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Original Article

Degradation of cardiac myosin light chain kinase by matrix metalloproteinase-2 contributes to myocardial contractile dysfunction during ischemia/reperfusion

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ABSTRACT

Although ischemia/reperfusion (I/R)-induced myocardial contractile dysfunction is associated with a prominent decrease in myofilament Ca^{2+} sensitivity, the underlying mechanisms have not yet been fully clarified. Phosphorylation of ventricular myosin light chain 2 (MLC-2v) facilitates actin–myosin interactions and enhances contractility, however, its level and regulation by cardiac MLC kinase (cMLCK) and cMLC phosphatase (cMLCP) in I/R hearts are debatable. In this study, the levels and/or effects of MLC-2v phosphorylation, cMLCK, cMLCP, and proteases during I/R were determined. Global myocardial I/R-suppressed cardiac performance in isolated rat hearts was concomitant with decreases of MLC-2v phosphorylation, myofibrillar Ca^{2+}-stimulated ATPase activity, and cMLCK content, but not cMLCP proteins. Consistently, simulated I/R in isolated cardiomyocytes inhibited cell shortening, Ca^{2+} transients, MLC-2v phosphorylation, and myofilament sensitivity to Ca^{2+}. These observations were reversed by cMLCK overexpression, while the specific cMLCK knockdown by short hairpin RNA (shRNA) had the opposite effect. Moreover, the inhibition of matrix metalloproteinase-2 (MMP-2, a zinc-dependent endopeptidase) reversed IR-decreased cMLCK, MLC-2v phosphorylation, myofibrillar Ca^{2+}-stimulated ATPase activity, myocardial contractile function, and myofilament sensitivity to Ca^{2+}, while the inhibition or knockdown of cMLCK by ML-9 or specific shRNA abolished MMP-2 inhibition-induced cardioprotection. Finally, the co-localization in cardiomyocytes and interaction in vivo of MMP-2 and cMLCK were observed. Purified recombinant rat cMLCK was concentration- and time-dependently degraded by rat MMP-2 in vitro, and this was prevented by the inhibition of MMP-2. These findings reveal that the I/R-activated MMP-2 leads to the degradation of cMLCK, resulting in a reduction of MLC-2v phosphorylation, and myofibrillar Ca^{2+}-stimulated ATPase activity, which subsequently suppresses myocardial contractile function through a decrease of myofilament Ca^{2+} sensitivity.

1. Introduction

Myocardial dysfunction or heart "stunning" resulting from ischemia/reperfusion (I/R) is a common clinical scenario in patients suffering from ischemic heart disease. Such dysfunction is associated with a prominent decrease of myofilament Ca^{2+} sensitivity because of covalent modifications or proteolytic injury to contractility-related proteins [1–5]. Thus, identification of roles of proteolytic enzymes, their targeting proteins and contributions to postischemic contractile dysfunction in acute myocardial I/R injury is fundamental to our understanding of the pathological processes and development of therapeutic approaches for ischemic heart disease.

Ventricular myosin regulatory light chain (MLC-2v) plays an important role in modulating motor properties of myofilaments [6,7]. Increasing phosphorylation of MLC-2v, bound to myosin at the head–rod junction, facilitates actin–myosin interactions and enhances myocardial myofilament Ca^{2+} sensitivity and maximizes Ca^{2+}-activated force [8,9], while ablation of MLC-2v phosphorylation results in a decrease in contractility and structural abnormality of the heart [6,10]. I/R induces a loss of MLC-2v protein in the myocardium [2], while the MLC-2v phosphorylation status after I/R still remains controversial. I/R-reduced myocardial performance is concomitant with a decrease of MLC-2v phosphorylation [11], but the decrease is not observed in the other study [2], making unclear the importance of the decrease of MLC-2v phosphorylation in I/R-suppressed myofilament Ca^{2+} sensitivity.

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MLC-2v is mainly phosphorylated by cardiac MLC kinase (cMLCK) and dephosphorylated by cardiac MLC phosphatase (cMLCP) [6,7,10]. cMLCK is highly expressed in the heart, regulating myocardial contraction and sarcomere organization and belongs to a family of genes encoding a number of serine/threonine kinases [10,12,13]. cMLCP is composed of 3 subunits: a catalytic subunit of type 1 phosphatase, PP-1cδ; a targeting subunit, termed myosin phosphatase target subunit, mainly MYPT-2 [14]; and a small 21 kDa unit of the holoenzyme which exists as a cardiac specific isoform, HS-M21 [6,15]. The expression level of cMLCK is reduced in animal myocardial infarction models [12,16], however, little is known about the expression level and regulators of cMLCK and cMLCP proteins and their effects on the MLC-2v phosphorylation during I/R.

Several proteases such as calpains, matrix metalloproteinases (MMPs, especially MMP-2), caspases (especially caspase-3), and proteasome have been found to be activated in heart diseases and degrade a series of contractility-related myofilament proteins and regulating kinases, resulting in serious contractile dysfunction [17–19]. We recently confirmed that the inhibiting of MMP-2 activation appears to be a common downstream mediator in cardioprotection-triggered intrinsic adaptive responses against acute I/R injury [5]. However, whether those proteases, especially MMP-2, affect MLC-2v regulatory kinases during I/R remains unclear.

To address these issues, this study aimed to determine (i) the status and regulation of MLC-2v phosphorylation during myocardial I/R; (ii) the relationship between these changes and decreased myofilament Ca^{2+} sensitivity in postischemic myocardium; and (iii) the effects of proteases on MLC-2v regulatory kinases in I/R hearts.

