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Molecules and Cells

p-Hydroxybenzyl Alcohol Prevents Brain Injury and Behavioral Impairment by Activating Nrf2, PDI, and Neurotrophic Factor Genes in a Rat Model of Brain Ischemia

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The therapeutic goal in treating cerebral ischemia is to reduce the extent of brain injury and thus minimize neurological impairment. We examined the effects of *p*-hydroxybenzyl alcohol (HBA), an active component of *Gastrodia elata* Blume, on transient focal cerebral ischemia-induced brain injury with respect to the involvement of protein disulphide isomerase (PDI), nuclear factor-E2-related factor 2 (Nrf2), and neurotrophic factors. All animals were ovariectomized 14 days before ischemic injury. Ischemic injury was induced for 1 h by middle cerebral artery occlusion (MCAO) followed by 24-h reperfusion. Three days before MCAO, the vehicle-treated and the HBA-treated groups received intramuscular sesame oil and HBA (25 mg/kg BW), respectively. 2,3,5-Triphenyltetrazolium chloride (TTC) staining showed decreased infarct volume in the ischemic lesion of HBA-treated animals. HBA pretreatment also promoted functional recovery, as measured by the modified neurological severity score (mNSS; $p < 0.05$). Moreover, expression of PDI, Nrf2, BDNF, GDNF, and MBP genes increased by HBA treatment. *In vitro*, H_2O_2-induced PC12 cell death was prevented by 24 h HBA treatment, but bacitracin, a PDI inhibitor, attenuated this cytoprotective effect in a dose-dependent manner. HBA treatment for 2 h also induced nuclear translocation of Nrf2, possibly activating the intracellular antioxidative system. These results suggest that HBA protects against brain damage by modulating cytoprotective genes, such as Nrf2 and PDI, and neurotrophic factors.

INTRODUCTION

Stroke, along with cancer and cardiovascular diseases, is one of the most common causes of death in many countries. Administration of neuroprotective agents within 6 h of stroke onset reduces neuronal cell death caused by ischemia. However, no effective neuroprotective agent is available for treating cerebral ischemia. Ischemic stroke results from a transient or permanent reduction in cerebral blood flow (Dirnagl et al., 1999; Won et al., 1999). In most cases, the reduction in flow is caused by the occlusion of a cerebral artery because of an embolism or local thrombosis.

Gastrodia elata Blume (GEB) has been used as a folk medicine in Asian countries for centuries, and based on previous studies, *p*-hydroxybenzyl alcohol (HBA) and vanillin are believed to be the major components of GEB (Kim et al., 2007; Liu and Mori, 1993; Taguchi et al., 1981). HBA facilitates memory consolidation and retrieval (Hsieh et al., 1997). Recently, Kim et al. (2007) reported that HBA blocks oxidative stress and excitotoxicity through increased GABA transaminase. We previously showed that HBA treatment 3 days before middle cerebral occlusion (MCAO) reduces infarct volume and that this treatment increases expression of genes encoding the antioxidant proteins protein disulphide isomerase (PDI) and 1-Cys peroxiredoxin (1-Cys Prx) (Yu et al., 2005). However, the exact mechanism of HBA action in neuroprotection has not been elucidated.

PDI is a multifunctional protein located primarily in the endoplasmic reticulum. PDI catalyzes thiol/disulphide exchanges, such as disulphide bond formation and rearrangement reactions during protein folding (Freedman et al., 1995). Accumulation of misfolded protein has been linked to neurodegenerative disorders (Uehara, 2007). Overexpression of the PDI gene in neurons protects cells from apoptotic cell death induced by hypoxia or ischemia (Ko et al., 2002).

The transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), regulates an expansive set of antioxidant/detoxification genes (Jeong et al., 2006). When activated, Nrf2 specifically targets genes that contain antioxidant response elements (AREs) within their promoters (Itoh et al., 1997). The

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ability of Nrf2 to upregulate the expression of antioxidant genes suggests that increasing Nrf2 activates a defense system to combat oxidative stress. Mattson and Cheng (2006) suggested that neurohormetic phytochemicals protect neurons against injury and disease by stimulating the production of antioxidant enzymes and neurotrophic factors.

