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European Journal of Pharmacology 485 (2004) 105–110
www.elsevier.com/locate/ejphar

Wogonin inhibits excitotoxic and oxidative neuronal damage in primary cultured rat cortical cells

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Received 24 July 2003; received in revised form 19 November 2003; accepted 25 November 2003

Abstract

The present study evaluated effects of wogonin (5,7-dihydroxy-8-methoxyflavone) on excitotoxic and oxidative stress-induced neuronal damage in primary cultured rat cortical cells. Wogonin was shown to inhibit the excitotoxicity induced by glutamate or *N*-methyl-*D*-aspartic acid, whereas it showed no effects on the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid- or kainate-induced toxicity. In addition, wogonin inhibited the oxidative neuronal damage induced by H_2O_2, xanthine/xanthine oxidase, and by a glutathione depleting agent D,L-buthionine [*S*,*R*]-sulfoximine. Furthermore, wogonin dramatically inhibited lipid peroxidation initiated by Fe^{2+} and L-ascorbic acid in rat brain homogenates. It also exhibited 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity. Taken together, these results demonstrate that wogonin exhibits neuroprotective actions in cultured cortical cells by inhibiting excitotoxicity and various types of oxidative stress-induced damage, and that its antioxidant actions with radical scavenging activity may contribute, at least in part, to the neuroprotective effects.

Keywords: Wogonin; Neuroprotection; Cortical neuron; Antioxidant; Excitotoxicity; Oxidative damage

1. Introduction

L-Glutamate (Glu) is the principal amino acid neurotransmitter in the mammalian central nervous system. Accumulating evidence indicates that excessive release of Glu is known to be the major cause for neuronal damage in many acute and chronic neurodegenerative disorders including cerebral ischemia, epilepsy, and Alzheimer's disease (Sauer and Fagg, 1992). Overstimulation of glutamatergic system results in a massive influx of Ca^{2+}, which activates neurotoxic mechanisms including the generation of reactive oxygen species such as superoxide radicals and hydrogen peroxide (Dugan et al., 1995; Reynolds and Hastings, 1995; Sengpiel et al., 1998). Another lines of evidence indicate that reactive oxygen species play an important role in many neurodegenerative diseases (Halliwell, 1992). Given the suggested roles of Glu and reactive oxygen radicals in neuronal death (Sauer and Fagg, 1992; Halliwell, 1992), antioxidants capable of inhibiting Glu-induced neurotoxicity, and thereby, protecting neurons from excitotoxic and oxidative injuries may provide useful therapeutic potentials for the prevention or treatment of neurodegenerative disorders.

Flavonoids are a group of polyphenolic compounds found ubiquitously in plants. They have been reported to exhibit a variety of biological effects including antiviral, antibacterial, anti-inflammatory, and vasodilatory actions (Cook and Samman, 1996). Wogonin (5,7-dihydroxy-8-methoxyflavone), a flavonoid isolated from *Scutellaria baicalensis* Georgi, is known to exhibit potent anti-inflammatory effects through the inhibition of nitric oxide (NO) and prostaglandin E_2 production in macrophages (Kim et al., 1999; Wakabayashi and Yasui, 2000). In addition, antioxidant activities of wogonin have been reported (Gao et al., 1999, 2001; Shieh et al., 2000). It was shown to scavenge hydroxyl radicals generated in vitro by Fenton reaction (Gao et al., 1999), inhibit xanthine oxidase activity, and reduce cytochrome *c* (Shieh et al., 2000). It also showed significant inhibition of NADPH-induced lipid peroxidation in rat brain cortex mitochondria (Gao et al., 1999). Further-

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doi:10.1016/j.ejphar.2003.11.064

more, in cultured human neuroblastoma SH-SY5Y cells, wogonin was shown to attenuate H_2O_2-induced oxidative stress (Gao et al., 2001).

In the present study, we investigated protective effects of wogonin on excitotoxicity and various types of oxidative stress-induced neuronal cell damage in primary cultured rat cortical cells. We also evaluated its effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and lipid peroxidation initiated by Fe^{2+} and L-ascorbic acid in rat brain homogenates.

