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Neuroprotective activity of honokiol and magnolol in cerebellar granule cell damage

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Abstract

The aim of the present study was to investigate the neuroprotective effects of honokiol and magnolol, two major bioactive constituents of the bark of *Magnolia officinalis*, against neuron toxicity induced by glucose deprivation, excitatory amino acids and hydrogen peroxide (H_2O_2) in cultured rat cerebellar granule cells. Cell membrane damage was measured with a lactate dehydrogenase (LDH) release assay and 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to assess mitochondrial activity, reflecting cell survival. Results showed that honokiol and magnolol alone did not affect mitochondrial function and cell damage, but significantly reversed glucose deprivation-induced mitochondrial dysfunction and cell damage. The glutamate receptor blocker MK-801 and antioxidant vitamin E also provided protection against this damage. Furthermore, honokiol was more potent than magnolol in protecting against glutamate-, *N*-methyl-D-aspartate (NMDA)- and H_2O_2-induced mitochondrial dysfunction. These results demonstrated that the neuroprotective effects of honokiol and magnolol may be related to their anti-oxidative actions and antagonism of excitotoxicity induced by excitatory amino acids, suggesting that both compounds may be potential therapeutic agents for neurodegenerative diseases.

Keywords: Honokiol; Magnolol; Oxidative stress; Excitotoxicity; Cerebellar granule cell

1. Introduction

The bark of the root and stem of various *Magnolia* species has been used in traditional herbal medicine to treat a variety of disorders including anxiety and nervous disturbances (Maruyama et al., 1998; Watanabe et al., 1983). Honokiol and magnolol, isomers of hydroxylated biphenolic compounds ($C_{18}H_{18}O_2$, MW=266.33), are the major bioactive constituents of the bark of *Magnolia officinalis* (Fujita et al., 1973; Li, 1985). Previous studies have indicated that honokiol possesses a neuroprotective effect against focal cerebral ischemia–reperfusion injury (Liou et al., 2003a,b,c). Magnolol was also shown to protect cortical neuronal cells from chemical hypoxia and to attenuate heat stroke-induced neuronal damage (Chang et al., 2003; Lee et al., 1998). Recently, we also demonstrated that honokiol and magnolol increased the seizure threshold and inhibited excitatory amino acid (EAA)-induced cation influx (Lin et al., 2005).

Based on evidence from experimental models in vitro and in vivo, oxidative stress, excitotoxicity induced by excitatory amino acids and ionic imbalance are considered to play causal roles in neuronal death induced by several environmental insults, such as ischemia, stroke and other neurodegenerative diseases (Coyle and Puttfarcken, 1993; Mahura, 2003). There is extensive interaction and overlap between multiple mediators of cell injury and cell death under pathological conditions. Excessive glutamate release can stimulate glutamate receptors, resulting in an intracellular cation imbalance followed by reactive oxygen species (ROS) generation, inflammation and cell death (Bettler and Mulle, 1995). Blockade of glutamate receptors is reported to prevent glutamate-induced reactive

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oxygen species and inflammatory responses (Gunasekar et al., 1995). Since glucose insufficiency increases glutamate toxicity and neuronal death, glucose deprivation in an in vitro model partly mimics the effects of stroke and neuronal degeneration (Ioudina et al., 2004). It is presumed that glucose deprivation can induce ATP depletion, extracellular accumulation of excitatory amino acids, loss of neuronal homeostasis, and neuronal degeneration and death (Cavaliere et al., 2001; Delgado-Esteban et al., 2000; Wieloch, 1985). Since honokiol and magnolol possess potent antioxidant activity against and inhibitory effects on excitatory amino acid-induced Ca^{2+} influx, we examined the neuroprotective effects of honokiol and magnolol against glucose deprivation-, H_2O_2- and excitotoxin-induced neurotoxicity in cultured cerebellar granule neurons, using MTT assay and LDH assay. Additionally, the neuroprotective effects were compared with those of glutamate receptor blocker MK-801 and antioxidant vitamin E. We thus investigated the neuroprotective activities of honokiol and magnolol against neuronal damage directly induced by oxidative stress and excitatory amino acids.

