PEP-1-GSTpi protein enhanced hippocampal neuronal cell survival after oxidative damage

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Reactive oxygen species generated under oxidative stress are involved in neuronal diseases, including ischemia. Glutathione S-transferase pi (GSTpi) is a member of the GST family and is known to play important roles in cell survival. We investigated the effect of GSTpi against oxidative stress-induced hippocampal HT-22 cell death, and its effects in an animal model of ischemic injury, using a cell-permeable PEP-1-GSTpi protein. PEP-1-GSTpi was transduced into HT-22 cells and significantly protected against H2O2-treated cell death by reducing the intracellular toxicity and regulating the signal pathways, including MAPK, Akt, Bax, and Bcl-2. PEP-1-GSTpi transduced into the hippocampus in animal brains, and markedly protected against neuronal cell death in an ischemic injury animal model. These results indicate that PEP-1-GSTpi acts as a regulator or an antioxidant to protect against oxidative stress-induced cell death. Our study suggests that PEP-1-GSTpi may have potential as a therapeutic agent for the treatment of ischemia and a variety of oxidative stress-related neuronal diseases. [BMB Reports 2016; 49(7): 382-387]

INTRODUCTION

Glutathione S-transferase (GST) exists in all cells of higher organisms and is known as a reactive oxygen species (ROS) scavenger and detoxifying protein. GST has been identified as a member of the multigene family of isozymes which exist in three forms in accordance with substrate specificity; these include the basic hepatic form (alpha), near neutral hepatic form (mu), and acidic placental form (pi) (1-4). Several studies have demonstrated that GSTpi is expressed in the kidney, the placenta, and the fetal liver, as well as being highly expressed in several human cancers including liver, colon, and stomach cancer. Overexpressed GSTpi increased the resistance to anticancer drugs, and is recognized as a tumor marker (5-7). However, other studies have shown that GSTpi plays an important role in the cell survival against carcinogens, anti-neoplastons, and cytotoxic effects. In addition, GSTpi is known to regulate cellular signaling pathways, including c-Jun NH2-terminal kinases (JNKs) and inhibits tumor necrosis factor alpha (TNFα)-induced apoptosis (8-12).

Oxidative stress, which induces the production of ROS, is one of the major risk factors in neuronal diseases. It is well known that ROS is produced naturally during cell metabolism, and excessive ROS production significantly contributes to neuronal damage. Thus, excessive ROS production is considered a hallmark of various neuronal diseases, including ischemia (13-16). In addition, several studies have demonstrated that excessive ROS levels are markedly increased during ischemic injury, and eventually lead to neuronal cell death via apoptotic signal pathways (17-19). However, little is known about the effects of GSTpi protein against oxidative stress-induced hippocampal neuronal cell death and ischemic injury.

To develop therapeutic agents as drugs to treat neuronal diseases, efficient transduction methods are important, since the transduction efficiency is restricted by low cell membrane permeability in the application of therapeutic agents. However, many studies have shown that therapeutic proteins transduce into the cells and provide protective effects against various diseases using protein transduction domains (PTDs) or...
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Purified PEP-1-GSTpi protein was transduced into the HT-22 cells
GSTpi protein is known to play a crucial role in protecting against oxidative stress-induced cell death, and has antioxidant and anti-inflammatory functions (27, 28). Although GSTpi has been shown to have beneficial roles in these physiological processes, its role in ischemic injury remains unclear. To examine the effects of GSTpi protein against oxidative stress-induced HT-22 cell death and in an animal model of ischemia, we generated a PEP-1-GSTpi protein with the ability to transduce into cells. As shown in Fig. 1A, cell permeable PEP-1-GSTpi and control GSTpi proteins were prepared; these were purified using a Ni²⁺-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. SDS-PAGE and Western blot analysis were used to confirm the purified proteins.

