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E-p-Methoxycinnamic acid protects cultured neuronal cells against neurotoxicity induced by glutamate

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1 We previously reported that four new phenylpropanoid glycosides and six known cinnamate derivatives isolated from roots of *Scrophularia buergeriana* Miquel (Scrophulariaceae) protected cultured cortical neurons from neurotoxicity induced by glutamate. Here, we have investigated the structure-activity relationships in the phenylpropanoids using our primary culture system.

2 The α,β-unsaturated ester moiety and the *para*-methoxy group in the phenylpropanoids appeared to play a vital role in neuroprotective activity. This suggested that *E-p*-methoxycinnamic acid (*E-p*-MCA) might be a crucial component for their neuroprotective activity within the phenylpropanoid compounds. *E-p*-MCA significantly attenuated glutamate-induced neurotoxicity when added prior to an excitotoxic glutamate challenge.

3 The neuroprotective activity of *E-p*-MCA appeared to be more effective in protecting neurons against neurotoxicity induced by NMDA than from that induced by kainic acid. *E-p*-MCA inhibited the binding of [propyl-2,3-^{3}H]-CGP39653 and [2-^{3}H]-glycine to their respective binding sites on rat cortical membranes. However, even high concentrations of *E-p*-MCA failed to inhibit completely [propyl-2,3-^{3}H]-CGP39653 and [2-^{3}H]-glycine binding.

4 Indeed, *E-p*-MCA diminished the calcium influx that routinely accompanies glutamate-induced neurotoxicity, and inhibited the subsequent overproduction of nitric oxide and cellular peroxide in glutamate-injured neurons.

5 Thus, our results suggest that *E-p*-MCA exerts significant protective effects against neurodegeneration induced by glutamate in primary cultures of cortical neurons by an action suggestive of partial glutamatergic antagonism.

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Keywords: *Scrophularia buergeriana*; *E-p*-methoxycinnamic acid; primary neuronal culture; neuroprotective activity; glutamatergic antagonism; structure-activity relationship; neurodegenerative disorders

Abbreviations: APV, DL-2-amino-5-phosphonovaleric acid; $[Ca^{2+}]_i$, content of intracellular calcium; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DCF-DA, 2,7-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; *E-p*-MCA, *E-p*-methoxycinnamic acid; HBSS, Hank's balanced salt solution; KA, kainic acid; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium; NO, nitric oxide

Introduction

Glutamate is widely known to be associated with central excitatory neurotransmission – neuronal survival, synaptogenesis, neuronal plasticity, learning, and memory processes – by acting on NMDA and non-NMDA receptors in the brain (Albright *et al*., 2000). However, glutamate is also recognized to cause neuronal cell loss within the central nervous system (Choi, 1988; Coyle & Puttfarcken, 1993) by two distinct forms of response, acute and delayed (Choi, 1985; Choi *et al*., 1987). Abnormalities in glutamate neurotransmitter systems may be involved in neurological disorders such as seizures (Lipton & Rosenberg, 1994), ischaemia and spinal cord trauma (Chase & Oh, 2000; Heintz & Zoghbi, 2000) and neurodegenerative disorders such as Alzheimer's disease (Choi & Rothman, 1990) and Parkinson's disease (Lee *et al*., 1999). Thus, neuroprotection against glutamate-induced neurotoxicity has been an appropriate therapeutic strategy for preventing and/or treating both acute and chronic forms of neurodegeneration (Muir & Lees, 1995; Trist, 2000).

We previously employed primary cultures of rat cortical cells as an *in vitro* assay system to isolate neuroprotective compounds from natural products which protect against glutamate-induced neurotoxicity (Kim *et al*., 1998). In our previous report, we found that phenylpropanoids isolated from *Scrophularia buergeriana* roots had significant neuroprotective activities in our *in vitro* system (Kim & Kim, 2000). In the present study, the structure-activity relationship of these isolated compounds (**1**–**13** shown in Figure 1, Category A) was compared to structurally-related compounds (**14**–**24** shown in Figure 1, Category B). Furthermore, we attempted to elucidate the neuroprotective mechanisms for *E-p*-methoxycinnamic acid (*E-p*-MCA) – the putative vital structure for this neuroprotective activity – by employing our primary culture system. Two interrelated pathways were assessed in an attempt to reveal possible mechanisms of *E-p*-MCA for neuroprotection.