2. Methods and materials

2.1. Preparation of cerebellar granule cells

Cerebellar granule cells were prepared from 8-day-old Sprague–Dawley rat pups from the Laboratory Animal Center of Tzu Chi University, Hualien, Taiwan. The experimental protocol was approved by the Committee of Tzu Chi University for the Use of Animal Subjects. Briefly, cerebella were dissociated in Ca^{2+}- and Mg^{2+}-free Hank solution with trypsin and deoxyribonuclease I (DNase I) and seeded at a density of about 3×10^5 cells/well into poly-L-lysine-coated 96-well plates. The tissue culture plates contained basic Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and supplemented with 25 mM glucose, 2 mM glutamine, 25 mM KCl and penicillin (10 U/ml)–streptomycin (10 μg/ml). Cells were then maintained at 37 °C in 5% CO_2–95% air. Cytosine arabinoside (10 μM) was added 24 h after cell plating to inhibit glial proliferation. Then, after 7 days of culture, cells were used in the following experiments.

2.2. Neuronal survival analysis

2.2.1. Mitochondrial activity (MTT) assay

The 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay measures mitochondrial activity, which quantitatively reflects cell survival. The assay is based on the conversion, via dehydrogenase enzymes, of tetrazolium salt to the colored product formazan, the color of which can be measured by microplate reader (OPTLmax, Molecular Devices). After neurotoxin treatment, the culture medium was removed and cells were then incubated with 100 μl MTT solution (0.5 mg/ml) for 4 h in a humidified 5% CO_2 incubator at 37 °C. The incubation was then stopped by removing the culture medium and adding 100 μl dimethylsulfoxide (DMSO) to solubilize formazan. Then, absorbance was measured at 570 nm. Most data are expressed as percent control (i.e., the percentage of control values obtained with the basic medium without supplementation).

2.2.2. LDH release assay

Lactate dehydrogenase (LDH) levels in the extracellular medium were measured by using a LDH assay kit from Sigma (TOX-7). LDH is present intracellularly in all cell types, but its presence in the extracellular medium indicates a loss of cell membrane integrity, reflecting cellular damage or cell death. After neurotoxin application, the culture medium was removed and then sampled for LDH by measuring the absorbance at 490 nm, using a microplate reader (OPTLmax, Molecular Devices). Data are expressed as optical density (OD) or percent control (i.e., the percentage of control values obtained with the basic medium without supplementation).

2.3. Materials

Honokiol and magnolol, purchased from Nacalai Tesque (Kyoto, Japan), were dissolved in DMSO; the final DMSO concentration in basic DMEM medium was less than 1%, which had no effect on cell growth or caused damage to cultured cells. Glycine and potassium chloride (KCl) were purchased from J.T. Baker (Mallinckrodt Baker, Inc, Kentucky, USA). Fetal bovine serum was purchased from GIBCO (Paisley PA4 9RF, Scotland). Other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.4. Solutions

Glucose-free balanced salt contained (mM): 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 26.2 $NaHCO_3$, 1 NaH_2PO4, 1.8 $CaCl_2$. Phosphate-buffered saline was composed of (mM): 140 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 8.1 Na_2HPO_4.

2.5. Data analysis

Most data are expressed as means ± S.E.M. Statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls post-hoc test. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of honokiol and magnolol on neuronal survival

In the following experiments, neuronal survival was determined by measuring mitochondrial activity with the MTT assay and with an LDH (as a marker of cell membrane damage) assay. Tables 1 and 2 show that neuronal mitochondrial activity and LDH release into extracellular medium in these groups did not differ significantly from each other. Results indicated that MK-801, vitamin E, honokiol and magnolol at the concentrations used did not influence

mitochondrial activity or cell membrane integrity following 3 and 24 h treatment of cultured cerebellar granule cells.

3.2. Effects of honokiol and magnolol on neuronal survival following glucose deprivation (GD)

Granule cells were initially cultured with basic medium free of glucose for 3 h, and then glucose deprivation was ended by changing the medium to basic DMEM media with 25 mM glucose for 24 h prior to MTT and LDH release assays. In cells cultured under conditions of glucose deprivation for 3 h, mitochondrial activity was lower and LDH release was higher than in the controls, in which the mitochondrial activity was decreased to ~72% and LDH release was increased to ~234%, respectively (Fig. 1).