In the present study, we examined whether HBA regulates gene expression as a protective mechanism in ischemic rat brain. Specifically, we measured expression of the cytoprotective genes Nrf2 and PDI, as well as several neurotrophic factor genes, such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and myelin basic protein (MBP). To determine the mechanism underlying HBA action, we examined the involvement of PDI in H_2O_2-induced PC12 cell damage and HBA-induced nuclear translocation of Nrf2.

MATERIALS AND METHODS

Chemicals

TRI Reagent, *p*-hydroxybenzyl alcohol (HBA), bacitracin, and cresyl-violet were purchased from Sigma Chemical Co. (USA). The RT-PCR kit was obtained from Promega Co. (USA). All chemicals used were of analytical grade.

Animals and experimental protocol

All animals were handled in accordance to the animal care guidelines of the US National Institutes of Health. All experimental procedures were approved by the Committee for Animal Experimentation and Institutional Animal Laboratory Review Board of Inje University. We performed ovariectomy on female Sprague-Dawley rats weighing 300-350 g. Three days before middle cerebral artery occlusion (MCAO), the vehicle-treated and HBA-treated groups received intramuscular sesame oil and HBA (25 mg/kg BW), respectively. At least 3 animals were included in each group.

Ovariectomy

All rats were ovariectomized 14 days before MCAO to eliminate the endogenous supply of gonadal steroid hormones. The rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) intramuscularly under aseptic conditions. The surgical procedure was performed with a small ventral abdominal midline incision. The ovaries were bilaterally clamped and removed; the uterine horns were ligated; the uterus was left intact; and the abdominal wall and skin were sutured.

Middle cerebral artery occlusion

After administration of anesthetics, the left common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) on the left side were exposed and dissected through a midline cervical incision. A 4-0 monofilament suture was introduced into the left ICA lumen and gently advanced until resistance was felt, indicating MCAO and compromised blood flow. The suture was kept in place for 1 h and then reperfusion was allowed for 24 h. The operating procedure was performed within 20 min, with little bleeding. Sham animals were treated in the same manner as those undergoing MCAO, but without cutting the ECA or inserting the embolus.

Measurement of infarct volume

Each animal was decapitated 1 day after MCAO, and the brains were quickly removed and placed in a metallic brain matrix for slicing the tissue. Four slices were made at 7, 9, 11, and 13 mm posterior to the olfactory bulb. Each slice was incubated for 30 min in a 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution in physiological saline at 37°C and then fixed in 10% neutral buffered formalin. The stained slices were photographed using a digital camera and the surface area of the ischemic lesion was subsequently measured using a multi-format scanning imager (Typhoon 9400; Amersham Biosciences, USA). The infarct volume was calculated as the sum of the infarct areas × thickness (2 mm), and was expressed as a percentage of the lesioned half of the brain.

Neurological function tests

Functional tests were performed 1, 7, 14, 21, and 28 days after MCAO by an investigator who was blinded to the experimental groups. All rats were evaluated using a modified neurological severity score (mNSS) (Chen et al., 2001). The mNSS is a composite of motor (muscle status, abnormal movement), sensory (visual, tactile, proprioceptive), reflex, and balance tests. Neurological function was graded on a scale of 0 to 18 points (normal score, 0; maximal deficit score, 18). In the severity scores of injury, 1 point was awarded for the inability to perform the test or for the lack of a tested reflex; higher scores indicate more severe injuries.