2. Materials and methods

2.1. Materials

Materials used for cell cultures including minimum essential medium (MEM) and fetal calf serum were obtained from Gibco BRL (Grand Island, NY). *N*-methyl-*D*-aspartic acid (NMDA), Glu, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid, xanthine, xanthine oxidase, H_2O_2, DPPH, D,L-buthionine [*S*,*R*]-sulfoximine (BSO), and lactate dehydrogenase (LDH) diagnostic kit were purchased from Sigma (St. Louis, MO). Culture plates were obtained from Falcon (Franklin Lakes, NJ), and wogonin was from Wako (Japan). All other chemicals were reagent grade or better.

Timed-pregnant Sprague–Dawley rats for primary cultures and male Sprague–Dawley rats for preparations of brain homogenates were obtained from Daehan Biolink (Chungbuk, Korea). Animals were maintained with Purina laboratory chow and water ad libitum in our animal facility with a 12-h light cycle at a controlled temperature (22±2 °C) until used. All animal experiments were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Primary cultures of rat cortical cells

Cortical cell cultures containing neuronal and non-neuronal cells were prepared from the cerebral cortices of Sprague–Dawley rat embryos at 16–18 days of gestation and maintained as previously described (Cho et al., 2000). Proliferation of non-neuronal cells was arrested by the addition of 10 μM cytosine arabinoside at 7 days in vitro. All experiments were performed at 10–13 days after plating.

2.3. Treatment of cells and assessment of cell damage

Excitotoxicity was induced by the exposure of cultures to Glu (100 μM) or NMDA (100 μM) for 15 min in Mg^{2+}-free HEPES-buffered salt solution (HBSS) as described previously (Cho et al., 2000). Oxidative damage was induced by xanthine (0.5 mM)/xanthine oxidase (10 mU/ml) for 10 min or H_2O_2 (100 μM) for 5 min in HBSS as previously described (Jung et al., 2002). After the exposures, the cultures were rinsed with HBSS and maintained at 37 °C for 20–24 h in MEM supplemented with 21 mM glucose. Cell damage was then observed under phase-contrast microscope, and assessed by measuring LDH activities released into the culture media (Cho et al., 2000, 2001). The AMPA- or kainate-induced excitotoxicity and BSO-induced oxidative damage were respectively induced by the exposure of cultures for 24 h to 100 μM AMPA or kainate, or 1 mM BSO in MEM supplemented with glucose, and the cell damage was assessed by LDH assay.

To evaluate the effects of wogonin on the excitotoxicity and oxidative damage, the cultures were simultaneously exposed to wogonin during the respective insults. Stock solution was prepared in 100% dimethyl sulfoxide (DMSO) at 200× the highest concentration tested and then serially diluted to the desired concentrations. For sham treatment, sister cultures were exposed to 0.5% DMSO, which showed no effect on cell viability (Cho et al., 2000).

2.4. Assay of lipid peroxidation in the rat brain homogenates

Lipid peroxidation was initiated by Fe^{+2} (10 μM) and L-ascorbic acid (100 μM) in the rat forebrain homogenates, and assayed as previously described (Dok-Go et al., 2003) with minor modifications. In brief, the reaction mixture was incubated at 37 °C for 1 h in the absence or presence of various concentrations of wogonin. The reaction was stopped by the addition of trichloroacetic acid (28% w/v) and thiobarbituric acid (1% w/v) in succession, and the mixture was then heated at 100 °C for 15 min. After centrifugation to remove precipitates, absorbance was measured at 532 nm using $VERSA_{max}$ microplate reader (Molecular Devices, USA). The percent inhibition was calculated using the following formula:

$$\text{Inhibition } (\%) = 100 \times (\text{Abs}_{\text{control}} - \text{Abs}_{\text{wogonin}})/\text{Abs}_{\text{control}}$$

2.5. Assay for DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method previously described (Dok-Go et al., 2003) with some modifications. Briefly, the reaction mixture containing various concentrations of wogonin and DPPH methanolic solution (150 μM) was incubated at 37 °C for 30 min and absorbance was measured at 520 nm. The percent scavenging activity was calculated using the above formula.

2.6. Data calculation

All experiments were performed at least three times in duplicate. Data are expressed as means±S.E.M. IC_{50} values, 50% inhibitory concentrations, were determined by non-linear regression of the mean values using Prism (GraphPad Software, USA).

