Free Radical Biology and Medicine 67 (2014) 195–210

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contributions

# Tat-glyoxalase protein inhibits against ischemic neuronal cell damage and ameliorates ischemic injury

Min Jea Shin [a,1], Dae Won Kim [b,1], Yeom Pyo Lee [a], Eun Hee Ahn [a], Hyo Sang Jo [a], Duk-Soo Kim [c], Oh-Shin Kwon [d], Tae-Cheon Kang [e], Yong-Jun Cho [f], Jinseu Park [a], Won Sik Eum [a,*], Soo Young Choi [a,*]

[a] Department of Biomedical Sciences and Research Institute of Bioscience and Biotechnology, Hallym University, Chunchon 200-702, Korea
[b] Department of Biochemistry and Molecular Biology, Research Institute of Oral Sciences, College of Dentistry, Kangnung-Wonju National University, Gangneung 210-702, Korea
[c] Department of Anatomy, College of Medicine, Soonchunhyang University, Cheonan-si 330-090, Korea
[d] School of Life Sciences, College of Natural Sciences, Kyungpook National University, Taegu 702-702, Republic of Korea
[e] Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chunchon, Kangwon-Do 200-702, Republic of Korea
[f] Department of Neurosurgery, College of Medicine, Hallym University, Chunchon, Kangwon-Do 200-702, Republic of Korea

ARTICLE INFO

*Article history:*
Received 8 July 2013
Received in revised form
8 October 2013
Accepted 18 October 2013
Available online 16 November 2013

*Keywords:*
Methylglyoxal (MG)
Glyoxalase (GLO)
Oxidative stress
Protein therapy
Ischemic damage

ABSTRACT

Methylglyoxal (MG), a metabolite of glucose, is the major precursor of protein glycation and induces apoptosis. MG is associated with neurodegeneration, including oxidative stress and impaired glucose metabolism, and is efficiently metabolized to *S*-D-lactoylglutathione by glyoxalase (GLO). Although GLO has been implicated as being crucial in various diseases including ischemia, its detailed functions remain unclear. Therefore, we investigated the protective effect of GLO (GLO1 and GLO2) in neuronal cells and an animal ischemia model using Tat-GLO proteins. Purified Tat-GLO protein efficiently transduced into HT-22 neuronal cells and protected cells against MG- and $H_2O_2$-induced cell death, DNA fragmentation, and activation of caspase-3 and mitogen-activated protein kinase. In addition, transduced Tat-GLO protein increased D-lactate in MG- and $H_2O_2$-treated cells whereas glycation end products (AGE) and MG levels were significantly reduced in the same cells. Gerbils treated with Tat-GLO proteins displayed delayed neuronal cell death in the CA1 region of the hippocampus compared with a control. Furthermore, the combined neuroprotective effects of Tat-GLO1 and Tat-GLO2 proteins against ischemic damage were significantly higher than those of each individual protein. Those results demonstrate that transduced Tat-GLO protein protects neuronal cells by inhibiting MG- and $H_2O_2$-mediated cytotoxicity *in vitro* and *in vivo*. Therefore, we suggest that Tat-GLO proteins could be useful as a therapeutic agent for various human diseases related to oxidative stress including brain diseases.



## Introduction

Methylglyoxal (MG) is a highly reactive carbonyl compound derived from oxidative and nonoxidative reactions, increases in which lead to oxidative stress and tissue damage [1]. MG induces protein glycation, leading to the formation of AGE, which play important roles in the pathogenesis of aging as well as diseases including diabetes and neurodegeneration [2–4]. MG is efficiently detoxified by the glyoxalase system. In this system, glyoxalase 1 (GLO1) metabolizes MG to *S*-$_D$-lactoylglutathione, and GLO2 converts *S*-$_D$-lactoylglutathione to D-lactate [4]. Several studies have shown that the overexpression of GLO1 lessens the effects of various disorders such as diabetes, hyperglycemia, and Alzheimer's disease, as well as aging [5–8].

Oxidative stress in neuronal cells is one of the major causes of neurodegenerative diseases and is due to the production of highly reactive oxygen species (ROS). Oxidative stress also plays an important role in the pathological processes of various human diseases including ischemic injury [9–15]. Therefore, antioxidant enzymes such as CuZn-SOD and catalase are well known for their protective effects against oxidative stress and ischemic injury

*Abbreviations:* AGE, glycation end products; CV, cresyl violet; DAPI, 4′,6-diamidino-2-phenylindole; DCF-DA, 2′,7′-dichlorofluorescein diacetate; F-JB, Fluoro-Jade B; GLO, glyoxalase; IPTG, isopropyl-β-D-thiogalactoside; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MG, methylglyoxal; PBS, phosphate-buffered saline; PTDs, protein transduction domains; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfated-polyacrylamide gel electrophoresis

* Corresponding authors. Fax: +82 33 241 1463.
*E-mail addresses:* wseum@hallym.ac.kr (W.S. Eum), sychoi@hallym.ac.kr (S.Y. Choi).

[1] These authors contributed equally to this work.

0891-5849/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2013.10.815

[15,16]. However, the contribution of MG toxicity and the exact mechanisms of neuronal damage in ischemia remain unclear. Also, the neuroprotective effects of GLO proteins in ischemia are not yet well documented.

