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Meso-dihydroguaiaretic acid and licarin A of *Machilus thunbergii* protect against glutamate-induced toxicity in primary cultures of a rat cortical cells

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1 We previously reported that four lignans isolated from the bark of *Machilus thunbergii* Sieb. et Zucc. (Lauraceae) protected primary cultures of rat cortical neurons from neurotoxicity induced by glutamate.

2 Among the lignans, *meso*-dihydroguaiarectic acid (MDGA) and licarin A significantly attenuated glutamate-induced neurotoxicity when added prior to or right after the excitotoxic glutamate challenge.

3 The neuroprotective activities of two lignans appeared to be more effective in protecting neurons against neurotoxicity induced by NMDA than that induced by kainic acid.

4 MDGA and licarin A diminished the calcium influx that routinely accompanies with the glutamate-induced neurotoxicity, and inhibited the subsequent overproduction of cellular nitric oxide and peroxide to the level of control cells. They also preserved cellular activities of antioxidative enzymes such as superoxide dismutase, glutathione peroxidase and glutathione reductase reduced in the glutamate-injured neuronal cells.

5 Thus, our results suggest that MDGA and licarin A significantly protect primary cultured neuronal cells against glutamate-induced oxidative stress, *via* antioxidative activities.
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Abbreviations: AMPA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionic acid; APV, DL-2-amino-5-phosphonovaleric acid; BSO, buthionine sulfoximine; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DEM, diethylmaleate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GSH, reduced glutathione; GSH-px, glutathione peroxidase; GSSG-R, glutathione reductase; KA, kainic acid; LDH, lactate dehydrogenase; MDGA, *meso*-dihydroguaiaretic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartic acid; NO, nitric oxide; SOD, superoxide dismutase

Introduction

Glutamate, an excitatory amino acid, activates different types of ion channel-forming receptors and G-protein-coupled receptors and plays its essential roles – neuronal survival, synaptogenesis, neuronal plasticity, memory, learning and behavior – in the central nervous system (CNS) (Sucher *et al*., 1996; Michaelis, 1998). However, high concentration of glutamate cause neuronal cell death within the CNS, and may be involved in neuropsychiatric and neuropathological disorders such as Parkinson's disease, Alzheimer's disease, epilepsy, seizures, ischemic stroke and spinal cord trauma (Lipton & Rosenberg, 1994; Cacabelos *et al*., 1996; Lee *et al*., 1999). Thus, neuroprotection against glutamate-induced neurotoxicity has been a therapeutic strategy to treat neurodegenerative diseases (Rajendra *et al*., 2004).

We previously employed primary cultures of rat cortical cells injured by glutamate as an *in vitro* assay system to isolate neuroprotective compounds from natural products, which protect neurons against glutamate-induced neurotoxicity (Kim *et al*., 1998). In our previous report, we found that lignans isolated from the bark of *Machilus thunbergii* had significant neuroprotective activities in our *in vitro* screening system (Ma *et al*., 2004).

In the present study, we have investigated the structure–activity relationship of these isolated compounds (**1–6** shown in Figure 1). Furthermore, we attempted to elucidate the neuroprotective mechanisms of MDGA and licarin A, which showed the most potent protection against glutamate-induced toxicity equally among the isolated compounds, although their structures are quite different.

Methods

Materials

All chemicals for rat cortical cell cultures and biochemical assays were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, U.S.A.), unless stated otherwise. Fetal bovine serum was obtained from Hyclone Co. (Logan, UT, U.S.A.). DL-2-Amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and MK-801 (dizocilpine

