BMB
reports

Imipramine enhances neuroprotective effect of PEP-1-Catalase against ischemic neuronal damage

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The protein transduction domains have been reported to have potential to deliver the exogenous molecules, including proteins, to living cells. However, poor transduction of proteins limits therapeutic application. In this study, we examined whether imipramine could stimulate the transduction efficiency of PEP-1 fused proteins into astrocytes. PEP-1-catalase (PEP-1-CAT) was transduced into astrocytes in a time- and dose-dependent manner, reducing cellular toxicity induced by H_2O_2. Additionally, the group of PEP-1-CAT + imipramine showed enhancement of transduction efficiency and therefore increased cellular viability than that of PEP-1-CAT alone. In the gerbil ischemia models, PEP-1-CAT displayed significant neuroprotection in the CA1 region of the hippocampus. Interestingly, PEP-1-CAT + imipramine prevented neuronal cell death and lipid peroxidation more markedly than PEP-1-CAT alone. Therefore, our results suggest that imipramine can be used as a drug to enhance the transduction of PEP-1 fusion proteins to cells or animals and their efficacies against various disorders. [BMB reports 2011; 44(10): 647-652]

INTRODUCTION

Ischemia results from the exhaustion of myocardial ATP and ionic imbalance such as low intramitochondrial Ca^{2+} and high cytosolic Ca^{2+} concentration. Although these ischemic conditions are restored by reperfusion, high levels of reactive oxygen species (ROS) that are generated by abnormal mitochondrial function during ischemia/reperfusion result in cellular damage, apoptosis and necrosis (1). However, various anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase have functional ability to decrease the level of ROS, maintain a cellular redox balance and function as cellular defense mechanism (2-4). So, anti-oxidant enzymes are proposed to function as therapeutic agents against inflammation and ischemia as proved by some studies using animal models (5-7). Although several drug candidates including peptides, proteins and siRNA show excellent efficacies *in vitro*, their hydrophilic properties and large molecular size impede the delivery of these molecules into cells or tissues, thereby failing to exert their therapeutic activities (8, 9). Therefore, many research groups have sought for several ways to avoid the problems, proving that PTDs has the potential to deliver exogenous molecules to living cells and animal tissues (10-16). Earlier we reported that after fusion of SOD, CAT, and ribosomal protein S3 (rpS3) with PTDs such as Tat and PEP-1, they can be transduced efficiently into cells and animal tissues and exert their anti-oxidant activities (5-7). Concomitantly, we have searched for several molecules having potential to improve the transduction efficiency of PTDs fused proteins into cells and tissues, leading to enhancement of their therapeutic effects.

Imipramine is well-known for an anti-depressant by inhibiting neurons from the reuptake of neurotransmitter such as norepinephrine and serotonin. Peng *et al*. have provided evidence that imipramine exerts a neuroprotective activity through up-regulating the expression of brain-derived neurotrophic factor (BDNF), MAPK/ERK pathway and Bcl-2 cascade (17). It is recently reported that bulbectomy is a well-established depression animal model and leads to reduced cell proliferation in the hippocampal subgranular zone and the subventricular zone (SVZ), but subchronic administration of imipramine can restore neurogenesis in these regions (18). Also, it has suggested that chronic treatment of imipramine enhanced cell proliferation in the subgranular zone of dentate gyrus and re-

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http://dx.doi.org/10.5483/BMBRep.2011.44.10.647

Rceived 21 February 2011, Accepted 19 March 2011

Keywords: Imipramine, Ischemic damage, PEP-1-CAT, Protein transduction

duced the neurodegeneration in the CA1 of the hippocampus 14 days after transient global cerebral ischemia in rats (19). In addition, imipramine is reported to markedly inhibit the inflammation in the carrageenan-induced paw edema model (20). It was demonstrated that imipramine inhibits IL-6 and NO production by IFN-γ-induced microglia cells through affecting the cAMP-dependent pathway (21). Imipramine suppressed the expression of inducible nitric oxide synthase and proinflammatory cytokines such as interleukin-1β and TNF-α and also inhibited IκB degradation, nuclear translocation of the p65 subunit of NF-κB, and phosphorylation of p38 mitogen-activated protein kinase in LPS-stimulated microglia cells (22).

