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PEP-1-HO-1 prevents MPTP-induced degeneration of dopaminergic neurons in a Parkinson's disease mouse model

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Heme oxygenase-1 (HO-1) degrades heme to carbon dioxide, biliverdin, and Fe^{2+}, which play important roles in various biochemical processes. In this study, we examined the protective function of HO-1 against oxidative stress in SH-SY5Y cells and in a Parkinson's disease mouse model. Western blot and fluorescence microscopy analysis demonstrated that PEP-1-HO-1, fused with a PEP-1 peptide can cross the cellular membranes of human neuroblastoma SH-SY5Y cells. In addition, the transduced PEP-1-HO-1 inhibited generation of reactive oxygen species (ROS) and cell death caused by 1-methyl-4-phenylpyridinium ion (MPP^+). In contrast, HO-1, which has no ability to transduce into SH-SY5Y cells, failed to reduce MPP^+-induced cellular toxicity and ROS production. Furthermore, intraperitoneal injected PEP-1-HO-1 crossed the blood-brain barrier in mouse brains. In a PD mouse model, PEP-1-HO-1 significantly protected against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicity and dopaminergic neuronal death. Therefore, PEP-1-HO-1 could be a useful agent in treating oxidative stress induced ailments including PD. [BMB Reports 2014; 47(10): 569-574]

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease accompanied by symptoms such as bradykinesia, tremors, and rigidity (1). PD is characterized by marked loss of dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta. Various stimuli such as excessive generation of reactive oxygen species (ROS), oxidative stress, misfolded proteins, and impaired mitochondrial function are known causes of PD. In a animal models of PD, neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are often used to damage dopaminergic neurons and induce symptoms similar to PD.

The expression of heme oxygenase-1 (HO-1) is induced by the presence of various molecules including heme, endotoxin, hydrogen peroxide, and cytokines (2). HO-1 catalyzes the degradation of the heme to carbon monoxide (CO), iron, and biliverdin. Biliverdin is subsequently converted to bilirubin by bilirubin reductase. These heme degradation products have anti-apoptotic, anti-inflammatory and anti-oxidant activities (3-5). CO, in a similar fashion to nitric oxide (NO), functions as a signaling molecule (3). CO inhibits inflammation by decreasing synthesis of several pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF) while at the same time up-regulating expression of the anti-inflammatory molecule, IL-10 (3). Also, exogenous CO suppresses TNF-alpha-induced apoptosis in endothelial cells (6). Bilirubin scavenges hydroxyl radicals and superoxide anions and inhibits lipid and protein peroxidation (3,7). Also, HO-1-derived free iron, a very toxic oxidant, potentially induces expression of ferritin, an iron-storage anti-oxidant protein, and Fe-ATPase, which rapidly decreases the level of free iron in cells (8). As described above, many studies have demonstrated that HO-1 is implicated in the modulation of inflammation and apoptosis (3,9).

Methods to increase the level of HO-1 in specific cells and tissues could be useful for protection against oxidative stress. Protein transduction domains (PTDs) such as Tat and PEP-1 are known to effectively deliver exogenous molecules to cells and tissues (5,10). In addition, we demonstrated that various recombinant proteins including Annexin and ribosomal protein S3, when fused with Tat or PEP-1, show anti-apoptotic and an-

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ti-inflammatory activities against oxidative stress-induced damage (11,12).

Here, we examined whether the recombinant protein, PEP-1-HO-1, could penetrate into SH-SY5Y cells and the brain tissue and prevent MPP^+- or MPTP-induced damage to SH-SY5Y cells and DA neurons in the SN.

RESULTS AND DISCUSSION

PEP-HO-1 transduces into SH-SY5Y cells in a dose- and time-dependent manner

PEP-1 consists of a hydrophobic tryptophan-rich and a hydrophilic lysine-rich domain with a spacer domain. PEP-1 is a well-known PTD used to transport various proteins into cells and tissues (5). We previously reported that PEP-1-HO-1 has the ability to spontaneously transduce into Raw 264.7 cells (9). In this study, we examined whether PEP-1-HO-1 can transduce into SH-SY5Y neuroblastoma cells. The schematic structures of HO-1 and PEP-1-HO-1 are shown in Fig. 1A. HO-1 protein consists of His and HO-1, while PEP-1-HO-1 contains a PEP-1 peptide as well as His and HO-1. We purified HO-1 and PEP-1-HO-1 proteins as previously described (9). Following the purification of both proteins, their purities were confirmed by sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using an antibody against His (Fig. 1B).

