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Research report

Differential effects of natural polyphenols on neuronal survival in primary cultured central neurons against glutamate- and glucose deprivation-induced neuronal death

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Abstract

Neuronal injury in the central nervous system following ischemic insult is believed to result from glutamate toxicity and glucose deprivation. In this study, polyphenols isolated from *Scutellaria baicalensis* Georgi, including baicalin, baicalein, and wogonin, were investigated for their neuroprotective effects against glutamate/NMDA (Glu/NMDA) stimulation and glucose deprivation in primary cultured rat brain neurons. Cell death was accessed by lactate dehydrogenase (LDH) release assay for necrosis, and mitochondrial activity was accessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction activity assay. It was found that both baicalin and baicalein decreased LDH release of the cultured neurons after 24 h treatment, whereas wogonin profoundly increased LDH release after 2 h treatment and resulted in neuronal death after 24 h. Glu/NMDA treatment profoundly increased LDH release and moderately decreased MTT reduction activity in an NMDA receptor-dependent manner. Both baicalin and baicalein significantly reduced Glu/NMDA-increased LDH release, in which baicalein is much more potent than baicalin. Glu/NMDA-increased intracellular calcium was also significantly attenuated by baicalin and baicalein. Baicalin and baicalein did not affect glutamate receptor binding activity, but baicalein did moderately decrease Glu/NMDA-induced nitric oxide (NO) production. In the glucose deprivation (GD) study, baicalein but not baicalin showed significant protective effects on the GD-increased LDH release, without affecting the GD-induced NO production, in cultured rat brain neurons. These results suggest that baicalein is the most effective compound among three polyphenols tested in preventing neurotoxicity induced by both glutamate and GD, whereas baicalin was only effective in preventing glutamate toxicity. Wogonin might have a neurotoxic effect on the brain.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Polyphenol; Neurotoxicity; Glutamate; Calcium; Glucose deprivation

1. Introduction

Neuronal damage and degeneration following acute brain injury, ischemic stroke, and chronic oxidative stress, etc., are believed to be mediated mostly by excitotoxicity, energy depletion, and oxidative stress [23,24]. Glutamate is an excitatory neurotransmitter at the majority of excitatory synapses in the mammalian CNS [3,26,39]. Intense exposure to glutamate can be neurotoxic. Two mechanisms contribute to the glutamate neurotoxicity: glutamate receptor-mediated excitotoxicity [4] and glutamate receptor-independent oxidative glutamate toxicity [28,35]. The excitotoxicity is mainly via activation of the NMDA receptor, which resulted in excessive elevation of intracel-

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lular calcium and subsequent induction of necrotic and apoptotic cell death [12,13]. Oxidative glutamate toxicity is induced by high concentration of external glutamate in a calcium-independent manner, which inhibits the cystine/glutamate antiporter x_c^- and in turn reduced uptake of cystine required for the synthesis of the potent intracellular-reducing agent glutathione (GSH). Depletion of GSH by glutamate causes accumulation of oxidative free radicals and leads to neuronal apoptosis [5,31,35]. Recent reports have shown that oxidative glutamate toxicity can be a downstream component of the NMDA receptor-mediated excitotoxicity because the necrotic effect of the latter increased membrane permeability and resulted in increase of external glutamate concentration to the millimolar range. The NMDA receptor-increased extracellular glutamate concentration hence blocks the cystine/glutamate antiporter and causes the oxidative stress [32].

Hypoglycemia is a common cause of neuronal damage in ischemic stroke [25]. The pathological mechanism is believed to be energy depletion, mitochondrial dysfunction, and subsequent external glutamate accumulation due to depolarization-induced glutamate release [9,17,34]. Glucose deprivation is a commonly used in vitro model to study hypoglycemia-induced neuronal death, and was found containing two components: glutamate receptor-dependent component with necrotic feature, and the other mitochondrial dysfunction with apoptotic feature [6].

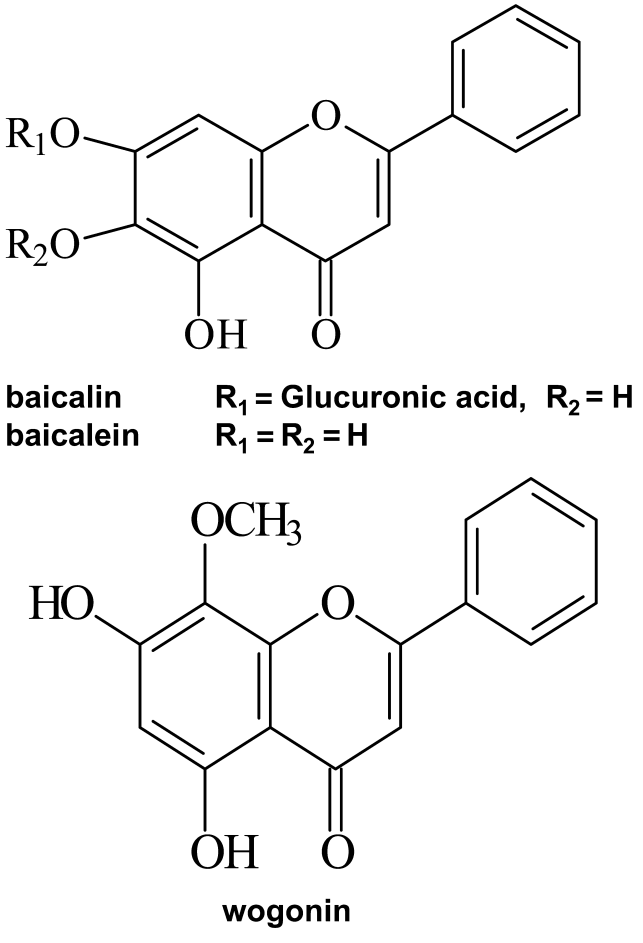

Fig. 1. Structure of baicalin, baicalein, and wogonin extracted from *Scutellaria baicalensis*.

