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Molecules and Cells

In Vivo Protein Transduction: Delivery of PEP-1-SOD1 Fusion Protein into Myocardium Efficiently Protects against Ischemic Insult

You-en Zhang[1,2], Jia-ning Wang[1,*], Jun-ming Tang[1], Ling-yun Guo[1], Jian-ye Yang[1], Yong-zhang Huang[1], Yan Tan[1], Shou-zhi Fu[2], Xia Kong[1], and Fei Zheng[1]

Myocardial ischemia-reperfusion injury is a medical problem occurring as damage to the myocardium following blood flow restoration after a critical period of coronary occlusion. Oxygen free radicals (OFR) are implicated in reperfusion injury after myocardial ischemia. The antioxidant enzyme, Cu, Zn-superoxide dismutase (Cu, Zn-SOD, also called SOD1) is one of the major means by which cells counteract the deleterious effects of OFR after ischemia. Recently, we reported that a PEP-1-SOD1 fusion protein was efficiently delivered into cultured cells and isolated rat hearts with ischemia-reperfusion injury. In the present study, we investigated the protective effects of the PEP-1-SOD1 fusion protein after ischemic insult. Immunofluorescecnce analysis revealed that the expressed and purified PEP-1-SOD1 fusion protein injected into rat tail veins was efficiently transduced into the myocardium with its native protein structure intact. When injected into Sprague-Dawley rat tail veins, the PEP-1-SOD1 fusion protein significantly attenuated myocardial ischemia-reperfusion damage; characterized by improving cardiac function of the left ventricle, decreasing infarct size, reducing the level of malondialdehyde (MDA), decreasing the release of creatine kinase (CK) and lactate dehydrogenase (LDH), and relieving cardiomyocyte apoptosis. These results suggest that the biologically active intact forms of PEP-1-SOD1 fusion protein will provide an efficient strategy for therapeutic delivery in various diseases related to SOD1 or to OFR.

INTRODUCTION

Myocardial infarction is a leading cause of death and disability, with a direct correlation between infarct size and prognosis. Early reperfusion is an absolute prerequisite for the survival of ischemic myocardium. However, reperfusion has been referred as the "double-edged sword" because reperfusion itself may lead to accelerated and additional myocardial injury beyond that generated by ischemia alone. This results in a spectrum of reperfusion-associated pathologies, collectively called reperfusion injury (Yellon et al., 2000).

The underlying pathophysiological mechanisms of ischemia-reperfusion have not been fully elucidated. The mechanisms proposed to cause reperfusion injury include formation of oxygen free radicals (OFR), calcium overload, neutrophils mediated myocardial and endothelial injury, progressive decline in microvascular flow to the reperfused myocardium and depletion of the high-energy phosphate store (Simes et al., 2002). The overproduction of OFR during the first minutes of reperfusion might be a key point. About 25% of cardiomyocyte death after reperfusion of acute myocardial infarction is caused by reperfusion injury (Yellon et al., 2000). In the heart, OFR may be generated by several mechanisms, such as mitochondrial respiration, activated neutrophils and, in some species, by xanthine oxidase activity. Excessive OFR can damage cellular lipids, proteins, and DNA. Consequently, oxidative stress has been implicated in various pathological conditions involving cardiovascular diseases, ischemia-reperfusion, carcinogenesis, neurological disorders, diabetes, inflammation/immune injury and aging (Dalle-Donne et al., 2006; Dhalla et al., 2000; Jenner, 2003; Sayre et al., 2001). The key antioxidant enzyme Cu, Zn-superoxide dismutase (Cu, Zn-SOD, also called SOD1) provides a defense against oxidative stress by catalyzing the dismutation of the superoxide anion into hydrogen peroxide plus oxygen, thus protecting cells from oxidative damage (Nelson et al., 2006). The endogenous antioxidant activity is severely damaged after ischemia-reperfusion, rendering the myocardium extremely vulnerable to OFR. However, exogenous SOD1 can not be delivered into live cells because of the poor permeability and selectivity of the cell membrane, thus its use for protecting cells/tissues from oxidative stress damage is greatly limited.

There is a growing effort to circumvent these problems by designing strategies to deliver full-length proteins into a large number of cells. A family of short, positively charged peptides, commonly known as cell-penetrating peptides (CPPs) or protein transduction domains (PTDs), have been used in numer-

[1]Institute of Clinical Medicine, Renmin Hospital, Yunyang Medical College, Shiyan 442000, China; [2]Department of Emergency, Renmin Hospital, Yunyang Medical College, Shiyan 442000, China
*Correspondence: rywjn@vip.163.com

