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PEP-1-SOD1 protects brain from ischemic insult following asphyxial cardiac arrest in rats

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Abstract

Aim of the study—Reperfusion following cerebral ischemia leads to overproduction of reactive oxygen species (ROS) and consumption of endogenous antioxidants. Antioxidant enzymes are considered to have beneficial effects against various diseases mediated by ROS. Cu, Zn-superoxide dismutase is one of the major means by which cells counteract the deleterious effects of ROS after ischemia. However, exogenous SOD1 can not be delivered into living cells because of the poor permeability and selectivity of the cell membrane, thus its usage for protecting cells/tissues from oxidative stress damage is greatly limited.

Methods—The purified SOD1 or purified PEP-1-SOD1 fusion proteins were injected into mice via their tail veins, the transduction ability of PEP-1-SOD1 was detected with immunofluorescence method and SOD1 activity. Moreover, we studied whether PEP-1-SOD1 can protect brain from ischemic injury in an experimental asphyxia-cardiac arrest rat model through detecting of the levels of histopathology, MDA, NSE and S100β.

Results—SOD1 protein was detected in PEP-1-SOD1-treated animals and the SOD1 activity levels were significantly increased. However, fluorescence signals were not detected in control SOD1-treated animals. The transduced PEP-1-SOD1 could significantly attenuate cerebral ischemia-reperfusion damage, inhibited ischemia-induced lipid peroxidation, and protected neurons in hippocampus from ischemic damage induced by transient global ischemic insults.

Conclusions—We propose that PEP-1-SOD1 fusion protein has potential use as a therapeutic agent against various disorders mediated by ROS and may be useful as a therapeutic agent for treating transient global cerebral ischemia.

Keywords

Cardiac arrest; cerebral ischemia; cell-penetrating peptide; PEP-1; SOD1

1. Introduction

Reactive oxygen species (ROS) are formed as by-products of normal cellular processes involving interactions with oxygen. Reperfusion following cerebral ischemia leads to overproduction of ROS and consumption of endogenous antioxidants. ROS are excessively

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produced in several disease states; their injurious effects contribute to the pathogenesis of many clinical conditions such as atherosclerosis, myocardial infarction, stroke, ischemia/reperfusion injury, chronic and acute inflammatory conditions such as wound healing, central nervous system disorders such as forms of familial amyotrophic lateral sclerosis (ALS), Parkinson's disease and Alzheimer's dementia.¹ Previous studies have provided evidence for the occurrence of oxidative stress in cerebral ischemia.² Neurons are especially vulnerable to ROS damage due to their high basal metabolic rate and their relatively low levels of antioxidant defense.

Copper, zinc-superoxide dismutase (Cu, Zn-SOD, also called SOD1) provides a defense system against oxidative stress by catalyzing the dismutation of the superoxide anion into oxygen and hydrogen peroxide, which is subsequently detoxified to water by glutathione peroxidase (GPX) or catalase (CAT), thus protecting cells from oxidative damage.³ This ability of neurons to upregulate antioxidant defense suggests that therapeutic means to increase such defenses might be protective. However, exogenous SOD1 can not be delivered into living cells because of the poor permeability and selectivity of the cell membrane, thus its usage 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. Several small regions of proteins, called protein transduction domains (PTD) or cell-penetrating peptides (CPPs), have been developed to allow the delivery of exogenous proteins into living cells. These include carrier peptides derived from HIV-1 TAT protein, *Drosophila* Antennapedia (Antp) protein, and herpes simplex virus VP22 protein.⁴ To increase the biological activity of transduced protein into cells, Morris group has designed a novel peptide carrier, PEP-1 (KETWWETWWTEWSQ PKKKRKV), which consists of three domains: a hydrophobic tryptophan rich motif (KETWWETWWTEW) and a hydrophilic lysine-rich domain (KKKKRKV), connected by a spacer (SQP).⁵ 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, catalase, EGFP, and HSP27.⁵⁻⁸

In a previous study, we have shown that PEP-1-SOD1 fusion protein could be transduced into myocardium in its active form, which protected myocardium from ischemia-reperfusion injury.⁹ In the present study, we observed PEP-1-SOD1 fusion proteins being transduced directly across the blood-brain barrier and efficiently protecting brain against ischemic insult induced by asphyxial cardiac arrest. The data suggest that the PEP-1-SOD1 fusion proteins may be useful as a therapeutic agent for transient global cerebral ischemia.