The ability of HO-1 and PEP-1-HO-1 to transduce into SH-SY5Y cells, cells were assessed at various doses (1-5 μM) of proteins for 1 h and assessed 5 μM of the proteins at various time periods (10-60 min). PEP-1-HO-1 transduced into SH-SY5Y cells in a dose- and a time-dependent manner, as shown in Fig. 2A and 2B. Furthermore, fluorescence microscopy analysis showed that PEP-1-HO-1 was markedly distributed in SH-SY5Y cells (Fig. 2C). To investigate the stability of PEP-1-HO-1 in SH-SY5Y cells after entering into the cells, the level of the transduced PEP-1-HO-1 was monitored by immunoblot analysis. The presence of PEP-1-HO-1 in SH-SY5Y cells was evident up to 24 h (Fig. 2D). This data indicates that PEP-1-HO-1 was effectively delivered to SH-SY5Y cells within 20 min and remains in the cells at significant levels for 24 h.

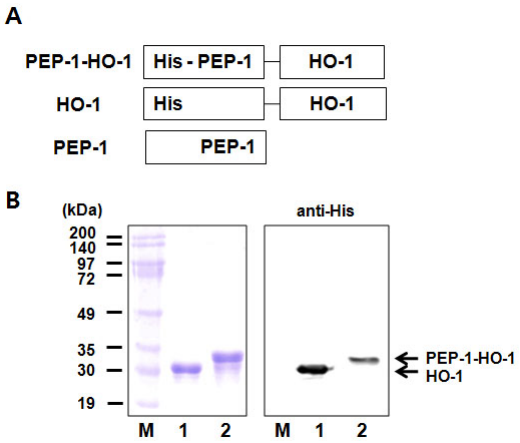

Fig. 1. Purified HO-1 and PEP-1-HO-1 proteins. (A) Schematic sequences of HO-1 and PEP-1-HO-1. His was used for purification and identification of HO-1 and PEP-1-HO-1. (B) After purification of PEP-1-HO-1 and HO-1, purified proteins were analyzed by 12% SDS-PAGE and Western blot analysis. Left panel, Coomassie blue staining; right panel, Western blot analysis using an anti-His antibody and horseradish peroxidase conjugated secondary antibody. Lane M, molecular weight markers (EBM-1035, Elpisbiotech, Korea); lane 1, purified HO-1; lane 2, purified PEP-1-HO-1.

PEP-1-HO-1 suppresses MPP^+-induced death of SH-SY5Y cells

MPTP is a neurotoxin which induces symptoms similar to PD (13). MPTP traverses the blood-brain barrier (BBB) and is converted into 1-methyl-4-phenylpyridinium ion (MPP^+) by monoamine oxidase B (14). MPP^+ causes cell degeneration by

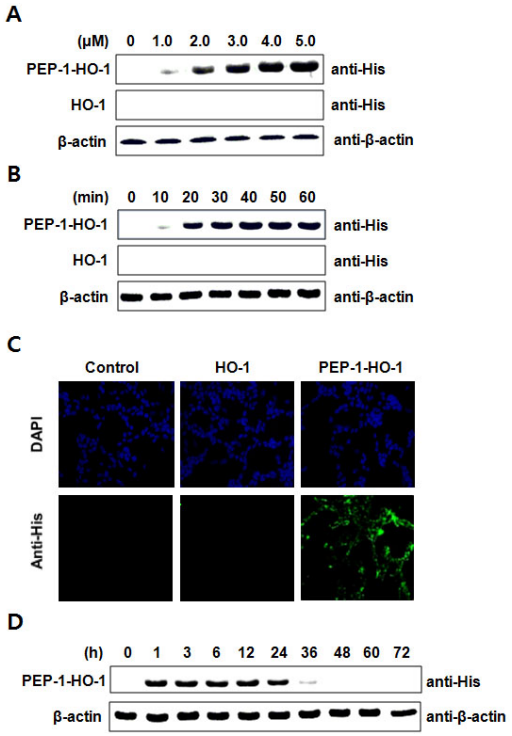

Fig. 2. PEP-1-HO-1 is delivered to SH-SY5Y cells. SH-SY5Y cells were incubated (A) with PEP-1-HO-1 and HO-1 (1.0-5.0 μM) for 1 h or (B) with PEP-1-HO-1 and HO-1 (5 μM) for 10-60 min. The levels of PEP-1-HO-1 or HO-1 in each sample were analyzed by Western blot. (C) PEP-1-HO-1- and HO-1-treated SH-SY5Y cells (5 μM) were incubated with a His antibody and sequentially Alexa fluoro 488-conjugated secondary antibody. The cells were imaged under a fluorescence microscopy. (D) To examine the cellular stability of PEP-1-HO-1 in SH-SY5Y cells, the cells were treated with PEP-1-HO-1 (5 μM) for 1 h and washed with PBS. Then, the cells were collected with trypsin at desired time points. The cellular level of PEP-1-HO-1 was measured by Western blot analysis.