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ous applications in biology and medicine since the first synthetic cell-permeable sequence was identified two decades ago. These include carrier peptides derived from the human immunodeficiency virus (HIV-1) TAT protein, Drosophila Antennapedia (Antp) protein, and herpes simplex virus VP22 protein (Fawell et al., 1991; Prochiantz, 2000; Vives et al., 1997). Many proteins fused with these CPPs have been shown to cross biological membranes efficiently and independently of transporters or specific receptors (Deshayes et al., 2005). The TAT protein from HIV-1 is able to deliver biologically active proteins *in vivo* and has considerable potential for protein therapeutics (Frankel and Pabo, 1988; Jin et al., 2001). However, protein transduction using CPP-TAT fusion protein systems may require denaturation of the protein before delivery to increase the accessibility of the TAT-CPP domain. This requirement introduces an additional delay between the time of delivery and intracellular activation of the protein (Schwarze and Dowdy, 2000). To increase the biological activity of transduced protein into cells, Morris group (Morris et al., 2001) designed a novel peptide carrier, PEP-1 (KETWWETWWTEWSQPKKKRKV), which consists of three domains: a hydrophobic tryptophan rich motif (KETWWETWWTEW), a spacer (SQP), and a hydrophilic lysine-rich domain (KKKRKV). To date, many researchers have demonstrated the successful delivery of full-length PEP-1 fusion proteins into cultured cells and the nervous system by protein transduction technology. These include β-Gal, full-length specific antibodies, human copper chaperone for Cu,Zn-SOD, EGFP and SOD (An et al., 2008; Choi et al., 2005; Dong et al., 2007; Morris et al., 2001).

In the present study, two prokaryotic expression plasmids of pET15b-SOD1 and pET15b-PEP-1-SOD1 were constructed using the TA-cloning method. The recombinant proteins of SOD1 and PEP-1-SOD1 were expressed and purified. The PEP-1-SOD1 fusion protein was successfully transduced into the rat myocardium in a native, enzymatically active form *in vivo* and efficiently protected against cardiomyocyte damage induced by ischemia-reperfusion injury. Therefore, we suggest that the PEP-1-SOD1 fusion protein would be useful as a therapeutic agent for myocardial ischemia-reperfusion injury.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 250-300 *g* (Certificate No. 420171) were obtained from the Hubei Animal Center (Wuhan, China) and allowed to acclimatize in the institutional animal house for more than 5 days before use, with standard rat food and water ad libitum. All animals were treated humanely, and the study protocols were in accordance with the Regulations of Good Laboratory Practice for non-clinical laboratory studies of drugs issued by the State Food and Drug Administration of China.

Expression and purification of PEP-1-SOD1

Two prokaryotic expression plasmids, pET15b-SOD1 and pET15b-PEP-1-SOD1, were successfully constructed using the TA-cloning method. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells (Novagen, USA). The transformed bacterial cells were grown in 100 ml of LB medium at 37°C to a OD_{600} value of 0.5-1.0 and induced with 0.83 mM isopropyl-β-D-thiogalactoside (IPTG) (Promega, USA) at 30°C for 12 h. Harvested cells were lysed by sonication at 4°C in a binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), and the recombinant SOD1 and PEP-1-SOD1 formed were purified. Briefly, clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid sepharose affinity column (Qiagen, USA) under native conditions. After the column was washed with 10 volumes of the binding buffer and 6 volumes of a wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), the fusion proteins were eluted using an eluting buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The fusion-protein-containing fractions were combined and the salts were removed using aPD-10 column. The protein concentration was estimated with the Bradford method using bovine serum albumin (BSA, 1 mg/ml) as a standard.

Transduction of PEP-1-SOD1 into rat myocardium

Seventy-two male Sprague-Dawley rats were randomly divided into two groups: SOD1-treated group and PEP-1-SOD1-treated group. 500 μg of purified SOD1 and 500 μg of purified PEP-1-SOD1 fusion proteins were administered in the rats through their tail veins (Eum et al., 2004), respectively. The animals were anesthetized with 10% chlorali hydras (250-300 mg/kg, i.p.) at designated times (0.5, 1, 2, 4, 8 and 24 h) after the protein injection, the hearts were removed and post-fixed in 4% paraformaldehyde solution at 4°C for 6 h (n = 6 for each group at each time point). Thereafter the frozen tissues were serially sectioned on a cryostat into 7 μm coronal sections. The sections were sequentially treated with a mixture of rabbit anti-His-probe (diluted 1:100) (Santa Cruz Biotechnology, USA) and mouse anti-Troponin T (diluted 1:100) (Santa Cruz Biotechnology, USA) antibodies overnight at 4°C, and washed 3 times for 5 min with PBS. Thereafter, the tissues were exposed to tetraethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (diluted 1:100) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:100) at 25°C for 2 h, washed 3 times for 5 min with PBS, then incubated with 4, 6-Diamidino-2-phenylinole (DAPI) (Sigma, USA) for 10 min. Finally, the sections were covered with glass cover slips and the immunoreactions were observed under a fluorescent microscope (Nikon, Japan). The fluorescent intensity was evaluated through Image Pro software.

Induction of myocardial ischemia

Sixty rats were randomly divided into five groups as follows: sham-operated group, control group, and PEP-1-SOD1 pretreated (100 μg, 300 μg, and 500 μg) groups (n = 12, each group). PEP-1-SOD1 fusion protein was injected into the rats via their tail veins in various concentrations 1 h before the occlusion of the left anterior descending artery in the PEP-1-SOD1-pretreated groups. Instead of PEP-1-SOD1 fusion protein, the same volume of physiological saline was injected into control animals via their tail veins. Sham-operated animals were subjected to the same surgical procedures except that the left anterior descending arteries were not occluded.

