Gastrodia elata Blume and an Active Component, p-Hydroxybenzyl Alcohol Reduce Focal Ischemic Brain Injury through Antioxidant Related Gene Expressions

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Ishamic stroke is a leading cause of death and long-lasting disability. Gastrodia elata blume (GEB) is a Chinese herb that is widely used to treat convulsive disorders, such as epilepsy, and p-hydroxybenzyl alcohol (HBA) is the active ingredient in GEB. The present study was conducted to evaluate the effects of GEB and HBA on the brain damage and transcriptional levels of Protein disulfide isomerase (PDI) and 1-Cys peroxiredoxin (1-Cys Prx) genes known to play a role in antioxidant systems after transient focal ischemia in the rat brain. Focal ischemia was induced in rats by middle cerebral artery occlusion (MCAO). All animals underwent ischemia for 1 h, followed by 24 h of reperfusion. Coronal brain slices were stained with 2,3,5-triphenyltetrazolium chloride or total RNA was extracted for the analysis of gene expression. Histopathologic analysis revealed a significant (p<0.05) decrease in infarct size in the ipsilateral brain with GEB extracts or HBA. Moreover, the levels of PDI and 1-Cys Prx transcription were significantly increased in the GEB extract- or HBA-treated group compared with the untreated group (p<0.05). This study therefore indicated that GEB and HBA provide neuroprotection by preventing brain damage through the increased expression of genes encoding antioxidant proteins after transient focal cerebral ischemia and may be effective as neuroprotective agents at the cellular and molecular levels in the brain.

Key words 1-Cys peroxiredoxin; Gastrodia elata blume; ischemia; p-hydroxybenzyl alcohol; protein disulfide isomerase

Ischemic stroke is a leading cause of death and long-lasting disability and therefore neural cell survival is a concern in aging brains as well as in many diseases of the central nervous system (CNS). Ischemic stroke results from a transient or permanent reduction in cerebral blood flow that is restricted to the territory of a major brain artery.1–3 The reduction in flow is, in most cases, caused by the occlusion of a cerebral artery either by an embolus or by local thrombosis. Transient focal cerebral ischemia initiates a cascade of detrimental events including accumulation of intracellular Ca2+ and formation of free radicals. When blood flow is restored, oxygen can enhance the biochemical reactions that generate free radicals. The formation of free radicals also plays an important role in the pathogenesis of ischemic cell damage. Free radicals damage the lipid constituents of cellular and organelle membranes, which leads to neuronal death.5–7 This cell death can be divided into two broad categories: early necrotic death of cells in the ischemic core and delayed death of susceptible neurons in other neighboring regions, the so-called penumbra. Because the second category of cellular death occurs over an extended time, these neurons have the potential to be rescued by pharmacologic agents.5,6

Gastrodia elata blume (GEB) is a traditional herbal agent that has been used as an anticonvulsant, analgesic, and sedative to treat general paralysis, epilepsy, vertigo, and tetanus in Oriental countries for centuries.2–10 An earlier study showed that GEB can reduce lipid peroxide levels and has free radical-scavenging activities in rats with ferric chloride-induced seizure. The results also suggested that these effects of GEB result mainly from the action of its major components, vanillin and p-hydroxybenzyl alcohol (HBA).11,12 The aqueous extract of GEB improved d-galactose-induced memory impairment in mice and performance deficits in senescent mice on the step-down passive avoidance task.13 Recently, it has been reported that compounds found in GEB inhibit glutamate-induced apoptosis in neuronal cells.14 Additionally, after pentylentetrazol induced seizure activity, the ether fraction of GEB has been shown to attenuate the decrease in γ-aminobutyric acid (GABA) and the increase in glutamate content, as well as to have anticonvulsant effects.15,16 Compounds in the ethyl ether fraction of GEB dramatically reduce the extent of neuronal cell death in IMR-32 neuroblastoma cells treated with amyloid-beta peptide.17 The improving effect of HBA after acute administration on learning and memory processes might be related to the decrease in dopamine concentration or other monoamine concentration and prevent DNA degradation.18 HBA isolated from the ethyl acetate fraction of the methanol extract also significantly prolonged the shortened step-through latency induced by scopolamine in the passive avoidance task.19 Exposure of tissues to oxygen free radicals results in lipid peroxidation, protein oxidation, and DNA damage. The final result induced by these oxidatively damaged macromolecules is to give rise to neurodegeneration. Therefore it appeared to be important to investigate the expression level of antioxidant proteins in brain disease. It was reported that protein disulfide isomerase (PDI) serves as a repair system for oxidatively damaged proteins via reduction of oxidized thiols in vascular endothelial cells.20 Upregulated PDI may play a critical role in resistance to ischemic damage, and the elevation of levels of this protein in the brain may have beneficial effects in brain stroke.21 The 1-Cys peroxiredoxin (1-Cys Prx) protein...
showed dysregulated expression in sporadic Creutzfeldt-Jacob disease (sCJD) brains.\textsuperscript{22} Furthermore, recent studies have shown that thioredoxin reductase can also scavenge reactive oxygen species.\textsuperscript{21} indicating that these proteins constitute a novel cellular defense system against oxidative injury in addition to the conventional antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase. In the present study, we investigated the effects of GEB and HBA on neuroprotection in vivo and whether the effects of GEB and HBA in preventing brain damage were associated with the induction of PDI and 1-Cys Prx gene expressions.