Although antioxidant enzymes play a pharmacologic role in cells, the therapeutic application of proteins is very difficult owing to low delivery efficiency [17]. Protein transduction technology is a commonly used method for delivering exogenous proteins into living cells and tissues via protein transduction domains (PTDs). Among the various PTD peptides, Tat peptide is well known for its ability to deliver exogenous proteins into cells and tissues and has been used for a number of clinical applications [18–20]. In previous studies, we have shown that various PTD fusion proteins efficiently transduced into cells and tissues, where they protected against cell damage *in vitro* and *in vivo* [21–30].

In this study, we investigated the protective effects of Tat-GLO proteins in MG- and $H_2O_2$-induced HT-22 neuronal cell death and *in vivo* in an animal model of ischemia. Tat-GLO proteins directly transduced into HT-22 neuronal cells, where they protected against cell death. In addition, the neuroprotective effects of Tat-GLO proteins against ischemic damage were evident *in vivo*. Therefore, we suggest that Tat-GLO proteins could be a potential therapeutic agent for the treatment of transient forebrain ischemia.

## Materials and methods

### *Materials*

MG was purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies and actin were obtained from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. $Ni^{2+}$-nitrilotriacetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, USA). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa (Budapest, Hungary). Plasmid pET-15b and *Escherichia coli* strain BL21 (DE3) were obtained from Novagen (La Jolla, CA, USA). Human GLO1 and GLO2 cDNA were isolated using the polymerase chain reaction (PCR) technique. The GLO1 mutant (M157A) and GLO2 mutant (Y175F) cDNA were obtained from Bioneer (Daejeon, Korea). A D-lactate colorimetric assay kit was obtained from BioVison (Milpitas, CA, USA). AGE ELISA and MG ELISA kits were purchased from Cell BioLabs (San Diego, CA, USA). All other chemicals and reagents, unless otherwise stated, were of the highest analytical grade available.

### *Purification of Tat-GLO fusion proteins*

In a previous study, we demonstrated Tat-GLO protein construction and purification [30]. Briefly, Tat-GLO proteins were constructed using the following sense and antisense primers: wild-type GLO1 and mutant GLO1 (M157A) sense primers, 5′-CTCGAGATGGCAGAACCGCAGCCCCCGTCC-3′; wild-type GLO1 and mutant GLO1 (M157A) antisense primer, 5′-GGATCCCTACATTAAGGTTGCCATTTTGTT-3′; wild-type GLO2 and mutant GLO2 (Y175F) sense primers, 5′-CTCGAGATGA AGGTAGAGGTGCT;GCCTGCC-3′; wild-type GLO2 and mutant GLO2 (Y175F) antisense primer, 5′-GGATCCTCAGTCCCGGGGCATCTTGAACTG-3′. After Tat-GLO proteins were overexpressed by 0.5 mM IPTG for 4 h, they were purified using a $Ni^{2+}$-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. The Bradford procedure was used to estimate protein concentration using bovine serum albumin as a standard [31].

### *Cell culture and transduction of Tat-GLO proteins*

Mouse hippocampal HT-22 cells were cultured in Dulbecco's minimum essential medium (DMEM; Lonza BioWhittaker, MD, USA) containing 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37 °C under humidified conditions of 95% air and 5% $CO_2$.

For transduction of Tat-GLO protein, cells were grown on a six-well plate for 12 h, after which they were exposed to various concentrations (0.5–3 μM) of Tat-GLO proteins over various durations (10–120 min). The cells were treated with trypsin-EDTA, washed with phosphate-buffered saline (PBS), and harvested for the preparation of cell extracts to perform Western blot analysis.

### *Western blot analysis*

The equal cell lysates were separated by 12% sodium dodecyl sulfated-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. After blocking with 5% nonfat dry milk in TBS buffer including 0.1% Tween 20 (TBST), the membrane was immunoblotted with the indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The protein bands were detected with enhanced chemiluminescent reagents [25,26].

### *Fluorescence microscopic analysis*

Transduced protein distribution fluorescence was performed as described previously [22,23]. Briefly, the cells were grown on coverslips and treated with 3 μM Tat-GLO protein for 2 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. After the cells were permeabilized, blocked, and washed, they were exposed to the primary antibody (His-probe, 1:2000; Santa Cruz Biotechnology) for 1 h at room temperature. The secondary antibody (Alexa-Fluor 488, 1:15000; Invitrogen) was applied for 45 min at room temperature in the dark. Nuclei were stained for 5 min with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland). The distribution of fluorescence was analyzed by confocal microscopy using a Model FV-300 microscope (Olympus, Tokyo, Japan).

### *Cell viability assay*

The biological activity of transduced Tat-GLO proteins was assessed by measuring the cell viability of HT-22 cells treated with MG and $H_2O_2$. A colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) assay was performed as described previously [21–23]. The cells were pretreated with Tat-GLO protein (0.5–3 μM) for 2 h before MG (1 mM) and $H_2O_2$ (0.65 mM) was added to the culture medium for 12 h. Cell viability was defined as the percentage of untreated control cells.

### *Measurement of D-lactate, AGE, and MG*

The cells ($1 \times 10^6$) were pretreated with Tat-GLO proteins (3 μM) for 2 h, after which they were treated with MG (1 mM) and $H_2O_2$ (0.65 mM) for 6 h. Then, the D-lactate assay was performed by using a D-lactate colorimetric assay kit (BioVison) as per the manufacturer's instructions. AGE and MG levels were analyzed using a AGE and MG enzyme-linked immunosorbent assay ELISA kit (Cell BioLabs) according to the manufacturer's instruction.