The animals were anesthetized with 10% chlorali hydras (250-300 mg/kg, i.p.). Surgery was performed under sterile conditions. A tracheotomy was performed and an intubating cannula was connected to a rodent ventilator (Chengdu TME Technology Co, Ltd., China). The animals were ventilated artificially with room air. Respiratory rate was synchronized with the rat's spontaneous rate (60-80 strokes/min, 1 ml/100 g body weight). After a left-side thoracotomy was performed at the fifth intercostal space, the pericardium was incised and the heart was exteriorized. A ligature (6/0 silk suture) was placed around the left main coronary artery close to its origin. The thread was then made into a knot as an occluder and another thread was tied to the first knot as a releaser. The ends of both threads were brought outside the thoracic cavity. Thus, the occlusion could be tightened or loosened by pulling the thread of the re-

leaser. The animals were then allowed to stabilize for 30 min. The coronary arteries were occluded for 1 h followed by 2 h of reperfusion.

Measurement of hemodynamics

Hemodynamic data were obtained after reperfusion as detailed below. Rats were anesthetized with 10% chlorali hydras (250-300 mg/kg, i.p.). A carotid artery and femoral artery were isolated. The two catheters filled with heparinized (10 U/ml) saline solution were connected to a Statham pressure transducer (Gould, Saddle Brook, USA). The carotid arterial catheter was advanced into the left ventricle to record ventricular pressure for a brief period. The femoral artery catheter was inserted into an isolated femoral artery to monitor hemodynamics. Hemodynamic parameters were monitored simultaneously and recorded on a thermal pen-writing recorder (RJG-4122, Nihon Kohden, Japan) and on an FM magnetic tape recorder (RM-7000, Sony, Japan). After the measurements, the heart was rapidly removed from the killed rats.

Assessment of infarct size

Six animals in each group were used for this experiment. After the completion of the infarct protocol and infusion, the hearts were quickly removed and frozen at -20°C for 1-2 h, 2 mm thick sections were cut from the apex to the level of the coronary suture, then incubated in 1% triphenyltetrazolium chloride (TTC) in a phosphate buffer (pH 7.4) at 37°C for 15-20 min. In a globally ischemic heart, the whole ventricle is at risk of infarction, the area of left ventricle and the infarcted tissue were measured by an independent blinded observer using planimeter. The volumes of the infarcted zone were calculated by multiplying the planimetered areas by slice thickness. Infarcted volume was expressed as a percentage of left ventricular volume for each heart.

Measurement of Cu, Zn-superoxide dismutase (SOD1), creatine kinase (CK), lactate dehydrogenase (LDH) activity, and malondialdehyde (MDA) levels

SOD1 activity of purified SOD1 and PEP-1-SOD1 fusion protein were measured by monitoring the capacity to inhibit the reduction of ferricytochrome c by xanthine/xanthine oxidase as described by McCord and Fridovich (McCord and Fridovich, 1969). The principle of the method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generator. Blood was sampled from abdominal aorta and serum obtained after centrifugation at 2,500 × *g* for 10 min. Activities of CK and LDH were determined spectrophotometrically at 340/660 nm and 340 nm, respectively. Malondialdehyde (MDA), an end-product of peroxidation of cell membrane lipids caused by OFR, is considered a reliable marker of cardiomyocyte oxidative damage and was determined by measurement of the chromogen generated from the reaction of MDA with 2-thiobarbituric acid. The above biochemical analyses were measured using commercial kits (JianCheng Bioengineering Institute, China).

Apoptosis assay

Cardiomyocyte apoptosis can be quantitatively analyzed by detection of DNA fragmentation via a fluorescence assay based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (Beyotime Institute of Biotechnology, China). This method takes advantage of DNA fragmentation, characteristic of apoptosis. The DNA breaking points (nicks) expose the 3′OH ends of DNA, which are labeled, thus allowing the identification of apoptotic cells. Briefly, terminal deoxynucleotidyl transferase was used to incorporate residues of digoxigenin nucleotide into the 3′OH ends of DNA fragments. Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed according to the manufacturer's instructions. For each slide, 5 fields were randomly chosen under the microscope on each section. The index of apoptosis was determined (i.e., number of positively stained apoptotic cardiomyocytes/total number of cardiomyocytes counted × 100). Assays were performed in a blinded manner.

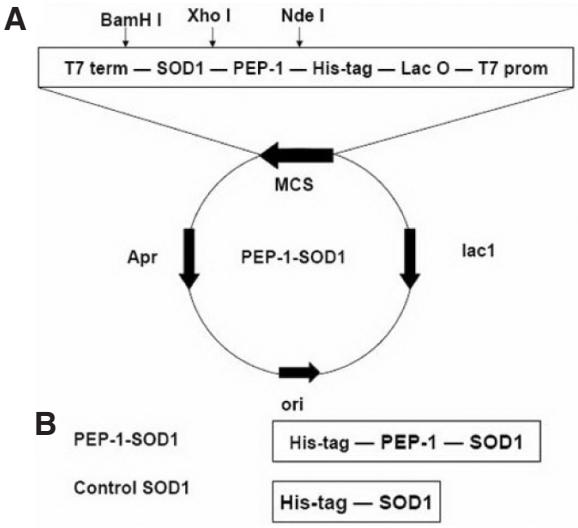

Fig. 1. Expression vector for PEP-1-SOD1 fusion protein. (A) Construction of PEP-1-SOD1 expression vector system based on the vector pET-15b. Synthetic PEP-1 oligomer was inserted between *Nde*I and *Xho*I, human Cu, Zn-SOD cDNA was inserted between *Xho*I and *Bam*HI sites of pET-15b. (B) Diagram of expressed PEP-1-SOD1 and control SOD1 proteins. The coding frame of human SOD1 is represented by an open box along with 6 His-tag and the PEP-1 peptide. The resulting vector was named pPEP-1-SOD1. Expression was induced by adding IPTG.