RT-PCR analysis

The hippocampus of the lesioned side was harvested for RT-PCR. Total RNA was extracted using the acid guanidinium isothiocyanate-phenol-chloroform method with TRI Reagent. The nucleotide sequences of the PCR primer sets were designed using the Primer3 software (Whitehead Institute, MIT Center for Genome Research, USA) on the basis of sequences obtained from the National Center for Biotechnology Information GenBank database. The sequences of the primer pairs used were PDI: 5′-TCT GGA GGA GGA GGA CAA C-3′, 5′-TGG AAA ACA CAT CGC TAT T-3′; Nrf2: 5′-AAG GAA GCT GGA AAA CAT TG-3′, 5′-AAA TGG TGC CCA AGA AAT TA-3′; GDNF: 5′-TGG GCT ATG AAA CCA AGG AG-3′, 5′-ATA CAT CCA CAC CTT TTA GCG-3′; BDNF: 5′- CAC CAG ATA AAC AAA TGG CAG TGC-3′, 5′- CGA CAG GTC ATC ATC AAA GGC G-3′; NGF: 5′- ATA CAG GCG GAA CCA CAC GCA G-3′, 5′-GTC CAC AGT AAT GTT GCG GGT C-3′; MBP: 5′-ACA CGG GCA TCC TTG ACT CCA TCG G-3′, 5′- TCC GGA ACC AGG TGG TTT TCA GCG-3′, and GAPDH: 5′-CAT GAC CAC AGT CCA TGC CAT CAC T-3′, 5′-TGA GGT CCA CCA CCC TGT TGC TGT A-3′. Total RNA samples (10 μg) were reverse-transcribed using 2.5 U Moloney murine leukemia virus (MMLV) reverse transcriptase and 0.5 μg of oligo (dT) primer in the presence of 1 mM dNTP and 1 U RNasin in a final volume of 50 μl. PCR amplification was performed on each cDNA subpopulation. To validate the mRNA levels using RT-PCR, serially diluted total RNA samples (0.1-1.0 μg) were tested with 25 cycles of PCR amplification. A linear relationship was observed between cycle number and the amount of amplification product, with a correlation coefficient of 0.98. A linear relationship was also observed between increasing number of PCR cycles (15–35) and the levels of amplification products, with a correlation coefficient of 0.96. PCR was performed for 30 (PDI, Nrf2, GDNF, BDNF, NGF, and MBP) and 25 (GAPDH) cycles of 60 s at 95°C, 60 s at each annealing temperature, and 60 s at 72°C using 1 μl of the RT product in a 25 μl reaction mixture containing 25 mM $MgCl_2$, 10× PCR buffer, 2 U Taq polymerase, 10 mM dNTP, 100 pM of each gene-specific primer, and nuclease-free water. The PCR products were mixed with 6× loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose) and separated on 2% agarose gels. Gels were stained with ethidium bromide, and the DNA bands were

viewed under ultraviolet light. Sequences were verified using a PCR sequencing system (Promega, USA).

Cell culture and drug treatment

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker Inc., USA), supplemented with 5% fetal bovine serum, 10% horse serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C, in a humidified atmosphere of 5% CO_2/95% air. Experiments were carried out 24-48 h after cells were seeded. The cells were treated with H_2O_2 (0.5 mM) for 30 min. After H_2O_2 treatment, the cells were gently harvested and stained in 0.4% trypan blue solution. Viability was calculated based on the percentage ratio of the number of live cells to the total cell number. Cells were treated with HBA (0.25, 0.5, and 1.0 mM in culture medium) for 24 h before H_2O_2 treatment. Cells were co-treated with various doses of bacitracin, a PDI inhibitor (Mandel et al., 1993), and HBA 24 h before H_2O_2 treatment, and cell viability was calculated.

Immunocytochemistry

After drug treatment, the media were discarded, and the cells were washed twice with pre-warmed phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 30 min. Cells were then blocked with reaction buffer (0.1% Triton X-100 (v/v), 0.6% bovine serum albumin (v/v), and 0.04% sodium azide (w/v), and were diluted in 0.02 M phosphate buffered saline) containing 10% normal goat serum. They were then incubated with rabbit Nrf 2 antibody (Santa Cruz biotech, USA) overnight at 4°C. Cells were washed 4 times with 1% Triton X-100-PBS and then incubated with Cy3 labeled goat anti-rabbit IgG (Amersham bioscience, USA) at 4°C for 30 min. They were then washed 4 times with 1% Triton X-10-PBS and coverslipped with a mounting medium containing DAPI (Vector laboratories, USA). The signals were detected by an Axioskop2 plus (Carl Zeiss, Germany).

