ORIGINAL ARTICLE

A Neuroprotective Mechanism of YGY-E in Cerebral Ischemic Injury in Rats

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Keywords

Apoptosis; Bcl-2; Hypoxia-reoxygenation; Stroke; YGY-E.

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Received 2 September 2011; revision 28 September 2011; accepted 2 October 2011

SUMMARY

Aims: To investigate the anticerebral ischemic properties of YGY-E (apigenin-7-O- β -D-glucopyranosyl 1-4'-O- α -L-rhamnopyranoside, a flavonoid glycoside extracted from plant phoenix-tail fern), focusing on its effects on neuronal apoptosis. **Methods:** *In vitro* YGY-E treatment was examined in primary cultured rat hippocampal neurons subjected to hypoxia-reoxygenation (H-R) injury. In addition, *in vivo* effects of YGY-E on neuronal apoptosis were measured by Hoechst staining and *in situ* DNA end labeling (TUNEL). Finally, B cell lymphoma/leukemia-2 (Bcl-2) level in ischemic rat brain tissue was evaluated with immunohistochemistry and western blot analyses. **Results:** *In vitro* YGY-E (50–100 μ g/mL) treatment increased the survival rate compared to that of the vehicle-treated group ($P < 0.05$ and $P < 0.01$, respectively). In *in vivo* experiments, YGY-E (2.5–10 mg/kg) decreased the percentage of apoptotic neurons ($P < 0.01$), increased Bcl-2 ($P < 0.01$) in ischemic rat brain tissue. These effects were dose dependent. **Conclusions:** Our findings indicate that YGY-E's neuroprotective effects may be because of its inhibition of neuronal apoptosis by increasing Bcl-2 expression.

doi: 10.1111/j.1755-5949.2011.00277.x

Introduction

Stroke, the rapid decline in brain function after an interruption of blood supply, is the third leading cause of death or disability in the world. In humans, inadequate blood supply to the brain (cerebral ischemia) initiates an ischemic cascade leading to severe brain damage and neural impairment. The complex mechanisms involved in the development and progression of cerebral ischemia include excitotoxicity, ionic imbalance, inflammation, and oxidative stress [1].

Tissue hypoxia-reoxygenation (H-R) causes cell injury and death, which can result in severe pathophysiological outcomes [2], such as tumor development [3], myocardial infarction [4], and cerebral ischemia [5]. Hypoxia alone or in combination with reoxygenation activates caspases and induces apoptosis [6–8]. Although reoxygenation supplies adequate oxygen for sustaining neuronal viability, it also provides oxygen as the substrate for oxidation reactions that produce reactive oxidants, including oxygen free radicals. These reactive oxidants can aggravate cells or cause secondary damage such that the neuronal impairments in reperfusion are even greater than those caused by hypoxia [9,10].

Apoptosis plays an important role in neuronal death after focal cerebral ischemia, especially in the penumbra. The ischemia-

induced signaling cascades, such as the loss of adenosine triphosphate, changes in intracellular ions, release of excitatory amino acids, and oxidative stress, may contribute to neuronal apoptosis [11]. Molecular mechanisms that attenuate these cellular dysfunctions may also reduce the incidence of neuronal apoptosis. B cell lymphoma/leukemia-2 (Bcl-2) has antiapoptotic properties [12,13], so the regulation of Bcl-2 expression is an important target for drug therapies against ischemic neuronal apoptosis [14].

Phoenix-tail fern (*Herba Pteridis Multifidae*) has potent analgesic, antiinflammatory effects, and antiischemic properties [15]. Pharmacodynamic screening of Phoenix-tail fern compounds in the *in vivo* animal model of cerebral ischemia revealed that YGY-E (Figure 1) was the main active ingredient for the treatment of stroke. It is a flavonoid glycoside can be acquired from *Ranunculus* (e.g., *Ranunculus sieboldii* and *Ranunculus sceleratus*). The procedure for the separation, purification, and identification of this effective ingredient has been established [16]. The purity of YGY-E is 99% by HPLC analysis and it is suitable for intravenous injection. However, few studies have investigated the neuroprotective effects of YGY-E. To evaluate the antiapoptotic effects of YGY-E, and to analyze its underlying mechanisms against cerebral ischemic injuries, we assessed how YGY-E affects neuronal apoptosis and Bcl-2 expression in ischemic rat brain tissue.

apigenin-7-O-β-D-glucopyranosyl-4'-O-α-L-rhamnopyranosid

Figure 1 The structure of YGY-E.