Statistical analysis

The reported data were expressed as means ± SD. Statistical analysis was performed by the program SPSS 13.0 for windows. For comparison between multiple groups, data were analyzed by unpaired Student's t-test and one-way analysis of variance (ANOVA) test followed by Student-Newman-Keuls post hoc analysis. *P* values less than 0.05 were considered statistically significant.

RESULTS

Expression and purification of PEP-1-SOD1 fusion protein

Two prokaryotic expression plasmids, pET15b-SOD1 and pET15b-PEP-1-SOD1, were successfully constructed using the TA-cloning method (Fig. 1A). Both the recombinant proteins (SOD1 and PEP-1-SOD1), with six histidine residues (His-tag) at the amino terminus, were expressed and purified (Fig. 1B), their enzyme activities were 15.87×10^3 U/g and 12.23×10^3 U/g, respectively. The expressed and purified proteins were further confirmed by 12% SDS-PAGE (Fig. 2A) and Western blot analysis using an anti-rabbit polyhistidine antibody (Fig. 2B). The results demonstrated that SOD1 and PEP-1-SOD1 proteins were highly expressed, with relative molecular mass of 22 and 26 kDa. In addition, the expressed products were present

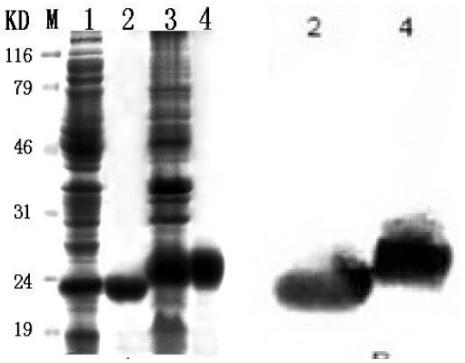

Fig. 2. Expression and purification of PEP-1-SOD1 fusion protein. Protein extracts of cells and purified fusion proteins were analyzed by 12% SDS-PAGE (A) and subjected to Western blot analysis with an rabbit anti-polyhistidine antibody (B). Lanes in A and B are as follows: M, pre-stained protein marker; lane 1, total proteins from SOD1 culture; lane 2, purified SOD1; lane 3, total proteins from PEP-1-SOD1 culture; lane 4, purified PEP-1-SOD1.

in a native and soluble form and the yields of the purified fusion proteins were about 158.5 g/L and 212.1 g/L LB cultures, respectively.

Transduction of PEP-1-SOD1 into the rat myocardium

To investigate the penetrating ability and subcellular localization of PEP-1-SOD1 fusion protein into the rat myocardium *in vivo*, the fusion protein was detected with rabbit anti-His-tag and analyzed by fluorescent microscopy. As shown in Fig. 3, native PEP-1-SOD1 fusion protein was successfully transduced into the myocardium in a time-dependent manner, and mainly localized to cytoplasm and nucleus (TRITC-labeled anti-His-tag). The levels of fluorescent signals increased gradually in a time-dependent manner, the brightest red fluorescent signals reached a peak at 1 h, whereas the control SOD1 was not transduced into the myocardium. The levels of SOD1 activities in the hearts were significantly increased when treated with PEP-1-SOD1 for 1 h and higher as compared with SOD1-treated group ($P < 0.01$) at each time point (Fig. 4). These results demonstrated that the PEP-1-SOD1 fusion protein was not only transduced into the myocardium in a native form, but the transduced protein was enzymatically stable for at least 24 h.

Effects of PEP-1-SOD1 fusion protein on hemodynamics and left ventricular function

In PEP-1-SOD1 pre-treated groups, the levels of heart rate (HR), left ventricular systolic pressure (LVSP), maximum rate of left ventricular pressure ($\pm$ dp/dt_{max}) at the end of 2 h reperfusion increased gradually on increasing the amount of fusion protein via vein injection, and significantly higher as compared with control animals at the same time-points ($P < 0.05$ or $P < 0.01$). Whereas the level of left ventricular end-diastolic pressure (LVEDP) declined gradually and was significantly lower as compared with control group ($P < 0.01$) as shown in Fig. 5.

***In vivo* transduced PEP-1-SOD1 fusion protein decreased infarct size**

To determine whether the transduced PEP-1-SOD1 fusion protein performed their biological roles *in vivo*, we tested the effects of PEP-1-SOD1 on myocardial infarct size with 1% TTC staining in a rat myocardial ischemia-reperfusion model. Infarct sizes were noticeably reduced in PEP-1-SOD1 pre-treated