To validate the permeability of PEP-1-GSTpi into HT-22 cell, we treated cells with various concentrations (1-14 μM) of PEP-1-GSTpi or control GSTpi proteins for 1 h; evaluation was also done at various times (10-60 min) of treatment with a consistent concentration (14 μM) of PEP-1-GSTpi or control GSTpi proteins. The resultant cells were analyzed by Western blotting. We found that the PEP-1-GSTpi protein was transduced into HT-22 cells in a dose- and time-dependent manner. Conversely, control GSTpi protein demonstrated no such transduction (Fig. 1B). In addition, we confirmed the intracellular stability and transduction using Western blotting and double fluorescence staining. As shown in Figs. 1C and Supplementary Fig. 1, PEP-1-GSTpi protein was transduced into HT-22 cells and remained stable within the cells for 48 h. Although transduction mechanisms are still not fully understood, various hypothesis of PTD transduction mechanism has been suggested by different routes such as PTD types or concentrations, endocytosis, direct and adaptive transduction (29). In addition, transduction efficiency of PTD fusion protein depends on several factors, including PTD types, protein size, and cell types (21, 22, 30). Further studies are needed to determine the exact transduction mechanism of the PTD fusion protein. In this study, we demonstrated that PEP-1-GSTpi protein was able to be efficiently transduced into HT-22 cells.

PEP-1-GSTpi inhibited the intracellular toxicity from oxidative stress
Hydrogen peroxide (H₂O₂) is known to be present in low concentrations under normal physiological conditions, but at markedly increased levels in cases of brain injury, including ischemia and spinal cord injury (31, 32). Excessive H₂O₂ levels are known to significantly contribute to neuronal cell death. To determine whether PEP-1-GSTpi protein inhibits cellular toxicities induced by H₂O₂, we examined cell viability.
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Fig. 2. Effects of PEP-1-GSTpi protein against H2O2-induced cellular toxicities. After pretreatment with 14 μM of PEP-1-GSTpi protein for 1 h into HT-22 cells, (A) cell viability was determined by WST-1 assay. HT-22 cells were pretreated with PEP-1-GSTpi protein (14 μM) for 1 h, following which they were exposed to H2O2 (0.5 mM) for 20 min. (B) intracellular ROS levels were detected by DCF-DA staining. Scale bar = 20 μm. *P < 0.01, compared with H2O2-treated cells.

and intracellular ROS production in HT-22 cells exposed to H2O2. WST-1 assay showed that H2O2 treatment decreased the cell survival from 100% to 50%, whereas transduced PEP-1-GSTpi dose-dependently inhibited the H2O2-induced cell death by up to 77% (Fig. 2A). We next performed DCF-DA staining to examine the effect of PEP-1-GSTpi protein against intracellular ROS production. As shown in Fig. 2B, H2O2-induced intracellular ROS production levels were markedly increased. In contrast, increased intracellular ROS production levels were significantly decreased by transduced PEP-1-GSTpi protein. Several studies have demonstrated that excessive H2O2 is associated with many central nervous system disorders, including ischemia and cancer. Also, increased GSTpi protein inhibited cell death via decrease in the intracellular H2O2 levels (33, 34). Consistent with other studies, our results show that transduced PEP-1-GSTpi protein protected against the oxidative stress-induced cell death.

PEP-1-GSTpi protein inhibited apoptotic signaling pathways
We further confirmed whether transduced PEP-1-GSTpi affects the oxidative stress-induced apoptotic signaling pathways. The mitogen-activated protein kinase (MAPK) signal pathway is known to be activated by a variety of external stimuli (including ROS), and is involved in apoptosis during ischemic injury. Akt is also highly involved in neuron survival in ischemia (35-37). As shown in Fig. 3A, phosphorylated p38, JNK, and Akt expression levels were increased in the H2O2 treated cells. PEP-1-GSTpi markedly decreased the phosphorylated p38, JNK, and Akt expression levels. In contrast, control GSTpi protein did not affect these signaling pathways (data not shown). We also examined the effects of PEP-1-GSTpi protein on the Bax, Bcl-2, and mitochondria membrane potential dysfunction which are all highly associated with ROS and cell survival (38-40). PEP-1-GSTpi protein significantly increased the expression levels of Bcl-2, whereas Bax expression levels were decreased in H2O2 treated cells (Fig. 3B). Also, PEP-1-GSTpi protein significantly inhibited mitochondria membrane potential dysfunction induced by oxidative stress (Supplementary Fig. 2). These results indicate that PEP-1-GSTpi protein increased the cell survival after oxidative stress by regulating apoptotic signaling pathways. However, further research is necessary to provide a better understanding of the mechanism or mechanisms involved.
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Fig. 3. Effects of PEP-1-GSTpi protein on H$_2$O$_2$-induced cellular signaling pathways and mitochondrial membrane potential in HT-22 cells. After treatment of HT-22 cells with 14 μM of PEP-1-GSTpi proteins, the cells were treated with H$_2$O$_2$ (0.5 mM) for 1 h (p38 and JNK), 40 min (Akt), and 6 h (Bcl-2 and Bax), respectively. Subsequently, (A) phosphorylated p38, JNK, and Akt levels and (B) Bcl-2 and Bax levels were measured by Western blot analysis; the band intensity were measured by densitometer. *P < 0.01, compared with H$_2$O$_2$-treated cells.