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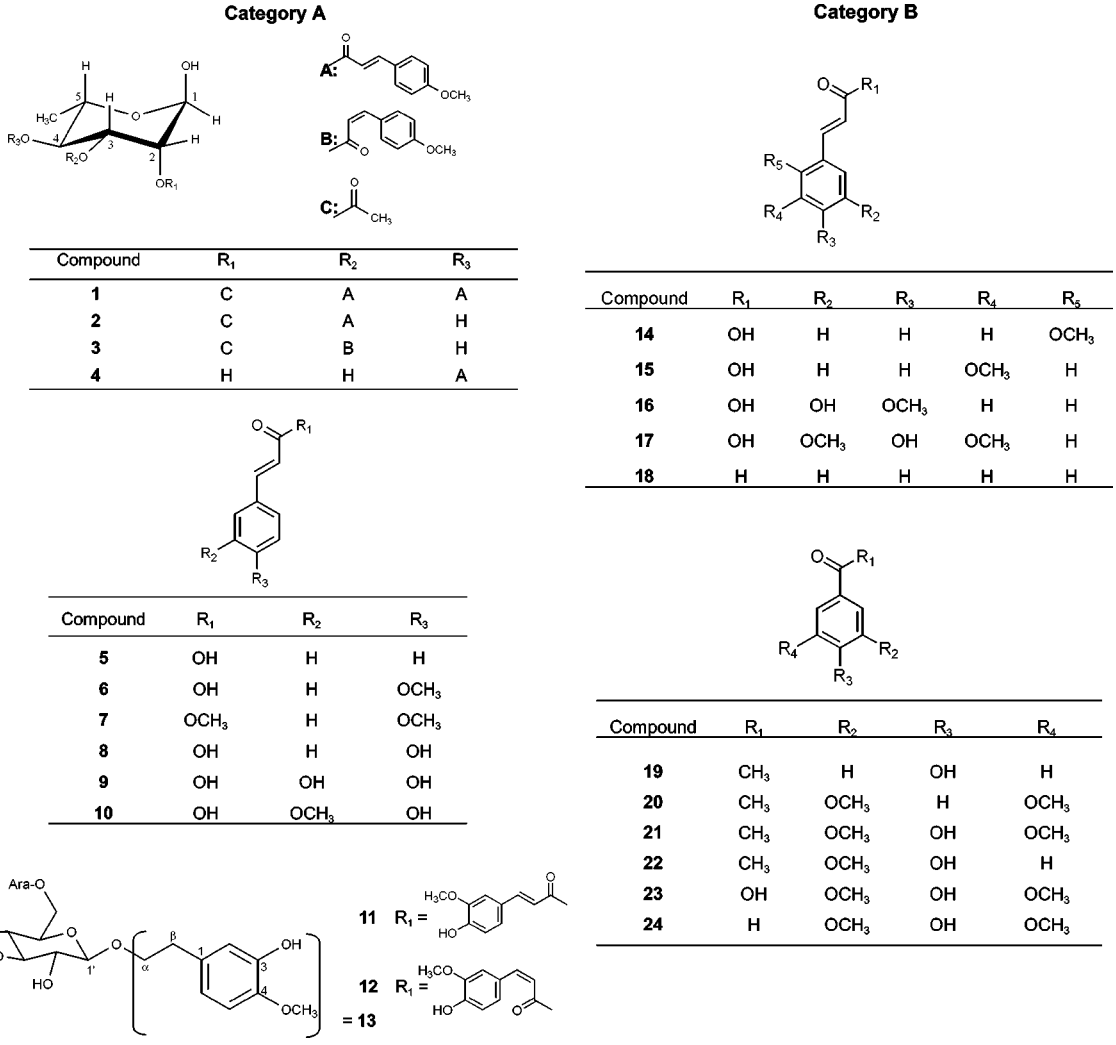

Compound	R_1	R_2	R_3
1	C	A	A
2	C	A	H
3	C	B	H
4	H	H	A

Compound	R_1	R_2	R_3
5	OH	H	H
6	OH	H	OCH_3
7	OCH_3	H	OCH_3
8	OH	H	OH
9	OH	OH	OH
10	OH	OCH_3	OH

Compound	R_1	R_2	R_3	R_4	R_5
14	OH	H	H	H	OCH_3
15	OH	H	H	OCH_3	H
16	OH	OH	OCH_3	H	H
17	OH	OCH_3	OH	OCH_3	H
18	H	H	H	H	H

Compound	R_1	R_2	R_3	R_4
19	CH_3	H	OH	H
20	CH_3	OCH_3	H	OCH_3
21	CH_3	OCH_3	OH	OCH_3
22	CH_3	OCH_3	OH	H
23	OH	OCH_3	OH	OCH_3
24	H	OCH_3	OH	OCH_3

Figure 1 Structures of *E-p*-MCA.