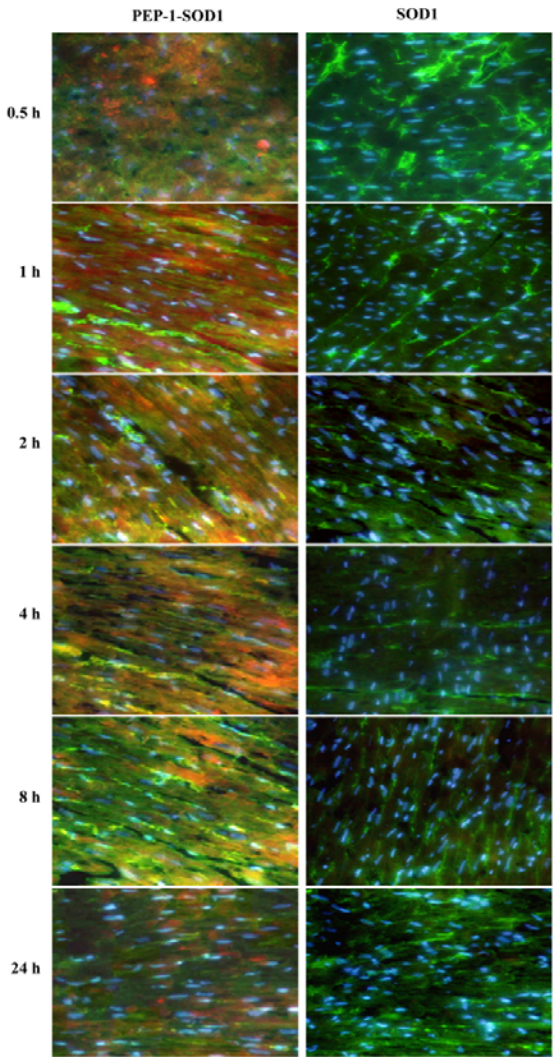

Fig. 3. Transduction of PEP-1-SOD1 into the myocardium. Rats were treated with 500 μg purified PEP-1-SOD1 and control SOD1 proteins for various time periods (0.5-24 h). Myocardium sections were incubated with rabbit-anti polyhistidine and mouse-anti Troponin T antibodies, and then visualized with a fluorescent microscopy. Red fluorescent signals represent TRITC-labeled anti-His-tag of SOD1; Green fluorescent signals represent FITC-labeled anti-Troponin T; Blue fluorescent signals represent DAPI-labeled nuclei.

groups compared with the control group, especially apparent for the 500 μg dose (Fig. 6).

Activities of CK and LDH

The activities of CK and LDH in serum were used to monitor the myocardial damage. Compared with the sham group, CK and LDH activities in the control group were markedly increased by ischemic insult. Treatment with PEP-1-SOD1 fusion protein could significantly decrease CK and LDH release as shown in Fig. 7.

Assessment of oxidative stress level

To determine whether the transduced fusion proteins had a functional role in OFR scavenging and lipid peroxidation, we tested the content of serum and myocardial MDA with the TBA

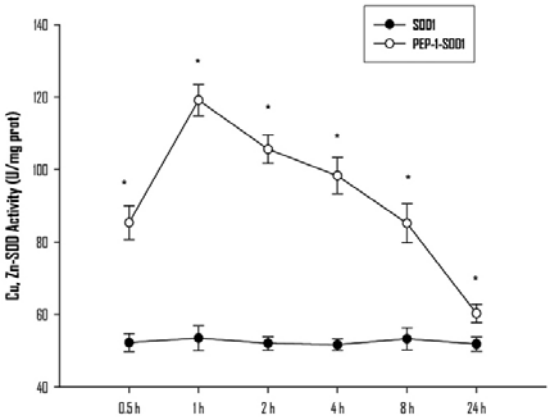

Fig. 4. Activity of PEP-1-SOD1 fusion protein transduced into rat myocardium ($n = 6$, $\bar{x} \pm s$). The 500 μg purified SOD1 and 500 μg purified PEP-1-SOD1 fusion proteins were administered in the rats through their tail veins for various time periods (0.5-24 h). * $P < 0.01$ *vs.* SOD1-treated group.

protocol. As shown in Fig. 8, compared with the control group, PEP-1-SOD1 fusion protein pre-treatment significantly decreased the levels of MDA content 2 h after reperfusion in a dose-dependent manner *in vivo* ($P < 0.05$ or $P < 0.01$).

Effects on cardiomyocyte apoptosis

TUNEL-positive cells showed typical apoptosis. As shown in Fig. 9, the sham group demonstrated a mean of 2.62 ± 1.04% TUNEL-positive cardiomyocytes per heart. However, the number of TUNEL-positive cells expressed as percentage of total normal nuclei was significantly increased subsequent to ischemia-reperfusion induced myocardial injury in the control group (11.38 ± 3.38%, $P < 0.01$). Treatment of various concentrations of PEP-1-SOD1 fusion protein markedly reduced TUNEL-positive cells *vs.* control group ($P < 0.05$ or $P < 0.01$).

DISCUSSION

It is widely accepted that myocardial ischemia-reperfusion induces the production of OFR. Furthermore, OFR reportedly contribute to the injury process of myocardial ischemia-reperfusion. Consequently, many investigators have demonstrated cardioprotective effects of various antioxidant enzymes or oxidant scavengers on myocardial ischemia-reperfusion injury. Increased understanding of the biological implications of redox and free radical chemistry/physics has challenged our fundamental beliefs on health and disease. The modern concept of redox balance implies that relatively low concentrations of OFR are thought to act as mediators or modulators of cell signaling and contribute to other key functions, such as regulation of activity of transcription factors and gene expression (Droge, 2002; Finkel, 2003; Nathan, 2003). However, activated cells with increased metabolism produce excessive OFR. Overproduction of OFR and their derivatives occurs in a number of diseases as previously noted. The Cu, Zn-SOD (SOD1) isoform of SOD appears to be expressed at relatively high levels in all cells including blood vessels, where it is the predominant isoform of SOD (when expressed as percent of total SOD activity). Superoxide anion does not readily penetrate through membranes, therefore these results imply that superoxide anion derived from cytosolic compartments contributes to myocardial ischemia-reperfusion injury. SOD1 is one of the key cellular enzymes that catalyze the dismutation of superoxide anion in cytosolic compartments, thus protecting them from oxidative stress. Although exogenous antioxidant enzymes have been considered potential therapeutic agents against OFR-mediated diseases, the lack of transduction shown by antioxidant enzymes into cells has limited their use for protecting cells/tissues from oxidative damage.

