Supplementary Information

Transduced Tat-CIAPIN1 reduces the inflammatory response on LPS- and TPA-induced damages

Hyeon Ji Yeo[1,§], Min Jea Shin[1,§], Ji Ho You[1], Jeong Su Kim[1], Min Young Kim[1], Dae Won Kim[2], Duk-Soo Kim[3], Won Sik Eum[1,*], Soo Young Choi[1,*]

[1]Department of Biomedical Science and Research Institute of Bioscience and Biotechnology, Hallym University, Chuncheon 24252, Korea. [2]Department of Biochemistry and Molecular Biology, Research Institute of Oral Sciences, College of Dentistry, Gangneung-Wonju National University, Gangneung 25457, Korea. [3]Department of Anatomy, College of Medicine, Soonchunhyang University, Cheonan-Si 31538, Korea.

FIGURES

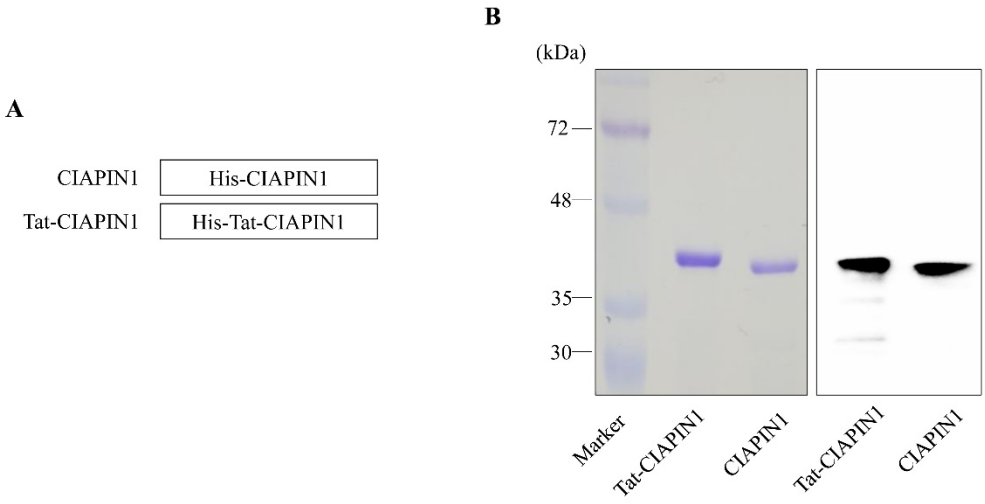

Fig. S1

Fig. S1. Purification of Tat-CIAPIN1 protein. Diagrams of the expressed Tat-CIAPIN1 and CIAPIN1 proteins (A). Purified Tat-CIAPIN1 and CIAPIN1 proteins were identified by 12% SDS-PAGE and detected by Western blotting (B).

