JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 863–870

Received October 11, 2006
Accepted March 05, 2007
DOI 10.1211/jpp.59.6.0013
ISSN 0022-3573

Decursinol and decursin protect primary cultured rat cortical cells from glutamate-induced neurotoxicity

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Abstract

We previously reported six neuroprotective decursinol derivatives, coumarins from *Angelica gigas* (Umbelliferae) roots. To elucidate the action patterns of decursinol derivatives, we investigated the neuroprotective effects of decursinol and decursin, which showed highly significant activity and were major constituents of *A. gigas*, using primary cultures of rat cortical cells in-vitro. At concentrations of 0.1–10.0 μM, both decursinol and decursin exerted a significant neuroprotective activity pretreatment and throughout treatment. In addition, decursin had a neuroprotective impact in the post-treatment paradigm implying that decursin might possess different action mechanisms from that of decursinol in the protection of neurons against glutamate injury. Both decursinol and decursin effectively reduced the glutamate-induced increased intracellular calcium ($[Ca^{2+}]_i$) in cortical cells, suggesting that these two coumarins may exert neuroprotection by reducing calcium influx by overactivation of glutamate receptors. This suggestion was supported by the result that decursinol and decursin protected neurons against kainic acid (KA)-induced neurotoxicity better than against that induced by *N*-methyl-D-aspartate (NMDA). Moreover, both decursinol and decursin significantly prevented glutamate-induced decreases in glutathione, a cellular antioxidant, and glutathione peroxidase activity. In addition, both compounds efficiently reduced the overproduction of cellular peroxide in glutamate-injured cortical cells. These results suggested that both decursinol and decursin protected primary cultured rat cortical cells against glutamate-induced oxidative stress by both reducing calcium influx and acting on the cellular antioxidative defence system. Moreover, decursin is considered to probably have a different action mechanism from that of decursinol in protecting cortical cells against glutamate injury.

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Funding: This research was supported by a grant (M103KV010024-06K2201-02410) from Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, the Republic of Korea.

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Introduction

Angelica gigas roots have been widely used traditionally in Korean herbal medicine not only for the treatment of anaemia, but also as a sedative, an anodyne and a tonic. The major compounds of *A. gigas* are known to be coumarins, such as decursinol and decursin (Han 1992). During our search for neuroprotective compounds against glutamate-induced excitotoxicity, from natural sources, we found that the methanolic extract and its methylene chloride fraction of *A. gigas* Nakai roots (Umbelliferae) exhibited significant neuroprotective activity. By activity-guided fractionation technique, decursinol derivatives, coumarins, were obtained from the methylene chloride fraction of *A. gigas* roots as active components against glutamate insult in primary cultured rat cortical cells (Kang et al 2001, 2005). In addition, our other previous studies revealed that coumarins isolated from the plant, especially decursinol, excellently inhibited acetylcholinesterase (AChE) in-vitro (Kang et al 2001), and both decursinol and decursin greatly improved scopolamine-induced amnesia in mice (Kang et al 2003). Particularly, decursinol has been recently reported to have an anti-amnesic effect against β-amyloid peptide (Yan et al 2004), an anti-excitotoxic effect against KA-induced toxicity in-vivo (Lee et al 2003) and antinociceptive (Choi et al 2003) activity in mice, suggesting that decursinol acts on the central nervous system or neurons in various ways. To date, however, there are no reports concerning the specific neuroprotective activity of decursinol or decursin in in-vitro cell cultures.

Therefore, this study was performed to demonstrate the neuroprotective activity of the active major compounds of *A. gigas* roots, decursinol and decursin, in glutamate-injured

primary cultures of rat cortical cells, and to elucidate the mechanisms responsible.

Materials and Methods

Plant material

The root of *A. gigas* was purchased in a local market for Oriental medicine in Chechon, Chung-Buk, Korea and voucher specimens (SNUPH-0415) were deposited in the Herbarium of the College of Pharmacy, Seoul National University.

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Hank's balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY). Sodium pyruvate, penicillin/streptomycin, trypsin and all other reagents were purchased from Sigma Chemical Co. (St Louis, MO). Dizocilpine maleate (MK-801), DL-2-amino-5-phosphonovaleric acid (APV), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) used as positive controls were purchased from Research Biochemicals International (Natick, MA).