First, we studied the effect of *E-p*-MCA on glutamate receptors. Blocking glutamate receptors is the most powerful anti-excitotoxic strategy in most cell culture models (Choi *et al*., 1988). Second, we evaluated the effect of *E-p*-MCA on cellular redox mechanisms in glutamate-injured cells. Glutamate may be directly toxic to cultured neuronal cells *via* either of two different processes, both of which result in the production of free radicals. The former process is the classical pathway, known as excitotoxicity (Olney, 1969), which can be blocked by known receptor antagonists; the latter process is known as the oxidative glutamate toxicity pathway (Murphy *et al*., 1989) which can be blocked by antioxidants (Miyamoto *et al*., 1989; Davis & Maher, 1994).

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum and Hank's balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY, U.S.A.). Glutamate, NMDA, kainic acid (KA), sodium pyruvate, penicillin/streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT), trypsin, Fura 2-acetoxymethyl ester (Fura 2-AM), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, hydrogen peroxide (H_2O_2) and 2,7-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma (St. Louis, MO, U.S.A.). DL-2-Amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and MK-801 used as positive control were purchased from Research Biochemicals International (Natick, MA, U.S.A.). [Propyl-2,3-^{3}H]-CGP39653 (specific activity, 44 Ci mmol^{-1}), [^{3}H]-kainate (specific activity, 58 Ci mmol^{-1}) and [2-^{3}H]-glycine (specific activity, 48.4 Ci mmol^{-1}) were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.). The compounds – *E-o*-methoxycinnamic acid, *E-m*-methoxycinnamic acid, *E*-isoferulic acid, *E*-3,5-dimethoxy-4-hydroxycinnamic acid, *E*-cinnamaldehyde and six acetophenones for studying structure-activity relationships – were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The antibodies against neuron-specific enolase and glial fibrillary acidic protein were obtained from DAKO (Denmark).

Biotinylated anti-rabbit IgG was purchased from Vector Laboratories (Burlingame, CA, U.S.A.).

Cell culture

Primary cultures of mixed cortical cells containing both neuronal and glial cells or hippocampal neuronal cells were prepared from 17~19-day-old foetal Sprague-Dawley rats as described previously (Kim *et al*., 1998). In brief, the trypsin-dissociated cortical and hippocampal cells were plated on 15 mm dishes (Falcon Primaria, Becton Dickinson, NJ, U.S.A.) coated with collagen at a density of 5×10^5 cells per dish and poly-L-lysine at a density of 1×10^5 cells per dish, respectively. The cortical or hippocampal cells were grown in DMEM containing 10% heat-inactivated foetal bovine serum with penicillin (100 IU ml^{-1}) and streptomycin (10 $\mu g\ ml^{-1}$) 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; Sigma). 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 (Hewett *et al*., 2000). All experiments were performed with Ethical Approval of Seoul National University.

Neurotoxicity

Test compounds were dissolved in DMSO (final culture concentration, 0.1%); preliminary studies indicated that the solvent had no effect on cell viability at the concentration used (Kim *et al*., 1998). Seventeen-day-old cortical cell cultures were used to assess compounds for neuroprotection from excitotoxin-induced oxidative damage. Cortical cell cultures were washed with DMEM and incubated with compounds for 1 h. The cultures were then exposed to 100 μM glutamate for 30 min and washed. After 24 h incubation in the presence of compounds, the cultures were assessed for the extent of neuronal damage (throughout treatment shown in Figure 2). In some experiments, the cultures were treated with the appropriate phenylpropanoid, *E-p*-MCA, either 1 h before exposure (pre-treatment shown in Figure 2), or after exposure (post-treatment shown in Figure 2) to 100 μM L-glutamate for 30 min. After an additional 24 h incubation in the absence (pre-treatment) or presence (post-treatment) of *E-p*-MCA, the cultures were assessed by measuring LDH in the media. In some experiments, cultures were pre-treated with *E-p*-MCA 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 *E-p*-MCA (Figure 2). 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.