Under glucose deprivation preconditioning, granule cells were simultaneously treated with MK-801 (10 μM), vitamin E (10 μM), honokiol (0.1–10 μM) or magnolol (0.1–10 μM) for 3 h, followed by 24 h culture in basic medium without any agents prior to MTT and LDH release assays. Results demonstrated that co-treatment with honokiol at 1 and 10 μM concentration-dependently reversed the glucose deprivation-induced decrease in mitochondrial activity (82.1±4.4% and 90.8±4.1% compared to control, respectively) (Fig 1A) and reduced the glucose deprivation-elicited increase in LDH release (131.2±16.5% and 108.0±6.7% compared to control, respectively) (Fig. 1B). Magnolol at 10 μM also significantly reversed the glucose deprivation-induced decrease in mitochondrial activity and the increase in LDH release. MK-801 at 10 μM significantly reversed the decrease in mitochondrial activity and the increase in LDH release caused by glucose deprivation. However, co-treatment with vitamin E (10 μM) during glucose deprivation preconditioning did not affect neuronal mitochondrial activity and LDH release (Fig. 1A and B).

In another experimental design, granule cells were cultured for 3 h with glucose deprivation preconditioning followed by the addition of MK-801, vitamin E, honokiol or magnolol to the basic culture medium for 24 h. The neuronal protective effects of honokiol and magnolol against glucose deprivation-induced granule cell toxicity were also observed at 1 μM and 10 μM.

Table 1
Effect of honokiol and magnolol treatment for 3 h on mitochondrial activity and LDH release in granule neurons

	Mitochondrial activity (OD) (n=5–10)	LDH release (OD) (n=6)
Vehicle	0.73±0.06	0.08±0.02
MK-801 10 μM	0.74±0.07	0.07±0.02
Vitamin E 10 μM	0.79±0.08	0.08±0.02
Honokiol 0.1 μM	0.65±0.05	0.08±0.02
Honokiol 1 μM	0.68±0.04	0.08±0.02
Honokiol 10 μM	0.66±0.04	0.08±0.02
Magnolol 0.1 μM	0.63±0.05	0.07±0.02
Magnolol 1 μM	0.64±0.05	0.08±0.02
Magnolol 10 μM	0.67±0.05	0.08±0.02

The granule cells in culture were exposed to MK-801 (10 μM), vitamin E (10 μM), honokiol (0.1–10 μM) and magnolol (0.1–10 μM) for 3 h, followed by a 24-h incubation with basic culture medium without any supplementation. Results are expressed as means±S.E.M.

Table 2
Effect of honokiol and magnolol treatment for 24 h on mitochondrial activity and LDH release in granule neurons

	Mitochondrial activity (OD) (n=13–42)	LDH release (OD) (n=6)
Vehicle	0.81±0.04	0.08±0.01
MK-801 10 μM	0.71±0.07	0.08±0.02
Vitamin E 10 μM	0.76±0.08	0.09±0.02
Honokiol 0.1 μM	0.75±0.05	0.09±0.02
Honokiol 1 μM	0.74±0.05	0.09±0.02
Honokiol 10 μM	0.76±0.05	0.09±0.02
Magnolol 0.1 μM	0.78±0.07	0.08±0.02
Magnolol 1 μM	0.81±0.08	0.09±0.02
Magnolol 10 μM	0.78±0.07	0.09±0.02

Cerebellar granule cells in culture were exposed to MK-801 (10 μM), vitamin E (10 μM), honokiol (0.1–10 μM) and magnolol (0.1–10 μM) for 24 h. Results are expressed as means±S.E.M.

Post-treatment with vitamin E (10 μM) significantly protected against the granule cell toxicity induced by glucose deprivation preconditioning, whereas MK-801 (10 μM) had no effect (Fig. 1C and D).

3.3. Effects of honokiol and magnolol on glutamate- and NMDA-induced excitotoxic injury in granule cells

When granule neurons were exposed to 300 μM glutamate or *N*-methyl-D-aspartate (NMDA) for 24 h, mitochondrial activity measured in the MTT assay was decreased to 67.8±3.2% or 74.6±1.9% compared to controls, respectively (Fig. 2). Co-treatment with the NMDA antagonist, MK-801 (10 μM), significantly inhibited the decrease in mitochondrial activity caused by 300 μM glutamate and NMDA. Consistent with the effects of MK-801, honokiol at 0.1, 1 and 10 μM concentration-dependently reversed the decrease in mitochondrial activity induced by glutamate and NMDA. Magnolol at 1 and 10 μM also concentration-dependently reversed the decrease in mitochondrial activity induced by glutamate (Fig. 2A), whereas magnolol reversed the decrease in mitochondrial activity induced by NMDA only at 10 μM (Fig. 2B).