MATERIALS AND METHODS

Chemicals  Tri-reagent and HBA were procured from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The RT-PCR kit was purchased from Promega Co. (Madison, WI, U.S.A.). All other chemicals used were of analytical grade.

Preparation of GEB Extract  The washed GEB was deep-frozen (\(-70^\circ\text{C}\)) until use. Freeze-dried GEB was powdered with a mixer. One hundred grams of dry fine powder was suspended in 600 ml of water and boiled for 3 h. This was repeated three consecutive times. The residue was removed by filtration and the extract evaporated at \(<40^\circ\text{C}\) under reduced pressure to yield a semisolid extract. The aqueous solution was extracted with methanol, and the methanol extract was resuspended in 10% methanol. The methanol fraction was reextracted with diethyl ether and divided into two fractions, the ethyl ether fraction and water fraction, and evaporated at 30°C under reduced pressure. The water layer was again extracted with chloroform. The remaining water fraction was extracted with ethyl acetate followed by \(\beta\)-butanol. Three fractions were collected: the ethyl acetate, methanol, and water fractions. The color of each fraction was light to dark brown. All fractions were dried in a rotary evaporator.

Experimental Protocol  Male Sprague-Dawley rats weighting 300—350 g at the time of surgery were used for the study. The animals were housed in pairs in cages with food and water in a temperature-controlled room (22±3°C) under a 12-h light–dark cycle. Three groups of three rats each were studied. Rats in groups I, II, and III were subjected to 1 h of temporary middle cerebral artery occlusion (MCAO), followed by 24 h of reperfusion. Group I was the untreated controls. Group II received GEB (500 mg/kg i.p.) extract with water, MeOH, or ethyl acetate beginning 3 d prior to MCAO and group III received HBA (Sigma Aldrich Co., U.S.A.) (25 mg/kg i.v.) beginning 3 d prior to MCAO.

Ischemic Surgery and Infarct Analysis  MCAO was achieved according to the methods described by others, with the following modifications.\textsuperscript{24,25} Briefly, rats were anesthetized with isoflurane (3% for induction and 2% for the surgical procedure) in a mixture of oxygen/nitrous oxide (30%/70%). Body temperature was monitored with a rectal probe, and maintained at 37±0.5°C with a thermostatically controlled warming plate. The left common carotid artery was exposed and carefully dissected free of the vagus nerve. The external and internal carotid arteries were also isolated. The external carotid artery was then tied and cut off. The middle cerebral artery was occluded with a 4–0 nylon thread (Ethilon; Ethicon Norderstedt, Germany) precoated with siliccon resin (Xantopren Bayer Dental, Osaka, Japan) introduced via the internal carotid artery. Then the skin incision was closed. After 1 h, the suture was withdrawn for reperfusion. All animals underwent ischemia for 1 h, followed by 24 h of reperfusion.

Each group of animals was decapitated 24 h after MCAO, and the brains were removed and placed in a metallic brain matrix for tissue slicing immediately after decapitation. For detection of the ischemia infarction area of the brain, four slices were made at 7, 9, 11, and 13 mm posterior to the olfactory nerve. Each slice was incubated for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiologic saline at 37°C and then fixed in 10% formalin. The stained slices were photographed with a digital camera (Sony DSC-F707) and subsequently the surface area of the slice and ischemic lesion were measured (Typhoon 9400). The ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the four slices divided by the total cross-sectional area of the four brain slices.