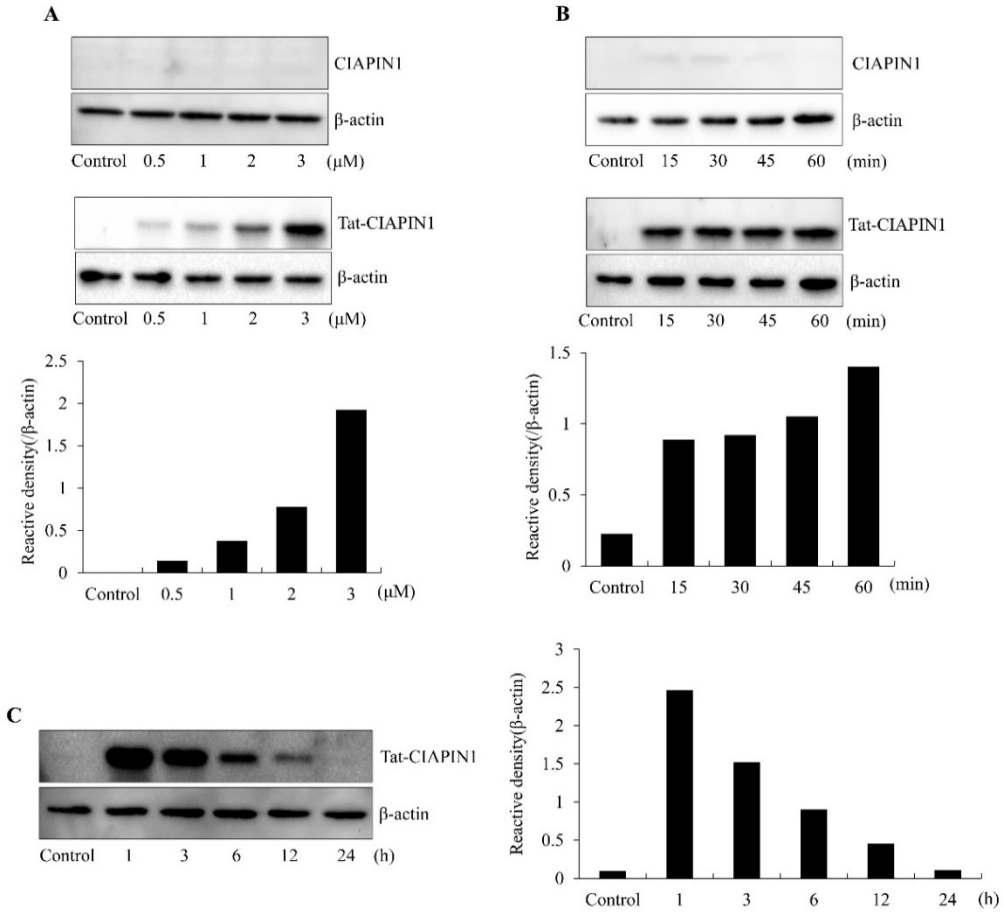

Fig. S2

Fig. S2. Transduction of Tat-CIAPIN1 proteins into Raw 264.7 cells. The cells were treated with Tat-CIAPIN1 protein at different doses (0.5-3 μM) or CIAPIN1 protein for 1 h (A). The cells were treated with Tat-CIAPIN1 protein (3 μM) or CIAPIN1 protein for different time periods (15-60 min) (B). Intracellular stability of transduced Tat-CIAPIN1 protein. The cell culture media were incubated for 24 h after transduction of Tat-CIAPIN1 protein for 1 h (C). Then, transduction of Tat-CIAPIN1 protein was measured by Western blotting and the intensity of the bands was measured by a densitometer.