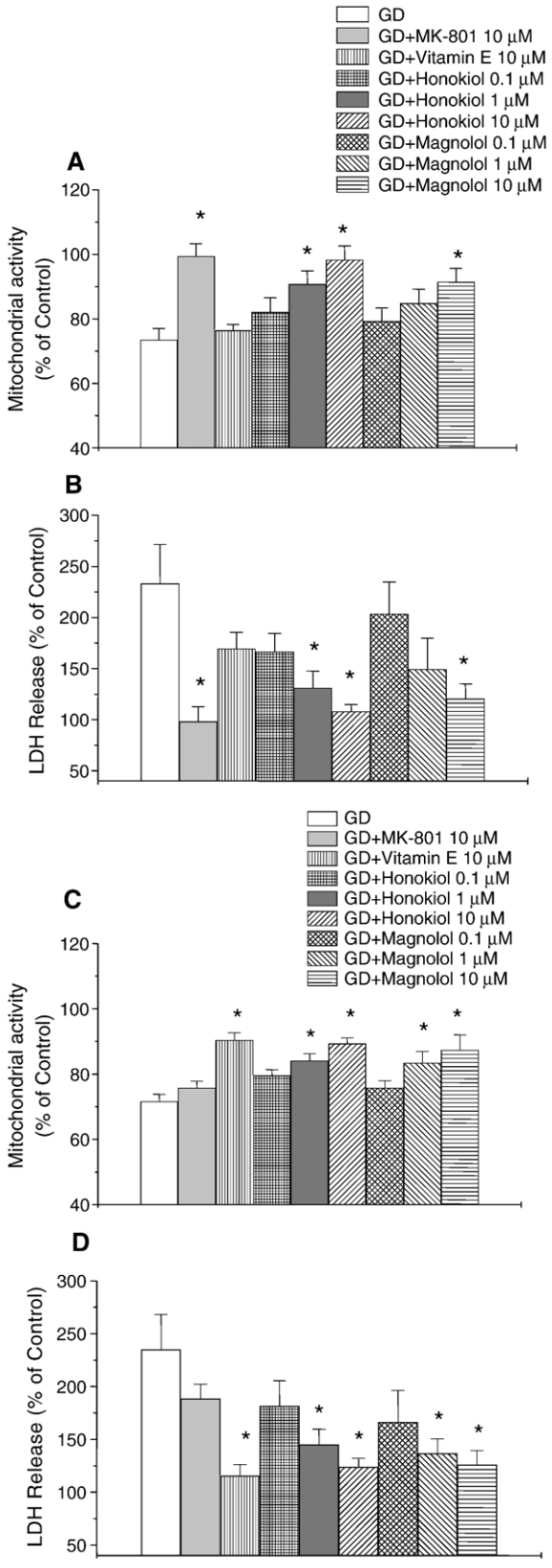
3.4. Effects of honokiol and magnolol on H_2O_2-induced neurotoxicity in granule cells

It was observed that mitochondrial activity was decreased to 73.8±2.7% after granule neurons were exposed to 600 μM H_2O_2 for 24 h. Co-treatment with vitamin E, but not MK-801, reversed the H_2O_2-induced mitochondrial dysfunction. Interestingly, honokiol at 1 and 10 μM significantly reversed the neurotoxicity induced by H_2O_2 (88.7±3.9% and 93.8±4.6% compared to control, respectively). Magnolol at 10 μM also significantly inhibited the decrease in mitochondrial activity caused by H_2O_2 (Fig. 3).