RT-PCR Analysis  The ipsilateral brains were harvested for RT-PCR. Total RNA was extracted using the acid guanidinium isothiocyanate-phenol-chloroform method using Tri-reagent. The nucleotide sequences of the PCR primers sets were designed using Primer3 software (Whitehead Institute/MT Center for Genome Research) on the basis of sequences deposited in the NCBI GenBank database. Briefly, total RNA samples (1 \(\mu\)g) were reverse-transcribed using MMLV reverse transcriptase 2.5 U (Perkin Elmer, Norwalk, CT, U.S.A.) and oligo(dT) primer 0.5 \(\mu\)g (Perkin Elmer) in the presence of dNTP 1 mM (Perkin Elmer) and RNasin 1 U (Perkin Elmer) in a final volume of 20 \(\mu\)l. Following a hot start (5 min at 95°C), PCR was performed for 30 cycles of 60 s at 95°C, 60 s at each annealing temperature, and 60 s at 72°C, using 10 \(\mu\)l of the RT product, 0.2 \(\mu\)l of the primers, Taq polymerase 2.5 U (Perkin Elmer), 0.2 mM of each dNTP, and MgCl\(_2\) 2 mM in a final volume of 50 \(\mu\)l. The sequence 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’; 1-Cys Prx: 5’-AAG AGG AGG CCA AAC AAC T-3’; 5’-AGC CCA CAC TGA CAC TAC C-3’; and GAPDH: 5’-CAT GAC GAC AGT CCA TGC CAT CAC T-3’; 5’-TGA GGT CCA CCA CCC TGT TGC TGT A-3’. A linear relationship was also observed between increasing number of PCR cycles and the levels of amplification products (data not shown). The resulting PCR products were visualized by electrophoresis and EtBr staining and cloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.). The sequences were verified using the fmol PCR sequencing system (Promega).

Statistical Analysis  The significance of differences in mean values among the experimental groups was determined using 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) was used to calculate probability values.

RESULTS

Effects of GEB Extracts on Brain Infarction  The borders of the TTC stain were well demarcated, enclosing a white infarct area in contrast to the normal red area.\textsuperscript{20} There was a significant difference in infarct size between the vehi-
The brain infarction was detected in brain slices cut 7 mm from the frontal pole using the TTC staining method. GEB extract (500 mg/kg) as given to the animals for 3 d before induction of ischemia. A, 1-h ischemia/24-h reperfusion (vehicle); B, 1-h ischemia/24-h reperfusion after GEB water-extract pretreatment for 3 d (water); C, 1-h ischemia/24-h reperfusion after GEB MeOH-extract pretreatment for 3 d (MeOH); D, 1-h ischemia/24-h reperfusion after GEB ethyl acetate-extract pretreatment for 3 d (ethyl acetate); E, neuroprotective effects of GEB were marked in the cerebral cortex. The regional infarct volumes were significantly reduced. Experiments were repeated three times and individual values are mean±S.E.M. The letters a and b on the bar indicate significant differences (p<0.05) compared with the vehicle and water groups, respectively.

**Effects of HBA on Brain Infarction**

HBA markedly decreased ischemic injury in the transient focal ischemia model. Figure 2 is a composite of representative coronal brain sections from control and HBA-pretreated rats following cerebral ischemia. HBA significantly reduced overall infarct volume as compared with vehicle. The greatest total infarct size was 78±5% in the vehicle group. Treatment with GEB extract for 3 d prior to the induction of ischemia attenuated the total brain infarction (29±3%, 43±4%, 40±4% in the GEB extract with water, MeOH, or ethyl acetate group, respectively) (Fig. 1). In the vehicle group, the infarct sizes were 45±4% and 36±4% of contralateral nonischemic cortex and striatum, respectively. Histologic examination revealed a significant (p<0.05) decrease in infarct sizes in the cortex and striatum in the HBA (23±2%, 15±1%) groups, respectively (Fig. 2).

**Effects of GEB Extract or HBA on PDI and 1-Cys Prx Gene Expressions**

To investigate further antioxidant systems in rat ischemic brain, we employed RT-PCR analysis to quantify the expression levels of PDI and 1-Cys Prx genes that encode antioxidant proteins. Since the TTC results showed that most of the brain was damaged by ischemia for 1 h, followed 24 h of reperfusion, the ipsilateral whole brain was used for RT-PCR analysis. Figure 3 shows the effects of GEB extract or HBA on PDI and 1-Cys Prx gene expressions in ischemic rat brains. PDI or 1-Cys Prx gene expression level significantly (p<0.05) increased in the GEB water-extract or HBA-pretreated group compared with the untreated vehicle group. Expression of the housekeeping gene, GAPDH, was unaltered.