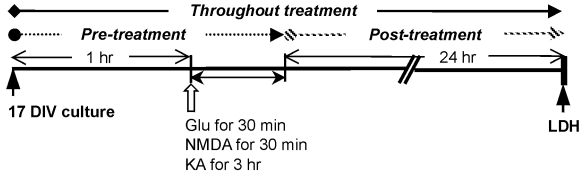

Figure 2 Experimental schedules.

Preparation of cerebral cortical membranes

Cerebral cortical membranes from adult male Sprague–Dawley rats (200−250 g) were prepared as described previously (Zhou *et al*., 1997). Cerebral cortices (whole brain minus cellebellum and brain stem) were collected in 0.32 M sucrose solution and homogenized (Omni glass homogenizer, setting 6, 30 s) in 35 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Homogenates were centrifuged at $850 \times g$ for 10 min at 4°C. The pellets were discarded, and the supernatant was re-centrifuged at $18{,}000 \times g$ for 20 min at 4°C. Supernatants were slowly decanted to retain the soft buffy coat on the pellet. Each pellet was briefly disrupted with a Polytron glass homogenizer in 35 ml of ice-cold filtered water. Homogenates were re-centrifuged at $7000 \times g$ at 4°C and the resulting pellet was washed three times by successive resuspension in 50 mM Tris-HCl and re-centrifugation at $20{,}000 \times g$ at 4°C. The final pellets were stored at −80°C until use. On the day of the assay, pellets were thawed and each was disrupted with the Omni homogenizer in 35 ml of the assay buffer at pH 7.4. Homogenates were incubated at 37°C for 30 min in a shaking water bath, followed by centrifugation at $40{,}000 \times g$ for 10 min at 4°C. This washing step without incubation was repeated three more times. For use in the assay, each pellet was resuspended using the homogenizer in 30 ml of assay buffer.

[Propyl-2, 3-^{3}H]-CGP39653, [^{3}H]-kainic acid or [2-^{3}H]-glycine binding

Assays were performed in a total volume of 500 μl containing membrane suspension (200 μg protein), [propyl-2,3-^{3}H]-CGP39653 (final concentration, 5 nM), [^{3}H]-kainate (final concentration, 5 nM) or [2-^{3}H]-glycine (final concentration, 10 nM) and the test compound, *E-p*-MCA. Non-specific binding was determined in the presence of 600 μM glutamate (for [propyl-2,3-^{3}H]-CGP39653 and [^{3}H]-kainate) and 10 μM 5,7-dichlorokynurenic acid (for [2-^{3}H]-glycine) as the binding antagonists. In some assays, membranes were pre-incubated with *E-p*-MCA at 4°C for 1 h before the addition of [propyl-2,3-^{3}H]-CGP39653, [^{3}H]-kainate or [2-^{3}H]-glycine. Alternatively, some membranes were incubated with *E-p*-MCA with the simultaneous addition of either [propyl-2,3-^{3}H]-CGP39653 or [2-^{3}H]-glycine. Assays were initiated by the addition of [propyl-2,3-^{3}H]-CGP39653 or [2-^{3}H]-glycine, incubated at 0°C for 30 min and then terminated by centrifugation for 5 min at $48{,}000 \times g$ (Beckman XL-100 ultracentrifuge, U.S.A.). This was followed by two 1-ml washes with ice-cold 50 mM Tris-HCl buffer. The radioactive pellets were then dissolved and counted in 3 ml of HydroSol scintillation cocktail at 54% efficiency in a Wallac system 1400 counter (An EG & G Co., Finland).

Measurement of nitrite and calcium contents

The level of nitric oxide (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. Intracellular calcium was determined by ratio fluorimetry using Ca^{2+} specific dye, Fura 2-AM (Kim *et al*., 1998). In brief, 1 h before exposure to 50 μM NMDA/10 μM glycine or 50 μM KA/10 μM MK-801, cultures grown on glass cover slides were treated with *E-p*-MCA 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. The change of $[Ca^{2+}]_i$ was measured 10 min after exposure to NMDA or KA. Cell culture slides were cut and mounted into spectrophotometer cuvettes containing 2.5 ml PBS (without bicarbonate). Fluorescence was measured with a spectrofluorometer by exciting cells at 340 and 380 nm and measuring light emission at 520 nm. Calcium concentration was calculated according to the method of Grynkiewicz *et al*. (1985).

Measurement of cellular peroxide

The relative level of free radicals, i.e. 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 by exciting cells with light at 485 nm and measuring light emitted at 530 nm.