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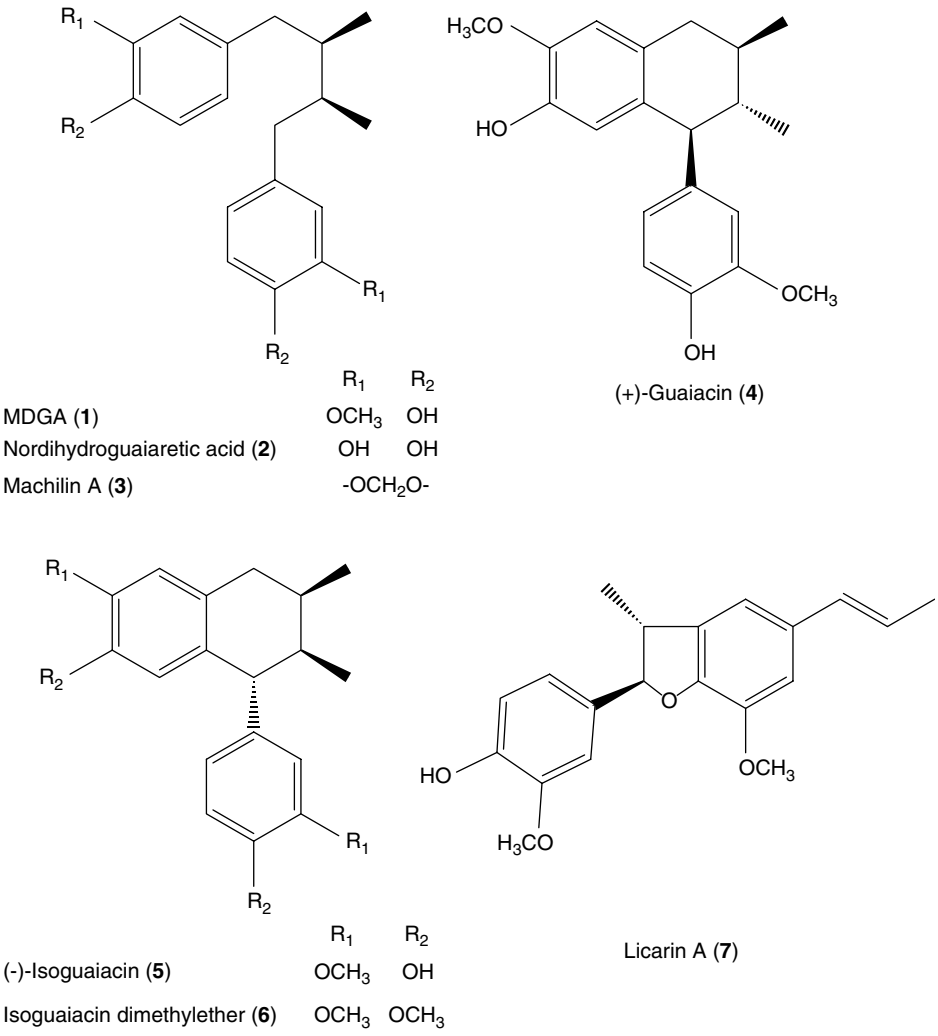

	R_1	R_2
MDGA (**1**)	OCH_3	OH
Nordihydroguaiaretic acid (**2**)	OH	OH
Machilin A (**3**)	$-OCH_2O-$	

	R_1	R_2
(-)-Isoguaiacin (**5**)	OCH_3	OH
Isoguaiacin dimethylether (**6**)	OCH_3	OCH_3

Figure 1 Structures of lignans isolated from *M. thunbergii*.

maleate) used as positive controls were purchased from Research Biochemicals International (Natick, MA, U.S.A.). Urethane and Triton X-100 were purchased from Junsei Chemical Co. (Tokyo, Japan) and Yakuri Chemical Co. (Osaka, Japan), respectively. All lignans were isolated from the bark of *M. thunbergii* and their purity were higher than 95.0%, respectively (Ma *et al*., 2004).

Cell culture

Primary cultures of mixed cortical cells containing both neuronal and glial cells were prepared from 17- to 19-day-old fetal Sprague–Dawley rats as described previously (Kim *et al*., 1998). In brief, the trypsin-dissociated cortical cells were plated on multiwell culture plates (Corning, NY, U.S.A.) coated with collagen at a density of 1×10^6 cells well^{-1} and poly-L-lysine at a density of 2×10^5 cells well^{-1}, respectively. The cortical cells were grown in DMEM containing 10% heat-inactivated fetal bovine serum with penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37°C in a humidified atmosphere of 95% air–5% CO_2. Cultures were allowed to mature for 17 days before being used for experiments. Our mixed cortical cultures consisted of approximately 70–75% cells immunopositive for neuron-specific enolase and 25–30% cells immunopositive for glial fibrillary acidic protein as determined by immunocytochemical staining methods (Kim *et al*., 2004). All experiments were performed with Ethical Approval of Seoul National University.

Neurotoxicity and cell viability

Test compounds were dissolved in DMSO (final culture concentration, 0.1%); preliminary studies indicated that the solvent had no effect on cell viability of control and glutamate-treated cells at the concentration used (Kim *et al*., 1998). Cortical cell cultures (17 days old) were washed with DMEM and incubated with compounds for 1 h. The cultured cells were then exposed to 100 μM L-glutamate for 30 min. After 24 h incubation in the presence of test compounds, the cultures were assessed for the extent of neuronal damage by measuring lactate dehydrogenase (LDH) in the media. In some experiments, the cultures were treated with the appropriate lignans, MDGA and licarin A, either 1 h before exposure or after exposure to 100 μM L-glutamate for 1 h. After an additional 24 h incubation in the absence (pretreatment) or presence (post-treatment) of lignans, the cultures were assessed by measuring LDH in the media. In some experiments, cultures were pretreated with lignans for 1 h before exposure to 50 μM

NMDA in HEPES-buffered salt solution containing 15 mM glucose and 10 μM glycine (pH 7.4) for 30 min or to 50 μM KA and 10 μM MK-801 (used to prevent NMDA receptor activation after the release of endogenous glutamate) for 3 h. After exposure to NMDA or KA, the cultures were then washed and further maintained in DMEM for 24 h in the absence of lignans. Neuronal viability was measured by the LDH assay, which reflects cellular integrity (Koh & Choi, 1987). Data are expressed as the percentage protection relative to vehicle-treated control cultures. Values shown are the mean $\pm$ s.d. of three experiments (three to four cultures per experiment).