blocking mitochondrial complex I in DA neurons of the SN (14). In this study, we examined whether transduced PEP-1-HO-1 had an effect on MPP^+-induced ROS or cell death in SH-SY5Y cells. SH-SY5Y cells were pretreated with HO-1 or PEP-1-HO-1 for 1 h, followed by exposure to MPP^+ (5 mM) for 24 h. An MTT assay demonstrated that PEP-1-HO-1 (1-5 μM) significantly inhibited MPP^+-induced toxicity in a dose-dependent manner. However cell viability at concentrations over 3 μM were similar to that at 3 μM (Fig. 3A). On the other hand, cell death was not prevented in cells pretreated with HO-1 (Fig. 3A). We also assessed whether PEP-1-HO-1 treatment inhibited generation of ROS induced by MPP^+. Fluorescence signals indicating cellular levels of ROS were significantly observed in MPP^+, HO-1 + MPP^+-, and PEP-1 + MPP^+-treated cells (Fig. 3B). However, PEP-1-HO-1-treated cells showed very weak fluorescence, similar to the untreated control (Fig. 3B). These results suggest that MPP^+-induced ROS generation and cell toxicity were significantly inhibited by PEP-1-HO-1.

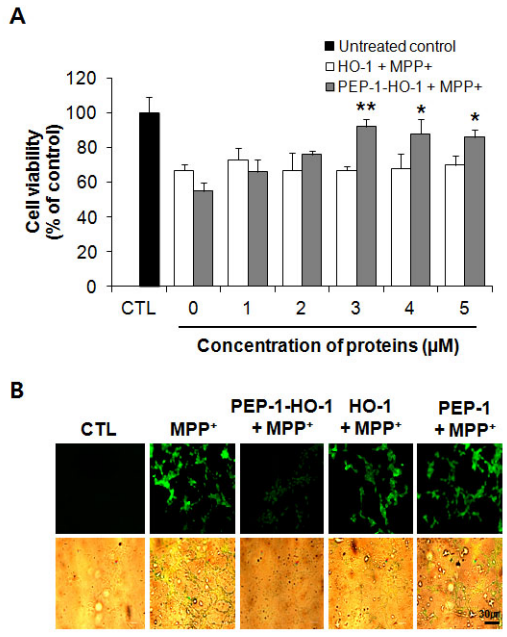

Fig. 3. PEP-1-HO-1 inhibits MPP^+-induced death of SH-SY5Y cells. (A) SH-SY5Y cells were treated with PEP-1-HO-1 or HO-1 for 1 h and intoxicated with MPP^+ (5 mM) for 24 h. The percentage of viable cells was measured with MTT assay. The data was expressed as mean ± SD. Black bar, CTL (untreated control cells); white bar, treated with HO-1 + MPP^+; gray bar, treated with PEP-1-HO-1 + MPP^+. *indicates a statistically significant difference between MPP^+-treated cells and PEP-1-HO-1 + MPP^+-treated samples (*$P < 0.05$; **$P < 0.01$). (B) SH-SY5Y cells were treated with PEP-1, PEP-1-HO-1 or HO-1 for 1 h, followed by treatment with MPP^+ (5 mM) for 1 h. The cells were stained with DCF-DA and fluorescence levels of each sample were measured with fluorescence microscopy.

PEP-1-HO-1 prevents death of DA neurons in the SN in the MPTP-induced PD model

The ability of intraperitoneal (i.p.) injected PEP-1-HO-1 to traverse the blood-brain barrier (BBB) was investigated. Immunohistochemistry analysis demonstrates that PEP-1-HO-1 was significantly distributed in the brain, including the SN of PEP-1-HO-1-injected mice, compared to the untreated control and HO-1-treated groups (Fig. 4A). This data demonstrates that PEP-1-HO-1 has the capacity to effectively cross the BBB.

Next, we examined the effect of the transduced PEP-1-HO-1 on the MPTP-induced death of DA neurons in mice. The existence of DA neurons was identified using an antibody against tyrosine hydroxylase (TH) and viable DA neurons were stained with cresyl violet together with an anti-TH antibody. HO-1 and PEP-1 did not prevent the MPTP-induced DA neuron death in the SN of mouse brains that is observed in mice treated with MPTP alone (Fig. 4B). Conversely, a considerable number of viable DA neurons were observed in PEP-1-HO-1-injected mice (Fig. 4B), demonstrating that PEP-1-HO-1 protect DA neurons in the SN of mouse brains from MPTP-induced toxicity.