Since the discovery was made that TAT was able to transport various proteins across cell membranes, thousands of studies have been performed characterizing and optimizing CPPs as cellular delivery agents. The usefulness of peptides as vehicles to introduce macromolecules into cells led to the development of many chimeric peptides. So far, these vectors have been used to translocate a wide range of macromolecules into living cells, including proteins, peptides, oligonucleotides, peptide nucleic acids and polysaccharides. Nanoparticles and liposomes have also been internalized by means of CPPs (Henriques et al., 2006). Some studies showed that transduced PEP-1-SOD fusion protein efficiently protect against ischemic insults (An et al., 2008; Eum et al., 2004), Gustafsson et al. demonstrated that TAT-fusion protein transduction into the myocardium is feasible and that transduction of TAT-fusion proteins is protective in cell culture and in the perfused heart (Gustafsson et al., 2002). However, protein transduction using TAT fusion protein systems may require denaturation of the protein before delivery to increase the accessibility of the TAT-CPP domain. This requirement introduces an additional delay between the time of delivery and intracellular activation of the protein (Schwarze and Dowdy, 2000).

Recently, PEP-1 technology has become a powerful tool for basic research, and several studies have demonstrated its usefulness for studying the role of proteins, or for targeting specific protein/protein interactions *in vitro* as well as *in vivo* (Eum et al., 2004; Hwang et al., 2005; Morris et al., 2001). In *in vitro* studies, PEP-1 localizes rapidly, in < 10 min, to the nucleus of human HS-68, murine NIH3T3 fibroblasts, or Cos cells (Morris et al., 2001). It was reported that PEP-1 strongly interacts with lipids and that this interaction is associated with a conformational transition to a helical form. The formation of PEP-1/cargo complexes does not involve any conformational changes of the carrier (Deshayes et al., 2004). In the present study, we first investigated the protective effect of transduced PEP-1-SOD1 fusion protein in a rat myocardial ischemia-reperfusion model. In our previous studies, we have observed that the PEP-1-SOD1 fusion protein was effectively delivered into cultured endothelial cells of the human umbilical vein (Wang et al., 2007) and isolated rat hearts with ischemia-reperfusion injury. In the present study we observed the delivery of PEP-1-SOD1 fusion protein into the myocardium region (as well as other organs, such as liver, spleen, lung, kidney of normal rat, data not shown) after 30 min via tail vein injection to open up new opportunities for an experimental protein therapy.

The properties of transduced proteins in tissues are key points for the therapeutic application of protein transduction. Therefore, the myocardial SOD1 activity was measured with the xanthine oxidase protocol. The enzyme activity of myocardial SOD1 in PEP-1-SOD1 group was found to increase in a time-dependent manner. Transduced SOD1 enzyme activity increased more than two-fold compared to that of the control.

Left ventricular global contractility was assessed by continuously monitoring the $\pm dp/dt_{max}$ and the LVEDP. In the present study, PEP-1-SOD1 pre-treatment caused significant changes in the recorded hemodynamic profiles, such as HR, blood pressure and left ventricular function, as compared with control animals. These results indicate that PEP-1-SOD1 plays an