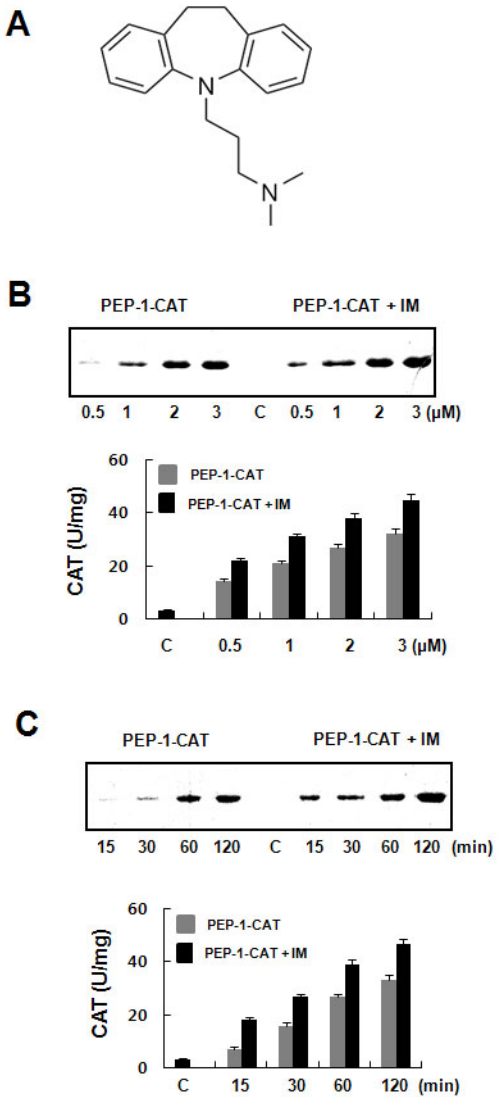

Fig. 1. Effect of imipramine on the transduction of PEP-1-CAT into astrocytes. (A) The structure of imipramine. (B) For dose-dependent transduction of PEP-1-CAT, cells were pretreated with or without imipramine (1.0 μg/ml) 1 h prior to treatment with PEP-1-CAT. (C) For time-dependent transduction of PEP-1-CAT, cells were preincubated incubated with PEP-1-CAT (3 μM) alone or with PEP-1-CAT and imipramine for 15-120 min. Transduced PEP-1-CAT was analyzed by Western blotting analysis. IM, imipramine.

In the present study, we investigated in what manner the transduction efficiency and thereby neuroprotective activity of PEP-1-CAT against ischemic damage change with or without imipramine and so attempted to examine whether imipramine has the potential to improve transduction efficiency of PEP-1-fusion proteins. Although the mechanism is not exactly understood until now, our study suggested that imipramine has another function as an enhancer of protein transduction as well as anti-depressive and neuroprotective functions, which were already reported.

RESULTS AND DISCUSSION

Effect of imipramine on transduction efficiency of PEP-1-CAT into astrocyte cells

Therapeutic efficiency of biologically active molecules can be improved by increasing their transduction efficiency into cells. So, searching for molecules to enhance the transduction of PTD fused proteins have been actively performed. Recently it was reported that treatment with 10% DMSO can facilitate the transduction of PTD fused proteins into cells (23). Also, we showed that bog blueberry anthocyanins (BBA) enhance the transduction efficiency of Tat-SOD into Hela cells and mice skin (24). Previously we reported that PEP-1-CAT proteins are significantly transduced into astrocytes in time- and dose-dependent manner and exert protective activity against ischemic neuronal damage (5).

The structure of imipramine, an anti-depressant drug, is shown in Fig. 1A. As shown in Fig. 1B and 1C, PEP-1-CAT proteins were transduced in dose- and time-dependent manner into astrocytes. In addition, when imipramine was added 1 h prior to treatment with PEP-1-CAT, the transduction efficiency of PEP-1-CAT was significantly increased. These results indicated that imipramine has a capability to increase the transduction efficiency of PEP-1-CAT into cells.

Effect of imipramine on cellular viability

Next we examined the effect of imipramine on the cellular viability after cellular toxicity was induced in control cells by treating with H_2O_2 (1.2 mM). As shown in Fig. 2, when treated with PEP-1-CAT (0.5-3 μM), cellular viability at each dose was dose-dependently increased. The single treatment with imipramine (1 μg/ml) for 1 h showed neither additional cellular toxicity nor protective activity, which was nearly similar to one of H_2O_2 treated control. On the other hand, when imipramine was added to cells 1 h prior to treatment with PEP-1-CAT, cell viability increased significantly than without addition of imipramine. Accordingly, our data suggested that the increase of

cellular viability might be mediated by the enhanced transduction of PEP-1-CAT promoted by imipramine rather than directly by imipramine.

