaMKII and its substrate phospholamban. Treatment with either KN-62 or KN-93 mitigated both the heart injury and the phosphorylation of CaMKII and phospholamban. The analysis of cell skeleton proteins revealed that IR injury resulted in an increase in the 150-kDa fragments resulting from the degradation of α-fodrin and dystrophin translocating from the sarcolemmal membrane to the cytosol and a decrease in the 220-kDa isoform of ankyrin-B. As expected, Evans blue dye staining showed an increase in membrane permeability or membrane rupture in the IR group. All of these alterations were alleviated by treatment with either KN-62 or KN-93. In addition, both KN-62 and KN-93 blocked the activity and membrane recruitment of calpain, a key protease responsible for destroying cell skeleton proteins during IR injury. In conclusion, our data provide evidence that damage to membrane skeleton proteins via calpain is a destructive downstream event of CaMKII activation in the setting of myocardial IR injury.

1. Introduction

Myocardial ischemia/reperfusion injury is a long-standing problem in clinical settings. Its mechanisms are still not fully understood, and treatment approaches are very limited [1]. Thus far, multiple lines of evidence have revealed that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) takes part in this process [2]. CaMKII is activated during the first few minutes of reperfusion, causing cell death, heart dysfunction, and arrhythmias [3–6]. Therefore, a comprehensive understanding of CaMKII action is necessary to evaluate its potential as a therapeutic target for heart protection.

One important characteristic of myocardial ischemia/reperfusion injury is the loss of membrane skeleton proteins [7–10]. In this scenario, fodrin, dystrophin, and ankyrin-B are prone to damage. Fodrin/non-erythroid spectrin is a tetrameric and elongated complex composed of two heterodimers of α and β subunits. It forms scaffold linings in pentagonal or hexagonal patterns under the intracellular side of the sarcolemmal membrane [11]. Dystrophin is a component of the dystrophin-glycoprotein complex located in the costamere [12]. Both fodrin and dystrophin provide a physical linkage between the actin-based cytoskeleton and the sarcolemmal membrane. Ankyrin-B is an adaptor protein, which is required for the sarcolemmal association of fodrin and dystrophin [11,13]. It has been established that damage to these proteins weakens the

* Corresponding author. Department of Physiology, Fourth Military Medical University, no. 169# Changle West Road, Xi'an 710032, China.
E-mail address: jjzhou@fmmu.edu.cn (J.-J. Zhou).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2017.07.128
0006-291X/

structural integrity of the sarcolemmal membrane and increases the vulnerability of the plasma membrane to mechanical stress. This consequently results in osmotic fragility and cell death [7,8,14]. Many laboratories, including ours, have previously demonstrated that CaMKII causes intracellular Ca^{2+} overload during ischemia/reperfusion by increasing Ca^{2+} leakage from the sarcoplasmic reticulum [5,15,16] and that calpain, a Ca^{2+}-dependent protease, destroys fodrin, ankyrin, and dystrophin [8,17,18]. However, the possibility that CaMKII activates calpain and results in a loss of membrane skeleton proteins has not been examined.

Isolated rat hearts were used in this study. We first evaluated the effects of both KN-62 and KN-93 [19], selective CaMKII inhibitors, in ischemia/reperfusion injury. Then, we detected the levels of fodrin, ankyrin, and dystrophin as well as the permeability of the sarcolemmal membrane. Third, we measured both the activity of calpain and the sarcolemmal membrane recruitment of calpain 1. These data provide evidence that CaMKII causes damage to membrane skeleton proteins via calpain, which contributes to myocardial ischemia/reperfusion injury.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (250–300 g body weight), provided by the Laboratory Animal Center at Fourth Military Medical University, were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals [National Institutes of Health (NIH) Publication No. 80-23, Revised in 1996]. The experimental procedures were approved by the Research Commission on Ethics of Fourth Military Medical University.

2.2. Drugs, chemicals and antibodies

Both KN-62 and KN-93 were purchased from Tocris Bioscience (Bristol, UK). Antibodies against phophso-CaMKII, CaMKII, phospholamban, calpain 1, cytochrome *c*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against phospholamban (pThr17), α-fodrin, dystrophin, and ankyrin-B were from Badrilla (Leeds, UK), Enzo Life Sciences (Plymouth Meeting, PA, USA), Abcam (Shanghai, China), and Santa Cruz (Shanghai, China) respectively. The calpain activity assay, LDH assay and caspase-3 activity assay kits were purchased from Calbiochem (San Diego, CA, USA), Abnova (Walnut, CA, USA), and Merck Millipore (Billerica, MA, USA) respectively. Tetramethylrhodamine (TRITC) goat anti-rabbit IgG was from Molecular Probes (Eugene, OR, USA). Evans blue dye, Triphenyltetrazolium chloride (TTC), 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI), and additional chemicals were obtained from Sigma (Shanghai, China).

