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Protective effects of protein transduction domain-metallothionein fusion proteins against hypoxia- and oxidative stress-induced apoptosis in an ischemia/reperfusion rat model

Kwang Suk Lim [a,1], Min-Ji Cha [c,1], Jang Kyoung Kim [a], Eun Jeong Park [a], Ji-Won Chae [a], Taiyoun Rhim [a], Ki-Chul Hwang [c], Yong-Hee Kim [a,b,*]

[a] Department of Bioengineering, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, Republic of Korea
[b] Institute for Bioengineering and Biopharmaceutical Research, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, Republic of Korea
[c] Cardiovascular Research Institute, Brain Korea 21 Project for medical Science, Yonsei University, College of Medicine, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-749, Republic of Korea

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ABSTRACT

Ischemic heart diseases caused by insufficient oxygen supply to the cardiac muscle require pharmaceutical agents for the prevention of the progress and recurrence. Metallothionein (MT) has a potential as a protein therapeutic for the treatment of this disease due to its anti-oxidative effects under stressful conditions. In spite of its therapeutic potential, efficient delivery systems need to be developed to overcome limitations such as low transduction efficiency, instability and short half-life in the body. To enhance intra-cellular transduction efficiency, Tat sequence as a protein transduction domain (PTD) was fused with MT in a recombinant method. Anti-apoptotic and anti-oxidative effects of Tat-MT fusion protein were evaluated under hyperglycemia and hypoxia stress conditions in cultured H9c2 cells. Recovery of cardiac functions by anti-apoptotic and anti-fibrotic effects of Tat-MT was confirmed in an ischemia/reperfusion (I/R) rat myocardial infarction model. Tat-MT fusion protein effectively protected H9c2 cells under stressful conditions by reducing intracellular ROS production and inhibiting caspase-3 activation. Tat-MT fusion protein inhibited apoptosis, reduced fibrosis area and enhanced cardiac functions in I/R. Tat-MT fusion protein could be a promising therapeutic for the treatment of ischemic heart diseases.

1. Introduction

Ischemic heart diseases caused by insufficient oxygen supply to the cardiac muscle due to the blockage of the coronary arteries resulting from atherosclerosis and thrombus are rapidly increasing causes of death worldwide [1]. They are accompanied with symptoms such as temporary pain (angina), irregular heart beat (arrhythmia), permanent heart muscle damage (myocardial infarction) and loss of muscle activity (heart failure). Although surgery based therapies including drug-eluting stents, coronary-artery bypass-graft surgery and anti-thrombosis have been highly advanced, appropriate biopharmaceutical drugs are required for the protection of cardiomyocytes and prevention of disease recurrence [2]. Various therapeutic protein drugs were developed by the recombinant protein technology; however, the application of these therapeutic proteins is limited by low transduction efficiency and short half-lives, all of which result in a loss of pharmacological action [3,4]. Drug delivery systems such as protein transduction domain (PTD), nanoparticles, and liposomes have been exploited for the improvement of the therapeutic efficacy of protein drugs [5–7].

Metallothionein (MT) is an intracellular anti-oxidative protein which consists of 61 amino acids including 20 cysteine residues [8,9]. MT is over-expressed under stressful conditions such as metal starvation, heat or inflammation and produced mainly in the liver and kidneys [10]. MT protects cells and tissues against diabetes and diabetic complications due to its anti-oxidative effects *in vitro* and *in vivo* [11]. MT null diabetic mice also shows diabetic cardiomyopathy and impaired ischemic heart contractility, while over-expression of MT decreases the infarction area and improved function of the heart [12]. Over-expression of MT by zinc displays protective effect in mice against diabetic damage caused by high blood glucose levels and progressive β-cell injuries, which are characteristics of type 2 diabetes [13,14]. Therapeutic effect of MT is highly related to its anti-oxidant effect because the overproduction of reactive oxygen species (ROS) under hyperglycemia and hypoxic states has been known to lead to diabetic complications [15].

Although MT was shown a potential to rescue cells and tissues from hypoxia- and oxidative stress-induced apoptosis, MT requires delivery systems for improvement of intracellular transduction efficiency. Therapeutic peptide and protein drugs face several challenges

* Corresponding author at: Department of Bioengineering, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, Republic of Korea. Tel.: +82 2 2220 2345.
E-mail address: yongheekim@hanyang.ac.kr (Y.-H. Kim).
[1] These authors contributed equally to this work.

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including enzymatic susceptibility, transduction efficiency, storage stability and efficacy after administration into the body [16]. Various drug delivery systems have been developed overcoming above limitations and prolonging the biological activity of these proteins [17,18]. Delivery systems maintain the protein activity *in vivo* by protecting proteins against proteolysis and anti-body neutralization. They also improve intracellular delivery of protein to be used as pharmaceuticals. Intracellular delivery technologies for therapeutic proteins need to be applied to overcome biochemical and anatomical barriers to protein drug transport and to target drug action to specific intra-cellular sites.

Protein transduction domains (PTD) among intra-cellular delivery systems opened a new challenging area of drug delivery research [19]. PTDs such as HIV-1 Tat and undecaarginine (11R) have been widely used for delivering bio-active cargos such as proteins, genes and particles due to their positively-charged amino acids such as lysine and arginine, which can bind strongly to the negatively-charged cellular membrane through electrostatic interaction [20,21]. Arginine residues in Tat are major components for enhancing the transduction of proteins and genes both *in vitro* as well as *in vivo*. Various delivery systems conjugated with Tat domains or poly-arginine have also been used to improve the transduction of heterologous proteins and peptide into cells [22,23].