Polyphenolic compounds, including a large group of flavonoids, are enriched in vegetables, fruits, tea, wine and Chinese herbs. *Scutellaria baicalensis* Georgi, also pronounced as Huang-Qin in mandarin, is one of the most important medicinal herbs in traditional Chinese medicine [11,15]. *S. baicalensis* Georgi contains three major polyphenolic components, namely wogonin, baicalin, and baicalein (Fig. 1). These three polyphenols have been demonstrated as free radical scavengers of hydroxyl radical, DPPH radical and alkyl radical [7]. Baicalein is also known as a selective inhibitor of 12-lipoxygenase, which accounts for the production of reactive oxygen species (ROS) during arachidonic acid metabolism [33]. However, to date, evidence regarding their effects on the central nervous system is still limited.

In this article, we used primary cultured rat brain neurons to study the effects of wogonin, baicalin, and baicalein extracted from *S. baicalensis* on neuronal survival. We found that only baicalin and baicalein had a significant effect in protecting neurons from glutamate toxicity and/or glucose deprivation-induced neuronal death. Possible mechanisms of baicalin- and baicalein-mediated neuroprotection were also examined.

2. Materials and methods

2.1. Extraction and purification of baicalin, baicalein, and wogonin from S. baicalensis Georgi

Baicalin, baicalein, and wogonin were extracted from dried *S. baicalensis*. In brief, dried *S. baicalensis* roots were cut into small pieces, immersed, and extracted with 10-times v/w acetone twice at room temperature for 2 weeks. After filtration, the residues were reflux-extracted with 4-times v/w of 50% aqueous ethanol twice. Acetone and 50% aqueous ethanol extracts were concentrated and recrystallized with aqueous ethanol to obtain baicalin. The acetone extracts were subjected to column chromatography on silica gel eluted with $CHCl_3$ and $CHCl_3$–MeOH, and rechromatographed on silica gel eluted with hexane–acetone to yield wogonin. A portion of the $CHCl_3$–MeOH elute was subjected to Sephadex LH-20 eluted with MeOH to yield baicalein. Each compound was identified by direct comparison of its ESI-Mass, ^{1}H- and ^{13}C-NMR spectroscopic data with authentic samples. Purity tests of baicalin, baicalein, and wogonin were performed by HPLC. The HPLC system consisted of a Shimadzu Model LC-10AT system equipped with a Shimadzu Model SIL-9A auto-injector and a Shimadzu Model SPD-10 A detector (Shimadzu, Kyoto, Japan). Peak areas were calculated with a Shimadzu Model C-R8A recorder. A LiChrospher 100

RP-18e reversed-phase column (Merck, Darmstadt, Germany) and a LiChrospher 100 RP-18e guard column (Merck) were used. The purity of all compounds was more than 99.5%. Commercially available wogonin, baicalin, and baicalein (Sigma, St. Louis, MO, USA) were used to confirm the results.

2.2. Primary cultured rat brain neurons

Primary cultured rat brain neurons were prepared as described previously [21]. Pregnant female Sprague–Dawley rats (National Institute of Experimental Animal Research, Taipei, Taiwan) at 17 days of gestation were deeply anesthetized with 60 mg/kg body weight of sodium pentobarbital. Fetal rats were collected followed by harvesting of fetal whole brains. Brain tissue was mechanically dissociated, and plated onto poly-L-lysine-coated culture plates at a density of 5×10^5 cells per well of 24-well culture plates with basal medium Eagle (BME, Life Technology) supplemented with sodium bicarbonate (13.1 mM), D-glucose (final concentration 33 mM), L-glutamine (2.0 mM), and 20% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA). Cells were initially incubated for 45 min in a humidified incubator with 5% CO_2 at 37 °C for monolayer attachment, after which the medium was replaced by serum-free BME. Cells were thereafter maintained in 5% CO_2 at 37 °C. Cultures obtained under these conditions appeared to contain mostly neurons [20]. The day of plating was counted as 0 days-in-vitro (DIV), and 9 or 10 DIV of cultured neurons were used in this study. Adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Neuronal survival analysis

Neuronal survival analysis was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction activity assay to access mitochondrial activity, and lactate dehydrogenase (LDH) release to access necrotic cell death. For MTT reduction activity assay, cultured neurons were incubated with MTT for 4 h, followed by addition of 0.3 ml of 0.4 N HCl in isopropanol to the mixture overnight to dissolve the formazan. The dissolved suspension was subjected to ELISA reader and the absorbance at a wavelength of 600 nm ($A_{600\ nm}$) was measured as previously described [27]. For LDH release assay [21], cultured neurons were equilibrated with newly changed BME medium for 1 h. Then, 500 μl of culture medium were collected and incubated with 0.2 mg of β-NADH in 2.4 ml of 0.1 M phosphate buffer for 5–15 min at room temperature. The absorbance at a wavelength of 340 nm was measured for 3 min immediately after 0.1 M sodium pyruvate was added to the mixture. The unit activity of LDH was defined as decrease of A_{340} in 1 min, multiplied by 1000 in 1 ml of sample. For the glutamate toxicity study, various concentrations of polyphenols were added to cultures to incubate for 30 min, followed by incubation with combined treatment of 50 μM of glutamate and 50 μM *N*-methyl-D-aspartate (Glu/NMDA). For the glucose deprivation study, cultured neurons were subjected to Earle's balanced salt saline (EBSS) containing 0 or 33 mM D-glucose for 1 h as the normal and hypoglycemic conditions, respectively, followed by 16 h incubation in BME medium containing 33 mM glucose. Polyphenolic compounds at various concentrations were included during the entire glucose deprivation and post-deprivation periods.