2. Methods

2.1. Animals

Male Kunming mice (weight 20-23 g) and male Sprague-Dawley rats (weight 250-300 g, Hubei Animal Center, Wuhan, China) were 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 all 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. According to these guidelines, efforts were made to minimize the number of animals and their suffering. Experimental details were provided as per Utstein guidelines.¹⁰

2.2. Generation of biologically active PEP-1-SOD1 fusion proteins

PEP-1-SOD1 fusion proteins were generated as described previously by our laboratory.⁹ Briefly, 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 OD₆₀₀ 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 were purified, clarified cell extracts were loaded onto a Ni²⁺-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 a PD-10 column. The protein concentration was estimated with the Bradford method using bovine serum albumin (BSA, 1 mg/ml) as a standard.

2.3. Transduction of PEP-1-SOD1 into mice

To test the transduction efficiency of PEP-1-SOD1 fusion proteins into cells *in vivo*, Kunming mice were randomly divided into SOD1-treated and PEP-1-SOD1-treated group. 50 µg of purified SOD1 or 50 µg of purified PEP-1-SOD1 fusion proteins were injected into mice via their tail veins, respectively. The animals were anaesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde at designated times (4 and 24 h) after the protein injection (*n*=5 for each group at each time point), brains were immediately removed and postfixed in 4% paraformaldehyde for 4 h. After cryoprotection in 30% sucrose, the brains were frozen and sectioned with a cryostat at 10 µm. For removal of non-specific immunoreactivity, free-floating sections were first incubated with 0.1% Triton X-100 and 10% normal goat serum in PBS for 1 h at room temperature. They were then incubated with a rabbit anti-histidine antibody (diluted 1:200, Santa Cruz Biotechnology, USA) and stored at 4 °C overnight, subsequently exposed to fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (diluted 1:200) at room temperature for 2 h and 4, 6-Diamidino-2-phenylindole (DAPI, Sigma, USA) for 15 min. Finally, the sections were covered with glass cover slips and the immunoreactions were observed under a fluorescent microscope (Nikon, Japan).

Cu, Zn-superoxide dismutase activity was assayed spectrophotometrically by measuring the capacity in inhibiting the reduction of ferricytochrome c by xanthine/xanthine oxidase as described by McCord and Fridovich.¹¹

2.4. *In vivo* experimental groups

Sprague-Dawley rats were randomized into three groups: Sham-operated group (*n*=6): Served as non-ischemic controls. Rats were underwent surgical procedure similar to cardiac arrest (CA) animals, but neither CA nor cardiopulmonary resuscitation (CPR). Vehicle-treated group (*n*=6): Under the same anesthetic conditions and preparations as the sham group, rats were subjected to asphyxial CA for 6 min, reversed by external CPR, mechanical ventilation, and drugs except preconditioning with PEP-1-SOD1. PEP-1-SOD1-treated group (*n*=6): Rats received the similar procedure as described in the vehicle group. To determine whether transduced PEP-1-SOD1 fusion protein protects against ischemic damage, animals were injected with PEP-1-SOD1 (2 mg/kg b.w.) via their tail veins 30 min prior to the asphyxia CA procedure.

2.4.1. Experimental asphyxia-CA model—Asphyxial CA and resuscitation protocol was performed as modified from Hendrickx et al.¹² Rats were fasted overnight except for free access to water. Anesthesia was initiated by intraperitoneal injection of 35 mg/kg pentobarbital sodium and supplemented with additional hourly doses of 10 mg/kg. Animals were placed on a surgical board in a supine position with extremities immobilized. A tracheotomy was performed and an intubating cannula mounted on a blunt needle was connected to a rodent ventilator (Chengdu TME Technology Co, Ltd., China).