PEP-1-GSTpi attenuated ischemic injury in an animal model
Several studies have demonstrated that PTD fusion protein is efficiently transduced into the brain crossing the blood-brain barrier (BBB). This transduction has been used to enhance the uptake of therapeutic proteins in various animal models of neurodegenerative diseases (21-26, 29, 30). Thus, we first examined whether the PEP-1-GSTpi protein transduces into the mice brain crossing the BBB in an ischemic injury animal model. As shown in Fig. 4A, GSTpi protein levels were markedly increased in the CA1 region of the brain in the PEP-1-GSTpi-treated mice. In contrast, GSTpi protein was not detected in the control GSTpi-treated mice. In addition, post ischemic injury neuronal cell survival in the hippocampal CA1 region was markedly increased after treatment with PEP-1-GSTpi protein, suggesting that the PEP-1-GSTpi protein was efficiently transduced into the animal brain and protected against neuronal cell death.

Next, we conducted immunohistochemistry experiments to better understand the protective effects of PEP-1-GSTpi protein in an animal model of ischemic injury. As shown in Fig. 4B, neuronal cell death significantly increased in the vehicle-treated mice. PEP-1-GSTpi protein drastically increased the neuronal cell survival. However, there were no differences between the control GSTpi-treated mice and the vehicle-treated mice. Also, PEP-1-GSTpi protein inhibited the activation of astrocytes and microglial cells. Other studies have demonstrated that GSTpi protein protected against oxidative stress-induced SH-SY5Y cell death. Also, GSTpi protein provided protection against dopaminergic neuronal cell death in MPTP-induced PD mouse models (41, 42). In previous studies, we have demonstrated that the PTD fused therapeutic protein crossed the BBB and significantly inhibited against ischemic injury (24-26).

In conclusion, we have shown that PEP-1-GSTpi protein was transduced into both HT-22 cells and mice brain, and it significantly protected against oxidative stress-induced cell death and ischemic injury, respectively. Based on these results, we suggest that PEP-1-GSTpi protein is a potential therapeutic agent for ischemia.
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Fig. 4. Protective effect of PEP-1-GSTpi protein in ischemic injury animal model. Gerbils were treated with a single injection of PEP-1-GSTpi protein (2 mg/kg) or control GSTpi protein. After 7 days, brain tissue was collected and immunohistochemistry performed. (A) Transduction of PEP-1-GSTpi into the gerbil brain and ischemic neuronal damage was determined using anti-His antibody and NeuN immunohistochemistry. Scale bar = 50 μm. (B) Protective effect of PEP-1-GSTpi protein on ischemia was confirmed using Cresyl violet, Iba-1, GFAP, and FJB immunostaining. Scale bar = 400 μm, 50 μm, and 25 μm.

MATERIALS AND METHODS
See supplementary information for this data.

ACKNOWLEDGEMENTS
This work was supported by a Priority Research Centers Program grant (NRF-2009-0093812) through the National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning in the Republic Korea and in part by a grant from Hallym University Specialization Fund (HRF-S-13). Also, this study was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (2015R1D1A3A01015668), and in part by BioGreen21 Program (PJ01121401) of Rural Development Administration.

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