Expression of HO-1 is normally low but can be induced in response to oxidative stress and toxin stimuli such as heme, H_2O_2, β-amyloid, UV light, lipopolysaccharide, and other pro-inflammatory cytokines (15). There are conflicting reports regarding the effect of increased HO-1 expression in various experimental models. For example, up-regulated HO-1 degrades modified heme to ferrous iron, biliverdin, and CO, which can exacerbate oxidative stress (16). Also, rats treated

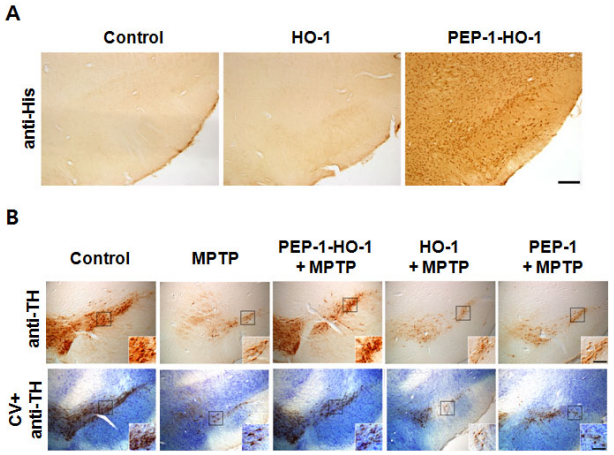

Fig. 4. PEP-1-HO-1 effectively prevents DA neurons death induced by MPTP. (A) HO-1 and PEP-1-HO-1 (1.5 mg/kg body weight) were i.p. injected into mice. Mice were sacrificed and mice brains were sectioned and stained with a His antibody. Scale bar, 100 μm. (B) In a MPTP-induced PD mice model, inhibitory effects of PEP-1-HO-1 and HO-1 against death of DA neurons were evaluated by immunohistochemical analysis. Representative images from all groups were shown in (B). Scale bar, 50 μm.

with HO-1 inductor and substrate hemin exhibited increased HO-1 expression and characteristics of neuroinflammation such as brain tissue loss, microglial activation, and neuronal death in the brains (17).

On the other hand, Panahian *et al*. (1999) demonstrate that elevation of HO-1 levels protect against oxidative stress-induced ischemic neuronal damage and suggest that HO-1 may constitute a novel therapeutic approach in the amelioration of ischemic injury (18). Also, overexpression of HO-1 protects neurons against glutamate toxicity through reducing ROS generation in transgenic mice (19) and adenovirus-mediated overexpression of HO-1 in the SN of rats prevents the death of DA neurons triggered by MPP^+ (20). Moreover, astrocytes from HO-1 knockout mice are more susceptible to heme-mediated damage (21) and ischemic preconditioning in HO-1 deleted mice failed to activate the HO-1-mediated pathway and subsequently reduced brain injuries (22). Also mollugin, a bioactive agent that up-regulates the HO-1 level in mouse hippocampal HT22 and BV2 cells, demonstrates neuroprotective and anti-inflammatory effects against glutamate-induced neurotoxicity and LPS-induced microglial activation (23). In addition, our data (Fig. 3 and 4B) shows that up-regulation of PEP-1-HO-1 suppresses MPP^+-induced toxicity in SH-SY5Y cells or MPTP-induced DA neuron degeneration in the SN of mouse brains. These findings are consistent with Hung *et al*. (2008) results (20) showing the neuroprotective functions of HO-1.

As described above, up- or down-regulation of HO-1 seems to produce contradicting results such as degeneration and survival of cells. However, many factors including experimental models, expression level of HO-1, long-term or temporary induction of HO-1, and levels of degradation products produced by HO-1 may be implicated in downstream signaling of HO-1 in central nervous system tissues. Consequently, it is likely that HO-1 shows contradictory roles in biological system.

Collectively, the present study demonstrates that PEP-1-HO-1 has the ability to transduce into cells and tissues and significantly inhibit ROS generation and cell toxicity caused by oxidative stress. This leads to the protection of DA neurons in the SN of mouse brains. Therefore, PEP-1-HO-1 may be useful for suppression of oxidative stress-induced neurodegeneration.