Isolation and identification of decursin and decursinol

The dried underground parts (5 kg) of *A. gigas* were extracted with methanol in an ultrasonic apparatus. Upon removal of solvent in-vacuo, the methanolic extract yielded 350 g. This methanolic extract was then suspended in water and partitioned successively with dichloromethane. We described the detailed process of isolating decursinol and decursin from dichloromethane fractions in a previous report (Kang et al 2001).

Cortical cell culture

Primary cultures of mixed cortical cells containing both neurons and glia were prepared from 17~19-day-old fetal rats (Sprague-Dawley) as described previously (Jang et al 2002). Sprague-Dawley rats, 200 ± 50 g, were provided by the Laboratory Animal Center, Seoul National University. Rats were kept on standard rat chow with free access to tap water in temperature- and humidity-controlled animal quarters under a 12-h light–dark cycle. The experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of Seoul National University. In brief, the trypsin-dissociated cortical cells were plated on a 48-well plate (Falcon Primaria, Becton Dickinson, NJ) coated with collagen at a density of 5×10^5 cells/well (for all biochemical assays except for the following) or on 60-mm dishes (Falcon Primaria, Becton Dickinson, NJ) at a density of 3×10^6 cells/dish (to assay GSH content and antioxidative enzymes activity). The cortical cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 IU mL^{-1} penicillin and 10 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere of 95% air–5% CO_2. After 3 days in culture, cell division of non-neuronal cells was halted by adding 5-fluoro-2′-deoxyuridine (50 μM). Cultures were allowed to mature for at least 2 weeks 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 (Jang et al 2002).

Assessment of neurotoxicity

Test compounds were dissolved in dimethyl sulfoxide (DMSO; final concentration in culture 0.1%). Cortical cell cultures (17 days in-vitro) were washed with DMEM and incubated with decursinol or decursin for 1 h. The cultures were then exposed to 100 μM L-glutamate and maintained for 24 h. After the incubation, the cultures were assessed for the extent of neuronal damage (throughout treatment). In some experiments, the cultures were treated with decursinol or decursin for 1 h before exposure (pretreatment), after exposure (post-treatment) to 100 μM L-glutamate for 30 min or simultaneously treated with 100 μM L-glutamate (coadministration). After an additional 24-h incubation in DMEM in the absence (pretreatment) or presence (post-treatment and coadministration) of decursinol or decursin, the cultures were assessed for viability by measuring lactate dehydrogenase (LDH) in the medium, which reflects the integrity of the cellular membrane (Kim et al 1998), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay, which reflects mitochondrial succinate dehydrogenase function (Mosmann 1983). MK-801, a noncompetitive antagonist of the NMDA receptor, and APV, a competitive antagonist of the NMDA receptor, were used as positive controls. CNQX, a non-NMDA receptor antagonist, was also used as a positive control. In some experiments, cultures were pretreated with decursinol or decursin 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 for 3 h. The cultures were then washed, and maintained in DMEM for 24 h in the absence of decursinol or decursin. Data are expressed as the percentage protection relative to vehicle-treated control cultures: in LDH assay, 100 × [(LDH released from excitotoxin-injured) − (LDH released from excitotoxin + test compound-treated)]/[(LDH released from excitotoxin-injured) − LDH released from control)]; in MTT assay, 100 × [(optical density (OD) from excitotoxin + test compound-treated) − (OD from excitotoxin-injured)]/[(OD from control) − (OD from excitotoxin-injured)].

Measurement of calcium content

The content of intracellular calcium ($[Ca^{2+}]_i$) was determined using the fluorescent dye, Fura 2-AM, by ratio fluorometry (Kim et al 2004). In brief, 1 h before exposure to 100 μM L-glutamate, cultures grown on glass cover slides were treated with decursinol or decursin and 5 μM FURA-2 AM in phosphate-buffered saline (PBS, pH 7.2) at 37 °C in a humidified atmosphere of 95% air–5% CO_2 and then washed with PBS. The change in $[Ca^{2+}]_i$ was measured 10 min after exposure to L-glutamate. Cell culture slides were mounted into spectrophotometer

