Life Sciences 167 (2016) 85–91

Contents lists available at ScienceDirect

Life Sciences

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

Cardioprotective effects of monocyte locomotion inhibitory factor on myocardial ischemic injury by targeting vimentin

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ARTICLE INFO

Article history:
Received 30 June 2016
Received in revised form 13 October 2016
Accepted 18 October 2016
Available online 20 October 2016

Keywords:
Monocyte locomotion inhibitory factor
Acute myocardial ischemic injury
Apoptosis
Vimentin

ABSTRACT

Monocyte locomotion inhibitory factor (MLIF), a heat-stable pentapeptide produced by *Entamoeba histolytica*, has anti-inflammatory function and protective effect on ischemic stroke. In this study, we evaluated the effect of MLIF on myocardial ischemia. Mice were subjected to ischemia/reperfusion by occlusion of the left anterior descending artery (LAD). After sacrifice, the serum concentrations of cardiac troponin I (cTnI), creatine kinase (CK), lactate dehydrogenase (LDH) as well as the heart infarct size were measured. HE and TUNEL staining were used to observe the pathological damage and the apoptotic cells. For in vitro study, the oxygen-glucose deprivation(OGD) model was established in H9c2 cells. MTT assay and flow cytometry assay were performed to evaluate cell viability and apoptosis. The expression of JNK and caspase 3 was assessed by western blot analysis. Pull-down assay was used to detect the specific binding protein of MLIF in myocardial cells. MLIF significantly reduced the infarct size, and the cTnI, CK and LDH levels, ameliorated pathological damage and reduced the apopotosis compared with the myocardial I/R model group. MLIF improved cell survival and inhibited apoptosis and necrosis by inhibiting the p-JNK and cleaved caspase3 expression. Furthermore, the binding protein of MLIF in myocardial cells was vimentin. Inhibition of vimentin expression by withaferin A or vimentin siRNA repressed the protective effects of MLIF in OGD-provoked H9c2 cells. Taken together, our results demonstrate that the cardioprotective effects of MLIF on myocardial ischemia injury are related to reductions in the inflammatory response and apoptosis by targeting vimentin.

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1. Introduction

Ischemic heart disease is the leading cause of disability-adjusted life-years (DAYL) worldwidely [1]. Cardiovascular disease, such as acute myocardial ischemia, which is usually caused by occlusion of the blood supply and a lack of oxygenation provision, quickly results in cardiomyocyte death, tissue injury and heart failure. Acute myocardial ischemic injury is associated with the inflammatory response and cell apoptosis. Inhibition of inflammation and apoptosis may be a promising therapeutic strategy for ischemia-reperfusion injury [2,3].

Monocyte locomotion inhibitory factor (MLIF), an anti-inflammatory heat-stable pentapeptide (Met-Gln-Cys-Asn-Ser) produced by *Entamoeba histolytica*, inhibited the locomotion of monocytes and mononuclear phagocytes *in vitro* [4]. In previous studies, MLIF reportedly down-regulated the expression of pro-inflammatory chemokines (MIP-1α, MIP-1β and I-309) and effectively improved the prognosis of collagen induced arthritis in mice and down-regulated *ex vivo* IL-1β expression in patients with rheumatoid arthritis. Microarray analysis showed that MLIF played important role as an immune regulator in a collagen-induced arthritis model by affecting apoptosis, cell adhesion, inflammation and chemotaxis, and extracellular matrix remodeling [5–8].

In previous study, we found that MLIF has a protective effect on ischemia stroke by decreasing ischemia volume and inhibiting the expression of inflammatory adhesion molecules by targeting eEF1A1/eNOS pathway in the cerebrovascular endothelium [9]. We also found MLIF ameliorates OGD-induced SH-SY5Y neuroblastoma injury by inhibiting the p-JNK/p53 apoptotic signaling pathway *via* eEF1A2 [10]. However, no reports have addressed the protective effect of MLIF on myocardial ischemia injury. In this study, MLIF contributed to attenuating acute myocardial ischemia reperfusion injury in mice and exhibited a protective effect against oxygen/glucose-deprivation-induced myocardial apoptosis by interacting with vimentin, an intermediate filament.

Abbreviations: MLIF, monocyte locomotion inhibitory factor; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; eEF1A1, eukaryotic translation elongation factor 1 alpha 1; eEF1A2, eukaryotic translation elongation factor 1 alpha 2; eNOS, endothelial nitric oxide synthase; MALDI-TOF-MS, matrix assisted laser desorption- ionization time of flight- mass spectrometry.

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http://dx.doi.org/10.1016/j.lfs.2016.10.021
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2. Materials and methods

2.1. Regents

MLIF was synthesized by Chengdu Shengnuo Biopharm Company (Chengdu, China), and biotinylated MLIF was synthesized by the Chinese Peptide Company (Hangzhou, China) with purity of >95%. A *Limulus* assay was performed to ensure that the preparations were endotoxin-free (lipopolysaccharide <0.3 pg). MLIF was stored at −20 °C and dissolved with physiological saline before usage. 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Sigma-Aldrich Co (St. Louis, MO, USA); the creatine kinase assay kit, lactate dehydrogenase assay kit and cardiac troponin assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, P.R. China).

2.2. Animal models

The animal procedures described in this study were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University (Shanghai, China). Male C57BL/6 mice aged 8–10 weeks were purchased from the SLAC Laboratory Animal Company (Shanghai, China) and housed in a standard room [temperature, 24 ± 3 °C; humidity, (60 ± 5) %] under a 12-h light/12-h dark cycle.