Materials and Methods

Animals

Newborn (male and female) Sprague–Dawley and male Wistar (weight: 200–300 g) rats were purchased from Shanghai Slack Laboratory Animal Co. Ltd (Shanghai, China). Animals were given food and water *ad libitum* and maintained at $24 \pm 1^\circ\text{C}$ with a 12/12 h light/dark cycle. All animals in this study received humane care in accordance with guidelines provided by our institutional animal care and use committee.

Primary Culture of Rat Neurons

Neurons were isolated and cultured as previously described [17,18] with minor modifications. In brief, the bilateral hippocampus was dissected from a newborn rat and immediately immersed in dissecting medium (4°C) for removal of blood vessels and meninges. The hippocampal tissue was mechanically fragmented to $\sim 1\text{ mm}^3$ pieces. The tissue fragments were incubated in 1–2 mL trypsin solution (0.125%) at 37°C for 20 min. The trypsin was removed and the tissue fragments were transferred to an inactivation solution (80% DMEM, 10% fetal bovine serum, and 10% equine serum, Gibco, Grand Island, USA) for 10 min. The treated tissue was triturated with a fire-polished Pasteur pipette to isolate individual neurons. These neurons were suspended in the inactivation solution and the supernatant was removed by vacuum after 10 min precipitation. Cells (2×10^5 per mL, $200\text{ }\mu\text{L}$ each well) were added to poly-L-coated 96-well culture plates (Sigma, VA, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 . After 24 h, the culture medium was removed and feeding medium (98% Neurobasal Medium and 2% B27; Gibco) was added. Every 3 days, half of the medium was replaced.

Assessment of Apoptosis Using TdT-mediated dUTP-biotin nick end labeling (TUNEL) and Hoechst Staining

Rats were deeply anesthetized with 12% chloral hydrate 24 h after middle cerebral artery (MCA) occlusion. Brains were perfused transcardially with 4% paraformaldehyde. The brains were isolated, fixed in 10% formalin, embedded in the paraffin, and sectioned ($5\text{ }\mu\text{m}$ thick). Apoptotic cells were identified by TUNEL-stained sections using an *In Situ* Cell Death Detection Kit (AP Rh Applied Science, Germany) according to the manufacturer's instructions. In brief, after dewaxing, the sections were washed

with 0.01 M phosphate buffered saline (PBS) and incubated in proteinase K ($20\text{ }\mu\text{g/mL}$; Promega, Madison, WI, USA) at 37°C for 30 min. After pretreatment, sections were blotted, incubated in a mixture solution containing TdT enzyme (Promega) and digoxigenin-tagged dUTP (Boehringer, Ingelheim, Germany) at 37°C for $\sim 4\text{ h}$, and counterstained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate. The stained sections were mounted with neutral gum. TUNEL-positive nuclei were counted in five randomly selected fields in each section by fluorescence microscopy (Leica, Hessen, Germany) with high-power magnification ($\times 200$). Apoptotic index was calculated as the percentage of TUNEL-positive cells (positive cells/total cells $\times 100\%$).

For Hoechst staining, sections were washed with 0.01 M PBS, and 0.5 mL Hoechst 33,258 ($25\text{ }\mu\text{g/mL}$; Sigma) was added with gentle shaking followed by staining at 37°C for 5 min. Dense, blue nuclei were detected by fluorescence microscopy with excitation and emission wavelengths of 350 and 460 nm, respectively [19]. The Hoechst-positive cells were counted in five randomly selected fields in each section and evaluated by the image analysis system.

