Protective effects of Tat-NQO1 against oxidative stress-induced HT-22 cell damage, and ischemic injury in animals

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MATERIALS AND METHODS
Materials
Ni-NTA His·Bind Resin was purchased from Novagen (Hilden, Germany). Fetal bovine serum (FBS) and antibiotics were obtained from Gibco BRL (Grand Island, NY, USA). The primary, secondary, and beta-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Cell Death Detection Kit was obtained from Roche Applied Science. (Basel, Switzerland). An ECL kit was obtained from Amersham (Buckinghamshire, UK). Human NQO1 cDNA was isolated using the polymerase chain reaction (PCR) technique and all chemicals or reagents used in this study were of the highest available analytic grade.

Construction and purification of Tat-NQO1 protein
Construction or purification of Tat-NQO1 protein was performed as described in a
previous study (1). Briefly, human NQO1 cDNA was amplified by PCR using the following primers. NQO1 sense, 5’-CTCGAGATGGTCGGCAGAAGAGC-3’ and NQO1 antisense, 5’-GGATCCTCATTTTCTAGCTTTGATCTGGTTGTC-3’. Next, the genes were cloned into a Tat expression vector. We also prepared control NQO1 protein without the Tat peptide. To prepare Tat-NQO1 and control NQO1 proteins, the overexpressed cells were harvested and purified by a Ni-NTA His affinity column and PD-10 column chromatography. The concentration of purified proteins was determined using the Bradford assay (2).

**Transduction of Tat-NQO1 protein into HT-22 cells**

To assess whether Tat-NQO1 protein transduced into the cells, HT-22 cells were treated with various quantities of Tat-NQO1 protein (0.5-3 μM) for 2 h or with Tat-NQO1 protein (3 μM) over various times (10-120 min). After the cells were washed twice with trypsin-EDTA and phosphate-buffered saline (PBS), transduced Tat-NQO1 protein levels were confirmed by Western blot analysis using anti-histidine antibody.

**Western blot analysis**

After cell lysates were prepared using a lysis buffer, equal amounts of cell lysates were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST buffer (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.5), and then incubated with primary and HRP-conjugated secondary antibodies. After washing the membranes thrice in TBST, the indicated protein bands were detected using an ECL kit (3,4).

**Fluorescence microscopy analysis**

Fluorescence microscopy analysis was performed as described previously (3). Briefly, HT-22 cells were grown on cover slips and treated with Tat-NQO1 protein (3 μM) for 2 h. Cells were washed twice with PBS, fixed, permeabilized and blocked. Following this, the cells were exposed to the His primary antibody and Alexa Flour 488 secondary antibody in the dark. Nuclei were stained for 5 min with 0.1 μg/ml DAPI. The
distribution of transduced Tat-NQO1 protein into HT-22 cells was determined using a FV-300 confocal microscope (Olympus, Tokyo, Japan).

**Cell viability assay**
A cell viability assay was performed as described previously (3,5). HT-22 cells were seeded into 96-well plates and treated with Tat-NQO1 (3 µM) and control NQO1 protein for 2 h. The cells were incubated for 10 h, after which they were treated with 1 mM hydrogen peroxide. Cell viability was then determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) assay. The absorbance at 570 nm was measured using a microplate reader (Labsystems Multiskan MCC/340), and cell viability was defined as the % of untreated control cells.

**Measurement of reactive oxygen species (ROS) and DNA fragmentation levels**
Intracellular ROS and DNA fragmentation levels were determined as described previously (3,6,7). Briefly, the HT-22 cells were treated with Tat-NQO1 (3 µM) and control NQO1 protein for 2 h and exposed to hydrogen peroxide (1 mM) for 10 min and 8 h, respectively. The cells were then washed, after which they were incubated for 30 minutes with DCF-DA (10 μM); DCF-DA and TUNEL staining were performed. Fluorescence intensity was detected at excitation 485 nm and emission 538 nm, using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

**Measurement of activation of Akt, MAPK, and caspase-3 signals**
HT-22 cells were treated with Tat-NQO1 (3 µM) and control NQO1 protein for 2 h and exposed to hydrogen peroxide (1 mM). The expression levels of p-Akt (4 h), p-JNK (6 h), p-ERK (6 h), p-p38 (6 h), cleaved caspase-3 (7 h) in whole cell lysates were then analyzed by Western blotting, using the specific antibodies. Band intensities were measured by a densitometer.

**Experimental animals**
Male gerbils (65-75 g) obtained from the Hallym University Experimental Animal Center were housed at a temperature of 23ºC, with humidity of 60%, and exposed to 12
hour periods of light and dark with 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 & Quarantine Service of Korea, and were approved by the Institutional Animal Care and Use Committee of Soonchunhyang University [SCH 14-0018].

To examine whether Tat-NQO1 protein protects against ischemic insults, we divided the gerbils into four groups (n=7 per group): sham-operated, vehicle-treated group, Tat-NQO1-treated group, and control NQO1-treated group. Tat-NQO1 or control NQO1 protein was intraperitoneally injected at a concentration of 2 mg/kg, 30 min before ischemia-reperfusion. After 7 days ischemia-reperfusion, brain tissue samples were extracted to perform histological analysis as described previously (3,6,7).

Quantitative and statistical analysis
The measurement of immunoreactive cells in all groups was conducted as described previously (8-10). We selected portions that involved the middle portion of the CA1 region in the tissue samples of each section. PC images of the tissue samples were obtained using a CCD camera of an Axiophot light microscope (Carl Zeiss, Jena, Germany). The images of positive neurons were obtained by Apple scanner. The number of neurons were measured using an image analysis system equipped with a computer based CCD camera (software: Optimas 6.5, CyberMetrics, USA). Data are expressed as the mean ± SEM of three different experiments. The data were analyzed using one-way ANOVA to determine statistical significance. Bonferroni’s test was used for post-hoc comparisons. A value of $P < 0.01$ was considered statistically significant.
References


