Effects of Soybean Isoflavone on the Notch Signal Pathway of the Brain in Rats with Cerebral Ischemia

Guowei HUANG1,2, Xiaohong CAO1,*, Xumei ZHANG2, Hong CHANG2, Yunxia YANG2, Wenpin DU2 and John X. WILSON3

1Tianjin Key Laboratory of Food Nutrition and Safety, College of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, China
2Department of Nutrition and Food Hygiene, School of Public Health, Tianjin Medical University, Tianjin 300070, China
3Department of Exercise and Nutrition Sciences, School of Public Health and Health Professions, University at Buffalo, Buffalo, New York 14214–8028, USA

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Summary The aim of this study was to test the hypothesis that gastric lavage with soybean isoflavone activates the Notch signaling pathway and prevents apoptosis in the cerebral cortex during experimental strokes. Sprague-Dawley rats were randomly assigned to four groups of 10 rats each: sham operation plus vehicle (Sham), middle cerebral artery occlusion plus vehicle (MCAO), MCAO plus low dose soybean isoflavone (10 mg/(kg·d)) and MCAO plus high dose soybean isoflavone (50 mg/(kg·d)). The vehicle (saline, 10 mL/(kg·d)) and soybean isoflavone were administered by gastric lavage for 28 d prior to sham or MCAO operation and for 7 d afterward. The mRNA and protein expression levels of components of the Notch signaling system (Notch1 and Hes5) were measured by in situ hybridization and western blotting, respectively, whereas apoptosis was quantified by TUNEL assay. The results showed that MCAO stimulated expression of Notch1 and Hes5, at both the mRNA and protein levels, and also increased apoptosis. Soybean isoflavone dose-dependently augmented the stimulatory effect of MCAO on Notch1 and Hes5 expression levels but decreased apoptosis. These results identify a possible mechanism by which soybean isoflavone confers neuroprotection in strokes.

Key Words soybean isoflavone, Notch, cerebral ischemia, rat

Ischemic stroke is the third most frequent cause of mortality in industrialized countries and the therapeutic options to treat it are limited (1). Epidemiological studies have established that habitual intake of dietary flavonoids, especially isoflavones, is inversely associated with stroke incidence (2). Dietary isoflavones also have been shown to improve experimental stroke outcome after transient focal cerebral ischemia (i.e., middle cerebral artery occlusion; MCAO), as indicated by smaller infarct volume and improved neurological status in rats fed a soybean-based diet compared to rats fed an isoflavone-free diet (3). Dietary soybean, which is rich in isoflavones, decreases stroke injury in both female and male rats (4) and the soybean isoflavone genistein confers neuroprotection in a mouse cerebral ischemia model (5). Importantly, dietary supplementation with soybean decreases apoptotic rate in the cerebral cortex of rats subjected to MCAO (6). However, the molecular mechanisms underlying these beneficial effects of isoflavones in the central nervous system are unknown (7, 8).

Recent findings suggest that Notch signaling in brain neurons, glia, and neural stem cells (NSCs) may be involved in the pathological changes that occur in stroke (9). A potential therapeutic target is suggested by the observation that Notch signaling modulates cell cycle time and thereby ensures that brain-derived neural stem cells retain their property of self-renewal (10–12). Hairy and enhancer of split 5 (Hes5) is an important effector for Notch signaling (12, 13). In the present study, we evaluated the hypothesis that gastric lavage with soybean isoflavone activates the Notch signaling pathway and prevents apoptosis in the cerebral cortex during experimental strokes.

MATERIALS AND METHODS

Animals and reagents. Male Sprague Dawley rats (body weight 160–180 g) were purchased from Peking Weitonglihua Laboratory Animal Center (Grade SPF II, Certificate No. SCXK (Jing) 20070001). Animal housing, care, and application of experimental procedures were in accordance with institutional guidelines under approved protocols.

Soybean isoflavone with a purity of 41% was obtained from Tianjin Jianfeng Natural Product Co. Ltd., China. Polyclonal antibodies against β-actin, Notch1 and Hes5 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All secondary antibodies were obtained from Zhongshan Goldbridge Biotechnology

*To whom correspondence should be addressed.
E-mail: guoweihuang@yahoo.com.cn
(Beijing, China). The TUNEL assay kit for measuring apoptosis and in situ hybridization kits for detecting Notch1 and Hes5 gene expression were supplied by Wuhan Boster Biological Technology Ltd., China.