Immunohistochemistry Assay for Bcl-2

Twenty-four hours after cerebral ischemia, brain sections were fixed in 4% paraformaldehyde for 1 h. Sections were incubated at 25°C for 30 min in methanol containing 0.5% hydrogen peroxide and then blocked in goat serum for 30 min at room temperature. Sections were incubated with the primary antibody anti-Bcl-2 (Santa Cruz Biotechnology, CA, USA) at 37°C for 1 h and the secondary antibody biotinylated goat anti-rat IgG (Santa Cruz) for 20 min. Bcl-2 positive cells were identified by a brown peroxidase reaction product after incubation with an avidin–horseradish enzyme complex for 20 min at 37°C using an ABC Kit (Sino-American Biotechnology Company, MA, USA) with DAB (Sino-American Biotechnology Company, MA, USA) as the chromogen. Bcl-2-positive cells were counted in five randomly selected fields in each section magnified at high-power ($\times 200$). The Bcl-2 protein expression level was quantified using a computerized image analysis system attached to the microscope.

Western Blot Analysis for Bcl-2

After centrifugation, brain tissue samples were separated into cytosol and mitochondrial fractions. The supernatant containing the mitochondrial fraction was resuspended in loading buffer. The mitochondrial proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane, which was blocked with nonfat dry milk in buffer. The membrane was incubated with the primary antibody anti-Bcl-2 (Santa Cruz Biotechnology) and the secondary antibody goat antimouse IgG-HRP (Santa Cruz Biotechnology). Proteins were visualized by electrochemiluminescence (Amersham BioSciences, Sweden) and analyzed by the Quantity One Analysis Software (Bio-Rad, CA, USA). GAPDH was used as the protein loading control.

Protocols

Experiment 1: Effect of YGY-E on H-R-Induced Injury of Primary Cultured Neurons

Primary cultured cells were divided into six groups ($n = 8$ per group): control, vehicle-treated, three YGY-E treatments (25, 50, and 100 $\mu\text{g/mL}$) and nimodipine treatment. YGY-E (Shanghai Institute of Pharmaceutical Industry, Shanghai, China) was dissolved in microdosis dimethyl sulfoxide (DMSO) and diluted to the treatment concentration with Earle's balanced salt solution.

Neuron culture medium was removed after 10 days, and the cells were washed twice with Earle's solution. One hour before induced-hypoxia, Earle's solution was added to the vehicle-treated cells and Earle's containing drug to the YGY-E and nimodipine cells. Neurons were cultured in a custom anaerobic culture system filled with 95% N_2 and 5% CO_2 at 37°C for 3 h and transferred to a 5% CO_2 incubator (Heraeus, Hessen, Germany). Samples

were analyzed under inverted phase contrast microscope (Nikon, Japan) 24 h after reoxygenation [20,21].

Methyl thiazolyl tetrazolium (MTT) was dissolved in PBS (pH: 7.2) to final concentration of 5 mg/mL and 100 μL was added to each well of the culture plate. Cells were incubated at 37°C for 4 h until purple crystals appeared. The supernatant was removed and precipitates were dissolved in 200 μL DMSO in each well. Absorbance was measured at 570 nm using an automatic ELISA reader (Labsystems Dragon, Finland) [22].

The cell survival rate was calculated as

$$\text{Cell survival rate (\%)} = \frac{\text{OD}_a - \text{OD}_b}{\text{OD}_c - \text{OD}_b} \times 100\%,$$

where OD_a is the optical density (OD) of the vehicle-treated or drug-treated cells, OD_b is the optical density of the reference well, and OD_c is the optical density of the control cells.

Figure 2 The effect of YGY-E on H-R injury in primary cultured hippocampal neurons. **(A)** MTT color development in neurons with H-R injury. Cytomorphology of neurons in six treatment groups were observed at $\times 400$. a: control; b: vehicle; c: nimodipine (5 $\mu\text{g/mL}$); d: YGY-E (25 $\mu\text{g/mL}$); e: YGY-E (50 $\mu\text{g/mL}$); f: YGY-E (100 $\mu\text{g/mL}$). **(B)** Cell survival rate normalized to the control group. Error bars show the SEM. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated group.