2.4. Measurement of intracellular calcium concentration

Cells were cultured on coverslips in 35-mm dishes and were loaded with 5 μM fura-2 acetoxymethyl ester (fura-2/AM, Molecular Probes, Eugene, OR, USA), a Ca^{2+}-sensitive dye at 37 °C for 45 min. At the end of the loading period, the coverslip was washed twice with physiological buffer solution (PBS) containing NaCl 125 mM, KCl 5 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 2 mM, NaH_2PO_4 0.5 mM, $NaHCO_3$ 5 mM, HEPES 10 mM, and glucose 10 mM, pH 7.4. Cells were incubated in PBS for another 30 min to complete dye deesterification. The coverslip was inserted at a 45° angle to the excitation beam into a quartz cuvette and placed in the thermostat holder of a Hitachi F-4500 spectrofluorometer. Fluorescence of Ca^{2+}-bound and unbound fura-2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at an emission wavelength of 510 nm. The ratios (R) of the fluorescence at the two wavelengths were computed and used to calculate changes in $[Ca^{2+}]_i$. Ratios of maximum (R_{max}) and minimum (R_{min}) fluorescence of fura-2 were determined by the addition of ionomycin (10 mM) in the presence of PBS containing 5 mM Ca^{2+} and by adding 5 mM EGTA at pH 8 in Ca^{2+}-free PBS, respectively. The intracellular calcium concentration was obtained from the following equation: $[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R)$, in which $K_d = 224$ nM and $\beta = 6.9352$ was assumed [10].

2.5. [^{3}H]L-Glutamate binding assay

Crude membrane used for glutamate receptor binding assays and the assay procedure were conducted as previously described [22]. In brief, the binding assay consisted of a 750-μl crude membrane (1 mg/ml in 50 mM Tris–acetate, pH 7.2), 100 μl of [^{3}H]L-glutamate (3.11 nM), and 50 μl of baicalin or baicalein for the final concentration of 3.5 μM, and 50 mM Tris–acetate, giving the final volume of 1 ml. For total binding, the binding mixture without

[^{3}H]L-glutamate was preincubated for 15 min at 4 °C, followed by a 45-min incubation with the addition of [^{3}H]L-glutamate. Nonspecific binding (NB) and drug binding (DB) were obtained by a preincubation of the binding mixture with 100 ml of 10 mM L-glutamate (NB), baicalin or baicalein (DB), for 15 min prior to the addition of [^{3}H]L-glutamate. The incubation was terminated by centrifugation. The pellet obtained was rinsed with double distilled water, and diluted in buffer for radioactivity counting.

2.6. Measurement of nitrite formation

We measured nitrite formation as an index of nitric oxide (NO) production as described previously [37]. In brief, cultured neurons were subjected to baicalin or baicalein along with Glu/NMDA stimulation for 24 h. Culture medium was collected and added to a 0.1 ml Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diaminedihydrochloride in 5% H_3PO_4) to form a purple azodye. $NaNO_2$ was used as a standard in the reaction to obtain a standard curve, and color development was measured by spectrophotometer at 530 nm.

3. Results

3.1. Cytotoxicity test of polyphenols on primary cultured rat brain neurons

We first tested cytotoxicity of baicalin, baicalein, and wogonin in primary cultured neurons. Fig. 2A shows that short-term exposure of wogonin at 3.5, 0.35, and 0.035 μM for 2 h significantly increased LDH release of primary cultured rat brain neurons, whereas baicalin and baicalein had no such detrimental effect. To further confirm the toxic effect of wogonin, we examined its effect on neuronal survival after 24 h exposure. Both Fig. 2B and C show that wogonin at the concentrations tested tended to increase the release of LDH and decrease the MTT reduction activity. It is noted that the increased LDH release at 24 h is far less than that at 2 h, which might be due to degradation or inactivation of early-released LDH in the culture medium. Morphological destruction, apoptotic features, and decreased cell density of the cultured neurons were apparent in the wogonin-treated condition as observed in Fig. 2C. These results suggest that wogonin might have detrimental effects on neurons, and hence was excluded in the following studies. Baicalin and baicalein were used in the following studies to investigate their possible neuroprotective activities.

3.2. Effects of baicalin, baicalein on neuronal survival in primary cultured central neurons against glutamate toxicity

We induced glutamate toxicity in primary cultured rat brain neurons with 50 μM NMDA and 50 μM glutamate (Glu/NMDA) to study the effect of baicalin and baicalein on glutamate excitotoxicity. Firstly, we examined whether the component of our Glu/NMDA toxicity is NMDA receptor-mediated. In Fig. 3A, the group treated with Glu/NMDA had significant increase of LDH release and moderate decrease of MTT reduction activity. MK-801, a specific channel blocker for the NMDA receptor at 10 μM, completely blocked Glu/NMDA-increased LDH release and Glu/NMDA-decreased MTT reduction activity. This result confirmed that the neurotoxic effect of Glu/NMDA is mainly mediated by the NMDA receptor. We then examined the effect of baicalin and baicalein on the survival of cultured neurons under control and Glu/NMDA stimulation. Fig. 3B and C show that both baicalin and baicalein tended to increase neuronal survival of primary cultured neurons but with different characteristics: baicalin at concentration from 3.5 nM to 3.5 μM significantly decreased LDH release but did not increase MTT reduction activity (Fig. 3B), whereas baicalein tended to increase MTT reduction activity at high concentrations but did not decrease LDH release (Fig. 3C). These results suggest that baicalin and baicalein might protect naturally occurring necrosis and mitochondrial dysfunction, respectively.

Under Glu/NMDA treatment, both baicalin and baicalein increase neuronal survival by significantly decreasing LDH release at concentrations from 3.5 nM to 3.5 μM, with slight attenuation of the Glu/NMDA-decreased MTT reduction activity. Baicalein also showed much more potent protective effect than baicalin in reduction of Glu/NMDA-increased LDH release. It is noted that baicalin at low concentrations (3.5 and 35 nM) had a similar degree of reduction of basal level LDH release (black bars in Fig. 3B-1) and reduction of Glu/NMDA-increased LDH release (gray bars in Fig. 3B-1). Higher concentrations of baicalin (350 and 3500 nM) had less protective effect in basal level cell death and their protective component against Glu/NMDA toxicity became more apparent. These results suggest that baicalin and baicalein have similar effective concentration but different potency in protecting brain neurons against NMDA receptor-mediated excitotoxicity.