Effect of imipramine on the protective activity of PEP-1-CAT against ischemic damage

Induction of transient ischemia in Mongolian gerbils produces neuronal loss in the CA1 region of the hippocampus. Histological changes of CA1 region from each group were evaluated immediately after induction of brain ischemic damage. The significant ischemia is induced in vehicle treated group, showing characteristic neuronal loss in the CA1 region, compared to sham operated animals (Fig. 3A and B). Neuronal protection in imipramine-treated group was observed slightly increased than that of vehicle group. Undoubtedly, treatment of animals with PEP-1-CAT (3 mg/kg) resulted in apparent protection in ischemia sensitive CA1 region. Furthermore, in imipramine pretreated condition, the PEP-1-CAT treatment showed more appreciable neuroprotection in the CA1 region as observed more or less on sham group (Fig. 3C, D and E).

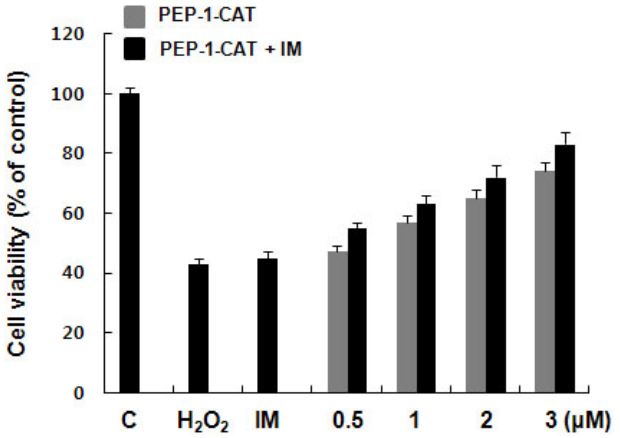

Fig. 2. Effect of imipramine on the viability of astrocytes. Cells were pretreated with imipramine (1.0 μg/ml) for 1 h and then incubated with PEP-1-CAT for additional 2 h. The cells were treated with H_2O_2 (1.2 mM) for 12 h. Cell viability was assessed using a MTT assay and is expressed as a percentage of H_2O_2 untreated control. IM, imipramine.

The extent of ischemic damage, which is known to be related with lipid peroxidation, can be evaluated by measuring the level of malondialdehyde (MDA) after each drug was administered and ischemic damage was induced (25). After ischemia induction, the level of MDA in vehicle-treated group markedly increased, compared to sham group (Fig. 4). While imipramine failed to decrease the level of MDA, PEP-1-CAT group showed to lower evidently the MDA level. Moreover, the MDA level of PEP-1-CAT + imipramine group declined much dramatically than that of PEP-1-CAT alone. Therefore, these results confirmed the notion that imipramine has the potential to improve the transduction efficiency of PEP-1-CAT proteins as the cellular uptake of PEP-1-CAT proteins increased.

Our data showed that preventive effect of imipramine + PEP-1-CAT against ischemic damage was superior to that of PEP-1-CAT alone. In the evaluation of histological analysis and MDA level, only slight preventive effect of imipramine against ischemic damage was observed by a single treatment with imipramine alone. On the other hand, recently imipramine was reported to be able to enhance the cell proliferation and protection by prolonged administration at once a day for 14 days after transient global cerebral ischemia (19). Peng *et al.*

