European Journal of Pharmacology 666 (2011) 87–92

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Neuropharmacology and Analgesia

Neuroprotective effect of Cu,Zn-superoxide dismutase fused to a TCTP-derived protein transduction domain

Jisun Lee, Sabin Kim, Dong Hae Shin, Hwa-Jung Kim, Kyunglim Lee *

College of Pharmacy, Center for Cell Signaling & Drug Discovery Research, Ewha Womans University, Seoul, Korea

ARTICLE INFO

Article history:
Received 7 September 2010
Received in revised form 16 May 2011
Accepted 22 May 2011
Available online 31 May 2011

Keywords:
Kainic acid
Paraquat
Protein transduction domain
Superoxide dismutase
Translationally controlled tumor protein

ABSTRACT

Previously, we have reported that a 10-amino acid peptide (MIIYRDLISH) derived from the NH_2-terminus of the human translationally controlled tumor protein (TCTP) functions as a protein transduction domain (PTD). In this study, we evaluated the transduction ability of SOD fused to TCTP-PTD (TCTP-SOD) into various cell lines. We also evaluated its ability to protect cells against paraquat-induced cell damage, in vitro and its neuroprotective effect in vivo against kainic acid-induced neuronal damage in an animal model. TCTP-SOD was transduced into various cell lines in a dose- and time-dependent manner without cytotoxic effect. Furthermore, TCTP-SOD showed cytoprotective activity in SH-SY5Y cells, and intraperitoneally, injected TCTP-SOD was delivered into the mouse brain and protected the cells in the hippocampal region against the damage induced by kainic acid. We propose TCTP-SOD as a potential candidate drug for treatment of brain diseases.

1. Introduction

Oxygen, which sustains life can also be life threatening when it becomes part of excessively produced reactive oxygen species. Under normal conditions, body's antioxidant defense mechanisms scavenge those molecules, but become impaired, if excessive oxidative stress is produced. Inefficient elimination of oxidative stress causes impaired cellular function. Since brain consumes approximately 20% of the total body oxygen, there is a direct correlation between oxidative damage and brain diseases (Barnham et al., 2004). When a ligand such as kainic acid, binds to the glutamate receptor, voltage-sensitive calcium channels in the membrane are activated and lead to influx of calcium into the cytoplasmic compartment of the cell. Increased calcium level in the brain activates enzymes that generate free radicals (Coyle and Puttfarcken, 1993), which in turn results in neuronal damage. Therefore, maintaining antioxidant defense mechanisms such as the enzyme, superoxide dismutase (SOD), which plays a major role as a first line of antioxidant defense mechanism are essential to life. However, SOD protein is not commonly used in clinical medicine because of its short half life in vivo, limited ability to cross the blood-brain barrier (BBB), among other pharmacological deficits (McCord and Edeas, 2005; Reddy and Labhasetwar, 2009). Availability of approaches that improve the ability of SOD to cross blood brain barrier can improve the potential usefulness of SOD in the therapy of brain diseases. Protein transduction domain (PTD) which can deliver therapeutic agents across BBB (Schwarze et al., 1999), has been suggested as a useful tool to treat brain diseases. Asoh and Ohta applied PTD technology to deliver SOD derivatives such as Cu,Zn-SOD into the brain (Asoh and Ohta, 2008).

PTD is a small peptide consisting of 10 to 16 amino acids with the ability to traverse biological membrane (Snyder and Dowdy, 2004). The first reported PTD which was able to cross the cell membrane and transactivate a viral genome was derived from human immnodeficiency virus (HIV) Tat protein (Denicourt and Dowdy, 2003). A subsequent study showed that the specific domain that possessed the ability to cross the cell membrane is TAT 48–60 (Fawell et al., 1994; Frankel and Pabo, 1988) and TAT 48–60 is the most widely studied PTD. Discovery of PTDs has opened up new strategies to deliver therapeutic agents to various cell lines and tissues.

Previously, we reported that a 10-amino acid peptide (MIIYRDLISH) derived from the NH2-terminus of human translationally controlled tumor protein (TCTP) functions as a PTD (Kim, 2011). In this study, we examined the transduction ability of TCTP-SOD into various cell lines, its ability to protect cells against paraquat-induced cell damage, in vitro and its neuroprotective effect in vivo against kainic acid-induced neuronal damage.