**DISCUSSION**

To determine whether the neuroprotective effects of GEB in ischemic rat brain were region specific, we analyzed treatment effects in the cerebral cortex and striatum. GEB pretreatment markedly reduced the cortical and striatum infarct sizes in both groups. The reduction was observed mainly in the cortical region. The magnitude of this reduction is similar to that seen in other studies involving antireperfusion injury agents in this model. In addition, all animals in the present study were evaluated at 24 h, and thus these results represent early ischemic injury events. Tissue injury following cerebral ischemia results from the interaction of complex pathophysiologic processes that are potential targets for therapy. Three routes have been identified as triggering neuronal death under physiologic and pathologic conditions. Excess activation of ionotropic glutamate receptors causes influx and accumulation of Ca$^{2+}$ and Na$^+$, resulting in rapid swelling and...
subsequent neuronal death within a few hours. The second route is caused by oxidative stress due to accumulation of reactive oxygen and nitrogen species.\(^3\) The apoptosis or programmed cell death that often occurs during the development process is an additional route to pathologic neuronal death in the mature nervous system. Evidence is being accumulated that oxidative stress contributes to neuronal loss following hypoxic-ischemic brain injury. Free radicals play an essential role in maintaining the physiologic condition of the body. However, oxidative stress induced by an excess accumulation of reactive oxygen species can damage basic components for cell function and survival. The brain accounts for about 20% of the aerobic metabolism. Neuronal cells are exposed to a minimum level of free radicals from both exogenous and endogenous sources under normal conditions. Since the brain has a minimum storage capacity for oxygen and a high probability of lipid peroxidation, brain cells are especially vulnerable to free radical-mediated injury, such as oxygen interruption and reperfusion.\(^{28}\)

The water, MeOH, or ethyl acetate GEB fraction alone significantly decreased ipsilateral brain infarct size compared with the untreated vehicle group. This result suggests that compounds in GEB are neuroprotective against brain injury induced by transient ischemia via an as yet unknown mechanism. We found that pretreatment with the antioxidant supplement HBA also produced a significant reduction in brain lesion sizes in the total ipsilateral brain, cortex, and striatum at 24 h in a focal CNS reperfusion model. It was previously reported that HBA from GEB extract protected the brain after neurotoxic insult.\(^{16–18}\) Although the exact neuroprotective mechanism of GEB and HBA is not known, there is strong support for their role as putative antioxidants. GEB components have been shown to be responsible for directly scavenging free radicals, decreasing lipid peroxidation, and reducing free radical generation.\(^{11,12}\) HBA has been reported to be the main component of GEB in chemical studies.\(^{15}\) Gastrodin penetrates easily through the blood-brain barrier and rapidly decomposes into HBA, through which its effect may eventually initiate cell death caused by endoplasmic reticulum dysfunction. Therefore PDI could be a target for unfolded protein response-induced gene expression.\(^{30}\) There is also evidence supporting a role for 1-Cys Prx in certain disease processes including atherosclerosis. Increasing 1-Cys

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Fig. 3. Semiquantitative RT-PCR of PDI and 1-Cys Prx Comparing Brain Tissue after Water Extract and HBA Treatment with Untreated Vehicle Tissue

All animals were underwent ischemia for 1 h, followed by 24 h of reperfusion. Specific expression was determined in relation to the expression of the housekeeping gene GAPDH. Typical paired results are documented (A). The levels of PDI and 1-Cys Prx transcription were significantly increased in the GEB extract- or HBA-treated groups compared with the vehicle group (B). Experiments were repeated three times and individual values are mean±S.E.M. The letters a and b on the bar indicate significant differences (\(p<0.05\)) compared with the vehicle and water groups, respectively.
Ppx may implicate not only increased oxidative stress in the sCJD frontal cortex but also suggest a role for 1-Cys Prx in CJD neuropathogenesis and/or signalling pathway in the sCJD brain.22,37 These findings support the hypothesis that increased oxidative stress and dysregulated antioxidant systems are closely associated with the pathogenesis of neurodegeneration. We found significant increases in PDI and 1-Cys Prx transcription levels in the GEB- and HBA-treated groups compared with the untreated vehicle group. Because the volume of brain infarct in the GEB- or HBA-treated groups did not fully recover to normal, the expression levels of PDI and 1-Cys Prx upregulated in the ischemic brain might lead to a better understanding of the link between oxidative stress and the roles of specific antioxidant systems are closely associated with the pathogenesis of neurodegeneration. 2) PDI and 1-Cys Prx exert neuroprotective effects against hypoxia.

In conclusion, these results suggest that oxidative stress in the brain may simultaneously trigger neuronal damage early after ischemia, and free radical damage may eventually promote neuronal death in this region. It is becoming more evident that although the etiologic factors of neurodegenerative diseases may differ, the neurodegenerative process in such disorders share some common factors such as oxidative stress. Our investigation of GEB and HBA in the ischemic brain might lead to a better understanding of the link between oxidative stress and the roles of specific antioxidant proteins such as PDI and 1-Cys Prx in the neuropathogenesis of brain tissue.

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