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RESEARCH NOTE

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Neuroprotective Effect of Scopoletin from *Angelica dahurica* on Oxygen and Glucose Deprivation-exposed Rat Organotypic Hippocampal Slice Culture

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Abstract This study examined the neuroprotective effect of scopoletin from *Angelica dahurica* against oxygen and glucose deprivation-induced neurotoxicity in a rat organotypic hippocampal slice culture. Scopoletin reduced the propidium iodide (PI) uptake, which is an indication of impaired cell membrane integrity. In addition, it inhibited the loss of NeuN, which represents the viability of neuronal cells. The results suggests that scopoletin from *A. dahurica* protects neuronal cells from the damage caused by oxygen and glucose deprivation.

Keywords: *Angelica dahurica*, scopoletin, oxygen-glucose deprivation, hippocampus slice culture, neuroprotection

Introduction

Since the brain consumes almost 20% of the oxygen inhaled, any deficiency in the supply of oxygen and glucose can cause serious injury. In particular, oxygen depravation in the hippocampus, which is quite vulnerable to ischemia, can lead to severe learning and memory impairments (1). Therefore, suitable strategies against ischemia are needed to improve the welfare of an ischemic patient and reduce its medical cost. In this study, coumarin of a number of *Angelica* have been screened for neuroprotecive activity, and one of the most potent of these agent, scopoletin was isolated from *Angelica dahurica* throughout the neuroprotective activity guided fraction. Scopoletin, 6-methoxy-7-hydroxycoumarin (Fig. 1), is a constituent of *Fraxinus rhynchophylla*, *Solanum lyratum*, *A. dahurica*, and *Artemisia feddei* (2-5). The pharmacological effects of scopoletin, which include its hepato-protective, anti-oxidant, and anti-inflammatory activity, have been reported (6, 7). However, the neuroprotective effects of scopoletin are unclear. An organotypic hippocampal slice culture has several advantages as a neuroprotective-agent-screening model. These include an intact network among the different cell types in the brain and some relevance to *in vivo* experiment models (8, 9). The aim of this study was to determine if scopoletin from *A. dahurica* protects neuronal cells against oxygen and glucose deprivation (OGD)-induced toxicity in an organotypic hippocampal slice culture.

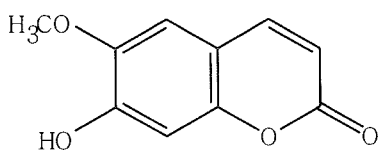

Fig. 1. Chemical structure of scopoletin.

Materials and Methods

Reagents The α-modified Eagle Medium (α-MEM), penicillin/streptomycin solution, Hank's balance salt solution (HBSS), and horse serum (HS) were purchased from Gibco BRL (Grand Island, NY, USA). Propidium iodide (PI), D-glucose, and all other chemicals used were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of scopletin from *A. dahurica* The air-dried powder of *A. dahurica* was extracted with MeOH 3 times. The resulting extract was suspended and partitioned in succession with hexane, $CHCl_3$, EtOAc, and BuOH. A portion of the $CHCl_3$ fraction was chromatographed on a silica gel 60 (0.063-0.200 mm, Merck Co., Damstalt, Germany) was used for open column chromatography. Silica gel plates (Kieselgel 60 F_{254}, Merck Co.) were used for TLC. Spots were detected by spraying with 20% H_2SO_4 in MeOH and heating. Infrared (IR) spectrum was recorded with Jasco FT/IR-300E instrument on KBr disc. ^{1}H-NMR spectrum was recorded with a Varian Gemini

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2000 (300 MHz) NMR spectrometer (Palo Alto, CA, USA). The column eluted using a stepwise gradient of *n*-hexane and $CHCl_3$ to give 10 sub-fractions. Repeated column chromatography of sub-fr. 3 on a silica gel (*n*-hexane-EtOAc) afforded compound **1**.

Compound **1**; IR ν_{max} (KBr)/cm : 3419 (OH), 1684 (C=O), 1621, 1559 (aromatic C=C); ^{1}H-NMR ($CDCl_3$) δ_H (ppm): 7.60 (1H, d, J = 9.1 Hz, H-4), 6.90 (1H, s, H-5), 6.83 (1H, s, H-8), 6.20 (1H, d, J = 9.1 Hz, H-3), 3.94 (3H, s, -OCH_3).