Two intramedic catheters (PE 50) were advanced through the surgically exposed left femoral artery and vein into the abdominal aorta and into the inferior vena cava for measurements of arterial pressures and for arterial and venous blood sampling. Electrocardiographic leads were attached to the limbs. Mean arterial blood pressure and electrocardiogram were continuously monitored. The head and body temperatures were maintained at 37 ± 0.5 °C using heating lamps.

Animals were then allowed to stabilize for at least 15 min before baseline. Five minutes before induction of asphyxial CA, rats received a dose of the short-acting muscle relaxant vecuronium (2 mg/kg b.w.) to prevent spontaneous breathing during asphyxia. Asphyxia was started by the interruption of controlled ventilation and clamping of the tracheal cannula at end-exhalation. CA was defined as a systolic aortic blood pressure decline to less than 25 mmHg. After 6 min of apnea, resuscitation was started by ventilator reconnection (100% O₂, 80 breaths/min), manual precordial compressions (200 compressions/min), intravenous bolus administration of epinephrine (0.02 mg/kg) and sodium bicarbonate (1 mEq/kg). Depth of compression was adjusted to decrease the anteroposterior diameter of the chest by 30%. Successful resuscitation (return of spontaneous circulation, ROSC) was defined as maintenance of a systolic aortic blood pressure of at least 60 mmHg for interval exceeding 10 consecutive minutes. If ROSC could not be achieved within 5 min, resuscitation procedures were stopped.

Hemodynamic measurements and mechanical ventilation after ROSC were continued for 3 h. The endotracheal tube and catheters were then removed. After recovery from anesthesia, animals were returned to their cages. At the end of 72 h of reperfusion, the animals were euthanized by anesthesia overdose.

2.4.2. Histopathology—At the end of 72 h of reperfusion, the animals were euthanized, transcardially perfusion. The brains were then removed and coronal brain blocks were embedded in paraffin, 10 µm coronal sections were cut and stained with hematoxylin and eosin (H&E). The entire hippocampus (anterior to posterior) was examined. Normal neuronal counts were made within the CA1 region of hippocampus at the level of 3.8 mm from posterior to bregma. Neurons exhibiting ischemic cell change were identified as: eosinophilic cytoplasm, dark-staining triangular shaped nuclei, and eosinophilicstaining nucleolus. Viable and nonviable neurons were counted manually, and the percentage of viable neurons was calculated for each area of interest.

2.4.3. Measurement of lipid peroxidation—Lipid peroxidation was measured as described previously.¹³ An aliquot (100 µl) of hippocampus supernatant was added to a reaction mixture containing 100 µl of 8.1% SDS, 750 µl of 20% acetic acid (pH 3.5), 750 µl of 0.8% thiobarbituric acid and 300 µl distilled water. Samples were then boiled for 1 h (95 °C) and centrifuged at 4000 g for 10 min. The absorbance of the supernatant was measured by a spectrophotometer at 532 nm.

2.4.4. Serum neuron-specific enolase (NSE) and S100β detection—Blood samples for NSE and S100β were collected from the femoral vein at 72 h after ROSC. Blood

was allowed to clot for 20 to 30 min at room temperature and then centrifuged and frozen to $\leq -18^{\circ}\text{C}$. Samples that showed visible hemolysis were not analyzed. Serum NSE and S100 β were quantified with an enzyme-linked immunosorbent assay (R&D, USA) according to the manufacturer's instructions.

2.5. Statistical analysis

Statistical analysis was performed with SPSS13.0 program. All data were expressed as mean $\pm$ SD. Measurements among groups were compared by analysis of variance (ANOVA), followed by Student-Newman-Keuls post hoc test. A *P* value of less than 0.05 was regarded as significant.