3.3. Effects of baicalin and baicalein on intracellular calcium increase induced by Glu/NMDA co-stimulation in cultured neurons

Since elevation of intracellular calcium is one of the major causes of glutamate excitotoxicity, we further examined the effect of baicalin and baicalein on Glu/NMDA-increased intracellular calcium at 10 DIV cultured neurons. Baicalin or baicalein was added 50 s before Glu/NMDA was applied. Both baicalin and baicalein by themselves at a range from 3.5 nM to 3.5 μM had no effect on the basal level of intracellular calcium concentration. Increasing concentration of these two polyphenols to 3.5 μM resulted in a significant reduction of

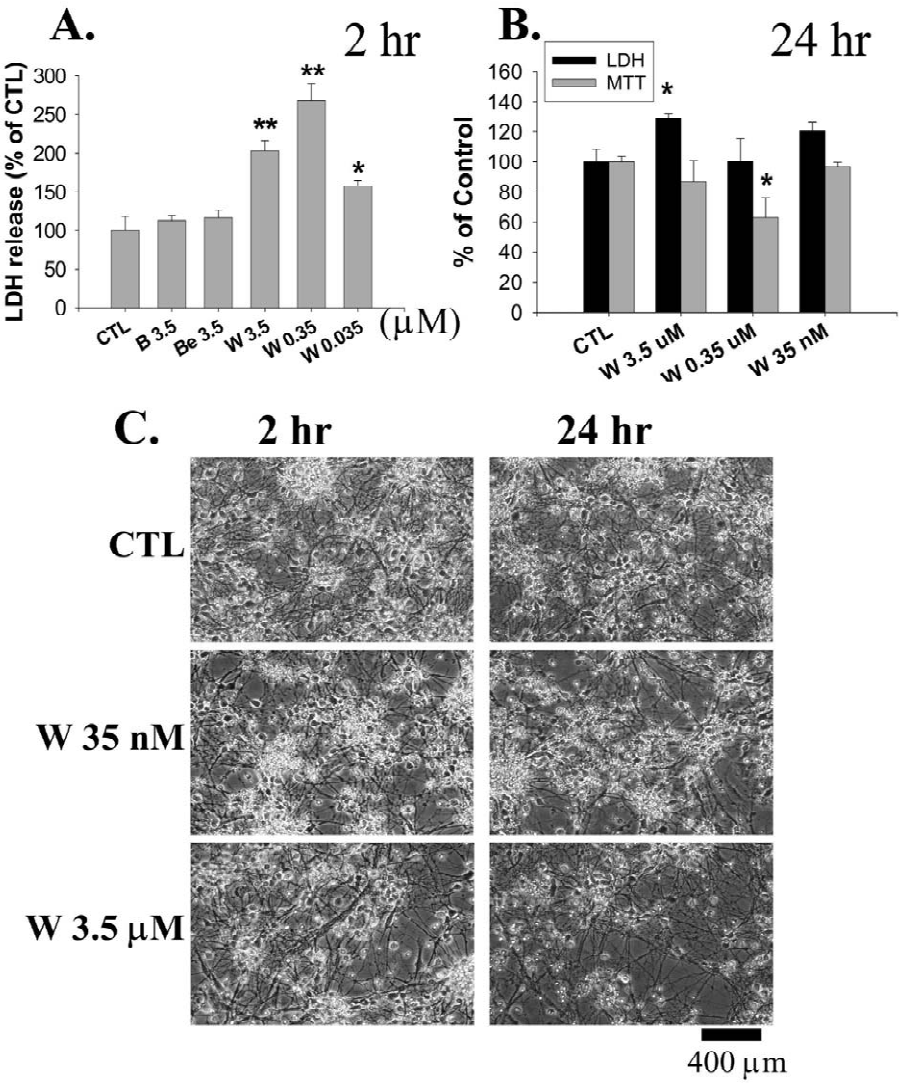

Fig. 2. Cytotoxicity test of polyphenols on primary cultured rat brain neurons. (A) Cells were treated with baicalin (B), baicalein (Be), and 3.5 μM wogonin (W) for 2 h in primary cultured brain neurons at 9 days-in-vitro (DIV). Two additional concentrations of wogonin at 0.35 and 0.035 μM were tested to further confirm the wogonin neurotoxicity. Culture medium in each condition was collected and subjected to LDH release assay. (B) The same culture treated in (A) continued to be incubated for another 22 h after the culture medium was collected at 2 h. At the end of 24-h incubation, culture medium was collected for LDH release assay, and cells were subjected to MTT reduction activity assay. The LDH value or MTT value obtained from the control group (CTL) served as 100%, and data obtained in other groups were calculated as percent of control accordingly. Data are expressed as the mean±S.E.M ($n=4$). *$P<0.05$, and **$P<0.01$ as compared with the vehicle-treated control group (CTL) by unpaired t-test. (C) Phase contrast photomicrograph of the wogonin-treated cultured neurons were taken at 2 h and 24 h after the treatment. Scale bar=400 μm.

Glu/NMDA-increased intracellular calcium (Fig. 4A and B). This result suggests that baicalin and baicalein might protect neurons against glutamate excitotoxicity at least in part by attenuating intracellular calcium elevation.

3.4. Effects of baicalin and baicalein on glutamate receptor binding and glutamate-induced nitric oxide production in cultured neurons

To examine if the neuroprotective effect of baicalin and baicalein against excitotoxicity is due to their direct effect on the glutamate receptor binding activity, we applied these two compounds to the [^{3}H]L-glutamate binding assay, with the source of glutamate receptors prepared from porcine brains. Fig. 5A shows that neither baicalin nor baicalein had appreciable effect on the glutamate receptor binding activity, excluding the possibility that these two compounds may directly modulate glutamate receptor activities. Furthermore, it is known that the activation of the NMDA receptor leads to increase of nitric oxide (NO) production [40]. Therefore we applied baicalin and baicalein onto the cultured neurons to examine their effects on the Glu/NMDA-induced NO production. Fig. 5B shows that Glu/NMDA stimulation did significantly increase NO production in cultured neurons. Baicalein but not baicalin at 350 μM caused moderate but not significant reduction of Glu/NMDA-increased NO for ~50%, suggesting that baicalein might reduce Glu/NMDA-increased