**Experimental design.** Sprague-Dawley rats were randomly assigned to four groups of 10 rats each. The groups were: sham operation plus vehicle (Sham), middle cerebral artery occlusion plus vehicle (MCAO), MCAO plus low dose soybean isoflavone (10 mg/(kg·d)) (MCAO+SI-L) and MCAO plus high dose soybean isoflavone (50 mg/(kg·d)) (MCAO+SI-H). The vehicle (saline, 10 mL/(kg·d)) and soybean isoflavone were administered by gastric lavage for 28 d prior to sham or MCAO operation and for 7 d afterwards. Then the rats were euthanized and their brains were collected for measurements of mRNA and protein expression levels and apoptosis.

**Induction of experimental stroke.** MCAO was induced by the intraluminal filament technique. Left common and external carotid arteries were ligated and the internal carotid artery was closed. A nylon monofilament was advanced through the left internal carotid artery to the origin of the MCA. Animals subjected to sham operation were treated similarly, except that the filament was not advanced to the origin of the MCA. Neurological deficit was assessed to confirm successful MCAO. A neurological score was assigned to each animal 5 min after waking up: 0, no deficit; 1, forelimb weakness; 2, circling to affected side; 3, partial paralysis on affected side; and 4, no spontaneous motor activity. MCAO rats with neurological deficit scores of 1–3 were used for experiments (14).

**Cell morphology and apoptosis.** The effects of treatments on cell morphology were observed by light microscopy. Brains were collected, fixed in 4% paraformaldehyde and then embedded in paraffin. Sections of 5 μm thickness were stained with hematoxylin and eosin for examination by light microscopy.

Apoptosis was quantified in deparaffinized coronal sections of the cerebral cortex using the TUNEL assay kit according to the manufacturer’s instructions. Subsequently, the sections were stained with dianaminobenzidine and counterstained with hematoxylin. The apoptotic index was the number of labeled apoptotic bodies expressed as the percentage of the total number of nuclei.

**Fluorescent in situ hybridization.** Hybridization was performed with biotin-labeled probe of Notch1 (probe 1: 5′-AACCTGCAACAATCCCACGATGGCTACAACACTCCGG-3′; probe 2: 5′-AAGTGTCGACAGACACAGTACACTGGGA-3′; probe 3: 5′-ACTTCAATGACCCCTTGGGAAGAACTGCCTCAGTCCTCC-3′), Hes5 (probe 1: 5′-GAGCAGCTGAAACTGCTGGAGCCAGAGTCCTGC-3′; probe 2: 5′-ACCAGAGTACAGCAGAGGGTACTGCTGGGTCCTCC-3′; probe 3: 5′-CAAGCGGAATGAGCTGGTTCACCTTCCAGGG-3′). In situ hybridization for detection of Notch1 and Hes5 transcripts was performed according to the kit protocol, with minor modifications. Deparaffinized slides were treated with 3% H2O2 for 20 min at room temperature, and washed 3 times in distilled water for 3 min each, then digested with trypsin for 10 s. After washing 3 times in PBS (pH 7.6) for 5 min each, the slide was incubated with 20 μL of pre-hybridization solution at 40°C for 3 h, followed by incubation with 20 μL of probe-containing hybridization solution at 40°C overnight. The slides were then washed sequentially in 2×SSC (twice for 5 min each), 0.5×SSC and 0.2×SSC (for 15 min each) to remove the unbound probes. Next, the slides were blocked at 37°C for 30 min and mixed with anti-DIG-biotin at 37°C for 1 h, then washed 4 times in PBS (pH 7.6) for 5 min each. The biotin-labeled probe was detected with avidin-fluorescein isothiocyanate (avidin-FITC; green color) conjugate. Hybridization signals were viewed with an Olympus BH-2 fluorescence microscope equipped with appropriate filters (Chroma Technology, Battleboro, VT) and Simple PCI version 1.0.

**Western blot analysis.** Left brain cerebral hemispheres were dissected out and homogenized in 10 volumes of cold homogenization buffer (50 mM Tris, 120 mM NaCl, pH 7.4) containing protease inhibitors and then were stored at −80°C. Subsequently, proteins in aliquots of the extracts (20 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk and incubated with a primary antibody (rabbit anti-hes5 antibody diluted 1:1,000, anti-notch1 diluted 1:1,000 or anti-β-actin antibody diluted 1:5,000) overnight at 4°C and then with a secondary antibody for 1 h at room temperature. Proteins were detected by chemiluminescence assay. Quantitation of proteins was done by densitometric analysis using NIH Image software (version 1.61).