cuvettes containing 2.5 mL PBS (without carbonate). Fluorescence was measured with a JASCO FP-6500 spectrofluorometer (Tokyo, Japan) by exciting cells at 340 nm and 380 nm and measuring light emission at 520 nm. Ionomycin and EGTA (final concentrations of 10 μM and 20 mM, respectively) were added to determine the emission of dye saturated with Ca^{2+} (10 mM $CaCl_2$) and free of Ca^{2+}, respectively. Calcium concentration was calculated according to the following equation (Grynkiewicz et al 1985):

$$[Ca^{2+}]_i = K_d[(R-R_{min})/(R_{max}-R)] \times F_0/F_s \quad (1)$$

Where K_d is 225 nM, R, the experimentally obtained ratio 340:380 of Fura 2 fluorescence, $R_{min,}$ the ratio 340:380 of Fura 2 in the Ca^{2+}-free solution, $R_{max,}$ the ratio 340:380 of Fura 2 in the presence of saturating Ca^{2+} concentration and $F_0/F_{s,}$ the ratio of 380 nm excitation fluorescence at zero and saturating Ca^{2+} levels.

Measurement of cellular peroxide

The relative levels of peroxides in cultured cells were measured with the oxidation-sensitive compound 2,3-dichlorofluorescein diacetate (DCF DA) by the method of Goodman & Mattson (1994). The cultured cells were loaded with DCF-DA (50 μM, 30 min incubation) followed by two washes with PBS. DCF fluorescence was then determined by exciting cells at 488 nm and measuring emitted light at 530 nm.

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

A 100-μL volume of a 200 μM solution of DPPH radicals in ethanol was added to 100 μL of a solution containing decursinol or decursin in ethanol. The contents were mixed and transferred into wells of a 96-well culture plate. After incubation for 4 h, the absorbance (OD) was determined using a microplate reader at 515 nm (Basly et al 2000). Authentic caffeic acid and butylated hydroxytoluene (BHT) were used as reference antioxidants.

Measurement of glutathione peroxidase (GPx) activity and glutathione (GSH) content

Cells from two 60-mm culture dishes 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 *g* at 4°C. The supernatant, consisting of the cytosolic + mitochondrial fraction, was used for assay. The activity of glutathione peroxidase was measured by the method of Flohe & Gunzler (1984). Total glutathione (GSH + GSSG) levels in the supernatant were determined spectrophotometrically by using the enzymatic cycling method of Tietz (1969). Protein content was measured by the method of Lowry et al (1951), with bovine serum albumin as a standard.

Statistical analysis

Data were evaluated for statistical significance using one-way analysis of variance and, if significant, group means compared by post-hoc analysis using Tukey multiple comparison of means.

Results

We previously reported the isolation of six decursinol derivatives from *Angelica gigas* roots and their neuroprotective activity in primary cultures of rat cortical cells injured by glutamate (Kang et al 2005). We therefore tried to investigate the action mechanisms of decursinol, a highly neuroprotective major coumarin of *A. gigas*, and its major derivative, decursin (Figure 1), using glutamate-injured primary cultures of rat cortical cells.

Decursinol and decursin protected cortical cells from glutamate-induced neurotoxicity

In our cultures, we found that >60% of neuronal cells died via necrosis in cortical cultures exposed to 100 μM glutamate for 24 h as determined under phase-contrast microscopy. Decursinol and decursin significantly attenuated glutamate-induced neuronal cell death at concentrations of 0.1–10 μM as measured by LDH assay in the throughout-treatment paradigm, showing protection degrees of >30% ($P<0.05$) to about 70% ($P<0.001$) of control (Table 1). In the MTT assay, we observed that there were similar trends in the neuroprotection by decursinol and decursin, although the efficacy of the two coumarins turned out to be lower than in the LDH assay. Decursin exhibited similar neuroprotective efficacy to decursinol, although it was less potent.

To elucidate the neuroprotective activity of decursinol and decursin, we evaluated their protective effect against glutamate-induced toxicity by a timed exposure to decursinol and decursin 30 min before (pretreatment), immediately after (coadministration) and 30 min after (post-treatment) excitotoxic challenge (Figure 2).

Decursinol and decursin exhibited similar neuroprotective activity in the pretreatment paradigm, showing protection degrees of 51% ($P<0.01$) and 57% ($P<0.001$), respectively, while they showed somewhat different protective effects in the post-treatment paradigms. Decursinol showed little neuroprotection in cortical cells already injured by glutamate. However, decursin exerted significant neuroprotective activity, showing protection degrees of about 40% ($P<0.05$) even in the post-treatment paradigm.