Measurement of intracellular calcium and nitric oxide (NO) contents

The intracellular calcium was determined by ratio fluorometry using Ca^{2+}-specific dye, Fura 2-AM (Kim *et al*., 1998). In brief, 1 h before exposure to 100 μM glutamate, cultures grown on 48-well plates were treated with lignans and 5 μM Fura-2 AM in phosphate-buffered saline (pH 7.2) at 37°C in a humidified atmosphere of 95% air–5% CO_2. The change of $[Ca^{2+}]_i$ was measured 10 min after exposure to glutamate. Fura-2 fluorescence was measured with a spectroflurometer by exciting cells at 340 and 380 nm and measuring light emission at 520 nm (Grynkiewicz *et al*., 1985). The level of NO formed was determined by measuring the content of nitrite released into the medium using the method of Dawson *et al*. (1994). The culture medium was reacted with Griess reagent and the absorbance was then read at 550 nm. The concentration was determined against a nitrite standard curve. Values shown are the mean $\pm$ s.d. of three experiments (three to four cultures per experiment).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

A measure of 100 μl of a 300 μM solution of DPPH in ethanol was added to 100 μl of a solution containing MDGA and licarin A in DMSO. The reaction mixtures were mixed and transferred into 96-well plates. After incubation for 30 min, the absorbance was determined using a microplate reader at 515 nm. The EC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, was calculated from the results (Kim *et al*., 2003). Values shown are the mean $\pm$ s.d. of three experiments (three to four cultures per experiment).

Measurement of cellular peroxide

The relative level of free radicals, that is peroxide, in cultured cells was measured with the oxidation-sensitive compound, 2,7-DCF-DA by the method of Goodman & Mattson (1994). Cells were loaded with DCF-DA (50 μM, 50 min incubation) followed by three washes in HBSS. DCF fluorescence was then determined after 3 h incubation by measuring light emitted at 530 nm of exciting cells with light at 485 nm. Values shown are the mean $\pm$ s.d. of three experiments (three to four cultures per experiment).

Assay for the activity of antioxidant enzymes

Cells from three culture plates were pooled in 2 ml of 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 30 min at $3000 \times g$ at 4°C and the supernatant (cytosolic and mitochondrial fractions) collected (Gibson & Skelf, 1988) for the measurements of antioxidative enzyme activity and glutathione (GSH) content. The activity of superoxide dismutase was determined according to the method of McCord & Fridovich (1969) by xanthine–xanthine oxidase reaction. GSSG reductase activity was measured according to the method of Carlberg & Mannervik (1975) based on the reduction of GSSG by GSSG reductase and NADPH. GSH peroxidase activity was determined by quantifying the rate of oxidation of GSH to GSSG by cumene hydroperoxide, a reaction catalyzed by GSH peroxide (Flohe & Gunzler, 1984). Values shown are the mean $\pm$ s.d. of three experiments (three to four cultures per experiment).

Total GSH content measurement

Total GSH in the supernatant was determined spectrophotometrically using the enzymatic cycling method (Tietz, 1969). Values shown are the mean $\pm$ s.d. of three experiments (three to four cultures per experiment).

Protein assay

Protein content was measured by the method of Lowry *et al*. (1951) with bovine serum albumin as a standard.

Statistical analysis

Statistical significance was determined by one-way ANOVA and, if significant, group means were compared by *post hoc* analysis using Tukey multiple comparison of means. Values shown are the mean $\pm$ s.d. of three experiments (three to four cultures per experiment).

Results

We previously reported isolation of lignans from the bark of *M. thunbergii* Sieb. et Zucc. (Lauraceae) and the neuroprotective activities of these compounds *in vitro* (Ma *et al*., 2004). Thus, we have investigated the structure–activity relationship of lignans isolated from *M. thunbergii* using a test system consisting of primary cultures of rat cortical neurons injured with glutamate (Table 1; an MTT assay showed the same trend as the LDH assay; data not shown). In our culture system, MK-801 and CNQX, well-known positive controls against glutamate-induced neurotoxicity, showed effective neuroprotective activities at a concentration of 10 μM (Table 1). The potency of neuroprotective activity throughout the treatment paradigm was in the order: MDGA $\geqslant$ licarin A $\geqslant$ nordihydroguaiaretic acid $>(+)$-guaiacin $=(-)$-isoguaiacin. The neuroprotective potency for compounds other than those described above is not given because their abilities to protect neurons in our assay system were lower than 30%.