In this study, the protective effect of Tat-MT fusion protein was verified in H9c2 cells under hyperglycemia, hypoxia and hydrogen peroxide stress conditions. Tat-MT fusion protein on damaged hearts was also evaluated by femoral vein injection of the protein into an ischemia/reperfusion rat model. The protective functions of Tat-MT fusion protein were confirmed in an ischemia/reperfusion rat model by evaluating apoptosis index, fibrosis area and cardiac functions.

2. Materials and methods

2.1. Materials

The pRSET vector was obtained from Invitrogen (La Jolla, CA). All restriction enzymes and polymerase chain reaction (PCR) cloning kits were purchased from Takara (Tokyo, Japan). An immobilized Ni-affinity chromatography column (IMAC) was obtained from Bio-Rad (Hercules, CA). Hydrogen peroxide (H_2O_2) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

2.2. Preparation of Tat-MT fusion protein

A plasmid vector for the expression of Tat-metallothionein (Tat-MT) was constructed using a pRSET vector. Tat-MT fusion construction was performed following a procedure described previously [24]. In brief, Tat oligonucleotide (TAG GGC AGG AAG AAG CGG AGA CAG CGA CGA CGA) was cloned into a pRSET vector. The amplified MT coding sequence was cloned into pRSET-Tat vector and verified by DNA sequence analysis (Cosmogenetech, Seoul, Korea). All fusion proteins were expressed from *Escherichia coli* strain BL21 (DE)pLysS (Novagen, Madison, WI) using isopropyl beta-D-thiogalactoside (IPTG) (1 mM) with zinc sulfate ($ZnSO_4$) (1 mM). They were purified by immobilized metal affinity chromatography (IMAC) using a Ni-NTA resin column with FPLC (Bio-Rad, Hercules, CA) and dialyzed against pH 7.4 phosphate buffered saline (PBS) using a 3500 molecular weight cut off (MWCO) membrane (Spectrum Laboratories, CA).

2.3. Cell culture

A mouse cardiomyocyte, H9c2 (Korean Cell Line Bank, Seoul, Korea) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Welgin, Daegu, Korea) containing 10% fetal bovine serum supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C under humidified conditions (5% CO_2 atmosphere).

2.4. Hyperglycemia and hypoxia induction

In order to evaluate the anti-oxidative effect of MT fusion proteins, hyperglycemia (HG) or hydrogen peroxide (H_2O_2) was treated to H9c2 cell for elevating intracellular reactive oxygen species (ROS) production. H9c2 cells were exposed to HG at 350 mM and H_2O_2 at 1.5 μM with MT fusion proteins for 24 h. Cell viability was measured by the MTT method following manufacture's protocol. Stock solutions of MT and Tat-MT fusion proteins were prepared at 3 μM (39 μg/ml) and 3.9 μg of protein was applied in each well.

2.5. Apoptosis assay

H9c2 cells were seeded in 96-well white plates and incubated for 24 h. After treatments of MT fusion proteins with HG and H_2O_2, H9c2 cells were further incubated for 8 h and 3 h, respectively. Intracellular apoptosis was measured by evaluating caspase-3 activity using a luminometric caspase-Glo 3/7 assay kit (Promega, Madison, WI), according to the manufacturer's protocol, with a luminescence plate reader.

2.6. Hypoxia induction and ROS fluorescence assay using H_2DCFDA

To determine anti-oxidative effects of MT fusion proteins, H9c2 cells were exposed to hypoxia conditions (1% O_2 and 5% CO_2) for 24 h. Cell viability was measured by the MTT method. A ROS detecting cell permeable probe, H_2DCFDA, was added at 10 μM to each well of the plates and incubated for 30 min at 37 °C. Fluorescence intensity was measured using UV/VIS fluorescence spectrophotometer (SpectraMax M2^{e-}, Molecular devices, Sunnyvale, CA) at 499 nm excitation and 522 nm emission.

2.7. Myocardial infarction model

All experimental procedures for animal studies were approved by the animal research committee of the Yonsei University College of Medicine and carried out following the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction in male Sprague–Dawley rats (200 ± 30 g) was performed by surgical occlusion in the left anterior descending coronary artery. Briefly, after rat was anesthetized by ketamine (10 mg/kg) and xylazine (5 mg/kg), it was opened in the chest *via* the space between the third and fourth ribs, and the heart was exteriorized through the intercostal space. The left coronary artery was ligated 2–3 mm from its origin with a 5–0 prolene suture (ETHICON, Somerville, NJ). The ligature ends to form snare was pressed onto the surface of the heart directly above the coronary artery. Ischemia was evaluated by the blanching of the myocardium and dyskinesis of the ischemic region. Tat-MT fusion proteins at 175 μg were suspended in 200 μl of PBS and injected into I/R rat model *via* femoral vein using a Hamilton syringe (Hamilton Co., Reno, NV) with a 30-gage needle during the occlusion. After occlusion for 1 h, the hemostat was eliminated, and the snare was untangled for reperfusion, with the ligature left loose on the surface of the heart. Throughout the operation, animals were ventilated with 95% O_2 and 5% CO_2 using a Harvard ventilator. The operative mortality was 10% within 48 h. Twenty animals per group (Normal, MI model, MI + MT, MI + (Tat-MT)) were used for morphologic analysis after occlusion of the left coronary artery.

2.8. Evaluation of myocardial infarct size (fibrosis area)

To measure infarct size, the heart was excised from rats after sacrifice and perfused with phosphate buffered saline (PBS) to remove the blood and then fixed in 10% formalin solution for 24 h at 4 °C. Sequentially, tissue sections were mounted onto gelatin-coated glass slides to guarantee that different stains could be used on successive tissue sections cut through the injection area. After the sections