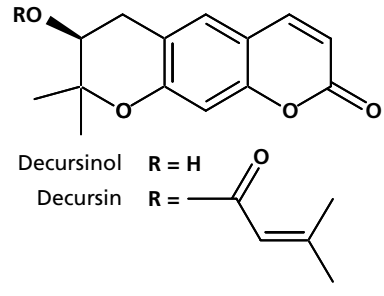

Figure 1 Chemical structure of decursinol and decursin isolated from *Angelica gigas.*

Table 1 Neuroprotective activity of decursinol and decursin on primary cultures of rat cortical cells injured by glutamate[a]

Compound	Cell viability (%)[b, e]		
	0.1 μM	1.0 μM	10.0 μM
Control[c,d]	100.0		
Glutamate-injured[c, d, f]	0.0		
Decursinol	32.4 ± 4.0*	67.1 ± 5.5***	47.5 ± 5.9*
	(23.0 ± 4.0)	(50.0 ± 4.0**)	(32.5 ± 2.5*)
Decursin	35.0 ± 3.6*	50.0 ± 3.3**	65.1 ± 1.6***
	(25.5 ± 3.0)	(39.1 ± 4.0*)	(52.0 ± 3.5**)
MK-801	35.0 ± 4.0**	66.0 ± 5.1***	75.0 ± 5.2***
	(31.0 ± 2.0*)	(54.4 ± 4.5**)	(66.0 ± 3.5***)
APV	10.0 ± 2.5	25.0 ± 3.0	39.0 ± 4.0*
	(9.1 ± 2.0)	(20.0 ± 2.5)	(33.0 ± 2.0*)
CNQX	29.0 ± 3.5*	40.5 ± 3.7*	50.5 ± 4.5**
	(20.0 ± 2.5)	(32.5 ± 2.2*)	(41.0 ± 3.1*)

[a]Rat cortical cell cultures were pretreated with test compounds for 1 h. The cultures were then exposed to 100 μM glutamate for 24 h. After the incubation, the cultures were assessed for the extent of neuronal damage. [b]Cell viability was measured by both LDH assay, and MTT assay (values in parentheses). [c]LDH released from control and glutamate-injured cultures were 14.0 ± 2.1 and 52.5 ± 5.5 U mL^{-1}, respectively. [d]ODs of control and glutamate-injured cultures were 1.49 ± 0.16 and 1.06 ± 0.04, respectively. [e]The values shown are the mean ± s.d. of three experiments (5–6 cultures). *P<0.05, **P<0.01, ***P<0.001, vs glutamate-injured group (one-way analysis of variance and Tukey's test). [f]Glutamate-injured value differed significantly from control at the levels of P<0.001 (LDH assay) and P<0.01 (MTT assay).

Decursinol and decursin inhibited Ca^{2+} influx induced by glutamate

Glutamate-induced neurotoxicity mostly results from Ca^{2+} influxed primarily inside of cells (Cacabelos et al 1999). Thus, we determined the effect of decursinol or decursin on the Ca^{2+} influx induced by glutamate. As shown in Figure 3, decursinol and decursin effectively blocked the influx of calcium ions provoked by glutamate, exhibiting inhibition degrees of over, or about, 50% (P<0.01 or P<0.001) at concentrations of 1–10 μM.

Decursinol and decursin were more protective against neurotoxicity induced by KA than NMDA

Glutamate exerts its effects by acting on both NMDA and non-NMDA receptors. To ascertain whether decursinol and decursin act on glutamate receptors, the neuroprotective activity of these two coumarins were evaluated in NMDA- or KA-injured cultures by the pretreatment paradigm (Figure 4). Decursinol protected rat cortical cells more selectively against KA-induced neurotoxicity than against NMDA, showing a protection degree of >45% (P<0.01 or P<0.001) of control at all treated concentrations. Decursin also exhibited selectivity in neuroprotection against KA insult, although showing lower potency than decursinol.