4. Discussion

In this study, we investigated the neuroprotective actions of honokiol and magnolol against glucose deprivation-, excitatory

amino acid- and H_2O_2-induced neuronal toxicity in cerebellar granule cells. Results showed that exposure to honokiol or magnolol alone for 3 or 24 h neither influenced mitochondrial functioning (MTT reduction) nor evoked cell membrane damage (LDH release). Co-treatment and post-treatment with



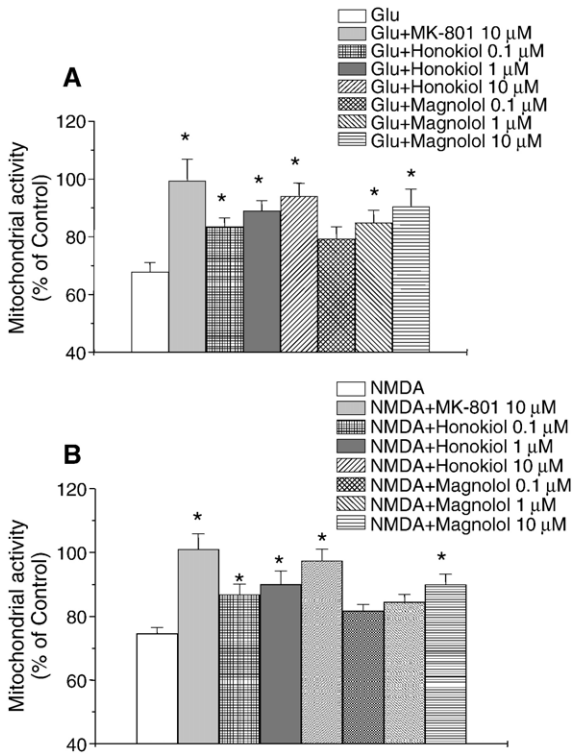


Fig. 2. Protective effect of honokiol and magnolol in granule cells against neuronal damage induced by glutamate and NMDA. Neuronal survival was evaluated by MTT assay. Granule cells in culture were treated with 300 μM glutamate (A) or 300 μM NMDA (B) in the absence or presence of MK-801 (10 μM) and various concentrations of honokiol and magnolol for 24 h. The absorbance of non-treated cells (control) was regarded as 100%. Results are expressed as means ± S.E.M. (n = 8–11). * indicates significant difference compared with glutamate- or NMDA-treated group, *P < 0.05.

honokiol and magnolol significantly and concentration-dependently reversed glucose deprivation-induced neuronal damage. Furthermore, honokiol and magnolol also protected against glutamate-, NMDA- and H_2O_2-induced granule cell toxicity. However, co-treatment with MK-801 reduced glucose deprivation-, glutamate- and NMDA-induced neuronal dysfunction and damage, whereas post-treatment and co-treatment with vitamin E decreased glucose deprivation- and H_2O_2-induced neuronal injury. Therefore, the neuroprotective effects of honokiol and magnolol were more comprehensive than those of MK-801 and vitamin E. Taken together; our data demonstrated that the neuroprotective effects of honokiol and magnolol may be attributed to their anti-oxidant activity and antagonism of excitotoxicity induced by excitatory amino acids.

Fig. 1. Protective effects of honokiol and magnolol in granule cells against neuronal toxicity induced by glucose deprivation. Neuronal toxicity was evaluated by MTT assay and LDH release assay. (A, B) Granule cells initially were cultured with glucose-free medium and treated with MK-801 (10 μM), vitamin E (10 μM) and various concentrations of honokiol and magnolol for 3 h, followed by 24 h culture in basic medium without any drug treatments. (C, D) After 3 h of glucose deprivation (GD), granule cells were cultured in basic medium with MK-801 (10 μM), vitamin E (10 μM) and different concentrations of honokiol and magnolol for 24 h. The absorbance of non-treated cells (control) was regarded as 100%. Results are expressed as means ± S.E.M. (n = 5–11). * indicates significant difference compared with glucose deprivation group, *P < 0.05.

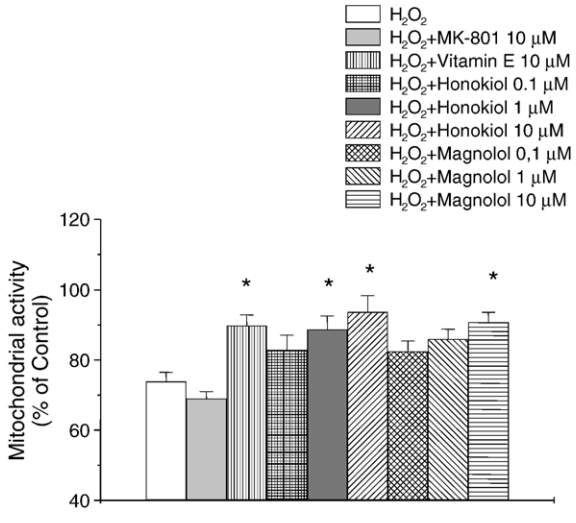


Fig. 3. Protective effect of honokiol and magnolol against H_2O_2-induced toxicity in granule neurons. Granule cells in culture were treated with 600 μM H_2O_2 with or without MK-801 (10 μM), vitamin E (10 μM) and various concentrations of honokiol and magnolol for 24 h. The absorbance of non-treated cells (control) was regarded as 100%. Results are expressed as means±S.E.M. ($n=8$). * indicates significant difference compared with H_2O_2-treated group, $*P<0.05$.