3. Results

3.1. Inhibition of the Glu- or NMDA-induced excitotoxicity by wogonin

To evaluate the effects of wogonin on the excitotoxicity, we employed primary cultured rat cortical cells maintained for 10–13 days in vitro. The exposure of cultured cells to 100 μM Glu or NMDA for 15 min produced prominent acute neuronal swelling and ultimately resulted in delayed neuronal death over 20–24 h. In agreement with previous reports (Choi et al., 1987; Cho et al., 2000, 2002), approximately 70–80% cells were found to be damaged based on LDH activity measurements in the culture media. Simultaneous treatments of the cultures with wogonin during the excitotoxic insults inhibited the Glu- or NMDA-induced toxicity in concentration-dependent fashions (Fig. 1). The calculated IC_{50} values were 143.3 and 72.8 μg/ml, respectively, exhibiting more potent inhibition of the NMDA-induced toxicity. In contrast, as shown in Fig. 1, the toxicity induced by AMPA or kainate was not inhibited by wogonin.

3.2. Inhibition of the H_2O_2-, xanthine/xanthine oxidase-, or BSO-induced oxidative damage by wogonin

To evaluate the effects of wogonin on various types of oxidative neuronal damage, cultures were exposed to H_2O_2 (100 μM) for 5 min or xanthine (0.5 mM)/xanthine oxidase (10 mU/ml) for 10 min in the absence or presence of wogonin. Consistent with our previous findings (Jung et al., 2002; Dok-Go et al., 2003), approximately 70–90% cells were damaged at 20–24 h after the exposure to H_2O_2 or xanthine/xanthine oxidase. As illustrated in Fig. 2, wogonin dramatically inhibited the H_2O_2- and xanthine/xanthine oxidase-induced oxidative damage. The respective IC_{50} were 9.0 and 23.7 μg/ml.

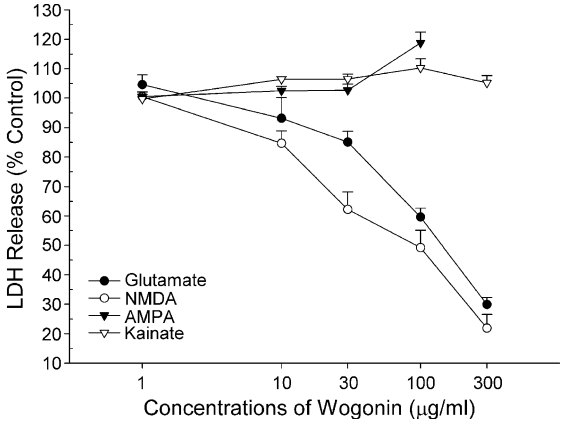

Fig. 1. Effects of wogonin on excitotoxic neuronal damage. Primary cultured rat cortical cells (10–13 days in vitro) were exposed to Glu, NMDA, AMPA, or kainate in the absence or presence of the indicated concentrations of wogonin and cell damage was assessed after 20–24 h by measuring LDH activity released into the culture media, as described in Materials and methods. Data were calculated as percent of control LDH activity measured in the medium exposed to the respective excitotoxic insults without wogonin. Each point represents the mean±S.E.M. from six measurements.

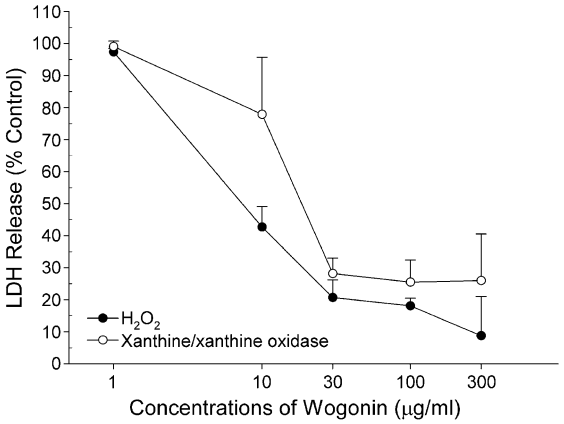

Fig. 2. Inhibition of the H_2O_2- or xanthine/xanthine oxidase-induced oxidative damage by wogonin. Primary cultured rat cortical cells (10–13 days in vitro) were exposed to H_2O_2 (100 μM) for 5 min or xanthine (0.5 mM)/xanthine oxidase (10 mU/ml) for 10 min in the absence or presence of the indicated concentrations of wogonin. Cell damage was assessed by LDH assay after 20–24 h and calculated as described in the legend of Fig. 1.