2. Materials and Methods

2.1. Construction and preparation of SOD, TAT-SOD, and TCTP-SOD

Cu,Zn-SOD sequence was amplified from the human Cu,Zn-SOD containing pET-17b vector (Lab Frontier, Korea), using sense 5'-CCG CTC GAG(*Xho I*) GCG ACG AAG GCC GTG TGC GTG-3' and the antisense 5'-CG GGA TCC(*BamH I*) TTA TTG GGC GAT CCC AAT TAC-3', and then subcloned into pET-15b expression vector which contains six His tag sequence (Kwon et al., 2000). PTD sequence from TAT or TCTP was

* Corresponding author. Tel.: +82 2 32773024; fax: +82 2 32772851.
E-mail address: klyoon@ewha.ac.kr (K. Lee).

0014-2999/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2011.05.040

inserted into the amino terminus of human Cu,Zn–SOD containing pET-15b vector. Primers used in this reaction are sense 5'-T AGG (*Nde I*) AAG AAG CGG AGA CAG CGA CGA AGA C (*Xho I*) -3', and the antisense, 5'-TC GAG (*Xho I*) TCT TCG TCG CTG TCT CCG CTT CTT CC (Nde *I*) -3' (for insertion of TAT-PTD): sense 5'-T ATG (*Nde I*) CTC ATC ATC TTC CGG ATC CTG ATC AGC CAC CAC CAC C (*Xho I*)-3', and the antisense, 5'-TC GAG (*Xho I*) GTG GTG GTG GCT GAT CAG GAT CCG GAA GAT GAT GAG CA (*Nde I*) -3' (for insertion of TCTP-PTD). The plasmids were transformed into E.coli BL21 (DE3), and the expressed proteins were purified by metal-affinity chromatography using TALON® Metal affinity Resin (Clontech, CA, USA). After replacing buffer with PBS using PD-10 column chromatography to remove salts, protein concentrations were determined according to Bradford assay.

2.2. In vitro experiments

2.2.1. Cell culture

HeLa, HaCaT, and SH-SY5Y cells were maintained in Dulbecco's minimal essential media (DMEM)/high glucose containing 20 mM HEPES/NaOH (pH 7.4), $NaHCO_3$, 10% fetal bovine serum (FBS), and antibiotics (100 U/ml streptomycin, 100 U/ml penicillin) at 37°C under a humidified conditions of 95% air and 5% CO_2.

2.2.2. Western blotting

Cells treated with each protein, were washed twice with PBS, twice with trypsin-EDTA and then incubated at 37°C for 10 min. Subsequently, the cells were lysed in sample buffer containing 1 mM 4-(2-Amino-ethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma, MO, USA), 50 mM Tris pH 6.8, 2.5% SDS, 22% glycerol, and 5% β-mercaptoethanol at room temperature for 20 min. The resulting cell extracts were heated at 100°C for 10 min and separated on a 15% SDS-PAGE gel. Then, the gel was electrophoretically transferred to a nitrocellulose membrane (Whatman, Germany), and after blocking with 3% non-fat dry milk, the transduced proteins were identified using polyclonal anti-human Cu,Zn-SOD antibody (Cell Signaling Technology, MA, USA; dilution 1:1,000) in TTBS containing 5% BSA. As a loading control, actin was identified by monoclonal anti-β-actin antibody (Sigma, MO, USA; dilution 1:5,000) in TTBS containing 3% BSA. Then the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody and visualized by enhanced chemiluminescence (ECL plus, Amersham Biosciences, Germany).

2.2.3. CCK-8 assay

Cellular viability was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacture's protocol. Briefly, cells were seeded into 96-well plate and then treated with various concentrations of SOD, TAT-SOD, or TCTP-SOD in serum-free media followed by assay reagent. After incubation for 2.5 h, absorbance was measured at 450 nm.