We tried to investigate the action mechanisms of MDGA and licarin A, the most potent neuroprotective lignans, using glutamate-injured primary cultures of rat cortical cells (see Table 1). Therefore, the neuroprotective activities of MDGA and licarin A against glutamate-induced neurotoxicity were initially investigated by a timed exposure to two lignans before or after glutamate treatment. As shown in Table 2, both

Table 1 Neuroprotective activities of lignans isolated from *M. thunbergii* on primary cultures of rat cortical cells injured by glutamate

Compounds	EC_{50} ($\times 10^{-6}$ M)	E_{max} (%)[a]
MDGA (**1**)	0.24±0.04	58.5±1.2*** at 1 μM
Nordihydroguaiaretic acid (**2**)	2.10±0.38	43.2±3.4** at 1 μM
Machilin A (**3**)	ND	23.6±2.1* at 10 μM
(+)-Guaiacin (**4**)	38.4±0.76	36.1±1.3** at 1 μM
(−)-Isoguaiacin (**5**)	56.2±0.82	37.9±2.6* at 10 μM
Isoguaiacin dimethylether (**6**)	ND	25.1±2.8* at 1 μM
Licarin A (**7**)	0.32±0.02	54.2±1.9*** at 1 μM
MK-801	0.38±0.01	83.4±5.1*** at 10 μM
APV	32.1±0.51	63.8±3.3*** at 50 μM
CNQX	1.2±0.07	60.8±2.4*** at 10 μM

ND = not determined.
Cortical cell cultures were washed with DMEM and incubated with test compounds for 1 h. The cultures were then exposed to 100 μM glutamate for 1 h. After 24 h incubation, cultures were assessed for the extent of neuronal damage (throughout the experiment). Values shown are the mean±s.d. of three experiments (three to four cultures per experiment).
[a]LDH released from control and glutamate-treated cultures were 117±3 and 287±9 mU ml^{-1}, respectively. Protection (%) was calculated as 100 × (LDH released from glutamate + test compound-treated cultures−LDH released from glutamate-injured cultures)/(LDH released from control cultures−LDH released from glutamate-injured cultures). Cell viabilities of control and glutamate-treated cells were representative as 100 and 0%, respectively. Glutamate-injured cells differ significantly from the control at a level of $P<0.001$. MK-801, dizocilpine maleate, a noncompetitive antagonist of the NMDA receptor; APV, DL-2-amino-5-phosphonovaleric acid, a competitive antagonist of the NMDA receptor; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione, non-NMDA receptor antagonist.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs glutamate-injured cells (ANOVA and Tukey).

Table 2 Neuroprotective activity of MDGA or licarin A on glutamate-induced neurotoxicity in primary cultures of rat cortical cells

	Concentration (μM)	*Protection* (%)	
		Pretreatment[a]	*Post-treatment*[b]
Control[c]		100.0±2.1	
Glutamate injured		0.0±0.5[d]	
MDGA + glutamate	0.1	30.6±3.7*	29.0±11.1
	1.0	46.7±0.7***	46.3±2.7**
	10.0	42.1±4.3**	43.1±3.8**
Licarin A + glutamate	0.1	34.5±2.8*	30.8±2.1*
	1.0	41.2±3.9**	40.5±4.1**
	10.0	38.1±1.8**	36.7±2.7**

[a]Pretreatment: MDGA or licarin A was pretreated for 1 h prior to the glutamate insult.
[b]Post-treatment: MDGA or licarin A was treated after the glutamate insult.
[c]The value of LDH released from control and glutamate-injured cultures was 117±3 and 328±7 mU ml^{-1}, respectively. Protection (%) was calculated as 100 × (LDH released from glutamate + test compound-treated cultures−LDH released from glutamate-injured cultures)/(LDH released from control cultures−LDH released from glutamate-injured cultures). The values shown are the mean±s.d. of three experiments (three to four cultures per experiment).
[d]Glutamate-injured cells differ significantly from the control at a level of $P<0.001$.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs glutamate-injured cells (ANOVA and Tukey).

