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Emodin from *Polygonum multiflorum* ameliorates oxidative toxicity in HT22 cells and deficits in photothrombotic ischemia

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ABSTRACT

Ethnopharmacological relevance: *Polygonum multiflorum* Thunb. has been used widely in East Asia in treatment of diseases associated with aging. Emodin, an active component from *Polygonum multiflorum* Thunb., provides benefits for brain disturbances induced by severe cerebral injury.
Aim of the study: We investigated the neuroprotective effect of emodin from *Polygonum multiflorum* Thunb. against glutamate-induced oxidative toxicity and cerebral ischemia.
Materials and methods: For examination of neuroprotective effects of emodin, cell viability, cytotoxicity, flow cytometry, and Western blot were performed in HT22 cells and infarct volume, behavioral tests and Western blot in a mouse model of photothrombotic ischemic stroke.
Results: Pretreatment with emodin resulted in significantly reduced glutamate-induced apoptotic cell death in HT22 cells. However, blocking of phosphatidylinositol-3 kinase (PI3K) activity with LY294002 resulted in significantly inhibited cell survival by emodin. Exposure of glutamate-treated cells to emodin induced an increase in the level of Bcl-2 expression, whereas the expression of Bax and active caspase-3 proteins was significantly reduced. In addition, treatment with emodin resulted in increased phosphorylation of Akt and cAMP response element binding protein (CREB), and expression of mature brain-derived neurotrophic factor (BDNF). This expression by emodin was also significantly inhibited by blocking of PI3K activity. In a photothrombotic ischemic stroke model, treatment with emodin resulted in significantly reduced infarct volume and improved motor function. We confirmed the critical role of the expression levels of Bcl-2/Bax, active caspase-3, phosphorylated (p)Akt, p-CREB, and mature BDNF for potent neuroprotective effects of emodin in cerebral ischemia.
Conclusions: These results suggest that emodin may afford a significant neuroprotective effect against glutamate-induced apoptosis through activation of the PI3K/Akt signaling pathway, and subsequently enhance behavioral function in cerebral ischemia.

1. Introduction

Traditional medicinal herb, *Polygonum multiflorum* Thunb. has been used widely in East Asia since ancient times in treatment of diseases commonly associated with aging (Lin et al., 2015b). Crude extracts and pure compounds of this plant possess various pharmacological activities including anti-aging, anti-hyperlipidaemia, anti-cancer, anti-inflammatory, and anti-oxidant effects (Lin et al., 2015b; Liu et al., 2011; Qiu et al., 2013). More than 100 chemical compounds have been isolated from *Polygonum multiflorum*, and stilbenes and anthraquinone are considered to be the indicator compounds for characterizing the quality and authenticity (Lin et al., 2015b).

Emodin, an anthraquinone derivative, is characterized as a strong apoptotic agent exacerbating oxidative injury at the highest concentration (Jackson et al., 2013; Tamokou Jde et al., 2013). Its toxicities are well detailed in the U.S. National Toxicology Program. However, emodin exhibits both protection and toxicity to various

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organs, which appears to be contradictory (Lin et al., 2015b). Although emodin is toxic to neurons, recent studies have shown that some natural emodin exerts neuroprotective effects as a promising therapeutic agent for treatment of neurodegenerative disease (Jackson et al., 2013; Zhou et al., 2011). Metabolites of emodin is found predominantly in the plasma in pharmacokinetic experiments (Lin et al., 2012).

Oxidative neuronal death contributes mainly to neuronal loss in neurodegenerative diseases, because neurons are fairly sensitive to oxidative stress by their high oxidative energy metabolism (Uttara et al., 2009; Xu et al., 2011). Excess level of extracellular glutamate is associated with development of neurodegenerative disorders or ischemic cell damage (Fonnum, 1984; Lau and Tymianski, 2010; Paschen, 1996). HT22 cells lack functional ionotropic glutamate receptor, but are sensitive to glutamate, which depletes glutathione and induces neuronal cell death (Fukui et al., 2009; Lee et al., 2012; Maher and Davis, 1996). This *in vitro* system is a good experimental model for oxidative neuronal toxicity by glutamate (Fukui et al., 2010; Jeong et al., 2011).

Phosphatidylinositol-3 kinase (PI3K) and its downstream effector Akt play critical roles in mediating survival signals under conditions of glutamate toxicity (Fukui et al., 2010; Qin et al., 2011). In addition, the PI3K/Akt pathway has an important role in both emodin-induced protection and toxicity (Jackson et al., 2013; Liu et al., 2010; Park et al., 2015). In our previous studies, extract from *Polygonum multiflorum* Thunb. exerted beneficial effects on glutamate-induced neurotoxicity or cerebral ischemic damage neurotoxicity via inhibition of apoptosis or an endothelial nitric oxide-dependent mechanism, respectively (Ahn et al., 2015; Jang et al., 2013; Lee et al., 2014). Because emodin from medicinal herbs decreases glutamate excitotoxicity in neurons (Gu et al., 2005), we isolated natural active emodin of the rhizome extract from *Polygonum multiflorum*.