2.3. In vivo experiments

2.3.1. Animals and treatment

Male ICR mice (26~30 g) were housed under a 12:12 h light/dark cycle with free access to food and water. 500 μg of SOD, TAT-SOD or TCTP-SOD in 500 μl PBS were injected intraperitoneally (i.p.) twice at an interval of 18 hours. Equal volume of normal saline was injected as a negative control. 6 h after the last injection, 0.3 μg of kainic acid (Cayman, U.S.A.) dissolved in 3 μl normal saline was administered (i.c.v.) at the hippocampal region using 10 μL Hamilton microsyringe (Hamilton Company, U.S.A.) fitted with a 30-gauge needle that was inserted to a depth of 2 mm. All animals were killed 3 days after the kainic acid injection, and their brains were dissected. For the examination of the transduction ability into the brain, 250 μg of SOD, TAT-SOD or TCTP-SOD in 500 μl PBS were injected intraperitoneally (i.p.) twice at an interval of 18 h and 6 h after the last injection, the mice were killed and the brains were dissected.

2.3.2. Cresyl violet staining and immunohistochemistry

Mice were anesthetized by intraperitoneal injection of Zoletil (Virbac, Carros, France) and Rompun (Bayer) complex and transcardially perfused with PBS-heparin (1 U/ml), followed by pre-chilled 4% paraformaldehyde in PBS. The brains were incubated in 4% paraformaldehyde, 30% sucrose at 4°C, and then cut into 30 μm coronal slices with a cryostat. The sections were then mounted on poly-l-lysine-coated microscope slides and air dried. Slides were incubated in 95% ethanol for 15 min to remove lipids and fixation chemicals, dehydrated (1 min) in 70% and 50% ethanol baths. Dehydrated slides were submerged in 0.5% cresyl violet (Sigma, U.S.A.) solution for 6 min, then washed by soaking in distilled water. Washed slides were dehydrated in a series of alcohol bath: 50% ethanol, 70% glacier acid ethanol, 95% ethanol, and 100% ethanol for 1 min each. The unstained parts of the tissue remained transparent following incubation in xylene for 5 min. After finishing the staining step, sectioned tissues were covered with coverglass using VectaMount Mounting Medium (Vector, U.S.A.) and then examined by light microscopy. Immunoreactivity was determined using the rabbit ABC staining system (Santa Cruz Biotechnology, USA) according to the manufacturer's protocol. Briefly, cryosections were incubated in 1.5 % blocking serum for 1 h and then incubated in polyclonal anti-histidine antibody (Santa Cruz Biotechnology, USA; dilution 1:100) overnight at 4°C. After incubation in primary antibody, sections were incubated in biotinylated secondary antibody for 1 h and subsequently with AB enzyme reagent for 30 min. Finally, sections were incubated with peroxidase substrate until the desired stain intensity developed. After covering with coverglass using mounting solution, the sections were examined by light microscopy.

2.4. Statistical Analysis

Statistical analysis was done by regression analysis for the significance of the dose- and time-dependence of the transduction levels or ANOVA followed by a post hoc test for group comparisons. $P<0.05$ was considered statistically significant. Data were expressed as means ± S.D.

3. Results

3.1. Transduction of SOD, TAT-SOD, and TCTP-SOD proteins into various cell lines

To evaluate the transduction ability of TCTP-SOD into cells (HeLa, HaCaT, and SH-SY5Y cells), 5 μM of each protein under denaturing conditions was incubated in serum-free media for 6 and 24 h with each of the cell lines: SOD and TAT-SOD were used as a negative and positive controls respectively.

With IPTG induction, target proteins were expressed as a major component of total soluble proteins (data not shown). The purified proteins were analyzed by 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue, and were further confirmed by Western blotting using polyclonal antibody to a human Cu,Zn-SOD (Fig. 1B). Calculated molecular weight of SOD, TAT-SOD, and TCTP-SOD was 18.34, 19.53, and 19.45 kDa respectively; however, each protein was detected at a higher molecular weight position than expected.

As shown in Fig. 1C, TAT-SOD and TCTP-SOD were successfully delivered into all three cell lines and the detected molecular weight position of TAT-SOD and TCTP-SOD was consistent with the position in Fig. 1B. Compared to 6 h treatment, TCTP-SOD transduction at 24 h was greater, suggesting that TCTP-SOD transduction requires longer time than TAT-SOD transduction.