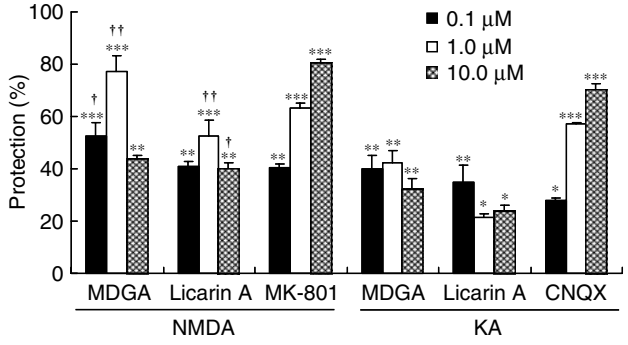

Figure 2 Neuroprotective activity of MDGA or licarin A on NMDA- or KA-injured rat cortical cells. The value of LDH releases from control, NMDA- or KA-injured cultures was 117±3, 210±8 and 230±6 mU ml^{-1}, respectively. Values shown are mean±s.d. of three experiments (three to four cultures per experiment). Protection (%) was calculated as 100 × (LDH released from NMDA/KA + test compound-treated cultures−LDH released from NMDA/KA-injured cultures)/(LDH released from control cultures−LDH released from NMDA/KA-injured cultures). NMDA- or KA-injured cultures differed significantly from control ($P<0.001$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs excitotoxin-injured cells; $^{\dagger}P<0.01$, $^{\dagger\dagger}P<0.001$ vs KA + each lignan (ANOVA and Tukey).

MDGA and licarin A significantly attenuated neurotoxicity induced by glutamate in the pre- or post-treatment paradigm at the concentration ranging from 0.1 to 10 μM, but a strict-concentration dependence was not observed. The neuroprotective activity of MDGA reached a plateau at the concentration ranging from 1 to 5 μM. The activities of MK-801 and CNQX also reached a plateau at the high concentration (100 μM) in our primary culture systems.

In order to reveal how MDGA and licarin A protected against glutamate-induced neurotoxicity, two excitotoxins, NMDA and KA, were used to induce selective receptor-mediated neurotoxicity in primary cultures of rat cortical cells. Both MDGA and licarin A showed neuroprotective activities on cortical cultures regardless of whether NMDA or KA was used as a neurotoxicant. However, they protected cultures slightly more selectively against NMDA-induced neurotoxicity of primary cultured rat cortical cells than from KA-induced neurotoxicity (Figure 2). Furthermore, this was confirmed by the results of MDGA and licarin A on the cellular calcium level in the early stages of glutamate-induced neurotoxicity. The $[Ca^{2+}]_i$ provoked by glutamate treatment was significantly and effectively inhibited by the treatment of MDGA and licarin A, respectively (Figure 3). Then, we estimated whether MDGA and licarin A could effectively reduce NO content in cultured cortical cells treated with glutamate since NO synthase could be overactivated by excess Ca^{2+} in cultured cells. Both MDGA and licarin A significantly reduced overproduction of NO in cortical cells exposed to glutamate (Figure 4).

Glutamate-induced toxicity is known to involve such free radicals as hydroxyl radicals and superoxide anions (Dykens *et al*., 1987; Lafon-Cazal *et al*., 1993). We measured the effects

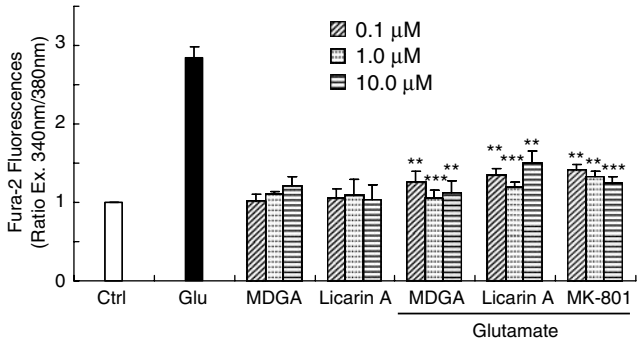

Figure 3 Effect of MDGA or licarin A on intracellular $[Ca^{2+}]$ in glutamate-injured rat cortical cells. Cultures were treated with lignans and 5 μM Fura-2 AM in phosphate-buffered saline (pH 7.2) 1 h before exposure to 100 μM glutamate. The change of $[Ca^{2+}]_i$ was measured 10 min after the exposure to glutamate. The values shown are mean$\pm$s.d. of three experiments (three to four cultures per experiment). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs glutamate-injured cells (ANOVA and Tukey).