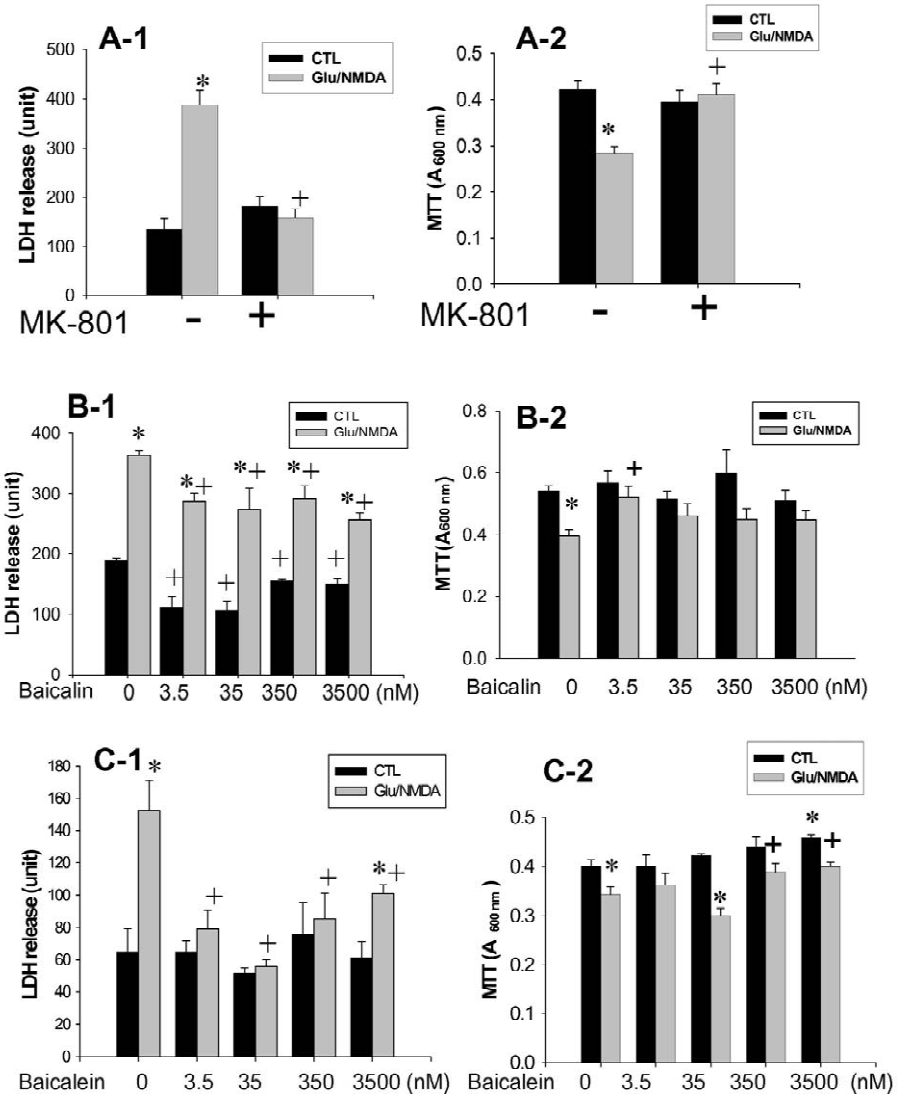

Fig. 3. Effect of baicalin and baicalein on Glu/NMDA-induced neuronal death in primary cultured brain neurons. (A) Cultured neurons at 9 DIV were treated with 10 μM of MK-801 in the absence (control group, CTL, black bars) or presence (Glu/NMDA group, gray bars) of 50 μM glutamate and 50 μM NMDA for 24 h. At the end of treatment, culture medium was collected for LDH release assay (A-1) and cells were subjected to MTT assay (A-2). (B, C) Baicalin and baicalein at 3.5, 35, 350, and 3500 nM were added in the absence (CTL, gray bars) or presence (black bars) of Glu/NMDA as described above for 24 h. Culture medium was harvested for the LDH release assay (B-1 and C-1), and treated cells were subjected to MTT assay (B-2 and C-2). Data are expressed as the mean±S.E.M ($n=4$). *$P<0.05$ as compared with the respective CTL group, and $^{+}P<0.05$ as compared with the CTL group and the Glu/NMDA group without additional treatments (unpaired t-test).

NO formation to provide additional protection to the central neurons.

3.5. Effects of baicalin and baicalein on glucose deprivation-induced neurotoxicity

We further examined the effect of baicalin and baicalein on glucose deprivation-induced neuronal death. SD rat brain cultured neurons at 9 DIV were subjected to 0 mM glucose deprivation condition (GD) or 33 mM normal glucose condition (NG) for 1 h, followed by recovering in the regular culture medium for 16 h. Fig. 6A shows that GD-treated neurons resulted in decrease of neuronal survival by increasing LDH release but not by decreasing MTT reduction activity, implicating the occurrence of necrosis. However, baicalin and baicalein had distinct effects on neuronal survival: GD-increased LDH release was significantly reduced by baicalein, but slightly increased by baicalin. Therefore, we further examine the protective effect of baicalein at different dosage in the cultured neurons. Fig. 6B shows that baicalein completely blocked GD-increased LDH release, but became neurotoxic at high concentration (35 μM) since it greatly reduced MTT reduction activity. However, baicalein did not reduce GD-increased NO production (Fig. 6C), im-