Decursinol and decursin reduced oxidative stress by improving the cellular antioxidative defence system

Glutamate-induced neurotoxicity is known to be due to free radicals, including hydroxyl radicals and superoxide anions. The mammalian brain has defensive mechanisms against oxidative stress, such as glutathione (GSH), glutathione peroxidase (GSH-px), catalase, glutathione disulfide reductase and superoxide dismutase (Warner et al 2004). Glutathione (GSH) has an important role in scavenging free radicals, produced heavily in the course of oxidative stress, as endogenous antioxidant. Glutamate-induced oxidative stress is also known to deplete intracellular GSH (Almeida et al 1998).

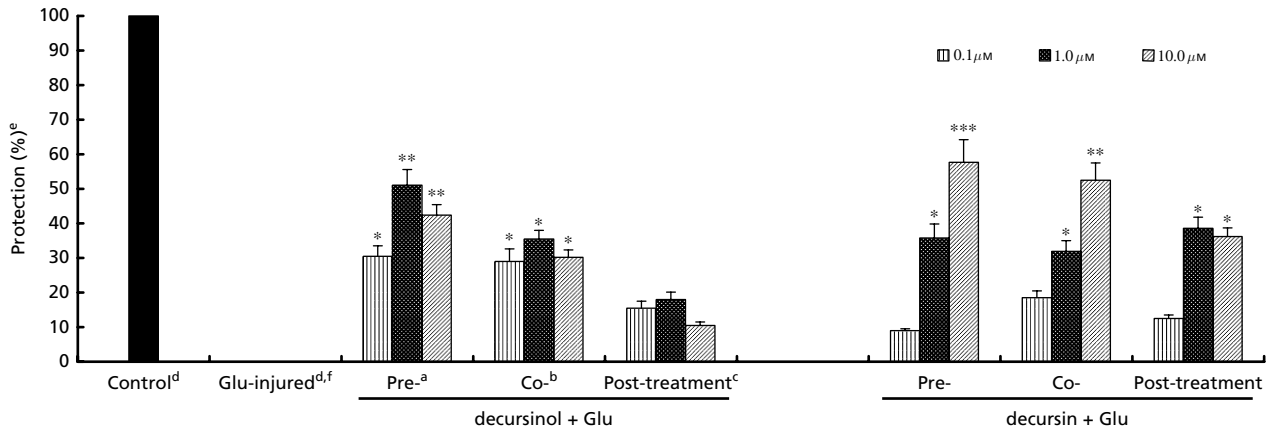

Figure 2 Neuroprotective activity of decursinol and decursin on glutamate-induced neurotoxicity. [a]Pretreatment: rat cortical cell cultures were treated with decursinol or decursin from 1 h before glutamate insult to glutamate wash-out. [b]Coadministration: rat cortical cell cultures were exposed to glutamate immediately after treatment with decursinol or decursin for 24 h. [c]Post-treatment: decursinol or decursin was added from the glutamate wash-out to the end of the experiment. [d]LDH released from control and glutamate-injured cultures was 16.0 ± 1.3 and 65.5 ± 6.5 U mL^{-1}, respectively. [e]Protection (%) was calculated as described for Table 1. The values shown are the mean ± s.d. of three experiments (5–6 cultures). *P<0.05, **P<0.01, ***P<0.001, vs glutamate-injured group (one-way analysis of variance and Tukey's test). [f]Glutamate-injured value differed significantly from control at a level of P<0.001.