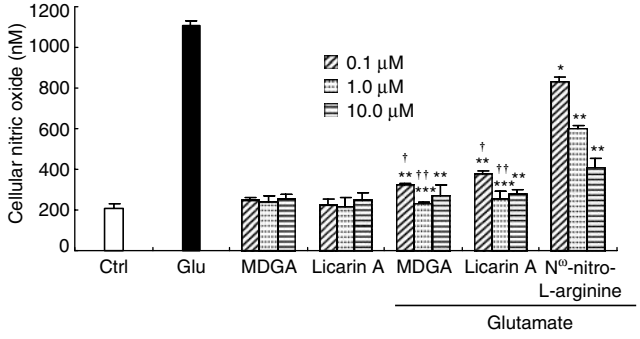

Figure 4 Effect of MDGA or licarin A on intracellular NO in glutamate-injured rat cortical cells. Cortical cultures were pretreated with MDGA or licarin A 1 h before glutamate-induced neurotoxicity. The values shown are mean$\pm$s.d. of three experiments (three to four cultures per experiment). N^{ω}-nitro-L-arginine is a well-known NOS inhibitor. Glutamate-injured value differs significantly from the control at a level of $P<0.001$. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs glutamate-injured cells; $^{\dagger}P<0.001$ vs each value of N^{ω}-nitro-L-arginine-treated cells (ANOVA and Tukey).

of MDGA and licarin A on the content of cellular peroxides using the specific fluorescent dye, 2,7-DCF-DA (Figure 5). Indeed, MDGA and licarin A effectively reduced cellular peroxides in cultured rat cortical cells exposed to glutamate. When cultured cortical cells were insulted with glutamate, the cellular peroxide content was increased up to 3 h after the insult (Kim *et al*., 2002a). However, the cortical cells were pretreated with MDGA or licarin A; the content of cellular peroxides at 24 h after the glutamate insult was significantly reduced. Furthermore, we determined direct free radical scavenging activities of MDGA and licarin A using DPPH radical. We found that MDGA directly scavenged DPPH free radical in our studies (IC_{50}: 15.0 μM); however, licarin A showed weak direct scavenging activity of DPPH free radicals (IC_{50}: 75.0 μM).

Glutamate-induced oxidative stress is also known to deplete intracellular GSH (Almeida *et al*., 1998). As such, we further investigated the effect of MDGA and licarin A on GSH level using such GSH depletors as BSO and DEM. Treatment with

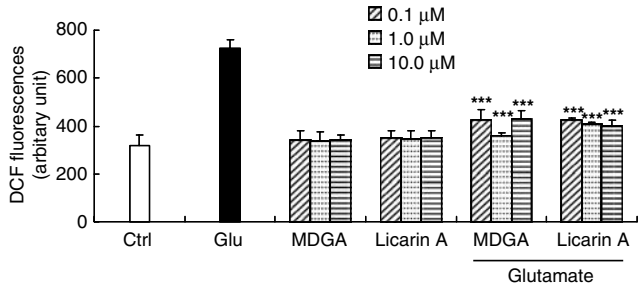

Figure 5 Effect of MDGA or licarin A on cellular oxidation in primary cultures of rat cortical cells. Cultures were pretreated with lignans 1 h before the glutamate insult. The relative content of intracellular peroxide was determined using the fluorescent dye 2,7-DCF-DA. The values shown are mean$\pm$s.d. of three experiments (three to four cultures per experiment). Glutamate-injured value differs significantly from the control at a level of $P<0.001$. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs glutamate-injured cells (ANOVA and Tukey).

BSO or DEM caused the GSH depletion in cells regardless of whether the cells were insulted with glutamate or not (Table 3). The pretreatment of the cultured cortical cells with DEM or BSO also rendered these cells more susceptible to glutamate insult. MDGA or licarin A itself did not influence the GSH level in the normal control cultures. They did not restore the reduced level of GSH induced by the treatment of BSO or DEM. However, these two lignans significantly prevented the GSH depletion in glutamate-induced toxicity (Table 3).

Furthermore, treatment with MDGA or licarin A significantly preserved the activities of superoxide dismutase (SOD), GSH peroxidase (GSH-px) and GSH reductase (GSH-R) to the control level in primary cultures of rat cortical cells injured with glutamate (Table 4).

Discussion

We previously reported that MDGA and licarin A were found to have neuroprotective activities against glutamate-induced neurotoxicity in cultured rat cortical cells by our activity-guided isolation system (Ma *et al*., 2004). MDGA significantly attenuated glutamate-induced neurotoxicity when added prior to or after an excitotoxic glutamate challenge in our culture system. At high concentration (over 10 μM), however, MDGA did not show the improvement in the cell survival rate due to inherent cytotoxicity. For example, when only MDGA was administered to normal control cultures at concentrations ranging from 10.0 to 50.0 μM, the percentage cell viability significantly dropped (10.0 μM: 84.3$\pm$2.4%, $P<0.5$; and 50.0 μM: 73.9$\pm$1.6%, $P<0.01$ of untreated normal control, respectively). However, we found that the decrease of neuroprotection by the treatment of MK-801 or CNQX also happens in our culture system even though they did not show any significant cytotoxicity at higher concentrations (over 100 μM).