Experiment 2: Effect of YGY-E on Neuronal Apoptosis in Ischemic Rat Brains

Male Wistar rats were randomly divided into 5 groups ($n = 5$ per group): vehicle-treated, YGY-E treatment (2.5, 5, and 10 mg/kg), and nimodipine treatment (5 mg/kg). MCA electrocoagulation was performed in animals as described before [23–25]. Animals were injected intravenously 3 h after cerebral ischemia. At 24 h after the MCA electrocoagulation, brains were dissected, and apoptosis was detected by TUNEL and Hoechst staining.

Experiment 3: Effect of YGY-E on Bcl-2 Levels in Ischemic Rat Brains

Bcl-2 levels were measured by immunohistochemistry and western blot analyses in brains from rats in Experiment 2.

Statistics

Scientists were blind to tissue treatment group when evaluating cell morphology, apoptosis, and Bcl-2 levels. Differences between treatment and vehicle groups were determined by one-way ANOVA followed by Bonferroni *post hoc* test. P values <0.05 were considered statistically significant.

Results

YGy-E Protects Against H-R-Induced Injury in Primary Cultured Neurons

Normal neurons were polygon with smooth edges and clear nuclei. They formed a network with surrounding neurons. After H-R injury, neurons swelled and significantly reduced in numbers (Figure 2A). Their nuclei drifted, shrank, or even disappeared. At concentrations 25–100 $\mu\text{g/mL}$, YGY-E protected against H-R induced neuron injury in a dose-dependent manner. Normalized to survival in the control group, cell survival of YGY-E treatment groups increased in a dose-dependent manner (Figure 2B). Compared with vehicle group, YGY-E (50–100 $\mu\text{g/mL}$) significantly increased cell survival rate ($P < 0.05$ or $P < 0.01$). The observed increase in cell survival in the nimodipine treated cells was not significant ($P > 0.05$). These results support the idea that YGY-E has protective action against H-R induced injury of primary cultured hippocampal neurons.

YGy-E Reduces Neuronal Apoptosis in Cerebral Ischemic Rats

YGy-E at 2.5 mg/kg ($P < 0.05$) and 5–10 mg/kg ($P < 0.01$) significantly reduced the percentage of TUNEL-positive cells compared to the vehicle group (Figure 3A). The slight reduction in the percentage of apoptotic cells in the nimodipine treatment was not

Figure 3 Effect of YGY-E on apoptosis in ischemic brains of rats. Brains were isolated 24 h after MCA electrocoagulation. Apoptosis was evaluated by (A) TUNEL and (B) Hoechst staining of sections at $\times 200$. The percentage of stained cells was calculated as the apoptotic index. Error bars are the SEM. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated group. a: vehicle; b: nimodipine (5 mg/kg); c: YGY-E (2.5 mg/kg); d: YGY-E (5 mg/kg); e: YGY-E (10 mg/kg).

Figure 4 Bcl-2 in ischemic brains of rats detected by immunohistochemistry in the (A) hippocampus and (B) cortex. The presence of Bcl-2 was indicated by a brown peroxidase reaction product at $\times 200$. Error bars are the SEM. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated group. a: vehicle; b: nimodipine (5 mg/kg); c: YGY-E (2.5 mg/kg); d: YGY-E (5 mg/kg); e: YGY-E (10 mg/kg).

statistically significant ($P > 0.05$). Hoechst staining also revealed a significant YGY-E dose-dependent decrease in neuronal apoptosis ($P < 0.01$) that was not observed in the nimodipine treatment (Figure 3B).

YG-E Increased Bcl-2 Levels in Ischemic Rat Brain

Bcl-2 positive cells were concentrated in the hippocampus and cerebral cortex. All concentrations of YGY-E dramatically increased the percentage of Bcl-2 positive cells compared to the vehicle group ($P < 0.01$), suggesting that YGY-E promotes Bcl-2 synthesis. Nimodipine treatment also significantly increased Bcl-2 levels compared to vehicle group ($P < 0.01$; Figures 4 and 5).