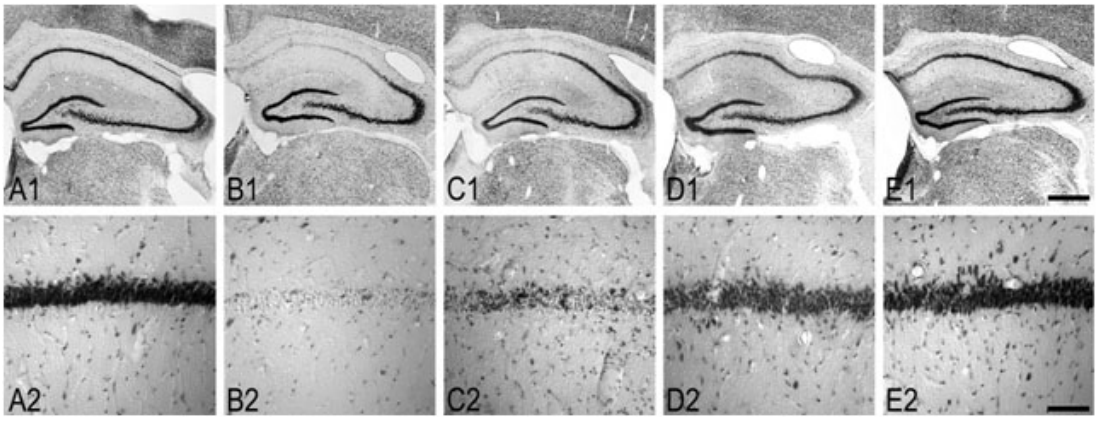

Fig. 3. Histological evaluation of hippocampus brain damage in Mongolian gerbil after ischemic insult. Imipramine was given intraperitoneally (i.p. 10 mg/kg) to gerbils. Then, PEP-1-CAT (3 mg/kg) was administered i.p. 30 min before ischemic damage. At once after ischemia-reperfusion, hippocampus from each group was stained with cresyl violet. The tissue damage is localized in the CA1 area of the hippocampus. (A) sham operated group, (B) vehicle treated group, (C) imipramine treated group, (D) PEP-1-CAT treated group, (E) PEP-1-CAT and imipramine treated group. Scale bar are 280 μm for A1-E1 and 50 μm for A2-E2.

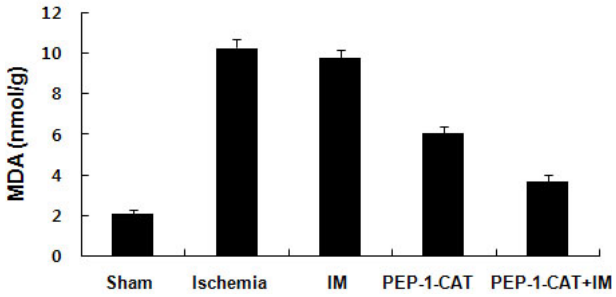

Fig. 4. Effects of transduced PEP-1-CAT on brain MDA level in the presence of imipramine. Imipramine was administered 30 min before i.p. injection of PEP-1-CAT, followed by ischemia 30 min later. At once after ischemic insult, hippocampi were dissected for measurement of MDA. IM, imipramine.

have been reported that imipramine alone can exert anti-inflammatory and neuroprotective effect in central nervous system and prevent directly ischemic damage (17). We surmised that different administration schedule and doses of imipramine employed in each study may contribute to these conflicting results, so in order to observe the neurogenetic and neuroprotective effects of imipramine, a longer time and higher concentrations might be required than those we used.

Many cationic amphiphilic drugs (CADs) are known to induce drug-induced phospholipidosis (DIPL) in animal and human. CADs are taken up into the lysosomes and make complex with phospholipids within the lysosomal membranes, leading to DIPL, a storage disease to abnormally accumulate phospholipids in some tissues. Also, imipramine is reported to be an anti-depressant, which can induce phospholipidosis (26, 27). Until now, it is not clear in what manner imipramine affects the membrane integrity and the delivery mechanism of PTDs. However, it is possible that imipramine might cause membrane disturbance such as phopholipidosis and consequently these change of membrane integrity can allow PEP-1-CAT to access to the interior of cells with ease.

Taken together, we demonstrated that a single treatment with imipramine failed to prevent the ischemic damage but the transduction efficiency of PEP-1-CAT significantly enhanced in the presence of imipramine, compared to that without imipramine. So PEP-1-CAT proteins level was increased in neural cells, and that could prevent effectively the ischemic damage and lipid peroxidation. In conclusion, our data suggested that imipramine can be used as a potentially valuable agent to promote the transduction of PTD fusion proteins into target cells and their therapeutic efficacy.

MATERIALS AND METHODS

Materials

Imipramine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against histidine and peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were the highest analytical grade available.

Cell culture and protein preparation

The astrocytes were cultured in Dulbecco's modified Eagle's medium containing 20 mM Hepes/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% heat-inactivated fetal bovine serum and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) at 37°C under a humidified atmosphere of 5% CO_2. PEP-1-CAT was overexpressed and purified as described previously (5).