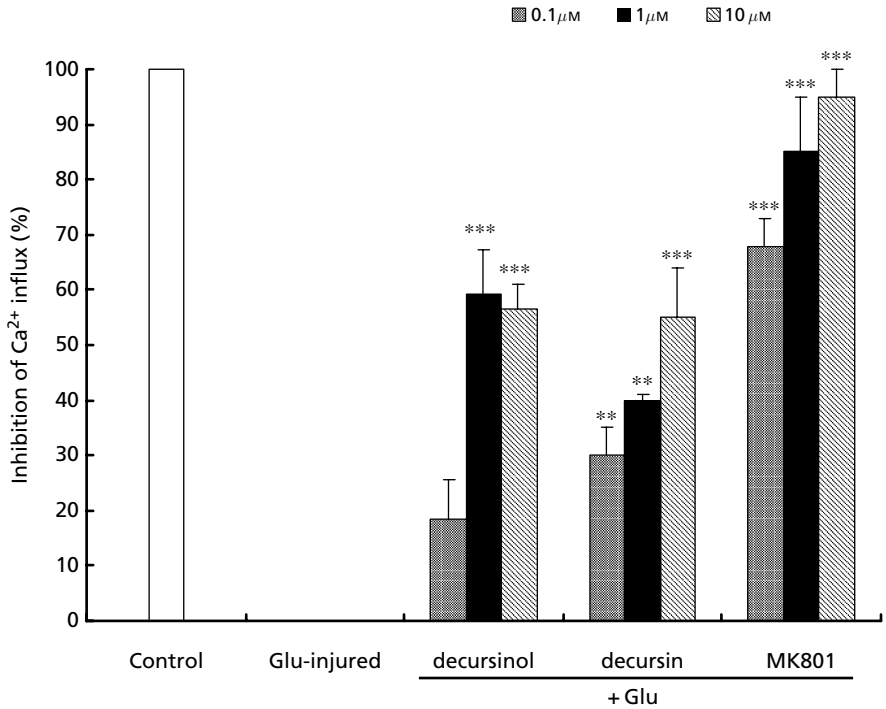

Figure 3 Effects of decursinol and decursin on intracellular [Ca^{2+}] in glutamate-injured cortical neurons. Rat cortical cell cultures were treated with decursinol or decursin and 5 μM fura-2 AM 1 h before exposure to 100 μM glutamate. The change in [Ca^{2+}]i was measured 10 min after glutamate exposure. Values shown are the mean ± s.d. of three experiments (5–6 cultures). 340 nm/380 nm ratio in Fura-2 fluorescence: Ctrl, 1.47 ± 0.13; glutamate-injured, 2.50 ± 0.31. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs glutamate-injured group (one-way analysis of variance and Tukey's test).