**Statistical analysis.** The results are presented as mean ± standard deviation (SD) for n number of experi-

![Fig. 1. Pictures of pathological rat brain cerebral cortex. Stained with HE, original magnification ×200 under a light microscope.](image-url)
ments. Differences between means were evaluated by one-way ANOVA using GraphPad Prism version 3.0, followed by Tukey’s multiple range test. \( p \leq 0.05 \) was considered statistically significant.

**RESULTS**

**Cell morphology and apoptosis**

Compared with sham operated control, MCAO rat brains showed pronounced changes in cell morphology (Fig. 1). In coronal sections of the cerebral cortex, more cells with pyramidal shapes and larger intercellular spaces were observed after MCAO. Consistently, the MCAO group also showed cell shrinkage with nuclear and cytoplasm condensation. However, the low and high doses of soybean isoflavone largely prevented these morphological effects of MCAO (Fig. 1).

Pronounced treatment effects on apoptotic rate were also observed in coronal sections of the cerebral cortex (Fig. 2). Compared with sham operated control, the apoptotic rate was much higher after MCAO. Soybean isoflavone partially prevented the stimulation of apoptosis by MCAO. This effect did not differ significantly between the low and high doses of soybean isoflavone (Fig. 2).

Taken together, these results indicated that soybean isoflavone attenuated significantly the changes in brain cell morphology and apoptosis induced by MCAO.

**Expression of Notch1 and Hes5**

We performed in situ hybridization to evaluate the expression of Notch1 and Hes5 mRNAs in coronal sections of the cerebral cortex (Fig. 3). Compared with sham operated control, expression of Notch1 and Hes5 at the mRNA level was upregulated after MCAO. Soybean isoflavone dose-dependently increased further the
abundance of Notch1 and Hes5 transcripts (Fig. 3).

Western blot analysis demonstrated that the expression of Notch1 protein was increased significantly in the ischemic brain tissue after MCAO, compared with the sham operation (Fig. 4). However, no difference in Hes5 protein expression was observed between the MCAO and sham-operated groups. Both Notch1 and Hes5 protein expression were increased significantly by...
soybean isoflavone in a dose-dependent manner (Fig. 4). These results indicated that the Notch signaling pathway was upregulated by soybean isoflavone in the rat MCAO model of stroke.

**DISCUSSION**

Stroke is a life-threatening disease characterized by rapidly developing clinical signs of cerebral dysfunction due to ischemia. Dietary isoflavones have been shown in rats to improve stroke outcome after transient focal cerebral ischemia, as indicated by smaller infarct volume and improved neurological status (3). A decrease in apoptotic rate in the cerebral cortex accompanies the decrease in brain infarct size conferred by a soybean-enriched diet in rats subjected to MCAO (6). In vitro experiments have confirmed that apoptosis is a potential target of soybean isoflavones, because genistein prevents glutamate- and thapsigargin-induced apoptosis in embryonic cerebral cortical cell cultures (15–17). In the present experiment, gastric lavage with soybean isoflavone attenuated the changed cerebral cortical morphology and apoptosis induced by MCAO. We also observed that the prevention by soybean isoflavone of apoptosis during experimental strokes was associated with activation of the Notch signaling pathway in the cerebral cortex.

The balance between proliferation and differentiation of neural stem cells is critical to generate the appropriate numbers and types of neurons and glia. Notch signaling maintains the progenitor pool throughout this process (10). The hope of developing new transplantation therapies for brain diseases is limited by inefficient stem cell growth and immunological incompatibility with the host (11). Because Hes5 antagonizes proneural genes and neuronal differentiation (13), its upregulation by soybean isoflavone may maintain the proliferative capacity of neural stem cells and thus lessen cerebral infarct volume and neurologic dysfunction after a stroke.

In conclusion, our results show that gastric lavage with soybean isoflavone decreases the stimulation of apoptosis by ischemia in the cerebral cortex. This effect of soybean isoflavone was associated with activation of the Notch signaling pathway in the cerebral cortex. These results identify a possible mechanism by which soybean isoflavone confers neuroprotection in strokes.

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