Statistical analyses

The significance of the differences in mean values among the experimental groups was determined using the t-test and one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. The level of statistical significance was set at $p < 0.05$. SPSS for Windows (version 10.0; SPSS Inc., USA) was used to calculate probability values.

RESULTS

HBA pretreatment decreased brain infarct volume in both the cortex and the striatum

Three rats randomly selected from each experimental group were killed after 1-h ischemic injury followed by 24-h reperfusion, and the extracted brains were analyzed for ischemic damage. The borders of the TTC stain enclosing the white infarct area were readily distinguishable in contrast to the red color of the normal area. The infarct volumes observed in the vehicle-treated group were significantly larger than those of the HBA-treated rats (Fig. 1A). The greatest total infarct volume was seen in the vehicle group, occupying 83 ± 10% of the lesioned side of the brain. HBA pretreatment for 3 days before the induction of ischemia dramatically reduced the total volume of brain infarction (36 ± 7%). This reduction was observed in both the cortex and the striatum (Fig. 1B).

HBA pretreatment improved functional recovery after MCAO

We examined whether HBA administration before MCAO reduced functional deficits caused by ischemic brain injury. The mNSS test showed that motor and sensory functions were impaired by ischemic insult. However, significant differences in the recovery of neurological functions were observed from 7 days after MCAO between the 2 groups. HBA pretreatment significantly improved functional recovery after MCAO compared with the vehicletreatment (Fig. 2).

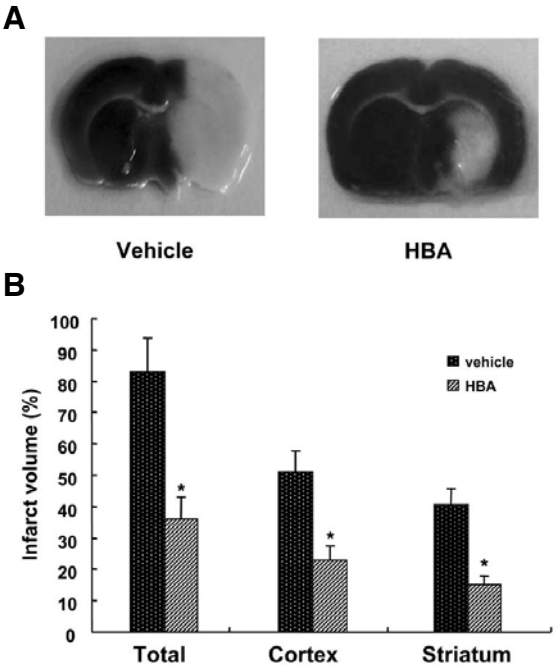


Fig. 1. Effects of HBA on the infarct volume at 24 h after MCAO. TTC-stained coronal sections (1 mm posterior to the bregma) from rats pretreated with vehicle or HBA (25 mg/kg, i.m.) for 3 days before MCAO for 1 h followed by 24 h reperfusion are represented (A). Non-ischemic region is red, and infarct region appears as white color. The infarct volume was calculated as the infarct areas x thickness (2 mm), and expressed as a percentage of the lesioned half of the brain. Each bar in the graph represents the mean ± S.E.M. (n = 3) (B). * $p < 0.05$ vs. vehicle.

HBA stimulated expression of PDI and Nrf2 genes in the hippocampus

We performed RT-PCR to analyze the effects of HBA on the expression of the PDI and Nrf2 genes in the hippocampus. After a 1 h ischemic insult followed by 24 h of reperfusion, the whole hippocampus of the lesioned area was subjected to RT-PCR analysis. The expression levels of PDI and Nrf2 genes decreased significantly in the vehicle-treated group compared to the sham group ($p < 0.05$). Furthermore, the expression levels of PDI and Nrf2 genes were significantly elevated in the HBA-treated group compared to the vehicle-treated group ($p < 0.05$; Fig. 3).