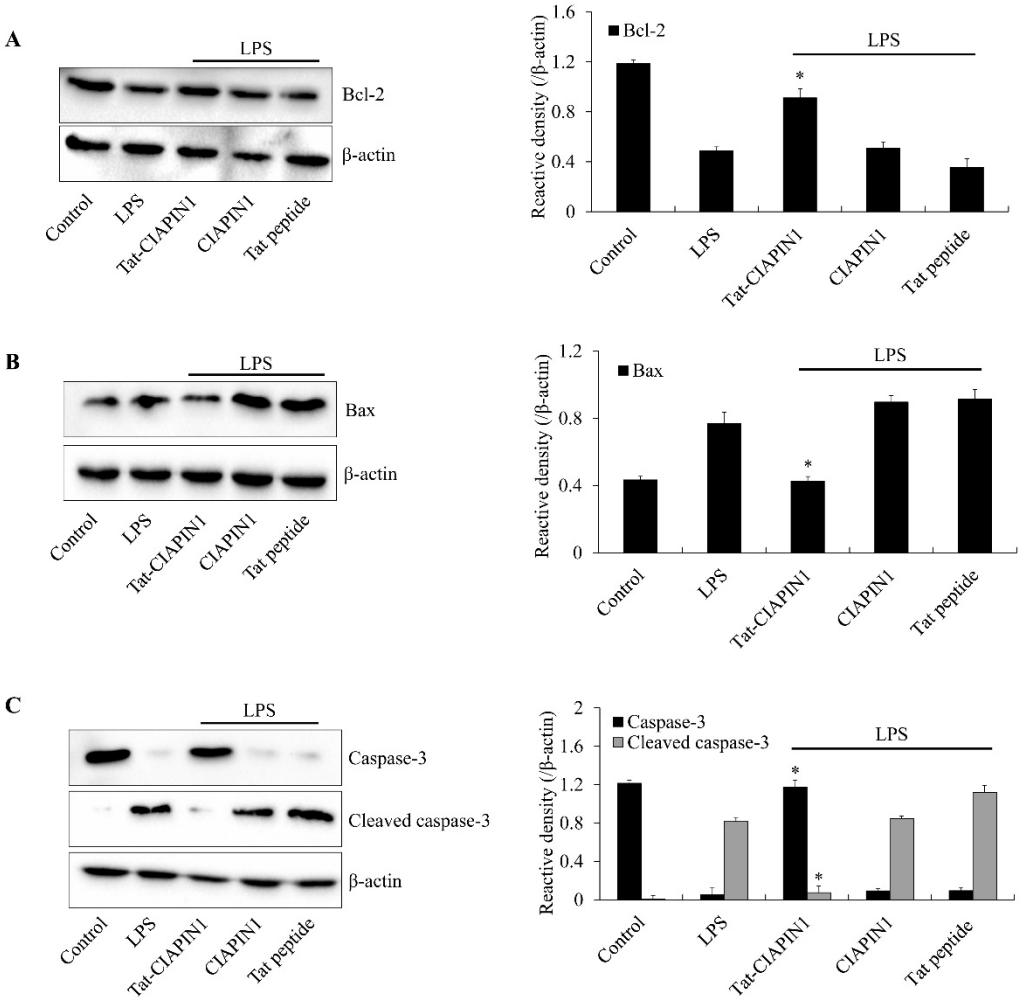

Fig. S3

Fig. S3. Effects of Tat-CIAPIN1 protein on LPS-induced apoptosis in Raw 264.7 cells. The cells were treated with Tat-CIAPIN1 (3 μM) or CIAPIN1 protein for 1 h before being treated with LPS (1 μg/ml). Bcl-2 (A), Bax (B) and caspase-3 (C) expression levels were analyzed by Western blotting. The band intensity was measured by densitometer. $^{*}P < 0.05$, compared with LPS treated cells.

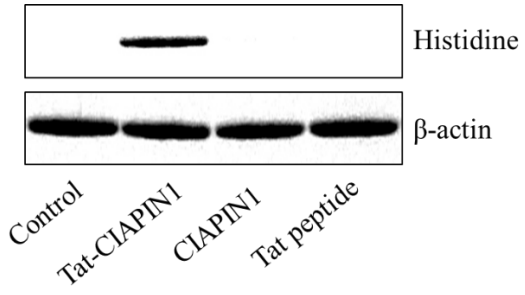

Fig. S4. Transduction of Tat-CIAPIN1 protein into ear of mouse tissue. Tat-CIAPIN1 (10 μg), CIAPIN1 and Tat peptide was topically applied to mice ears 1 h. Then the transduction of Tat-CIAPIN1 protein was analyzed by Western blotting using a Histidine antibody.

MATERIALS AND METHODS

Materials

Ni^{2+}-nitrilotriacetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, USA). PD-10 columns were purchased from Amersham (Brauncschweig, Germany). Fetal bovine serum (FBS) and antibiotics (streptomycin and penicillin) were obtained from Gibco BRL (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Lonza/BioWhittaker (Walkersville, MD, USA). LPS, TPA, and 2',7'-Dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Histidine antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence agent was purchased from Amersham (Franklin Lakes, NJ, USA). The indicated antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Unless otherwise stated, all other agents were of the highest grade available.