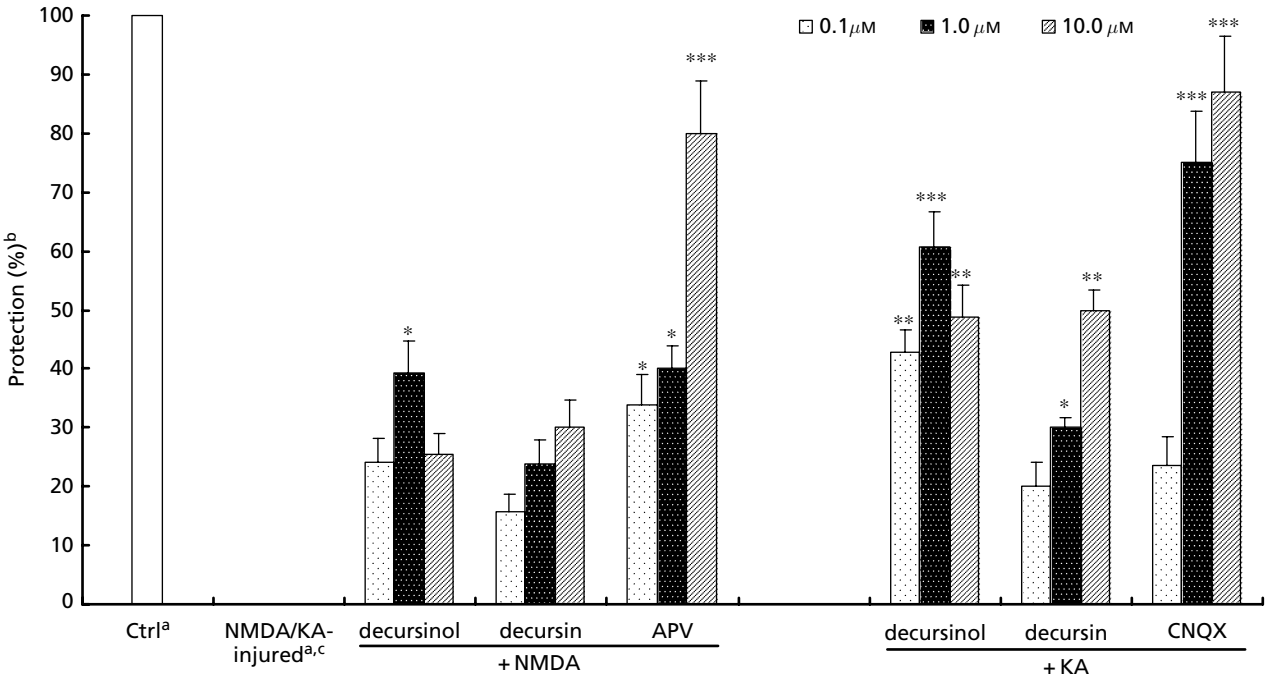

Figure 4 Neuroprotective activity of decursinol and decursin on NMDA- or KA-injured rat cortical cells. Rat cortical cell cultures were treated with decursinol or decursin for 1 h before exposure to NMDA (50 μM for 30 min) or KA (50 μM for 3 h). [a]LDH released from control, NMDA or KA-injured cultures was 15.0 ± 1.0, 65.5 ± 6.5 and 60.0 ± 5.5 U mL^{-1}, respectively. [b]Protection (%) was calculated as described for Table 1. The values shown are the mean ± s.d. of three experiments (5–6 cultures). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs glutamate-injured group (one-way analysis of variance and Tukey's test). [c]NMDA- or KA-injured value differed significantly from control at a level of $P < 0.001$.

Table 2 Effect of decursinol and decursin on glutathione peroxidase activity, and the content of glutathione and cellular peroxide in glutamate-injured cortical cell cultures[a]

	Concn (μM)	GSH-px (μmol NADPH consumed/min/mg protein)	GSH (nmol (mg protein)$^{-1}$)	Cellular peroxide[b] (arbitrary units)
Control		15.1 ± 0.8	43.6 ± 6.1	220.3 ± 20.5
Glutamate-injured		10.0 ± 0.6	23.4 ± 2.5	455.6 ± 66.2
Decursinol	0.1	10.8 ± 0.5	28.2 ± 2.5	349.7 ± 40.0**
	1.0	12.9 ± 0.9*	34.2 ± 0.5**	290.4 ± 21.0***
	10.0	13.8 ± 0.9**	27.5 ± 3.7*	297.5 ± 20.5***
Decursin	0.1	12.3 ± 0.4*	26.5 ± 1.2	373.2 ± 40.0*
	1.0	12.1 ± 0.3*	28.5 ± 1.4*	362.0 ± 36.8*
	10.0	14.5 ± 1.2**	32.0 ± 2.0**	286.2 ± 27.0***

[a]Decursinol or decursin treatment was by throughout-treatment paradigm as in Table 1. Values shown are the mean ± s.d. of three experiments (5–6 cultures). [b]Glutamate-injured/Control in DCF fluorescence: 1.93 ± 0.03. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs glutamate-injured group (one-way analysis of variance and Tukey's test).

Thus, we tried to determine whether decursinol or decursin affected the glutathione defence system in our cortical cell cultures. In our cultures, decursinol and decursin significantly attenuated the decreases in both GSH-px activity and GSH content in cultures damaged by glutamate, retaining > 70% ($P < 0.01$) of control at the most effective concentration (Table 2). Furthermore, we measured the content of cellular peroxide using the peroxide-specific fluorescence dye, 2, 7-DCF-DA (Table 2). Indeed, decursinol and decursin excellently attenuated the increased cellular peroxides produced by glutamate in cultured rat cortical cells to a degree of about 40 ($P < 0.05$) to 70% ($P < 0.01$ or $P < 0.001$) at concentrations of 1–10 μM. We therefore examined whether decursinol or decursin had direct free radical scavenging activity through the DPPH radical scavenging assay. It was found that these two coumarins did not have the ability to directly scavenge free radicals, such as the DPPH radical, showing a scavenging activity of 0% at concentrations of 0.1–10 μM (positive control: caffeic acid, 45% ($P < 0.01$) at 10 μM and BHT, 42% ($P < 0.01$) at 100 μM).

Discussion

Glutamate produces its effects by acting on NMDA and non-NMDA receptors (Lipton & Rosenberg, 1994). Glutamatergic neurotoxicity is propagated by various events, including Ca^{2+}-mediated neurotoxicity due to NMDA and non-NMDA receptor-mediated changes of the intracellular Ca^{2+} concentration and oxidative stress-related neurotoxicity through free radical formation and membrane lipid peroxidation. Our study on the neuroprotective activity of decursinol and decursin in glutamate-injured cultures of rat cortical cells was focused on these two processes in neurotoxicity induced by glutamate. In our culture system, both decursinol and decursin significantly prevented neuronal cell death in glutamate-injured rat cortical cells.