We next induced oxidative damage by the treatment of cultures for 24 h with 1 mM BSO, a glutathione (GSH) depleting agent by inhibition of γ-glutamylcysteine synthetase (Griffith and Meister, 1979). The BSO-induced damage was partially inhibited by wogonin (Fig. 3). Approximately 50% cells were protected from the BSO-induced damage by wogonin at the concentrations of 30 μg/ml and above.

3.3. Inhibition of lipid peroxidation by wogonin and its DPPH radical scavenging activity

Wogonin potently inhibited lipid peroxidation initiated by Fe^{2+} and L-ascorbic acid in rat brain homogenates

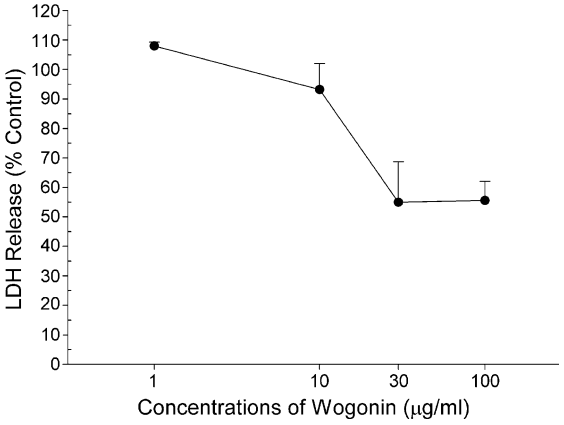

Fig. 3. Attenuation of the D,L-buthionine [*S*,*R*]-sulfoximine (BSO)-induced oxidative damage by wogonin. Primary cultured rat cortical cells (10–13 days in vitro) were exposed to 1 mM BSO for 24 h in the absence or presence of the indicated concentrations of wogonin. Cell damage was assessed by LDH assay and calculated as described in the legend of Fig. 1.

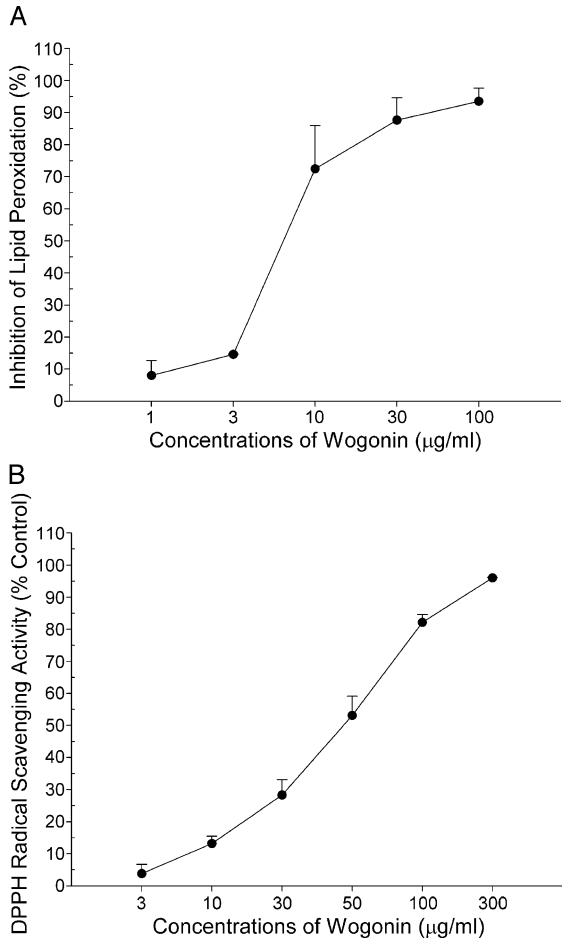

Fig. 4. Effects of wogonin on lipid peroxidation and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. (A) Lipid peroxidation initiated in rat brain homogenates by Fe^{+2} and L-ascorbic acid was assessed as described in Materials and methods in the absence or presence of the indicated concentrations of wogonin. (B) DPPH radical scavenging activity was determined in the absence or presence of the indicated concentrations of wogonin as described in Materials and methods. Each point represents the mean±S.E.M. from three measurements performed in duplicate.