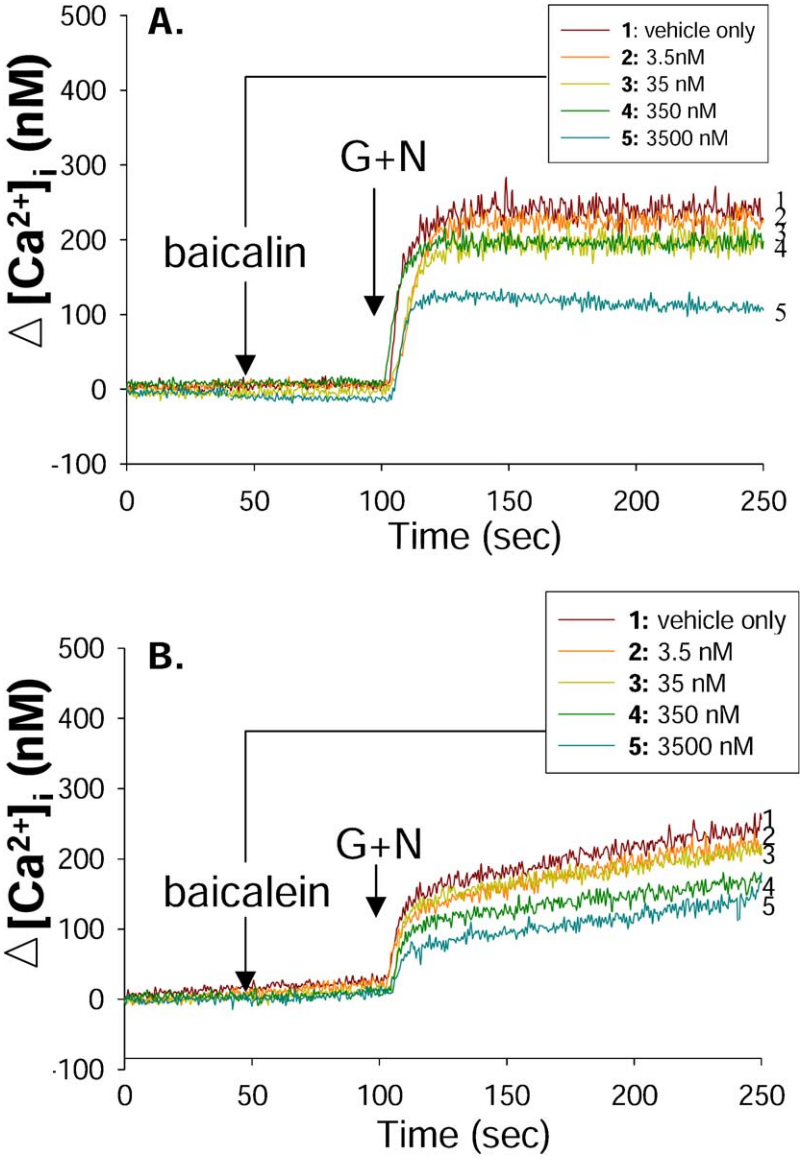

Fig. 4. Effects of baicalin and baicalein on Glu/NMDA-increased intracellular calcium in primary cultured brain neurons. Cultured neurons at 10 DIV were loaded with 5 μM Fura-2 AM, treated with 3.5, 35, 350, and 3500 nM of baicalin (A) or baicalein (B) for 50 s, followed by stimulations with 50 μM glutamate and 50 μM NMDA (Glu/NMDA). Fluorescent detection of intracellular calcium was carried out for 600 s in a fluorescence spectrophotometer as described in Section 2. Intracellular calcium increased over the basal level was expressed as $\Delta[Ca^{2+}]_i$ (nM) for the *Y*-axis. Four batches of experiments were performed with similar results.

plicating that its neuroprotective effect is mediated by other mechanisms.

4. Discussion

In this paper we demonstrated that among three polyphenols tested, baicalin and baicalein are the safest compounds and exert significant but distinct neuroprotective effects in central neurons. We also demonstrated that both baicalin and baicalein reduced glutamate excitotoxicity, but only baicalein can protect neurons from glucose deprivation-induced cell death. On the other hand, wogonin was reported as a free radical scavenger, but its detrimental effect to CNS neurons shown in this study suggests that not all free radical scavengers have neuroprotective effects. The differential nature of baicalin and baicalein and their possible neuroprotective mechanisms are discussed as follows.

4.1. Structure–activity relationship of polyphenolic compounds

Among three polyphenolic compounds tested in this study, only baicalin and baicalein have neuroprotective effects raising a question of possible specificity of the

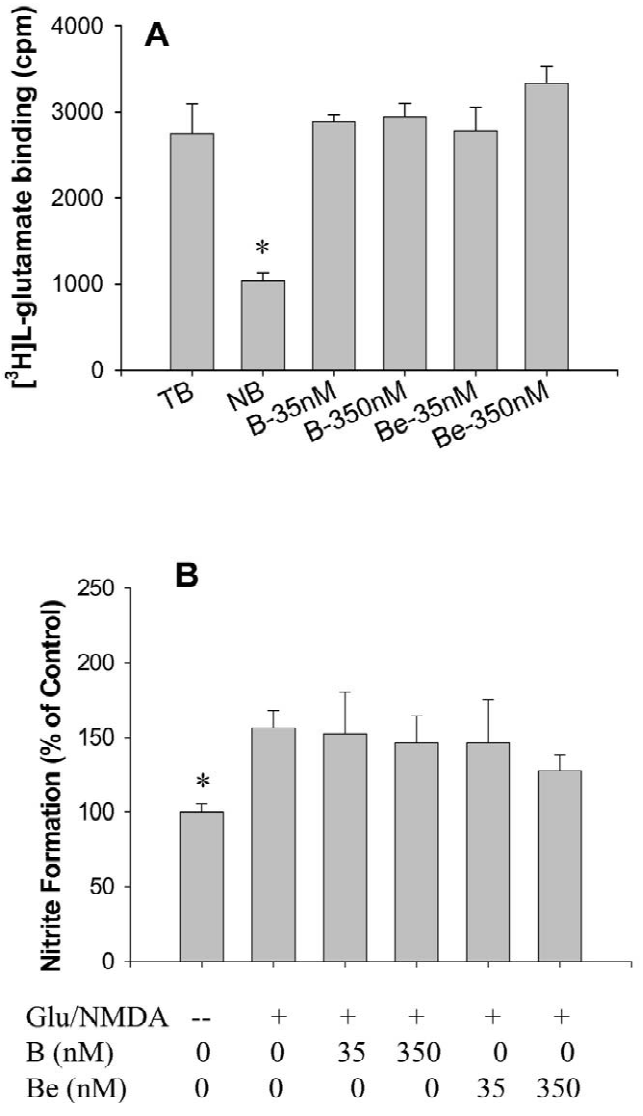

Fig. 5. Effects of baicalin and baicalein on glutamate receptor binding and Glu/NMDA-induced NO production in cultured neurons. (A) Baicalin (B) and baicalein (Be) at 35 and 350 nM were applied 15 min prior to the addition of [^{3}H]L-glutamate to the crude membrane preparation for glutamate receptor binding assay, followed by procedures described in Section 2. Isotopic counts (cpm) obtained in each group were directly plotted. TB, total binding; NB, non-specific binding. (B) Cultured brain neurons at 9 DIV were treated with B and Be at 35 and 350 nM in the presence or absence of Glu/NMDA for 24 h. At the end of treatment, culture medium was harvested and subjected to the nitrite formation assay as described in Section 2. The nitrite formation value obtained from the control group (without Glu/NMDA, B or Be treatment) served as 100%, and data obtained in other groups were calculated as percent of control accordingly. Data are expressed as the mean±S.E.M ($n=4$). *$P<0.05$ as compared with other groups by one-way ANOVA with Newman–Keuls multiple comparison post-test.