2.3. Langendorff perfusion

The hearts were quickly removed following anesthesia application and the aortas were connected to a Langendorff apparatus using a three-way stopcock (Radnoti Glass Technology Inc., Monrovia, CA, USA). The perfusion pressure was maintained at 80 mmHg. A modified Krebs-Henseleit (KH) solution, equilibrated with 95% O_2/5% CO_2, was maintained at 37 °C and used for perfusion. The KH solution contained (in mmol): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 25, and glucose 11. A latex balloon connected to a pressure transducer was inserted into the left ventricle through the atria, and adjusted to obtain a left ventricular end-diastolic pressure (LVEDP) at 5–10 mmHg by inflating the balloon. Left ventricular pressure was monitored by Labchart 7 software (ADInstruments) and stored in a computer. LVEDP, recorded at the end of the experiment, and left ventricular developed pressure (LVDP), which was calculated as the difference between the left ventricular peak systolic pressure and LVEDP, were used to reflect heart function as described previously [18]. All hearts were stabilized for 20 min before the experiments started.

2.4. Experimental protocol

The hearts were randomly divided into four groups of at least six hearts each. In the ischemia/reperfusion group, hearts were subjected to 45 min of global ischemia. Cardiac function and myocardial injury area were evaluated using 30 and 120 min of reperfusion, respectively [18]. In the control group, the hearts received no treatments but were perfused with normal KH solution for the same amount of time as that in the ischemia/reperfusion groups. For the KN-62 and KN-93 groups, drugs were administered to hearts 1 min before ischemia and during the first 5 min of reperfusion. The concentrations of KN-62 and KN-93 used were based on previous studies [3,4,20,21].

2.5. Measurements of myocardial injury area

At the end of each experiment, hearts were frozen at −80 °C for 1 h and cut into 6 slices perpendicular to the apex-base axis. The slices were incubated with 1% 2,3,5-triphenyltetrazolium (TTC) at 37 °C for 15 min, and fixed with 4% polyformaldehyde for 24 h. Viable tissue stained brick red and irreversibly injured tissue was white. Myocardial injury area was calculated by a computerized planimetry technique (OPTIMAS v.5.2, BioScan Inc, Edmonds, WA, USA) and expressed as a percentage of the total area [18].

2.6. Assays for LDH release, calpain activity and caspase-3 activity

During the first 5 min of reperfusion, the coronary effluent was collected, and LDH release was measured with a kit from Abnova [18]. To evaluate calpain activity, left ventricular tissues were excised at 5 min following reperfusion. The measurement was carried out following the manufacturer's instructions. MDL-28170 was used at 10 μM to determine the specificity of the assay, as described previously [8,22]. The tissue caspase-3 activity was detected with a colorimetric assay kit at the end of reperfusion [18].

2.7. Confocal image analysis

To detect membrane recruitment of calpain 1, left ventricular tissues were excised at 5 min following reperfusion and paraffin-embedded tissue sections (3-μm thick) were prepared. The sections were incubated with antibodies against calpain 1 and dystrophin (1:100) at 4 °C overnight, followed by a TRITC-labeled secondary antibody for 1 h at room temperature [17,18,23]. DAPI was used to label the nuclei. To assess membrane permeability, the hearts were reperfused for 5 min with a membrane-impermeable Evans blue dye at a concentration of 0.01%, and a 4-μm thick frozen section was prepared [10,24]. After that, the images were examined with a laser-scanning confocal microscope equipped with an FV10-ASW system (Olympus FV1000, Tokyo, Japan). The excitation wavelength was 543 nm and the barrier filter (590 nm) was used to view TRITC and Evans blue dye staining [24]. The percentage of positive cells was calculated as described previously [18].

2.8. Western blot analysis

Left ventricular tissues were snap-frozen immediately, and stored at −80 °C until analyzed. To detect cytochrome *c* and

Please cite this article in press as: L.-H. Kong, et al., CaMKII inhibition mitigates ischemia/reperfusion-elicited calpain activation and the damage to membrane skeleton proteins in isolated rat hearts, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.07.128