On the basis of the results in Table 1, we suggest that the substitution on the phenyl nucleus of a lignan might contribute to exert neuroprotective activity. When the neuroprotective activities of structural derivatives (**1–6**) of MDGA, dibenzylbutane and arylnaphthalene lignans, were compared, the

activity of machilin A (**3**), which has two methylenedioxy moieties, was lower than that of MDGA (**1**) or nordihydroguaiaretic acid (**2**) by 35%. Moreover, (−)-isoguaiacin (**5**) showed stronger activity than isoguaiacin dimethylether (**6**), which has four methoxy groups without hydroxy group.

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and can activate three ionotropic receptors, NMDA, AMPA and KA. Treatment of primary cultured rat cortical cells with NMDA induces NMDA receptor-mediated neurotoxicity characterized by acute influx of calcium (Choi *et al*., 1988). On the other hand, KA-induced neurotoxicity is attributed to an initial rapid influx of Na^+, leading to passive influx of Cl^- and H_2O and excessive generation of ROS (Farooqui *et al*., 2001). Both MDGA and licarin A significantly attenuated glutamate-induced neurotoxicity during the pre- and post-treatment paradigms in our culture system. Therefore, we postulate that MDGA and licarin A might act on both of the early stage and the consequent receptor-mediated responses in our culture system.

Table 3 Effect of MDGA or licarin A on glutathione content in glutamate-injured rat cortical cell cultures

	Glutathione (μmol mg^{-1} protein)		
	w/o Lignan	*MDGA*	*Licarin A*
Control	2.42±0.21	2.38±0.24	2.26±0.14
BSO treated	1.15±0.10	1.17±0.19	1.32±0.20
DEM treated	1.14±0.24	1.18±0.15	1.21±0.25
Glu treated	0.86±0.12	2.05±0.11**	1.91±0.21**
BSO+Glu treated	0.75±0.19†	1.52±0.17**	1.44±0.17**
DEM+Glu treated	0.73±0.17†	1.55±0.21**	1.46±0.23**

At 1 h before the treatment of 1 μM MDGA or licarin A, cortical cells were pretreated with 50 μM BSO or DEM for 1 h. The cultures were then exposed to 100 μM glutamate and maintained for additional 24 h. The values shown are mean±s.d. of three experiments (three to four cultures per experiment).
*$P<0.01$; **$P<0.001$ vs each value in the absence of lignans; $^{\dagger}P<0.001$ vs each value of BSO or DEM only (ANOVA and Tukey).

MDGA and licarin A protected neuronal cells more selectively against toxicity induced by NMDA compared to KA-induced toxicity (Figure 2). Overactivation at NMDA receptors triggers an excessive entry of Ca^{2+}, initiating a series of cytoplasmic and nuclear processes that promote neuronal cell death. Indeed, Ca^{2+}-dependent enzymes like calpains and endonucleases can degrade essential proteins and DNA, respectively, which can induce cell death through necrosis. Ca^{2+} also activates NO synthase, increasing the presence of NO in the neuron and in surrounding areas. NO reacts with superoxide anion (O_2^-) to form the strong oxidizing compound, peroxynitrite, which causes nitration in proteins and oxidation of lipids, proteins and DNA, leading to cell death. In our culture system, MDGA and licarin A could effectively inhibit the increase of Ca^{2+} influx in the early stages of glutamate-induced neurotoxicity and subsequent NO overproduction (Figure 4) (McDonald & Johnston, 1990). In addition, we could suppose that peroxynitrite radical was also suppressed by the treatment with MDGA and licarin A. Superoxide anion has a higher affinity for NO than for SOD under certain conditions (Kohno *et al*., 1995). The decrease in O_2^- inactivation *via* a reduction in SOD activity promoted the overproduction of peroxynitrite (Greene & Greenamyre, 1996). Our results in Table 4 showed that MDGA and licarin A significantly preserved SOD activity in glutamate-injured cells. This might lead to the scavenging of potent free radicals and keeping the level of $^{\bullet}O_2^-$ low. Thus, we could suggest that the retention of SOD activity by the treatment with MDGA and licarin A promotes O_2^- inactivation and, in turn, inhibits overproduction of NO and peroxynitrile radical.

Even though MDGA and licarin A almost completely inhibited Ca^{2+} influx and NO overproduction probably induced by overactivation of NMDA receptor, their neuroprotective activities against the glutamate-injured neurons in culture was lower than expected. This observation could be explained by the fact that our cultured cells intoxicated with glutamate were more effectively protected from excitotoxicity when NMDA- and non-NMDA receptor-mediated cellular responses were blocked at the same time (Kim *et al*., 2002b).