HBA upregulated expression of neurotrophic factor genes in ischemic rat brain

We examined whether HBA had any effect on endogenous neurotrophic activity. To examine the potential involvement of neurotrophic factors in the mechanism of neuroprotection by HBA, mRNA levels of neurotrophic factors, such as GDNF, BDNF, NGF, and MBP, were analyzed by RT-PCR. The results showed increased expression of all genes in the HBA-pre-

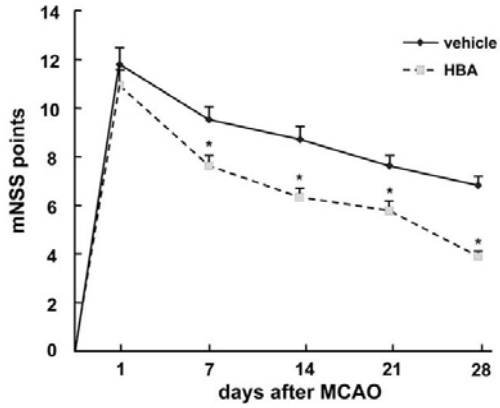


Fig. 2. Effect of HBA on the functional behavioral recovery after MCAO. Neurological functional tests were performed at 1, 7, 14, 21, and 28 days after MCAO. HBA-treated group (-■-) shows significantly lower mNSS score than vehicle-treated group (-◆-) after the seventh day (n = 3). *p < 0.05 vs. vehicle

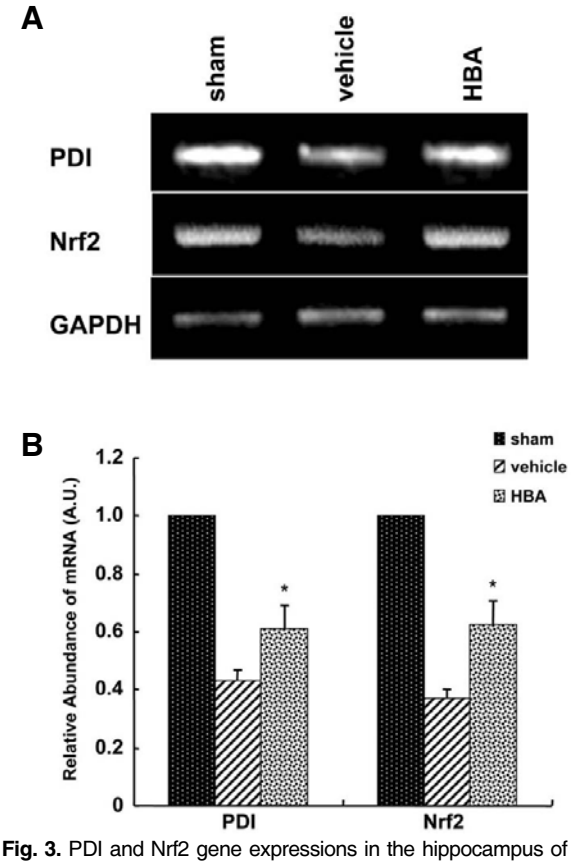


Fig. 3. PDI and Nrf2 gene expressions in the hippocampus of rats treated with vehicle or HBA. After vehicle or HBA was administered daily for three days, each group of animals were underwent 1 h ischemic injury followed by 24 h reperfusion. Representative RT-PCR results are documented (A). The band intensity of PDI and Nrf2 were normalized by the band intensity of GAPDH and individual values are expressed as the mean ± S.E.M. (B). Experiments were repeated three times. *p < 0.05 vs. vehicle.