structure–activity relationship. Indeed, Ishige et al. [14] demonstrated three structural features of flavonoids, including the hydroxylated C3, an unsaturated C ring, and hydrophobicity, might be required for protection of neurons from oxidative glutamate toxicity. Although none of the tested compounds contain hydroxylated C3, baicalin, baicalein, and wogonin do have an unsaturated C ring and are rather hydrophobic. Furthermore, baicalein is a hydrolyzed form of baicalin, which usually occurs in the digestive tract when baicalin is orally administered [41]. Since conversion of baicalin to baicalein increases membrane permeability and antioxidant activity, it is possible that baicalein might be more potent than baicalin in protecting neurons based on this nature.

4.2. Possible mechanisms of baicalin- and baicalein-mediated neuroprotection—reduction of Glu/NMDA-increased intracellular calcium

It is interesting to note that both baicalin and baicalein attenuated Glu/NMDA-increased intracellular calcium in cultured brain neurons. This result coincides well with other reports on the baicalein-decreased intracellular calcium elevation by other stimulants in the C6 glioma cell line and SH-SY5Y neuroblastoma cells [8,18]. The minimal concentration of baicalin and baicalein effective in lowering the Glu/NMDA-increased calcium is 3.5 μM and 0.35 μM, respectively, coinciding with other findings that baicalein is more potent than baicalin in reducing stimulant-induced intracellular calcium concentration [18]. Baicalin at 3.5 μM is also the concentration required for protecting Glu/NMDA-stimulated neurons, but the effective concentration of baicalein in protecting neurons is far less than its minimal concentration in reducing intracellular calcium. This information suggests that the neuroprotective effect of baicalin might be closely associated with its calcium-reducing effect, whereas baicalein might have other components, such as antioxidant activity, playing a more important role in mediating its neuroprotective activity. In addition, baicalin and baicalein had no effect on glutamate receptor binding, implicating that these two compounds might reduce Glu/NMDA-increased intracellular calcium downstream of the glutamate receptor activation, such as calcium channels or phospholipase C signaling cascade [16,18].

4.3. Possible mechanisms of baicalin- and baicalein-mediated neuroprotection—reduction of Glu/NMDA-increased but not GD-increased nitric oxide production

In our study, both Glu/NMDA and GD profoundly increased NO production, which may in turn form peroxynitrite with hydroxyl free radical and superoxide anions to cause neuronal death [19]. Baicalein at 350 nM decreased Glu/NMDA-increased, but not GD-increased NO production, implicating that it might inhibit certain isoforms of nitric oxide synthase (NOS) activity. It has been reported that glutamate receptor-mediated NO production is mediated by neuronal NOS (nNOS) in a calcium-dependent manner, whereas GD-increased NO production is mainly mediated by increasing activity of inducible NOS (iNOS) in a calcium-independent manner [6,36]. Baicalein at 350