The mice were randomly divided into 4 groups of 10 mice each: 1 sham group, 1 myocardial I/R model group and 2 MLIF treated groups (1 mg/kg and 5 mg/kg). The MLIF-treated groups were injected MLIF dissolved in saline solution in tail vein 5 min prior to reperfusion. The other groups were treated with an equal volume of saline solution.

All mice were anesthetized with ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg), orally intubated, and connected to a rodent ventilator. Myocardial I/R model was induced using a 8–0 silk suture to ligate the left arterial descending coronary artery (LAD) with a slip knot for 30 min followed by 3 h reperfusion. The sham group was subjected to thoracotomy and pericardiotomy without ligation in coronary artery. The chest wall was closed in sutured layers. The animals were kept warm at 37 °C during the period of ischemia and reperfusion. The infarct size was evaluated by Evans blue and 2,3,5-triphenyltetrazolium chloride (TTC) staining.

2.3. Evans blue and TTC double staining

Myocardial infarct size was assessed by double staining with Evans Blue/TTC as described previously [11]. Briefly, after the hearts were perfused with saline, the left coronary artery (LCA) was reoccluded, 1% Evans blue dye (Sigma-Aldrich, USA) was injected via the aorta and coronary arteries, in order to delineate the non-ischemic area. After the tissues were frozen at −20°C for several hours, hearts were cut into 2mm thick slices. The slices were subsequently stained with 1% TTC for 15min at 37°C. Then, the slices were fixed by immersion in 10% neutral buffered formalin. The normal tissue (blue), the infarct area (pale white) and the risk area (red) were calculated by planimetry using Image-Pro Plus software. Infarct size was expressed as the percentage of risk area.

2.4. Measurement of cardiac troponin I (cTnI), creatine kinase (CK), lactate dehydrogenase (LDH)

At the end of 3 h reperfusion period, mice were anesthetized. Blood samples were collected from the aorta abdominalis and stored at −80 °C. The biological activities of CK and LDH and the concentrations of cTnI were determined according to the instructions in the kits.

2.5. Cell culture and OGD experiment

H9c2 myoblast cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% FBS, and 1 × penicillin (100 U/mL) -streptomycin (100 μg/mL) (Thermo Scientific, Waltham, MA, USA) in a humidified incubator with 5% CO_2 and 95% air at 37 °C.

The OGD model was induced by placing the myocardial cells into a hypoxic chamber (Billups-Rothenberg; Del Mar., CA, USA) with 5% CO_2 and 95% N_2, at 37 °C for 4 h. MLIF (100 μg/mL) was added into the culture media after OGD treatment [12].

2.6. Flow cytometry assay

The number of cells that underwent apoptosis or necrosis was detected by the Annexin V-FITC and propidium iodide Analysis kit (Carlsbad, CA, USA) according to the manufacturer's instructions. The H9c2 cells were harvested, washed in cold 1 × phosphate-buffered saline (PBS, PH7.4) twice, and suspended in 100 μL 1 × binding buffer containing 5 μL of propidium iodide and 5 μL of Annexin V-FITC. They were then incubated for 15 min in the darkness at room temperature, and 1 × binding buffer was added to 500 μL of the solution. The stained cells were subjected to analysis by a flow cytometer (BD Biosciences, San Jose, *CA*) [13].

2.7. Western blot analysis

The total protein was extracted from cells with M-PER Protein Extraction Reagent (Pierce, Rockford, IL, USA) supplemented with protease inhibitor cocktail (Beyotime, shanghai, China) according to the manufacturer's instructions. Cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electrotransferred onto PVDF membranes (Milipore). After blocking with 5% BSA for 2 h at room temperature, the membranes were probed with first antibodies at 4 °C overnight. The blots were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were detected by ECL western blotting substrate (Thermo Scientific). Rat monoclonal antibodies to JNK, p-JNK, caspase3 and cleaved-caspase3 were purchased from Cell Signaling Technology (Danvers, MA, USA), and vimentin antibody was purchased from Abcam (Cambridge, MA, USA). The levels of those blots for target proteins were normalized to β-actin as a loading control.

2.8. Pull-down assay

The cells were lysed as described in western blot and the cell debris was removed by centrifugation at 12,000 *g* for 10 min. The biotinylated MLIF (40 μL, 5 mg/mL, bio-MLIF) or control solution was incubated with the protein supernatant for 6 h at 4 °C. Streptavidin-agarose beads (Invitrogen, Carlsbad, CA, USA) were added and stirred gently at 4 °C for 8 h after recovery by a brief centrifugation three times with 1 mL of PBS. The mixture was centrifugated at 12,000 *g* for 5 min and washed twice with 1 mL of PBS by centrifugation at 500 ×*g* for 2 min to obtain a pellet (S1). The supernatant was used to repeat the binding assay twice to obtain sample2 (S2) and sample3 (S3). The control groups were *C*1, C2, and C3, respectively. Bound proteins were separated by SDS-PAGE followed by Coomassie Brilliant Blue staining and analyzed by MALDI-TOF-MS (Voyager DESTR mass spectrometer, Applied Biosystems, Waltham, MA, USA) after in-gel digestion. Protein identification was performed using the MASCOT search engine (Matrix Science, Boston, MA, USA) [9,14,15].

2.9. RNA interference

The sequences of vimentin siRNA used in this study were 5′-GCGUGAAAUGGAAGAGAAUTT-3′ and 5′- AUUCUCUUCCAUUUCACG CTT-3′. A scrambled siRNA was used as negative control (NC), which was purchased from GenePharma (Shanghai, China). siRNA transfections were performed using Lipofectamine™ 2000 reagent (Thermo

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