treated groups, although the increase in mRNA levels of NGF

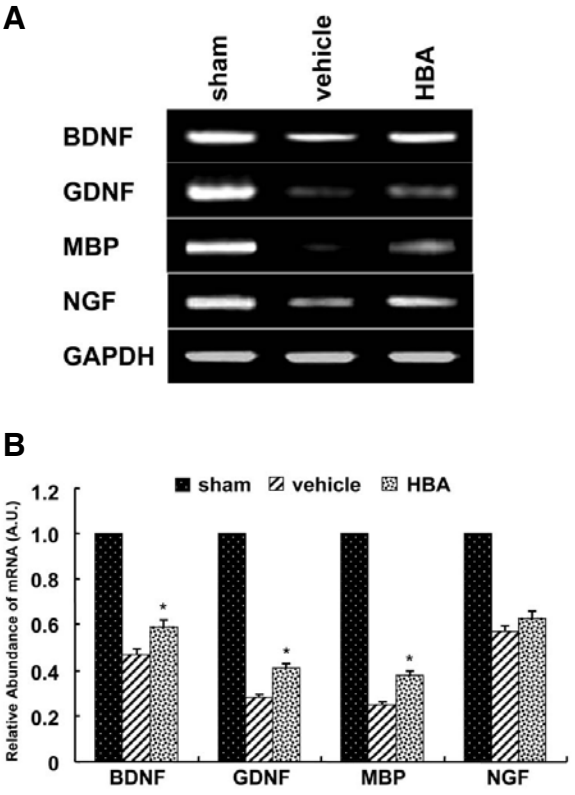


Fig. 4. Effects of HBA on gene expression of neurotrophic factors in the hippocampus. After vehicle or HBA was administered daily for three days, each group of animals were underwent 1 h ischemic injury followed by 24 h reperfusion. Representative RT-PCR results are documented (A). Specific gene expression was determined in relation to the expression of GAPDH. The levels of BDNF, GDNF, and MBP gene expression were significantly increased in the HBA-treated group compared with the vehicle group (B). Experiments were repeated three times and individual values are expressed as the mean ± S.E.M. *p < 0.05 vs. vehicle.

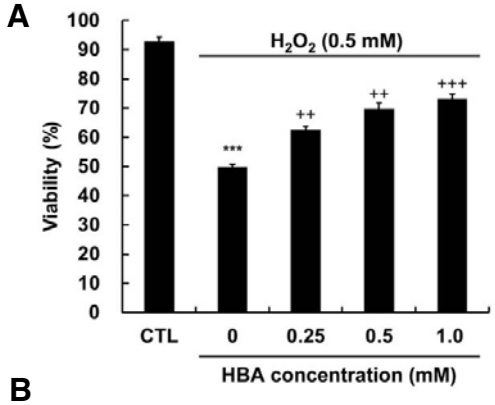
was not significant (Fig. 4).

HBA prevented H_2O_2-induced cell death by increasing PDI activity

To examine the neuroprotective effect of HBA on oxidative stress-induced cell death, cells were HBA-treated (0.25, 0.5, and 1.0 mM) 24 h before 0.5 mM H_2O_2 treatment. The reduction in cell viability induced by H_2O_2 was reduced by HBA treatment in a dose-dependent manner; 1.0 mM HBA prevented 23% of the cell death induced by H_2O_2 (Fig. 5A). To examine the involvement of PDI in HBA's action, cells were co-treated with bacitracin, a PDI inhibitor, and 0.5 mM HBA 24 h before H_2O_2 treatment. With 1, 2, and 5 mM bacitracin co-treatment, the effects of HBA were inhibited in a dose-dependent manner (Fig. 5B).

HBA induced nuclear translocation of Nrf2

Since Nrf2 translocates to the nucleus from the cytoplasm when it is activated, we examined whether HBA could induce Nrf2 nuclear translocation as a possible mechanism of its protective action. As shown in the immunocytochemistry figures, Nrf2 was localized predominantly in the cytoplasm in untreated cells (Figs. 6A, 6B, and 6C) and this localization was altered by



H_2O_2 (0.5 mM)	-	+	+	+	+	+
HBA (0.5 mM)	-	-	+	+	+	+
Bacitracin (mM)	-	-	-	1	2	5

Fig. 5. Effect of HBA treatment on H_2O_2-induced cell death of PC12 cells and effect of bacitracin on HBA action. Different doses of HBA were treated to the cells for 24 h before 30 min-H_2O_2 treatment (A). Different dose of bacitracin were cotreated with HBA for 24 h before H_2O_2 treatment (B). The viability was calculated from the percentage ratio of live cell number to the total cell number. Data represent as the mean ± S.E.M (n = 3). CTL: vehicle. ***$p < 0.001$ vs. CTL; $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. H_2O_2 only; #$p < 0.05$ vs. H_2O_2 + HBA.