Pretreatment with decursinol significantly attenuated glutamate-induced neurotoxicity, while the post-treatment exhibited little neuroprotective activity. From these results, we could postulate that decursinol more effectively protects cortical cells from glutamate insult when it exists in cortical cells in the status of inactivation of glutamatergic receptors than after glutamate insult. This suggestion was supported by the finding that decursinol protected KA-injured cultures much better than NMDA-injured ones. Lee et al (2003) reported that decursinol pretreated orally for 30 min significantly attenuated KA-induced lethal toxicity in ICR mice in a dose-dependent manner through affecting phosphorylation of ERK and dephosphorylation of CREB. However, they did not show that decursinol affected the death of pyramidal cells in the CA3 pyramidal region. Although this in-vivo data could not be directly applied to our in-vitro data, it partly supports the results of our study.

Similarly to decursinol, pretreatment with decursin significantly attenuated glutamate-induced neurotoxicity. It also exhibited better neuroprotection in KA-injured cultures than in NMDA-injured ones, showing a protection degree of about 50% at a concentration of 10 μM. Besides, both decursinol and decursin efficiently inhibited glutamate-induced Ca^{2+} influx. Neurodegeneration may occur secondary to glutamate-triggered Ca^{2+} influx through any of three routes: NMDA channels, voltage-sensitive Ca^{2+} channels and Ca^{2+}-permeable AMPA/kainite channels (Ca-A/K). It is well known that among them, Ca^{2+} influx through Ca-A/K is much less than that through NMDA channels (Lu et al 1996). In this study, at the concentration range of 0.1–10 μM, decursinol and decursin exhibited significant inhibition on increase of $[Ca^{2+}]_i$ in glutamate-injured cultures, although the degree of inhibition was lower than that by MK-801, a non-specific antagonist of NMDA receptors.

The above results, including this evidence, demonstrate that these two coumarins more effectively interfere with the neurotoxic processes developed after activation of glutamatergic receptors when it exists in the status of inactivation of glutamatergic receptors than after glutamate or KA insult.

However, in contrast to decursinol, decursin exhibited significant effects in protection against glutamate-induced neurotoxicity even in the post-treatment paradigm. Decursin has also been reported to be a protein kinase C (PKC) activator (Ahn et al 1996) and to activate conventional PKC isoforms,

PKC α and βII, in K562 human erythroleukaemia cells (Kim et al 2005). According to recent studies, the activation of PKC isoforms mainly exerts a neuroprotective effect, and conventional protein kinase C isoforms, PKC α, βI and βII, mediate neuroprotection induced by phorbol-12-myristate-13-acetate (PMA) and oestrogen in primary cerebrocortical neurons (Jung et al 2005; Siniscalchi et al 2005; Cordey & Pike 2006). Although this evidence was not produced from primary cultured cortical cells, it could be suggested that the neuroprotection by decursin observed even in the post-treatment paradigm might involve the activation of conventional PKC isoforms.

Decursinol and decursin significantly prevented decreases in GSH and GSH-px activity, and then reduced the level of cellular peroxide in glutamate-injured cultures. Through these actions, they may attenuate neuronal cell death. In a similar vein, it was reported that the over-expression of GSH-px protected cultured hippocampal and cortical neurons from necrotic insult by kainic acid, glutamate or sodium cyanide, as well as inhibited pro-apoptotic mediators, to protect neurons from experimental stroke (Hoehn et al 2003; Wang et al 2003). From these results, we suggest that both decursinol and decursin exert neuroprotective activity by retaining the content of GSH and activity of GSH-px, and then reducing the level of cellular peroxide in glutamate-injured rat cortical cells.

Conclusions

At present, the cellular and molecular mechanisms that underlie the action of decursinol and decursin are not fully understood. However, through this study, it was demonstrated that the decrease of glutamate-induced Ca^{2+} influx and the retention of the glutathione defence system by both decursinol and decursin are responsible for their neuroprotective effects in rat cortical cells. Therefore, we suggest that decursinol derivatives from *A. gigas* may offer a useful therapeutic choice in treatment of neurodegenerative disorders caused by excitotoxicity, and provide an important idea of the neuroprotective moiety in chemical structures.

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