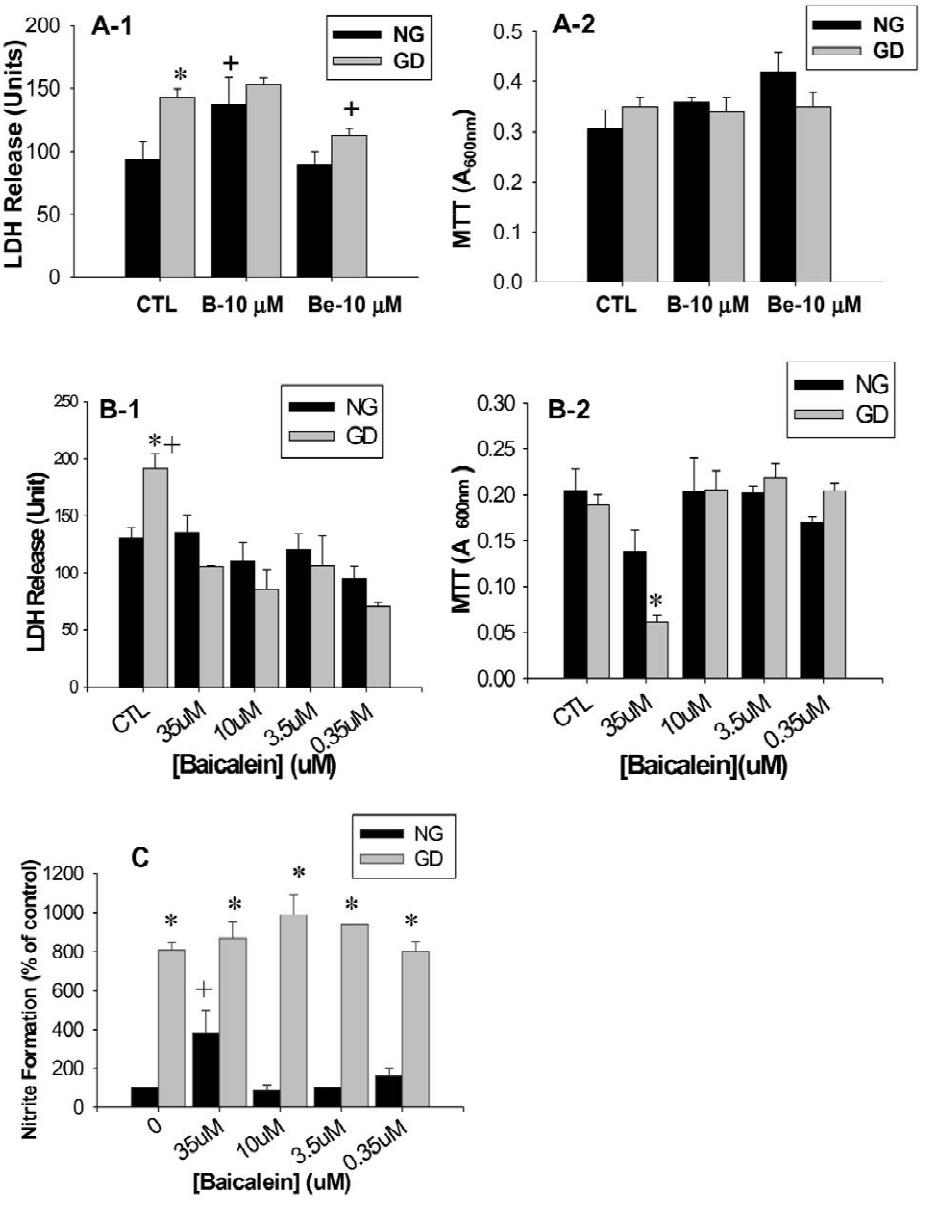

Fig. 6. Effects of baicalin and baicalein on neuronal survival and NO production under glucose deprivation. (A) Cultured neurons at 9 DIV were treated with 0 mM (GD, gray bars) or normal level of glucose concentration (NG, 33 mM) in EBSS solution for 1 h glucose deprivation in the presence or absence of baicalin (B) and baicalein (Be) at 10 μM for 24 h. At the end of treatment, cultures were subjected to LDH release assay (A-1) and MTT assay (A-2). (B) Baicalein at 0.35, 3.5, 10, and 35 μM were applied to cultured neurons under NG or 1 h GD condition for 16 h. At the end of treatment, cultures were subjected to LDH release assay (B-1) and MTT assay (B-2). (C) Cultured brain neurons at 9 DIV were treated with baicalein at 0.35, 3.5, 10, and 35 μM under NG or 1 h GD condition for 16 h. At the end of treatment, culture medium was harvested and subjected to the nitrite formation assay as described in Section 2. The nitrite formation value obtained from the control group (without baicalein treatment) served as 100%, and data obtained in other groups were calculated as percent of control accordingly. Data are expressed as the mean±S.E.M ($n=4$). *$P<0.05$ compared with the normal glucose control (CTL in black), and $^{+}P<0.05$ compared with the 0 mM glucose control (CTL in gray) by unpaired t-test. Data are expressed as the mean±S.E.M ($n=4$). *$P<0.05$ as compared with the respective NG group (unpaired t-test). $^{+}P<0.05$ as compared with other groups under the GD condition (A-1 and B-1) or under the NG condition (A-1 and C) by one-way ANOVA with Newman–Keuls multiple comparison post-test.

nM also significantly reduced Glu/NMDA-elevated intracellular calcium, which might in turn reduce nNOS activity and result in reduction of NO production. Combined with the finding that baicalein did not affect GD-increased NO production, these results suggest that baicalein might selectively reduce nNOS activity in central neurons.

4.4. Possible mechanisms of baicalin- and baicalein-mediated neuroprotection—inhibition of oxidative stress

Gao et al. [7] showed that baicalin and baicalein, but not wogonin, can scavenge hydroxyl radical, DPPH radical, and alkyl radical, which might be one of the important factors that contribute to their neuroprotective activities

since they may neutralize free radicals generated by Glu/NMDA stimulation. The NMDA receptor-mediated excitotoxicity was found to increase extracellular glutamate and subsequently induced high glutamate-induced oxidative glutamate toxicity [32]. It is possible that baicalin and baicalein might protect neurons against excitotoxicity by neutralizing oxygen free radicals produced in the downstream oxidative stress. Another possible mechanism of baicalein-mediated reduction of oxidative stress is its inhibition of 12-lipoxygenase activity, which is related to oxygen free radical formation [1,29]. However, baicalin had only a negligible effect on 12-lipoxygenase activity. Although some reports found that cultured brain neurons had relatively low lipoxygenase level [30], it is possible that baicalein might inhibit a limited amount of lipoxygenase in the cultured neurons to attenuate both the glutamate toxicity and the GD toxicity. Since glutamate excitotoxicity is believed to result from formation of peroxynitrite by reaction of NO with superoxide anion, it is conceivable that baicalein might be a potential agent in attenuating peroxynitrite formation by reducing both NO production and oxygen free radical formation to protect central neurons.

4.5. Possible adverse effect of polyphenolic compounds

Although wogonin was shown to be effective in inhibiting both NADPH-induced lipid peroxidation and lipopolysaccharide-induced nitric oxide production in other tissues [7,15], lack of a protective effect of wogonin in this study implies that these two effects might not be the major criteria for neuroprotection. To our surprise, wogonin at concentrations as low as 35 nM caused an immediate LDH release in the cultured neurons, implying that a possible adverse effect of polyphenolic compounds should be carefully examined in a future study. Cytotoxic effects of baicalin, baicalein, and wogonin were all reported previously in cancer cell lines [2,38], but only wogonin is neurotoxic as found in our study, making baicalin and baicalein better candidates in both cancer therapy and neuroprotection.

In summary, baicalin and baicalein have differential effects on protecting central neurons against naturally-occurring neuronal death, glutamate toxicity, and GD-induced neuronal death. The mechanism of baicalin- and baicalein-mediated neuroprotection might be different, because baicalein but not baicalin is effective in protecting neurons against both glutamate excitotoxicity and glucose deprivation. The neuroprotective activities of baicalin and baicalein might be mediated by reduction of glutamate-increased intracellular calcium and, for baicalein only, with additional activity in reduction of Glu/NMDA-increased NO production. These two polyphenols are also relatively safe for neurons up to 10 μM, which makes them potential candidates in therapeutic applications in ischemic stroke and preventive therapy for neurodegenerative diseases. Neurotoxicity of wogonin observed in this study further reveals an important issue regarding possible adverse effects of certain polyphenols in the central nervous system, and its pharmacotoxicological mechanism in the brain remains to be elucidated.

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