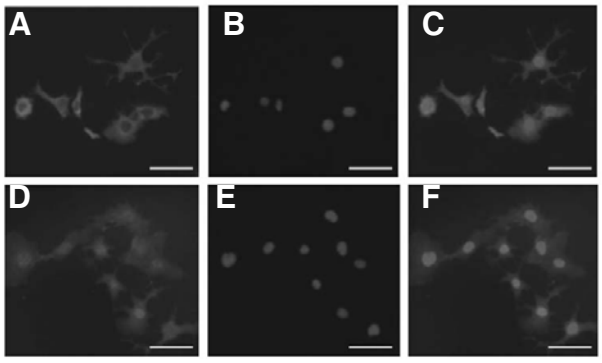


Fig. 6. Nuclear translocation of Nrf2 induced by HBA treatment. PC12 cells were treated for 2 h with 0.5 mM HBA. The representative cell figures in upper panels are for control (A, B, and C) and in lower panels are for HBA-treated cells (D, E, and F). Control cells show Nrf2-immunopostive signals are in cytoplasm (A) while the signals are clearly concentrated in the nucleus (D). DAPI staining represents for nuclei (B, E). Scale bar, 50 μm.

HBA treatment. Two hours after 0.5 mM HBA treatment, the immunopositive signals for Nrf2 were denser in the nucleus than the cytoplasm (Figs. 6D, 6E, and 6F).

DISCUSSION

Several studies have suggested that HBA possesses antioxidant and free radical scavenging activities (Jung et al., 2007; Liu and Mori, 1993). HBA treatment attenuates drug-induced learning deficits in a passive avoidance task, by suppressing dopaminergic and serotonergic function (Wu et al., 1996). In the present study, HBA pretreatment significantly decreased infarct volume compared with the vehicle-treated group and this reduction was observed in both the cortex and the striatum. The magnitude of the reduction was similar to that seen in our previous study (Yu et al., 2005). Because the animals in the present study were subjected to evaluations 24 h after MCAO, these results may represent acute ischemic injury events.

Our results indicate that PDI and Nrf2 genes are involved in the preventive effects of HBA on MCAO-induced stroke in rats. We performed RT-PCR to quantify the expression levels of these 2 genes in ischemic brain. The increased expression of both genes by HBA was found in homogenates of whole hippocampus. Overexpression of the PDI gene protected against loss of cell viability induced by hypoxia in human neuroblastoma SK-N-MC cells and a reduction in the number of DNA fragmented cells in the hippocampal CA1 regions of rats subjected to cerebral ischemia (Tanaka et al., 2000). Hypoxia caused the accumulation of immature proteins, which may eventually lead to cell death due to endoplasmic reticulum dysfunction. Therefore, PDI could be a target for unfolded protein response-induced gene expression (Ko et al., 2002) since it catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins. In addition, some researches support that this molecular chaperone is involved in the response to ischemic injury in glial cells (Tanaka et al., 2000) and neurons (Hwang et al., 2005). Our result shows that PDI gene expression decreased after the ischemic injury whereas Tanaka et al. (2000) showed PDI up-regulation in response to ischemic injury. This discrepancy seems to be due to the ovariectomized female rat model in this study considering that the previous studies used male animals. PDI gene expression is known to be affected by estrogen status (Fu et al., 2008). When we examined PDI gene expression after ischemic injury with *in situ* hybridization technique, we got similar result to one by RT-PCR (data not shown). We also investigated the involvement of PDI in HBA-mediated protection against oxidative stress-induced cell death in an *in vitro* system. High oxidative stress is thought to be dangerous to cells because it increases intracellular reactive oxygen species (ROS), which can cause widespread intracellular damage (Cadenas, 1989). The results showed that high concentrations of HBA markedly decreased cell death caused by H_2O_2 and greatly enhanced cell viability. In addition, the present study shows that bacitracin, a PDI inhibitor (Mandel et al., 1993) reduced the HBA effect, confirming that PDI mediates the cytoprotective action of HBA. This result coincides with that of a previous study (Descamps et al., 2009).