Transduction of PEP-1-CAT into astrocytes and Western blot analysis

For the concentration dependent transduction of PEP-1-CAT, cells were grown to confluence in a 6-well plate, pretreated with imipramine (1.0 μg/ml) for 1 h, and exposed to various concentrations of PEP-1-CAT (0.5-3 μM) for an additional 2 h. Also, for the time dependent transduction, cells were treated with PEP-1-CAT (3 μM) for various incubation times (15-120 min). The cells were treated with trypsin-EDTA (Gibco), washed with phosphate-buffered saline (PBS) and harvested. For Western blot analysis, an equal amount of proteins from each cell lysate was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a nitrocellulose membrane, which was then blocked with 5% nonfat dry milk in PBS. The membrane was incubated with rabbit anti-histidine polyclonal antibodies at 4°C overnight and sequentially incubated with horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham, Piscataway, NJ, USA).

MTT assay

To assess cell viability, cells were seeded into wells of a 6-well plate at grown to 70% confluence. The cells were pretreated with imipramine (1 μg/ml) for 1 h. After removal of imipramine, the cells were exposed to various concentrations of PEP-1-CAT (0.5-3 μM) for 2 h. The cells were washed with Dulbecco's phosphate buffered saline (DPBS) and treated with hydrogen peroxide (1.2 mM) for 4 h. Assessment of cell viability was performed according to the MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide assay protocol (28). Cellular viability was expressed as a percentage of untreated control.

Induction of cerebral forebrain ischemia in experimental model

Mongolian gerbils (*Meriones unguiculatus*) were purchased from the Experimental Animal Center, Hallym University. The animals were housed at a constant temperature (23°C) and relative humidity (60%) with alternating 12 h cycles of light and dark. They were permitted free access to food and water. All animal studies were conducted according to the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23), and

handling and experimental protocols were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

To determine whether the protective effect of PEP-1-CAT against ischemic damage affects in the presence or absence of imipramine, gerbils were randomly divided into 5 groups (n = 7), namely, sham-operated, vehicle-treated, imipramine-treated, PEP-1-CAT-treated, and PEP-1-CAT + imipramine treated group. Imipramine dissolved in PBS was injected i.p. (10 mg/kg). Then, PEP-1-CAT (3 mg/kg) was administered i.p. followed by the occlusion of common carotid arteries. For the occlusion of common carotid arteries, male Mongolian gerbils were placed under general anesthesia with a mixture of 2.5% isoflurane (Abbott Laboratories, North Chicago, IL, USA) in 33% oxygen and 67% nitrous oxide. A midline ventral incision was made in the neck, and the common carotid arteries were isolated, freed of nerve fibers, and occluded with nontraumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in the eyeball using an ophthalmoscope. After 5 min occlusion, the aneurysm clips were removed. The restoration of blood flow (reperfusion) was observed directly under the ophthalmoscope. Sham-operated animals (n = 7) were subjected to the same surgical procedures except that the common carotid arteries were not occluded. Rectal temperature was monitored and maintained at 37 ± 0.5°C before, during, and after the surgery, until the animals had recovered fully from anesthesia. At once after ischemia-reperfusion, animals from each group were euthanized for cresyl violet staining.

Tissue processing for histology

All animals from each group were anesthetized with pentobarbital sodium and perfused transcardially with PBS (pH 7.4), followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4 and 7 days (n = 7) after the surgery. Brains were removed and postfixed in the same fixative for 4 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. The tissues were then frozen and sectioned with a cryostat at 50 μm and consecutive sections were collected in six-well plates containing PBS.

Cresyl violet staining

Cresyl violet staining was used according to the previously described procedure (5). Sections of tissues were mounted on gelatin-coated microscopic slides. Cresyl violet acetate (Sigma, St. Louis, MO, USA) was dissolved at 1.0% (w/v) in distilled water, and glacial acetic acid was added to this solution. After being stained for 2 min at room temperature, the sections were washed twice in distilled water, dehydrated by placing in ethanol baths of 50, 70, 80, 90, 95, and 100% for 2 h each in succession at room temperature, and finally mounted with Canada balsam (Kato, Japan). Sham-operated animals were used as a control group.

Measurement of lipid peroxidation in the hippocampus of gerbil

Lipid peroxidation was measured according to the method described by Ahang *et al*. (29). An aliquot (100 μl) of brain supernatant was added to a reaction mixture containing 100 μl of 8.1% sodium dodecyl sulfate (SDS), 750 μl of 20% acetic acid (pH 3.5), 750 μl of 0.8% thiobarbituric acid, and 300 μl of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 4,000 g for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 532 nm.

Acknowledgements

This work was supported by the research grant from the Brain Research Center of the 21st Century Frontier Research Program (2010K000808) and by the Regional Research Universities Program/Medical & Bio-material Research Center grant and in part by the Priority Research Centers Program grant (2009-0093812) through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

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