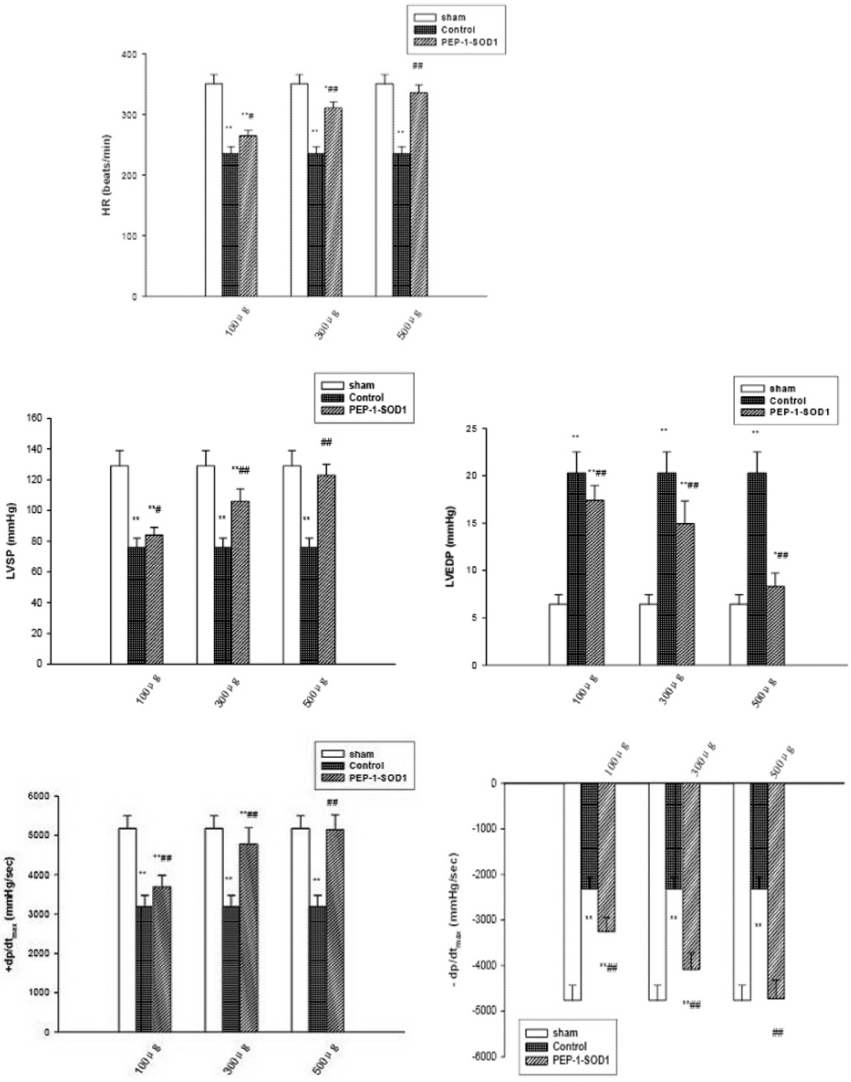

Fig. 5. Effects of PEP-1-SOD1 fusion protein on hemodynamics and left ventricular function ($n = 6$, $\bar{x} \pm s$). The levels of HR, LVSP and $\pm$ dp/dt$_{max}$ during ischemia were decreased in the control group. However, LVEDP increased significantly. The above parameters were markedly increased or decreased in PEP-1-SOD1 pre-treated groups, especially apparent for the 500 μg dose. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* control group.

important role in improving the post-ischemic recovery of left ventricular systolic and diastolic functions. Myocardial enzymes can be released from the injured cardiomyocytes induced by ischemia-reperfusion and myocardium specific enzyme analysis has proved considerably valuable in the diagnosis of myocardial infarction. Alterations in CK and LDH have been considered as the important markers of myocardial injury. In the present study, we found that the levels of CK and LDH in PEP-1-SOD1 pre-treated animals were markedly decreased, suggesting that PEP-1-SOD1 fusion protein can protect cardiomyocytes. MDA, an end product of peroxidation of cell membrane lipids caused by OFR, is considered a reliable marker of cardiomyocyte oxidative damage (Surh et al., 2003). The present study demonstrated that PEP-1-SOD1 fusion protein significantly restrained the increase of serum and myocardial MDA levels caused by acute ischemic injury, suggesting that PEP-1-SOD1 may exert its protective effect against the myocardial ischemic injury by reducing lipid peroxidation. Furthermore, the transduced PEP-1-SOD1 fusion protein could decrease infarct size caused by ischemia-reperfusion damage. All of these findings demonstrate that PEP-1-SOD1 fusion protein exerted a beneficial effect on the ischemic and reperfused heart.

Apoptosis is structurally and biochemically distinct from necrosis and is tightly controlled by the cellular genetic apparatus. Previously, apoptosis in terminally differentiated cells (e.g., cardiomyocytes and neurons) was considered controversial. However, since the report of cardiomyocyte apoptosis during myocardial ischemia-reperfusion, apoptosis has been documented in a variety of cardiovascular diseases, including myocardial infarction, ischemia-reperfusion, end-stage heart failure, arrhythmogenic right ventricular dysplasia, and adriamycin-induced cardiomyopathy (Haunstetter and Izumo, 1998). Cumulative evidence suggests that OFR, which have been implicated in cardiac pathophysiology, can trigger cardiomyocyte apoptosis by up-regulating pro-apoptotic proteins, such as Bax and caspases, and the mitochondria-dependent pathway. Oxidative stress is a major apoptotic stimulus in many cardiovascular diseases and the process can be inhibited by antioxidants both *in vitro* and *in vivo* (Kumar and Jugdutt, 2003). In the present study, using TUNEL staining of the ventricular myocardium, the TUNEL-positive cells of PEP-1-SOD1 pre-treated groups were significantly decreased. It is suggested that the PEP-1-SOD1 fusion protein inhibits cardiomyocyte apoptosis caused by ischemia-reperfusion injury. This may be re-

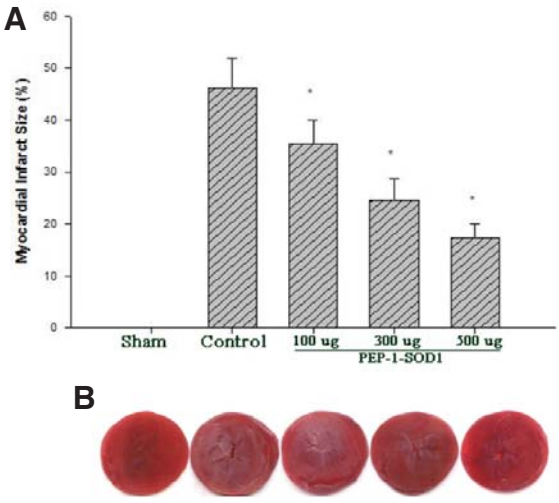

Fig. 6. Effects of transduced PEP-1-SOD1 fusion protein on myocardial infarct size (n = 6, $\bar{x} \pm s$). Representative photographs of the TTC-stained myocardium of rats 2 h after reperfusion (B). Sham-operated, control, and PEP-1-SOD1 fusion protein were injected into the rats via their tail veins as a single dose at 100 μg, 300 μg, and 500 μg. The infarcted volume was expressed as a percentage of left ventricular volume for each heart, infarct sizes in PEP-1-SOD1 pre-treated groups were significantly reduced induced by ischemia-reperfusion injury (A). $^{*}P < 0.01$ *vs.* control group.