Nrf2 plays a key role in ARE-mediated gene expression. Overexpression of Nrf2 upregulates ARE-mediated expression of reporter genes that are inducible by various antioxidants and chemopreventive agents (Jeong et al., 2005). Cellular antioxidants are important in reducing oxidative stress and preventing neuronal death. A pathway for inducing antioxidant enzymes involves transcriptional activation through the ARE (Itoh et al., 2004). These findings support the hypothesis that increased

oxidative stress and dysregulated antioxidant systems are closely associated with the pathogenesis of neurodegeneration. The present study shows that HBA induces nuclear translocation of Nrf2, which is a factor that promotes transcription of cytoprotective genes. Under basal conditions, Kelch-like ECH-associated protein-1 (Keap1) sequesters Nrf2 in the cytosol. When oxidative stress increases within the cell, Nrf2 escapes Keap1-mediated proteosomal degradation and translocates into the nucleus (Bloom and Jaiswal, 2003; Tong et al., 2006). In addition to conditions of oxidative stress, treatment with many cytoprotective molecules has been reported to induce Nrf2 translocation (Jeong et al., 2006; Surh et al., 2008). Therefore, HBA-induced translocation of Nrf2 suggests a role for Nrf-2 in HBA-mediated cytoprotection.

Numerous studies have reported behavioral deficits in rodents after focal stroke (DeVries et al., 2001). We examined whether HBA pretreatment protects functional loss due to ischemic brain injury. Neurological scores measured by mNSS showed clear differences between sham and vehicle-treated animals during the first days after MCAO in all subcategories of the behavioral test (data not shown). HBA pretreatment promoted recovery from neurological functional deficits induced by MCAO. The mechanism of the functional improvement in HBA-treated animals is still unknown. The effect of HBA on functional recovery may be related to the increased expression of neurotrophic factor genes. Neurotrophic factors are essential for neuronal survival and differentiation during development, as well as for the maintenance of normal neuronal function in adults (Snider, 1994). Neurotrophic factors have also been shown to protect neurons against oxidative stress by increasing the activity of antioxidant enzymes and modulating expression of apoptosis-related proteins that promote cell survival (Mattson et al., 1995). NGF and BDNF have been reported to play a neuroprotective role in the MCAO rat stroke model (Kokaia et al., 1995). NGF is the prototype of the neurotrophin family of growth factors that mediate survival pathways, proliferation, differentiation, and neuroprotection in the nervous system (Sofroniew et al., 2001). BDNF also plays an important role in the survival, differentiation, and outgrowth of peripheral and central neurons during development and adulthood (Conover and Yancopoulos, 1997). GDNF reduces ischemic insults, and several ischemic insult-reducing agents, such as FGF-2 and vitamin D2, induce protection through GDNF (Harvey et al., 2005). Miyazaki et al. (2001) suggested that ischemia-induced reactive astrocytes produce GDNF to protect against neuronal death. Myelin basic protein (MBP) is an abundant myelin membrane lipoproteinproduced by oligodendrocytes and may assist in the clinical assessment of stroke (Hill et al., 2000). Many neurotrophic factors may play a role in formation or modification of neural circuits. In the present study, HBA pretreatment increased expression of these neurotrophic factor genes, including BDNF, GDNF, NGF, and MBP and the upregulation of these factors may be responsible for the observed improvements in behavioral function. In addition, a report recently proposed that anti-apoptosis underlies the neuroprotective effect of HBA in focal cerebral ischemia in rats (Yu et al., 2010).

Given these findings, we suggest that (1) PDI and Nrf2 upregulation by HBA in the ischemic brain is involved in the mechanism of tolerance to ischemic stress, (2) PDI and Nrf2 exert neuroprotective effects against hypoxia, and (3) the improved neurological function induced by HBA is related to neurotrophic factors such as BDNF, GDNF, and MBP. Indeed, the neuroprotective effect of HBA may help in understanding the link between oxidative stress and the roles of specific proteins such as PDI and Nrf2, as well as neurotrophic factors in the pathogenesis of the ischemic brain.

In conclusion, our results suggest that HBA protects against ischemic brain injury through upregulation of gene expression of PDI, Nrf2, and several neurotrophic factors, as well as the modulation of PDI and Nrf2 activities.

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