MATERIALS AND METHODS

Preparation of PEP-1-HO-1

PEP-1-HO-1 protein was prepared as described in a previous report (9). Briefly, PEP-1-HO-1 and HO-1 expression vectors were constructed and transformed into *E. coli* BL21 cells. Two proteins were overexpressed by induction with isopropyl- β-D-thiogalactoside at 37°C for 4 h. The harvested *E. coli* culture was lysed by sonication and cell debris was removed by centrifugation. PEP-1-HO-1 and HO-1 were purified by Ni^{2+}-nitrilotriacetic acid Sepharose column chromatography. Protein concentration was assessed using a Bradford assay. A PEP-1 peptide, consisting of a hydrophobic tryptophan-rich motif, a hydrophilic lysine-rich domain derived, and a spacer domain, was synthesized by Peptron (Daejeon, Korea).

SDS-PAGE

Gel was stained with Coomassie staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% glacial acetic acid) for 1 h with gentle agitation. The gel was then treated with destaining solution (40% methanol and 10% glacial acetic acid). The destaining solution was changed several times until background of the gel was fully destained.

Immunoblot analysis

Equal amounts of protein lysates were resolved by 12% SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked in 5% skim milk solution and then incubated with an anti-His antibody (1:5,000 dilution) and horseradish peroxidase-conjugated secondary antibody (1 : 5,000 dilution). The antibody complex was detected using an enhanced chemiluminescence detection kit.

Fluorescence microscopy analysis

Fluorescence microscopy was performed as previously described (24). SH-SY5Y cells, grown on coverslips in a 12-well plate, were incubated with PEP-1-HO-1 and HO-1 (5 μM) for 1 h. The cells were fixed with 4% paraformaldehyde, made permeable with 0.1% Triton X-100, and incubated with an anti-His antibody (1 : 2,000) and an Alexa Fluor conjugated secondary antibody (1 : 10,000). Nuclei were stained with 0.1 μg/ml 4′6-diamidino-2-phenylindole (DAPI). The cells were observed under a fluorescence microscope. Intracellular ROS levels were measured by incubating HO-1- or PEP-1-HO-1-treated SH-SY5Y cells with MPP^+ (5 mM) for 30 min. The treated cells were subsequently washed with PBS. The cells were exposed to 2′,7′-dichlorofluorescein diacetate (DCF-DA) for 30 min. The fluorescence was detected under a fluorescence microscope (Nikon, Tokyo, Japan).

MTT assay

SH-SY5Y cells were seeded into each well of 96-well plate. The next day the cells were incubated with various concentrations (1-5 μM) of PEP-1-HO-1 and HO-1 for 1 h and, after addition of MPP^+ (5 mM), the cells were additionally incubated for 24 h. The cells were incubated with MTT for 4 h and formazan crystal was dissolved in DMSO. Optical density was measured at 595 nm.

Animal study

Male, 8-week-old, C57BL/6 mice were purchased from the Hallym University Experimental Animal Center. Mice were housed at a constant temperature (23ºC) and relative humidity (60%) under a 12 h light/dark cycle. All research 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 Hallym Medical Center Institutional Animal Care and Use Committee.

Immunostaining was performed as previously described (25). To investigate the *in vivo* transduction of PEP-1-HO-1 or HO-1 into mice brains, PEP-1-HO-1 and HO-1 (1.5 mg/kg body weight) were i.p. injected to mice (n = 7/group). Six hours later mice were euthanized by an i.p. injection of sodium pentobarbital (100 mg/kg body weight). The mouse brains were fixed transcardially with 4% paraformaldehyde, and sectioned. The sections were sequentially incubated with an anti-His antibody and a peroxidase-conjugated secondary antibody and visualized with 3,3-diaminobenzidine (DAB) in 0.1 M Tris buffer and mounted on gelatin-coated slides. Images were captured and analyzed using an Olympus DP72 digital camera and DP2-BSW microscope digital camera software.

To examine whether PEP-1-HO-1 inhibits MPTP induced DA neuron death, mice were i.p. injected with PEP-1-HO-1 or HO-1 (1.5 mg/kg body weight) (n = 7/group) and the following day MPTP (20 mg/kg body weight) was i.p. injected into mice 4 times each at 2 h intervals. One week after MPTP injection mice were euthanized by an i.p. injection of sodium pentobarbital (100 mg/kg body weight). The mouse brains were harvested as described above. To detect DA neurons in the SN, brain sections were incubated with TH antibody and peroxidase-conjugated secondary antibody. Also, to detect viable cells in the SN, the sections were counterstained with cresyl violet.

Statistics

Data are expressed as the means ± SD. Comparison between groups was performed by Student's *t* test. Values of $*P < 0.05$ and $**P < 0.01$ were considered to be statistically significant.

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