Organotypic hippocampal slice culture The hippocampus slice culture was prepared and grown using a modified interface culture method (7). Sprague-Dawley rats (5-8 days old) were decapitated. The hippocampus was isolated and the dorsal halves were cut into 400 μm transverse sections using a Mcllwain Tissue Chopper (Mickle Laboratory Engineering Co., Surrey, UK). Six tissue slices were placed on an insert membrane in a random order (0.4 μm in porous, 30 mm in diameter, Millipore Co., Bedford, MA, USA). The inserts were transferred to 6 well culture trays, where each well contained 1 mL of the culture medium that consisted of 50% α-MEM, 25% HS, and 25% HBSS supplemented with 25 mM D-glucose. The medium was changed every 3 days and the experiments were carried out over a 14-day period.

OGD injury and scopoletin treatment The culture medium of the hippocampus slice culture was replaced with an Ischemic Balanced Salt Solution (IBSS: 143.4 mM NaCl, 5 mM HEPES, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 2 mM $CaCl_2$) in the presence or absence of scopoletin. The OGD was carried out in a chamber containing an anaerobic gas mixture (95% N_2 and 5% CO_2) for 40 min. OGD was terminated by removing the IBSS and adding a serum-free medium containing 7.5 μM PI with or without scopoletin. The cultures were incubated in a CO_2 incubator at 37°C for 48 hr.

PI staining The level of cell death was assessed using a fluorescent exclusion dye, PI, in which the fluorescence was excited at a wavelength of 514 nm using confocal laser scanning microscopy (LSM 510; Carl Zeiss, Heidenheim, Germany). The digital photos were analyzed using a NIH image program (version 1.29), which was obtained from the public domain. The area of cell death of the 40 min OGD-exposed hippocampal slice culture was used as the reference and was considered to be 100% cell death. The level of cell death in the scopoletin-treated hippocampal slice culture is reported as a percentage of this value.

Immunohistochemistry The slices were fixed for 2 hr by incubation with 4% phosphate buffered paraformaldehyde, and transferred to a 0.15 M phosphate buffer. After incubation with a mouse anti-mouse NeuN monoclonal antibody (1:150, Chemicon, Temecula, CA, USA) followed by a reaction with biotinylated anti-mouse serum and an avidin-biotin peroxidase system (Vectastain Elite kit; Vector Laboratories, Burlingame, CA, USA), the immunostaining was visualized using 3',3-diaminobenzidine tetrahydrochloride (DAB) for 3 min. The slices were dehydrated with ethanol, cleared in xylene, and treated with 0.3% H_2O_2 in absolute methanol to eliminate the endogenous peroxidase. The slides were rehydrated by soaking them in a graded series of ethanol and mounted them on microscope slides. All the slices were processed and stained collectively.

Statistical analysis The data is presented as the mean± SEM from 3 independent experiments. A statistical comparison between the different treatments was carried out using one-way ANOVA followed by a Turkey's test. A p value <0.05 was considered significant.

Results and Discussion

It was previously reported that 30 min of oxygen-glucose deprivation (OGD) was not enough to devastate the energy generation system, and the induction of cell death by 35 min of OGD was limited to the CA 1 region of the hippocampus (10). When the hippocampal slices were exposed to OGD for 40 min, cell death was detected in the pyramidal cell (CA 1 and 3 region) and granular cell layers (dentate gyrus) (Fig. 2B). This study examined the protective effect of scopoletin on the hippocampal slices that had been exposed to 40 min of OGD-induced toxicity by determining the PI-uptake and NeuN staining. PI penetrates the cell membrane and binds the DNA when the cell membrane is injured, which can be identified using confocal microscopy (11, 12). In this study, the area of cell death referenced to the vehicle-treated group was approximately 81, 73, 56, and 43% after a treatment with 50, 250, 500, and 1,000 μM scopoletin, respectively (Fig. 2. C, D, E, F, and G). In addition, staining with NeuN, which is a specific neuronal cell marker, showed that scopoletin rescued the neuronal cells injured by OGD (Fig. 3). Although it was demonstrated that scopoletin protected neuronal cells against OGD, the mechanism by which it reduced the level of neuronal cell death is unclear. However, there is some speculation as to the scopoletin mode of action. When the blood supply is blocked, a complicated series of events are involved in neuronal cell death. These include excitotoxicity, oxidative stress, and an inflammatory reaction. A deficiency in the oxygen and glucose supply causes a dysfunction of the neurotransmitter regulatory system, resulting in the release of a large amount of glutamate into the extracellular region (13). The binding of glutamate to the counter-receptor causes an influx of calcium ions, which has a deleterious effect on cell survival (14).