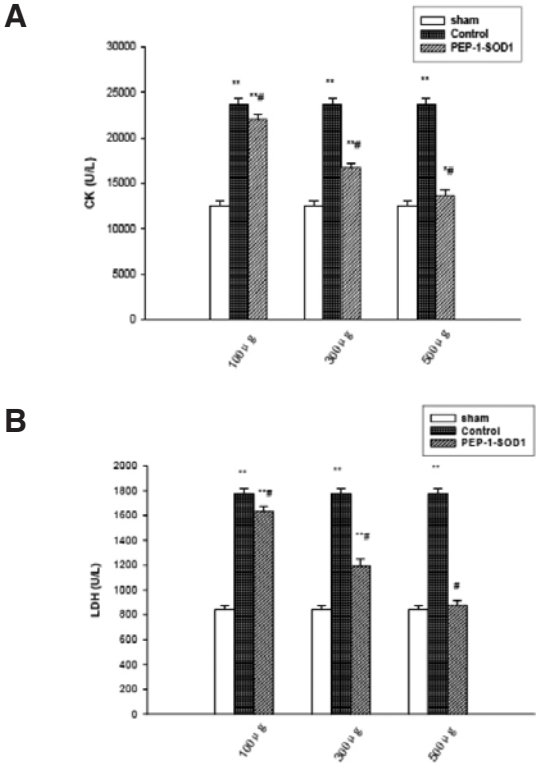

Fig. 7. Effects of transduced PEP-1-SOD1 fusion protein on CK (A) and LDH (B) of rats in ischemia-reperfusion injury (n = 6, $\bar{x} \pm s$). In the control group, the activities of CK and LDH were markedly increased after ischemia-reperfusion. PEP-1-SOD1 pre-treatment significantly decreased CK and LDH release as compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* control group.

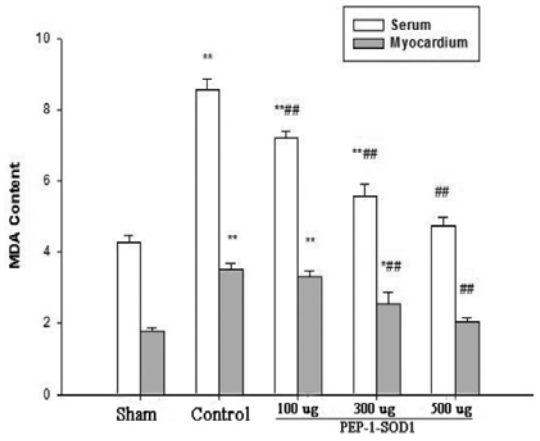

Fig. 8. Effects of transduced PEP-1-SOD1 fusion protein on the content of MDA after ischemic insult (n = 6, $\bar{x} \pm s$). MDA content of control group markedly increased post the ischemia-reperfusion process. Treatment with PEP-1-SOD1 could efficiently prevent the elevation of MDA content in serum and myocardium after ischemia-reperfusion injury. Serum: nmol/mL; Myocardium: nmol/mg·prot. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* control group.

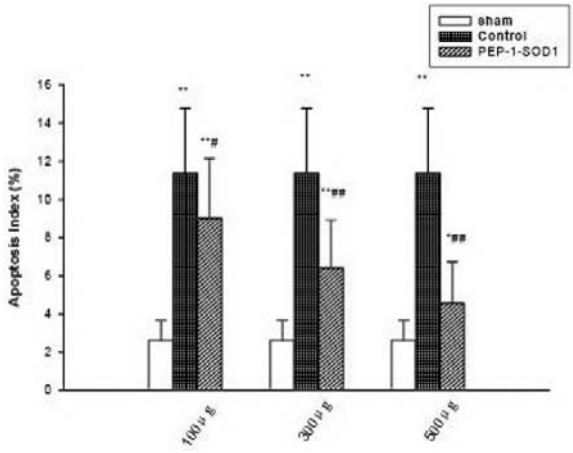

Fig. 9. Effects of PEP-1-SOD1 on apoptosis index of rats in ischemia-reperfusion injury (n = 6, $\bar{x} \pm s$). Apoptotic cells were significantly increased in the control group compared with the sham. PEP-1-SOD1 pre-treatment significantly reduced the apoptosis cells after ischemia-reperfusion. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* control group.

lated to its effect of scavenging the OFR or regulating apoptosis genes and synthesis of corresponding proteins, either of which possibility will require further research.

In conclusion, the present results suggest that a PEP-1-cargo fusion protein is efficiently delivered into cardiomyocytes in its native conformation *in vivo*, and PEP-1-SOD1 treatment in ischemic animals reduce ischemic damage. Our success in the protein transduction of PEP-1-SOD1 fusion protein may provide a new strategy for protecting against cardiomyocytes insult resulting from ischemic damage and therefore may provide an opportunity for therapeutic delivery for various diseases related to SOD1 or to OFR.

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