(Fig. 4A), with the IC_{50} of 6.8 μg/ml. In addition, it scavenged free radicals generated by DPPH (Fig. 4B). Fifty percent scavenging activity was achieved at 44.5 μg/ml.

4. Discussion

The radix of *S. baicalensis* is widely employed in traditional oriental prescriptions for various infections, inflammatory diseases, and stroke (Gong and Sucher, 1999). Its methanol extract has been reported to protect PC12 cells against H_2O_2-induced toxicity and CA1 hippocampal neurons against transient global ischemia induced in rats by four-vessel occlusion for 10 min (Kim et al., 2001). The main components of *S. baicalensis* are known to be baicalin, baicalein, wogonin, and wogonoside. These flavonoids have been shown to possess a broad spectrum of antiviral, anti-inflammatory, antiallergic, and antioxidant actions (Wakabayashi and Yasui, 2000; Shieh et al., 2000; Ma et al., 2002). The mixture of four flavones in extracts of *S. baicalensis* has been reported to markedly increase the survival of neuronal HT-22 cells from oxidative stress induced by low levels of H_2O_2 for 24 h (Choi et al., 2002). Although baicalein and baicalin are shown to exhibit remarkable antioxidant activities in neuronal cell lines and in vitro assays (Gao et al., 1999, 2001; Shieh et al., 2000), it is still unclear which particular flavonoid(s) included in *S. baicalensis* extracts is responsible for the neuroprotective actions (Kim et al., 2001; Choi et al., 2002).

In this study, we investigated effects of wogonin on excitotoxicity as well as various types of oxidative stress-induced neuronal damage using primary cultured rat cortical cells, and demonstrated its neuroprotective effects. As reported previously, brief and intense exposure of cultured neurons to Glu results in massive acute cell swelling followed by delayed neuronal disintegration over a period of time, which ultimately leads to excitotoxic cell death (Choi, 1992; Cho et al., 2000). We found in this study that the Glu-induced excitotoxicity was inhibited by wogonin (Fig. 1). When the toxicity was induced by the receptor subtype-selective ligands, NMDA, AMPA, or kainate, only the NMDA-induced toxicity was selectively inhibited by wogonin. Furthermore, the NMDA-induced toxicity was inhibited more potently than the Glu-induced toxicity (Fig. 1). These findings imply that wogonin may directly bind to NMDA receptors and inhibit the activity of receptor channels, thereby attenuating excitotoxicity. Receptor binding assays or electrophysiological recordings may be useful to determine this possibility.

Overactivation of NMDA receptors provokes multiple neurotoxic mechanisms including production of reactive oxygen species, which can cause cellular damage and subsequent cell death by oxidizing cellular components such as lipids, proteins, and DNA (Simonian and Coyle, 1996). In this study, we exposed cultured cortical cells to H_2O_2, xanthine/xanthine oxidase, or BSO in the presence of wogonin to investigate its effects on various types of oxidative neuronal damage. The exposure to H_2O_2 or xanthine/xanthine oxidase generates free radicals such as hydroxyl and superoxide radicals, which are believed to actively participate in the initiation of lipid peroxidation and eventually cause cell death (Halliwell, 1992; Simonian and Coyle, 1996; Dok-Go et al., 2003). Gao et al. (2001) reported in neuroblastoma cells that wogonin inhibited the H_2O_2-induced damage, but the effect was only marginal at the concentration they tested (10 μM). In our study, we found in cortical cells that wogonin dramatically inhibited the H_2O_2-induced oxidative damage within the concentration range of 1–300 μg/ml (Fig. 2). The calculated IC_{50} was 9.0 μg/ml, which corresponds to approximately 30 μM. The apparent discrepancy appears to be due to the different cell types employed in each study (neuroblastoma SH-SY5Y cells vs. primary cultured rat cortical cells), the concentration (400 μM vs. 100 μM) and duration (2 h vs. 5 min) of

H_2O_2 exposure, and above all, the concentrations of wogonin tested (10 μM vs. 1–300 μg/ml).