Table 4 Effect of MDGA or licarin A on activities of antioxidative enzymes in glutamate-injured rat cortical cell cultures

	Concentration (μM)	*SOD* (U mg^{-1} protein)	*GSH-R* (mU mg^{-1} protein)	*GSH-px* (mU mg^{-1} protein)
Control		34.7±2.7	39.7±0.4	37.7±0.5
Glutamate injured		15.8±2.1	19.8±2.1	21.8±2.4
MDGA	0.1	34.0±2.2	38.9±2.7	38.1±1.8
	1.0	33.9±1.7	39.1±2.9	37.5±1.5
	10.0	34.5±1.9	39.5±3.4	37.9±1.4
Licarin A	0.1	33.7±3.1	38.7±4.7	38.0±1.5
	1.0	34.1±2.7	39.1±2.9	37.9±2.4
	10.0	35.0±4.7	40.4±3.7	38.3±0.9
MDGA+glutamate	0.1	27.4±4.1**	31.5±2.4**	32.5±2.1**
	1.0	30.8±2.5**	33.4±3.8**	36.3±3.4***
	10.0	28.7±2.0**	27.1±1.8**	28.1±1.8**
Licarin A+glutamate	0.1	25.7±2.8*	22.7±2.8*	24.2±1.6*
	1.0	29.4±3.5**	27.6±2.4**	28.9±2.1**
	10.0	26.7±2.4*	24.9±2.5*	24.6±2.4*

Cortical cell cultures were pretreated with MDGA or licarin A for 1 h before exposure to 100 μM glutamate and then maintained for 24 h. The values shown are mean±s.d. of three experiments (three to four cultures per experiment). Glutamate-injured value differs significantly from the control at a level of $P<0.001$.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs glutamate-injured cells (ANOVA and Tukey).

In our cultures, cotreatment with both 10 μM MK-801 and 10 μM CNQX was more effective in protecting neurons from glutamate-induced toxicity (over 95% neurons survived) than treatment with either 10 μM MK-801 (80% neurons survived) or 10 μM CNQX alone (60% neurons survived). Indeed, MDGA and licarin A showed significant protection against NMDA-induced neurotoxicity in our culture system; however, they showed significant but weaker protection against KA-induced neurotoxicity in cultured cells (Figure 2).

All living cells have developed mechanisms for protection against oxidative stress. In general, GSH plays a major role in the elimination of a large number of nucleophilic toxicants such as oxidative radicals. In normal cells, GSH levels are decreased by oxidative radicals but are promptly restored to normal levels. The depletion of GSH alone did not result in a severe leakage of LDH from primary cultured cells (Casey *et al*., 1995); however, glutamate insult to cells rapidly and continuously decreased cellular GSH levels and inactivated many related antioxidant enzymes including superoxide dismutase, GSH-px and GSH-R (Yasuda *et al*., 1980). Thus, toxic free radicals such as $^{\bullet}O_2^-$, H_2O_2 were kept high in response (Yu *et al*., 2000). Such defects in GSH metabolism might cause oxidative stress, which has been implicated in several neurologic and neurodegenerative diseases (Bains & Shaw, 1997; Schulz *et al*., 2000). MDGA and licarin A significantly preserved the level of GSH in glutamate-injured rat cortical cultures (Table 3). However, these lignans might not accelerate GSH synthesis since they did not affect GSH depletion when the cultured cells were exposed to BSO and DEM. BSO is known to deplete GSH by inhibition of glutamylcysteine synthetase and DEM to deplete GSH *via* a reaction catalyzed by glutathione-*S*-transferase (Kim *et al*., 2002a). In our culture system, MDGA and licarin A maintained the levels of not only SOD but also GSH-px and GSH-R. Therefore, MDGA and licarin A could be supposed to facilitate GSH redox system in glutamate-injured cortical cells through preserving GSSG-R and GSH-px activities.

We also found that MDGA directly scavenged DPPH free radical, but licarin A has low DPPH free radical scavenging activity (IC_{50}; 15 and 75 μM, respectively). These results suggest that the difference in free radical scavenging activity resulted from the structural differences. MDGA is a dibenzylbutane lignan, but licarin A is a dihydrobenzofuran neolignan. However, free radical scavenging by MDGA was not supposed as a pivotal mechanism since licarin A having weak DPPH scavenging activity showed almost equal degree of neuroprotection in our culture system.

At present, the cellular and molecular mechanisms that underlie the action of MDGA and licarin A are not fully understood. However, our results show that MDGA and licarin A significantly protect primary cultured neuronal cells against glutamate-induced oxidative stress *via* antioxidative activities. Therefore, we conclude that MDGA and licarin A might offer useful therapeutic choices in the treatment of neurodegenerative disorders caused by excitotoxicity.

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