Transduction of Tat-CIAPIN1 into Raw 264.7 cells

Tat-CIAPIN1 and control CIAPIN1 protein was prepared as described in a previous study (1). Purified Tat-CIAPIN1 and control CIAPIN1 protein concentrations were determined by the Bradford assay (2). Murine macrophage Raw 264.7 cells were maintained in DMEM containing 20 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum (FBS)

and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) at 37℃ in a 5% CO_2 incubator.

To examine the transduction efficiency of Tat-CIAPIN1, Raw 264.7 cells were exposed to different concentrations of Tat-CIAPIN1 or CIAPIN1 (0.5-3 μM) for 1 h. Raw 264.7 cells were exposed to Tat-CIAPIN1 or CIAPIN1 (3 μM) for various time periods (15-60 min). Then, the cells were washed with PBS and treated with trypsin-EDTA (Gibco Grand Island, NY, USA). We also determined the intracellular stability of Tat-CIAPIN1. To confirm the stability of Tat-CIAPIN1, the cells were further cultured (1-24 h) after transduction. The levels of transduced proteins were measured by Western blotting using an anti-histidine antibody.

Western blot analysis

Equal amounts of proteins were loaded into 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore; Billerica, MA, USA). The membrane was blocked with a TBS-T (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.5) buffer containing 5% non-fat dry milk for 1 h. After being washed with TBS-T buffer, the membrane was incubated with the indicated primary and appropriate secondary antibodies. Then the membranes were washed with TBS-T buffer three times and the protein bands were identified using chemiluminescent reagents as recommended by the manufacturer

(3, 4).

Fluorescence microscopy analysis

To determine the intracellular distribution of transduced Tat-CIAPIN1 in Raw 264.7 cells, we performed confocal fluorescence microscopy as described previously (1, 4). Raw 264.7 cells were placed on coverslips and treated with 3 μM Tat-CIAPIN1 for 1 h. The cells were washed with PBS twice and fixed with 4% paraformaldehyde for 5 min. The cells were treated in PBS containing 3% bovine serum albumin, 0.1% Triton X-100 (PBS-BT) at room temperature for 30 min and washed with PBS-BT. The histidine primary antibody was diluted 1:1500 and incubated at room temperature for 3 h. The Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) was diluted 1:1500 and incubated in the dark for 1 h. Nuclei were stained with 1 μg/ml DAPI (Roche Applied Science, Mannheim, Germany) for 2 min. Then cells were analyzed by confocal fluorescence microscopy using a model FV-300 microscope (Olympus, Tokyo, Japan).

Measurement of intracellular reactive oxygen species (ROS) levels

Intracellular ROS levels were determined using 2'7'-dichlorofluorescein diacetate (DCF-DA) staining as described previously (4, 5). After being incubated with Tat-CIAPIN1 or CIAPIN1 (3 μM) for 1 h, Raw 264.7 cells were exposed to LPS (1 μg/ml) for 3 h. Cells were then

washed twice with PBS and stained with DCF-DA (20 μM) for 30 min. Then, fluorescent images were obtained by fluorescence microscopy (Nikon eclipse 80i, Japan) and the fluorescence intensity was detected with excitation at 485 nm and emission at 538 nm using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

TUNEL assay

To examine whether transduced Tat-CIAPIN1 protects against LPS-induced DNA damage, Raw 264.7 cells were pretreated with Tat-CIAPIN1, CIAPIN1, or Tat peptide (3 μM) for 1 h after which LPS (1 μg/ml) was added to the culture medium for 28 h. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using a Cell Death Detection kit (Roche Applied Science, Basel, Switzerland). Fluorescent images were obtained by fluorescence microscope (Nikon eclipse 80i, Tokyo, Japan). Fluorescence intensity levels were measured using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland) at 485 nm excitation and 538 nm emission (1, 4).