We also found in this study that wogonin inhibited the oxidative damage induced by xanthine/xanthine oxidase in cortical cultures (Fig. 2). This observation is in consistence with the previous report demonstrating superoxide radical scavenging activity and inhibition of xanthine oxidase activity by wogonin (Shieh et al., 2000). At this point, however, it is not clear whether wogonin inhibits the xanthine/xanthine oxidase-induced oxidative damage by blocking the generation of superoxide radicals, or acting directly on the generated radicals, or both. In addition to its protective actions against the H_2O_2- or xanthine/xanthine oxidase-induced damage, wogonin attenuated the oxidative cell damage induced by BSO, a GSH depleting agent (Fig. 3). Taken together, these results demonstrated neuroprotective actions of wogonin against excitotoxicity and various types of oxidative stress-induced damage in primary cultured rat cortical cells.

Recently, it has been reported that wogonin inhibits inflammatory activation of microglia by diminishing tumor necrosis factor-α, interleukin-1β, and NO production (Lee et al., 2003). In addition, Lee et al. (2003) found that wogonin protected cocultured PC12 cells against microglial cytotoxicity, and suggested an indirect role of wogonin in neuroprotection. In the present study, we demonstrated in cortical cultures that wogonin exerts neuroprotective effect by inhibiting excitotoxic and oxidative neuronal cell damage. Therefore, based on the report by Lee et al. (2003) and our findings in this study, wogonin may exert neuroprotective effects by not only inhibiting microglial activation, but also inhibiting excitotoxic and oxidative neuronal cell damage. Since NO is considered to be one of the crucial mediators of neuronal damage in NMDA-induced excitotoxicity (Dawson and Dawson, 1998), and since wogonin has been reported to inhibit NO production in microglia by suppressing inducible NO synthase (iNOS) induction (Lee et al., 2003), it would be interesting to investigate effects of wogonin on neuronal NOS activity.

We next assessed antioxidant activities of wogonin by cell-free assays. Previously, Gao et al. (1999) reported that wogonin (10 μM) exhibited significant inhibition of NADPH-induced lipid peroxidation in rat brain cortex mitochondria, but they found that it did not inhibit lipid peroxidation induced by Fe^{2+}–ascorbic acid nor by 2,2′-azobis (2-amidinopropane) hydrochloride. We tested wogonin at broader concentration range (1–100 μg/ml) in this study, and found that it exhibited marked inhibition of Fe^{2+}–ascorbic acid-induced lipid peroxidation in rat brain homogenates, with the IC_{50} of 6.8 μg/ml (Fig. 4A). Thus, based on the previous report by Gao et al. (1999) and our finding, wogonin may inhibit lipid peroxidation induced by NADPH as well as by Fe^{2+} and L-ascorbic acid. Similarly, Gao et al. (1999) found by electron spin resonance technique that wogonin scavenged hydroxyl radicals, but it had no effects on DPPH or alkyl radicals at 10 μM. In this paper, we showed DPPH radical scavenging activity of wogonin using higher concentrations (3–300 μg/ml) than they tested (Fig. 4B). These findings imply that wogonin exhibits variable degrees of antioxidant and free radical scavenging activities depending on the experimental systems and concentrations tested. In any event, the antioxidant actions of wogonin with radical scavenging activity may contribute, at least in part, to its neuroprotective effects.

In summary, this study confirmed and extended previous reports by Gao et al. (1999, 2001) and Shieh et al. (2000) on antioxidant and neuroprotective effects of wogonin. Most importantly, the present study demonstrated that wogonin exhibits neuroprotective effects in cultured cortical cells by inhibiting excitotoxicity and various types of oxidative stress-induced neuronal damage, and that it also exhibits antioxidant actions with radical scavenging activity which may contribute to the neuroprotective effects. These findings suggest that wogonin may act as one of the active neuroprotectants in *S. bacalensis* extracts, and therefore, it may provide advantage to prevent and/or treat neurodegenerative disorders associated with excessive Glu release and generation of reactive oxygen species. According to the recent report by Lee et al. (2003), wogonin attenuated ischemic death of hippocampal neurons in transient global ischemia induced by four-vessel occlusion. Currently, effects of wogonin on a focal cerebral ischemia model of rat induced by middle cerebral artery occlusion are under investigation in our laboratory.

Acknowledgements

This research was supported by a grant (PF0320202-01) from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology in Korea.

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