3. Results

3.1. PEP-1-SOD1 fusion protein can pass through blood-brain barrier and maintain enzyme activity

Our previous studies have shown that the PEP-1-SOD1 fusion protein was effectively delivered into cultured endothelial cells of the human umbilical vein and rat myocardium and have protected cardiomyocyte damage induced by ischemia-reperfusion injury.^{9,14} To determine whether the PEP-1-SOD1 fusion protein can pass through the blood-brain barrier, we performed immunostaining on brain sections of PEP-1-SOD1-treated and SOD1-treated mice. As shown in Fig. 1, SOD1 protein was detected in PEP-1-SOD1-treated animals and the SOD1 activity levels were significantly increased. However, fluorescence signals were not detected in control SOD1-treated animals. These results indicate that PEP-1-SOD1 fusion protein was efficiently transduced across the blood-brain barrier. We then examined the ability of PEP-1-SOD1 fusion protein in crossing the blood-brain barrier and transducing into mouse hippocampus. As shown in Fig. 2, the enzymatic activities of transduced PEP-1-SOD1 were more than 3-fold higher than those of control SOD1. These results demonstrate that PEP-1-SOD1 has potential use as a therapeutic agent against various disorders mediated by ROS.

3.2. Transduced PEP-1-SOD1 inhibits lipid peroxidation

Lipid peroxidation appears to be involved in the genesis of the post-ischemic hypoperfusion phenomenon, which is inhibited by SOD and deferoxamine.¹⁵ There is evidence implicating membrane damage by reperfusion-induced lipid peroxidation in both histological damage to neurons and in the genesis of the post-ischemic hypoperfusion syndrome.¹⁶ Therefore, we determined whether PEP-1-SOD1 inhibits ischemia-induced lipid peroxidation by measuring levels of malondialdehyde (MDA), a marker of lipid peroxidation, in the hippocampus. The level of MDA following ischemia-reperfusion injury was significantly increased. However, the PEP-1-SOD1-treated group showed a significant decrease in hippocampus MDA levels compared with the vehicle-treated group, which was not significantly different from normal controls (Fig. 3).

3.3. Transduced PEP-1-SOD1 protects brain against ischemic damage

To directly observe the protective effect of PEP-1-SOD1 against brain damage caused by ischemic injury, a histological assessment of neuronal death in the CA1 region of the hippocampus was performed 72 h after induction of transient global cerebral ischemia. In the vehicle-treated group, the percentage of viable neurons was significantly reduced compared to the sham-operated group. In the PEP-1-SOD1-treated groups, neuronal cell viability was increased significantly (Fig. 4).

S100 β is a calcium-binding protein mainly secreted from glial cells and Schwann cells.¹⁷ This protein has putative intra- and extracellular functions. Intracellular roles include

regulation of protein phosphorylation, serving cytoskeleton components or transcriptional factors. Extracellular S100 β plays a trophic role on neuronal and glial cells. S100 β level in peripheral blood samples is reportedly elevated in patients with various central nervous system disorders like ischemic stroke, subarachnoid hemorrhage, and traumatic brain injury.¹⁸ Serum S100 β levels have been used as markers of brain insult.¹⁹

NSE is a neuronal form of cytoplasmic glycolytic enzyme enolase. It is a dimeric enzyme composed of two γ subunits ($\gamma\gamma$ isomer). It is mainly located in neurons and neuroendocrine cells. Neuronal damage and impairment of the blood-brain barrier integrity can be detected by the release of NSE into cerebrospinal fluid (CSF) and eventually into the blood. Serum NSE levels have been reported after stroke, brain injury, and CA.²⁰⁻²² To further determine the protective role of PEP-1-SOD1 in brain damage, we detected serum S100 β and NSE levels after the ischemic injury. As shown in Fig. 5, serum S100 β and NSE levels were elevated significantly after cardiac arrest as compared to the sham-operated group. PEP-1-SOD1 treatment, however, significantly decreased the NSE and S-100 β levels compared to the vehicle-treated group (Fig. 5). These data demonstrate that transduced PEP-1-SOD1 proteins can effectively protect brain against ischemic damage.

4. Discussion

Cardiac arrest and resuscitation produce global ischemia and reperfusion damage to the brain, which lead to high mortality and delayed neuronal death. Despite advancement in cardiopulmonary resuscitation (CPR) techniques, survival rates have not improved for decades, a majority die within 72 h.²³ Among survivors, neurological complications represent the leading cause of morbidity and disability. Considerable evidence indicates that ROS, such as superoxide anion and hydrogen peroxide, mediate many of the pathophysiological events that cause several diseases.²⁴ Neurons, in particular, are vulnerable to ROS damage due to their relatively low levels of endogenous antioxidants. A well-established fact is that neurons continue to die over days and months after ischemia. Thus, development of novel therapies to protect the brain from the ravages of cerebral ischemia is a key to improvement in survival and better outcomes following such a devastating condition.