Measurement of MAPKs and apoptotic protein expression

Raw 264.7 cells were incubated in the absence or presence of Tat-CIAPIN1 (3 μM) for 1 h, and then treated with LPS (1 μg/ml) for various times. The expression of MAPKs, NF-κB, Akt and apoptotic protein expression levels were determined by Western blotting using the

indicated specific antibodies. The bands were quantified by Image J software (NIH, Bethesda, MD, USA) (1, 4).

RT-PCR analysis

Total RNA was isolated from Raw 264.7 cells using an Easy blue kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA (2 μg) from each sample was reversibly transcribed with reverse transcriptase and an oligo-(dT) primer. For amplification of cDNA the following sense and antisense primers were used. COX-2 sense, 5'-CTGGTGCCTGGTCTGATGATGT-3'; COX-2 antisense, 5'-AGTCTGGTTTGGAATAG TTGCT-3'; iNOS sense, 5'-GAATCTTGGAGCGAGTTGTGGA-3'; iNOS antisense, 5'-GT GAGGGCTTGGCTGAGTGAG-3'; IL-6 sense, 5'-AAGGAGTGGCTAAGGACCAAGAC-3'; IL-6 antisense, 5'-AGTGAGGAATGTCCACAAACTGATA-3'; TNF-α sense, 5'-CTTG TTGCCTCCTCTTTTGCTTA-3'; TNF-α antisense, 5'-CTTTATTTCTCTCAATGACCCGT AG-3'; and GAPDH sense, 5'-CTTTGGCATTGTGGAAGGGCTC-3'; GAPDH antisense, 5'-GCAGGGATGTTCTGGGCAG-3'. PCR products were analyzed on ethidium bromide 1% agarose gels staining (6, 7).

TPA-induced skin inflammation and histology

Male 4-6-week-old ICR mice were housed at a constant temperature (23°C) and relative

humidity (60%) with a fixed 12 h light:12 h dark cycle and free access to food and water. All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

Skin inflammation was induced according to the method described previously (6). In this study, each group consisted of five mice. TPA (1.0 μg) dissolved in 20 μl of acetone and Tat-CIAPIN1 (10 μg) were applied to the same area of mice ears every day for 3 days. After the final TPA and Tat-CIAPIN1 treatment, mice were sacrificed and 5 mm diameter ear biopsies were obtained. Ear biopsy weight and thickness were measured and identified by hematoxylin and eosin staining.

Statistical analysis

Data are expressed as the mean ± SEM of three different experiments. Differences between groups were analyzed by ANOVA followed by a Bonferroni's post-hoc test. Statistical significance was considered at $P < 0.05$.

REFERENCES

1. Yeo HJ, Shin MJ, Yeo EJ, et al (2019) Tat-CIAPIN1 inhibits hippocampal neuronal cell damage through the MAPK and apoptotic signaling pathways. Free Radic Biol Med 135, 68-78.
2. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254.
3. Han AR, Yang JW, Na JM, Choi SY and Cho SW (2019) Protective effects of N,4,5-trimethylthiazol-2-amine hydrochloride on hypoxia-induced β-amyloid production in SH-SY5Y cells. BMB Rep 52, 439-444.
4. Shin MJ, Kim DW, Lee YP, et al (2014) Tat-glyoxalase protein inhibits against ischemic neuronal cell damage and ameliorates ischemic injury. Free Radic Biol Med 67, 195-210.
5. Kim DH, Kim HM, Huong PTT, et al (2019) Enhanced anticancer effects of a methylation inhibitor by inhibiting a novel DNMT1 target, CEP 131, in cervical cancer. BMB Rep 52, 342-347.
6. Kim MJ, Park M, Kim DW, et al (2015) Transduced PEP-1-PON1 proteins regulate microglial activation and dopaminergic neuronal death in a Parkinson's disease model. Biomaterials 64, 45-56.
7. Kwon MY, Park J, Kim SM, et al (2019) An alpha-lipoic acid-decursinol hybrid compound attenuates lipopolysaccharide-mediated inflammation in BV2 and RAW264.7

cells. BMB Rep 52, 508-513.