Glucose deprivation is generally presumed to lead to depletion of cellular energy, induction of oxidative stress (Auer et al., 1984; Liu et al., 2003; Monyer et al., 1989), extracellular accumulation of glutamate (Hashimoto et al., 2003), loss of ion homeostasis (notable intracellular calcium increase) (Silver et al., 1997) and ultimately neuronal death. Our experiments consistently showed a decrease in neuronal mitochondrial activity associated with increased cell damage under conditions of glucose deprivation. We also discovered that co-treatment with MK-801, the NMDA receptor blocker, but not vitamin E, an antioxidant, reversed the changes in mitochondrial activity and cell damage induced by glucose deprivation preconditioning. In contrast, after glucose deprivation preconditioning vitamin E, but not MK-801, protected against the decline during the period of glucose reapplication. Previous reports indicated that aglycemia-induced glutamate release occurs during early glucose deprivation (Hashimoto et al., 2003), whereas oxidative stress occurs during oxygen/glucose-reapplication after oxygen/glucose deprivation (Jiang et al., 2004). These observations could explain the distinct effects of MK-801 and vitamin E in the presence of glucose deprivation. Both honokiol and magnolol have the ability to protect against neuronal injury induced by glucose deprivation under both treatment conditions. Magnolol has been reported to reduce glutamate release and free radical formation during heat stroke (Chang et al., 2003). Thus, we suggest that the neuronal protection provided by honokiol and magnolol against glucose deprivation-induced injury is at least in part associated with the inhibition of glutamate release and oxidative stress.

Similar to those of MK-801, the neuroprotective effects of honokiol and magnolol were also exhibited against glutamate- and NMDA-induced excitotoxic injury. These findings are consistent with an earlier observation that honokiol and magnolol reduce glutamate-induced Ca^{2+} influx in cerebellar granule cells (Lin et al., 2005). Together, it is suggested that honokiol and magnolol can also protect against neuron damage via blockade of the glutamate receptors. Furthermore, we found that H_2O_2-induced toxicity in cultured granule cells was reversed by vitamin E, honokiol and magnolol. These findings further support that the neuronal protection provided by honokiol and magnolol is associated with their antioxidant activity, since both compounds inhibit mitochondrial lipid peroxidation and oxidative stress-induced hepatotoxicity (Haraguchi et al., 1997; Park et al., 2003). It is also important to note that the neuroprotective potency of honokiol was higher than that of magnolol in the present study. This is also consistent to our previous finding that honokiol is a more selective blocker of NMDA receptors than magnolol (Lin et al., 2005). Furthermore, the antioxidative activity of honokiol is more potent than that of magnolol (Haraguchi et al., 1997).

It is known that inflammation, oxidative stress, excitotoxicity and ionic imbalance could play a causal role in the pathological neuronal death induced by several environmental insults, including hypoxia–ischemia. Recent evidence indicates that honokiol possesses neuroprotective effects against focal cerebral ischemia–reperfusion injury in animal models (Liou et al., 2003a,b,c), and improves the survival and growth of cortical neurons in serum-free medium (Fukuyama et al., 2002). In addition, magnolol has been reported to protect cortical neuronal cells from chemical hypoxia and to attenuate heat stroke-induced neuronal damage in rats (Chang et al., 2003; Lee et al., 1998). The present results demonstrated that, compared with MK-801 and vitamin E, honokiol and magnolol possess more comprehensive protective effects against NMDA-, glutamate- and H_2O_2-induced neuronal toxicity. Our findings support the hypothesis that the neuroprotective activity of honokiol and magnolol is due to their ability to block glutamate- and ROS-activated neurotoxicity. Therefore, the therapeutic application of honokiol and magnolol to protect against neurodegenerative disorders associated with excitotoxicity and oxidative stress should be considered.

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