Regulation of glutamate-induced neuronal death may provide a potential therapeutic strategy for neuroprotection in neurodegenerative disease such as cerebral ischemia. However, the beneficial effects and underlying mechanisms of emodin on neuroprotection are not clear. Therefore, we used murine hippocampal HT22 cells to study the efficacy and molecular mechanism of emodin-mediated neuroprotection under oxidative stress and confirmed its beneficial effects in a mouse model of photothrombotic ischemic stroke.

2. Materials and methods

2.1. Chemicals and antibodies

L-glutamate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ribonuclease A was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA) and LY294002 was purchased from Tocris Bioscience (Ellisville, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other cell culture reagents were purchased from Gibco-Invitrogen (Carlsbad, CA, USA). Antibodies recognizing pro-brain-derived neurotrophic factor (BDNF), Akt, and phospho-Akt (pAkt, Ser473) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Bcl-2, Bax, caspase-3, cAMP response element binding protein (CREB), and phospho-CREB (pCREB, Ser133) were supplied by Cell Signaling (Danvers, MA, USA). Antibody recognizing mature BDNF was supplied by Abcam (Cambridge, MA, USA). Secondary antibodies were supplied by ENZO Life Sciences (Farmingdale, NY, USA). A lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Promega (Madison, WI, USA). A FITC Annexin V apoptosis detection kit was purchased from BD Bioscience (San Diego, CA, USA).

2.2. Isolation of emodin and structural elucidation

Roots of dried *Polygonum multiflorum* Thunb. powder were collected in September 2012 from Sanchung, Korea. A voucher specimen (accession number PNUNPR-CRP) has been deposited in the Herbarium of Pusan National University. The plant was identified by one of the authors (Y.W. Choi). The dried roots of *Polygonum multiflorum* Thunb. (1.0 kg) were ground to a fine powder and were successively extracted at room temperature with *n*-hexane, ethyl acetate (EtOAc), and methanol (MeOH). EtOAc extract (32.26 g) was evaporated in vacuo and chromatographed on a silica gel column with CH_2Cl_2-Acetone (5% and 25% acetone in CH_2Cl_2 and 5%, 25%, 50% MeOH in $CHCl_3$) to give emodin (381.8 mg). ^1H and ^{13}C NMR spectral studies were performed using a Varian Spectrometer (Palo Alto, CA, USA) at 500 MHz with DMSO-d_6 for emodin and this purified compound was identified as emodin by gas–liquid chromatography–mass spectrometry and ^1H and ^{13}C NMR (Park et al., 2015). ^1H and ^{13}C NMR results were in agreement with data provided in the literature (Chu et al., 2005).

2.3. Cell culture

HT22 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a 5% CO_2 humidified incubator at 37 °C. The cells were incubated for 24 h prior to experimental treatments. After incubation, cells were pretreated with various concentrations of emodin for 1 h followed by co-exposure to 5 mM glutamate and emodin for 24 h. Cells were pretreated with inhibitors for 30 min before addition of emodin and then treated with glutamate.

2.4. Determination of cell viability and cytotoxicity

For MTT assay, cells were incubated with 0.5 mg/ml MTT solution and then left in a dark place for 4 h at 37 °C. Following incubation, the cells were treated with DMSO in order to dissolve the formazan crystals. Absorbance was determined at 595 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as a percentage of control. Release of LDH from damaged cells was performed according to the manufacturer's instructions for the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega). The absorbance at 490 nm was measured using a spectrophotometer (Molecular Devices). Data represent the percentage of LDH released relative to the assay positive controls.

2.5. Flow cytometric analysis

After treatment, cells were harvested and resuspended in binding buffer at a concentration of 1×10^4 cells/ml. For analysis of cell death types, 100 μl of the solution was transferred to a flow cytometric tube, followed by incubation with Annexin V-FITC and propidium iodide (PI) in the dark at room temperature for 15 min. Subsequently, 400 μl of binding buffer was added and analysis of the samples was performed using a flow cytometer (FACS Canto™ II; Becton-Dickinson, San Jose, CA, USA). For apoptotic DNA analysis, cells were washed with PBS, fixed in 1 ml ice cold 70% ethanol, and stored at 4 °C for 4 h. The fixed cells were harvested, and washed with PBS. Cells were incubated with 0.2 mg/ml ribonuclease A for 30 min and PI was added. The percentage of apoptotic cells in the sub-G1 phase was estimated by assessing the PI fluorescence using a flow cytometer.

2.6. Western blot analysis

The cell or tissue samples were homogenized in lysis buffer