SOD catalyzes the conversion of the highly reactive superoxide anion to oxygen and to hydrogen peroxide, which is subsequently detoxified to water by GPX or CAT. Necrotic insults, which place neurons under oxidative stress, can cause a compensatory increase in expression of GPX or CAT.²⁵⁻²⁷ In contrast, there is little evidence for SOD being upregulated under similar conditions. Further, ROS are known to be present inside cells as well as in the extracellular spaces. Therefore, it is essential that anti-oxidant enzymes permeate across cell membrane for greater efficacy.²⁸ Although exogenous supplementation of antioxidant enzymes has demonstrated therapeutic potential in minimizing the ROS-associated damage, the inability of antioxidant enzymes to transduce into cells has limited their use in protecting cells/tissues from oxidative damage.²⁹ Many techniques such as endocytosis, electroporation, microinjection, liposome- and viral-based delivery system have been designed to circumvent these limitations, but there are still disadvantages when put them into clinical practice.³⁰ Using either mice or rats overexpressing SOD1 or SOD1-knockout mice, has been shown that SOD1 exerts neuroprotection in models of traumatic brain injury and transient global or focal cerebral ischemia.³¹ Increasing endogenous expression of SOD1 through gene therapy provides neuroprotection following experimental stroke, including post-ischemic protection. However, the invasive method of vector delivery and the kinetics of vector expression may limit application of this technique in the clinical setting.³²

5. Conclusions

In this report, we demonstrate that human SOD1 fused with PEP-1 peptide can efficiently pass through blood-brain barrier in its native conformation. Most importantly, treatment with PEP-1-SOD1 fusion proteins has provided neuroprotection following experimental cardiac arrest and resuscitation. Although the detailed mechanism remains to be further elucidated, our results provide a foundation for a new strategy for protecting neurons against cell destruction resulting from ischemic damage and therefore provide an opportunity for therapeutic intervention using antioxidant enzymes for the treatment of ROS-mediated neuronal diseases.

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Fig 1. Transduction of PEP-1-SOD1 fusion protein across the blood-brain barrier

Transduction of PEP-1-SOD1 fusion protein into mouse brain was analyzed by immunostaining using an antibody against His-tag. a-c: Brain sections from mice treated with control SOD1 for 4 h. d-i: Brain sections from mice treated with PEP-1-SOD1 fusion protein for 4 h (d-e) and 24 h (g-i) . Green fluorescent signals represent FITC-labeled anti-His-probe of SOD1; Blue fluorescent signals represent DAPI-labeled nucleus.

Fig 2. Activity of PEP-1-SOD1 fusion protein transduced into mouse brain
50 μ g of purified SOD1 or PEP-1-SOD1 fusion proteins were administered in mice via their tail veins. Transduction efficiencies were analyzed by measuring specific enzyme activities of SOD1 in brain tissue with xanthine/xanthine oxidase method as described in Materials and Methods. * $P < 0.05$ and ** $P < 0.01$ vs SOD1 group.

Fig 3. Effects of transduced PEP-1-SOD1 fusion protein on MDA content in hippocampus after ischemic insult

Treatment with PEP-1-SOD1 could efficiently reduce the MDA levels after ROSC. * $P < 0.01$ vs sham group; # $P < 0.01$ vs vehicle group.

Fig 4. Effects of transduced PEP-1-SOD1 fusion protein on neuronal cell viability after ischemic insult

(A) Representative histological images of hippocampal CA1 region 72 h after ischemic insults (H&E staining). All images were captured at 200× magnification. (B) Numbers of normal neurons in the CA1 region of the rat hippocampus. * $P < 0.01$ vs sham group; # $P < 0.01$ vs vehicle group.

Fig 5. Effects of transduced PEP-1-SOD1 fusion protein on serum S100β and NSE levels
 S100β and NSE levels were measured as described in Materials and Methods. *P<0.05 and **P<0.01 vs sham group; #P<0.01 vs vehicle group.