Discussion

H-R induced neural injury not only plays an important role in causing stroke but is also the major cause of cerebral ischemia-reperfusion injury. The human brain requires a substantial volume of oxygen for proper function—one-fifth of total oxygen consumed by the body is used in the nervous system. As a result, our brains are particularly vulnerable to hypoxic damage. Within the brain, H-R injury increases the production of oxidants,

which can cause significant deoxyribonucleic acid (DNA) damage, protein oxidation, and lipid peroxidation [26,27].

Affecting a wide range of cell types [2,28,29], cell death after H-R injury because of primarily apoptosis rather than necrosis [30,31]. In apoptosis, a programmed sequence of events results in cell death without damaging neighboring cells. It likely has a significant role in both physiological regulation and pathological situations. Apoptosis is regulated by a variety of genes and allows cells to respond to external and internal stimuli. Although the underlying mechanisms of apoptosis are not completely understood, evidence suggests that it is triggered through a death receptor-mediated extrinsic pathway or a mitochondrial intrinsic pathway. The Bcl protein family, including Bcl-2, Bcl-x, Bax, and Bad, which promote or prevent apoptosis [12,32,33], are expressed in the mitochondria. The balance between antiapoptotic Bcl-2 levels and proapoptotic Bax levels plays a major role in regulating apoptotic cell death. Elevated expression of Bcl-2 inhibits apoptosis whereas elevated expression of Bax will promotes apoptosis. Thus, the gene encoding Bcl-2 is known as a longevity gene [34], which protects the nervous system by inhibiting intracellular Ca^{2+} overload and reducing the toxic effects of peroxides.

To study the pathological changes in neurons to hypoxic-ischemic injuries, we investigated neuroprotective effects of drugs in primary neuronal cultures. Using the MTT test, we analyzed cell survival rate and evaluated the protective

Figure 5 Bcl-2 expression in ischemic brains of rats by Western blot analysis in the (A) hippocampus and (B) cortex. Relative Bcl-2 (%) was calculated as the density of the Bcl-2 gel band/the density of the GAPDH gel band. Error bars are the SEM. * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated group. a: YGY-E (2.5 mg/kg); b: YGY-E (5 mg/kg); c: YGY-E (10 mg/kg) d: vehicle; e: nimodipine (5 mg/kg).

effect of drugs. This colorimetric assay measured the cell survival rate as a percentage of survived cells compared to untreated controls [35]. The results of Experiment 1 showed that YGY-E reduced H-R-induced neuron death and maintained normal neuronal morphology. Its dose-dependence effect was stronger than the effect of nimodipine. In Experiment 2, we used TUNEL staining to detect DNA fragmentation and Hoechst staining to evaluate apoptosis after H-R injury. After YGY-E treatment (2.5–10 mg/kg), the percentage of TUNEL-positive cells increased significantly, indicating YGY-E's neuroprotective properties come through inhibiting neuronal apoptosis. Experiment 3 showed that YGY-E treatment increased Bcl-2 levels in ischemic rat brains, suggesting that YGY-E inhibits neuronal apoptosis by changing the balance between Bcl-2 and Bax. Therefore, we conclude that YGY-E protects against cerebral ischemia diseases. This series of experiments demonstrated that YGY-E protected against ischemic injuries in rat brains. These findings suggest that YGY-E has a potential pharmacological role in the treatment of ischemia-related neurological disorders.

Most patients with ischemic stroke have limited treatment options because they arrive for care after the critical poststroke 3-h

time limit. Extension of the treatment time window for stroke will greatly increase treatment options [36,37]. Future studies should focus on the role of YGY-E in extending the treatment time window of cerebral ischemia.

Conclusions

In this study, YGY-E reduced H-R-induced neuron death by promoting Bcl-2 synthesis and reducing the incidence of neuronal apoptosis.

Acknowledgments

This study was supported by "Traditional Chinese Medicine Modernization Project" (No. 06DZ19711) grants from the Shanghai Science and Technology Commission of PR China.

Conflict of Interest

The authors have no conflict of interest.

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