The blockage of blood followed by its restoration triggers the overproduction of reactive oxygen species (ROS), which are responsible for the neuronal cell injury observed (15, 16). In addition, inflammatory reactions are known to aggravate neuronal cell death. In particular, nitric oxide (NO) generated from inducible nitric oxide synthase (iNOS) plays a key role in exacerbating the neuronal cell damage (17). Therefore, anti-oxidant and anti-inflammatory agents are expected to be useful for treating ischemia patients (18, 19).

It was reported that scopoletin inhibited the protein

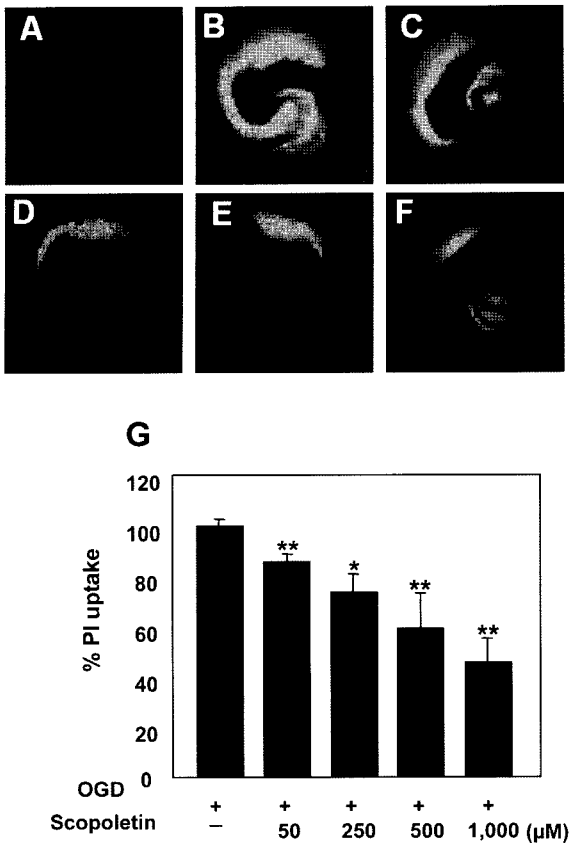

Fig. 2. PI fluorescence in a hippocampal slice culture. After a 2-day treatment with scopoletin in the OGD-exposed condition, the level of cell death was analyzed using the PI uptake and a public domain NIH image program (version 1.29). (A), Normoxia; (B), 40 min OGD and vehicle treatment; (C), OGD and scopoletin (50 μM); (D), OGD and scopoletin (250 μM); (E), OGD and scopoletin (500 μM); (F), OGD and scopoletin (1,000 μM); (G), Cell death of the OGD-induced hippocampal slice culture in the presence of scopoletin (n=5). The data is expressed as the mean± SEM of 3 independent experiments. The asterisk indicates a significant difference from the control (*p<0.05, **p<0.01).

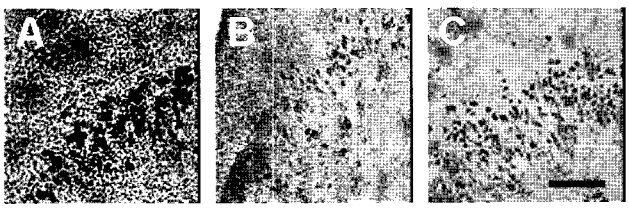

Fig. 3. NeuN immunohistochemistry in the hippocampal slice culture. After a 2-day treatment with scopoletin in the OGD-exposed condition, the hippocampal slices were stained with the NeuN antibody. (A), CA 1 subfield of normoxia; (B), CA 1 subfield of 40 min OGD and vehicle treatment; (C), CA 1 subfield of 40 min OGD and scopoletin (1,000 μM). Scale bar represents 100 μm.

oxidative modification induced by copper, represses the induction of iNOS expression in lipopolysaccharide (LPS)-stimulated macrophage cells, and acetylcholinesterase inhibitory activity (3, 20, 21). Scopoletin is ineffective for N-methyl-D-aspartate (NMDA) induced exicitotoxicity. The neuroprotective effect of scopoletin is believed to be partly due to its anti-oxidative, anti-inflammatory, and acetylcholinesterase inhibitory activity.

Acknowledgments

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