2. Methods

Expanded methods for in vivo siRNA delivery, measurement for cMLCK gene expressions, determination of myofibrillar ATPase activity, construction of recombinant adenoviruses, isolation, culture, and adenoviral infection of ventricular myocytes, simulated I/R in isolated cardiomyocytes, simultaneous measurement of Ca^{2+} transient and cell shortening, measurement of MMP activity in coronary effluent, cMLCK antibody preparation, Western blotting analysis, immunofluorescence analysis, co-immunoprecipitation assay, and expression of rat recombinant cMLCK protein are provided in Supplementary Methods.

2.1. Animals

All experimental procedures on rats conformed to the guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, Revised 1996), and were approved by the Institutional Review Board of the Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences and School of Medicine and Shanghai Jiao Tong University, China.

2.2. In vivo siRNA delivery

Male Sprague–Dawley rats (Shanghai Slac Laboratory Animal Co. Ltd, Shanghai, China) were anesthetized with pentobarbital sodium (45 mg/kg ip). Small interfering RNA (siRNA) targeting rat MMP-2 (siMMP-2) (30 μg) or nonspecific control siRNA (NS) were directly injected into the left ventricular (LV) myocardium. The surgical procedures and siRNA injection were carried out as described previously [20]. All experiments were performed after 48 h of siRNA injection.

2.3. I/R injury model in Langendorff-perfused rat hearts

Rats were anesthetized with pentobarbital sodium (45 mg/kg ip), and the hearts were rapidly excised and perfused with Krebs–Henseleit buffer at 37 °C by using the Langendorff technique under a constant pressure of 80 mm Hg as previously described [21]. After equilibration perfusion, the hearts were subjected to 30 min of global no-flow ischemia followed by 30 min of reperfusion. LV developed pressure (LVDP) were synchronously monitored with PowerLab system (AD Instrument, NSW, Australia).

2.4. Experimental protocols in isolated hearts

After equilibration, the hearts were randomly assigned to groups (Suppl. Fig. 1A). The MMP inhibitor doxycycline (100 μM, Sigma, St. Louis, MO, USA) [22] or ONO-4817 (50 μM, Tocris Bioscience, Ellisville, Missouri, USA) [23] and calpain inhibitor MDL-28170 (10 μM, Enzo, Meeting, PA, USA) [24] was perfused for 10 min prior to ischemia and 10 min from the beginning of reperfusion. The proteasome irreversible inhibitor lactacystin (2 μM, Sigma) was added for 10 min prior to ischemia as previously described [25,26]. The caspase-3 inhibitor Ac-DEVD-CHO (1 μM, Enzo) was applied for 10 min prior to ischemia and during the reperfusion as previously described [27]. The MLCK inhibitor ML-9 (15 μM, Enzo) was added during the reperfusion as previously described [28]. Doxycycline, Ac-DEVD-CHO, or lactacystin was dissolved in the deionized distilled water; MDL-28170, ONO-4817, or ML-9 was dissolved in dimethyl sulfoxide (DMSO) in a final perfusate concentration containing 0.05% DMSO (v/v). Vehicle controls of 0.05% DMSO were performed at the same time course as did by MDL-28170, ONO-4817, and ML-9 treatment. At the end of perfusion, coronary effluent collected at various time points and LV tissues were immediately frozen in liquid N_2 and stored at −80 °C.

2.5. Measurement for cMLCK gene expression

PCR for cMLCK was performed using cDNA generated from total RNA extracted from LV tissues as previously described [16].

2.6. Determination of myofibrillar ATPase activity

The myofibril isolation from LV tissue was prepared, and myofibrillar Ca^{2+} stimulated ATPase activity was determined as previously described [29].

2.7. Construction of recombinant adenoviruses

Recombinant adenoviruses over-expressing short hairpin RNAs (shRNA) of rat MMP-2 (Ad.shMMP-2) or cMLCK (Ad.shcMLCK), rat cMLCK protein (Ad.cMLCK), or GFP (Ad.GFP, as a negative control) were prepared as described previously [4,30] by using pAdMAX™ or pAdEasy™ vector system.

2.8. Isolation, culture, and adenoviral infection of ventricular myocytes

LV myocytes were isolated from adult rat hearts by using a standard enzymatic method as previously described [4,21]. Then, the isolated myocytes were cultured in vitro and infected with Ad.shMMP-2, Ad.shcMLCK, Ad.cMLCK, or Ad.GFP as previously reported [4]. All experiments were performed after 48 h of adenoviral infection.

2.9. Simulated I/R in isolated cardiomyocytes and experimental protocols

After equilibration perfusion, myocytes were perfused with ischemic solution containing (in mM) 123 NaCl, 8.0 KCl, 6.0 $NaHCO_3$, 0.9 NaH_2PO_4, 0.5 $MgSO_4$, 20.0 Na-lactate, and 1.8 $CaCl_2$, gassed with 95% N_2/5% CO_2 (pH 6.8) for 20 min to mimic ischemia with hypoxia, acidosis, lactate, hyperkalemia, glucose-free, which occurs in tissues in border zones adjacent to developing infarcts or central tissues with some collateral circulation [21,31]. Then myocytes were reperfused for 30 min. The MMP inhibitor ONO-4817 (50 μM) was applied for 10 min prior to ischemia and 10 min from the beginning of reperfusion, and the MLCK inhibitor ML-9 (15 μM) was added during the reperfusion as