DPPH and hydrogen peroxide radical scavenging assay

One hundred microliters of a 300 μM solution of DPPH in ethanol was added to 100 μl of a solution containing *E-p*-MCA 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 (Basly *et al*., 2000). To determine H_2O_2 radical scavenging activity, *E-p*-MCA was dissolved in 3.4 ml of 0.1 M phosphate buffer (pH 7.4) and mixed with 600 μl of a 43 mM solution of H_2O_2 prepared in the same buffer. The absorbance of reaction mixtures was recorded every 5 min up to 30 min at 230 nm. For each concentration, a separate blank sample (devoid of H_2O_2) was used for background subtraction. The concentration of H_2O_2 in the assay medium was determined using a standard curve. The H_2O_2-scavenging capacity of *E-p*-MCA was calculated as $100-((H_2O_2$ concentration of medium containing *E-p*-MCA/H_2O_2 concentration of the control medium) $\times 100)$ (Wettasinghe & Shahidi, 2000).

Protein assay

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 ANOVA and, if significant, group means were compared by *post hoc* analysis using Tukey multiple comparison of means. The confidence level for statistical significance was set at a probability value of 0.05.

Results

Structure-activity relationship of neuroprotective phenylpropanoids

We previously reported the isolation of 12 phenylpropanoids from the root of *Scrophularia buergeriana* Miquel (Scrophulariaceae) and the neuroprotective activity of these compounds *in vitro* (Kim & Kim, 2000). Therefore, we have investigated the structure-activity relationship of 12 phenylpropanoids (**1**–**12**) and a phenylalcohol (**13**) isolated from *S. buergeriana* with structurally-related compounds (**14**–**24**) using a test system consisting of primary cultures of rat cortical neurons injured with glutamate (see Table 1). In our culture system, MK-801 and CNQX, well-known positive controls against glutamate-induced neurotoxicity, showed effective neuroprotective activity at a concentration of 10 μM (Table 1). Interestingly, in our cultures, co-treatment 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 (84% neurons survived) or 10 μM CNQX alone (62% neurons survived). The potency of neuroprotective activity in the throughout treatment paradigm was in the order: buergeriside A_1 (**1**) $\geqslant$buergeriside B_1 (**2**) $\geqslant$*E-p*-MCA (**6**) $\geqslant$*E*-isoferulic acid (**16**) $\geqslant$*E*-3,5-dimethoxy-4-hydroxycinnamic acid (**17**) $>$buergeriside C_1 (**4**) $\geqslant$*E*-ferulic acid (**10**) $\geqslant$*E-o*-methoxycinnamic acid (**14**) $\geqslant$angoroside C (**11**) $>$*E-m*-methoxycinnamic acid (**15**) $\geqslant$buergeriside B_2 (**3**) $>$*E-p*-methoxycinnamoyl methyl ester (**7**) $>$*E-p*-coumaric acid (**8**) $>$*E*-caffeic acid (**9**). The neuroprotective potency for compounds other than those described above is not given because their ability to protect neurons in our assay system was lower than 50%. However, E_{max} of all compounds tested are listed in Table 1.

The neuroprotective activity of *E-p*-MCA against glutamate-induced toxicity was initially evaluated by a timed exposure to *E-p*-MCA before or after excitotoxic challenge. As shown in Table 2, *E-p*-MCA added as a pre-treatment at concentrations ranging from 100 nM to 10 μM significantly attenuated neurotoxicity induced by glutamate. The glutamate-induced increase in LDH release from cortical neurons (198 ± 10 mU ml^{-1}) was reduced after pre-treatment with 1.0 μM (126 ± 5 mU ml^{-1}) or 10.0 μM (134 ± 3 mU ml^{-1}). Furthermore, *E-p*-MCA protected primary cultures of rat hippocampal neurons against glutamate-induced neurotoxicity (Figure 3). Glutamate-induced morphological signs of necrosis including swelling of cell bodies and neurite fragmentation were prevented by pre-treatment with 1 μM *E-p*-MCA. However, the addition of *E-p*-MCA post-treatment did not effectively attenuate neurotoxicity induced by glutamate even at identical *E-p*-MCA concentrations (Table 2).

Table 1 Neuroprotective activities of phenylpropanoids (**1**–**18**) and acetophenones (**19**–**24**) on glutamate-challenged primary cultures of rat cortical cells[a]

Compounds	EC_{50} ($\times 10^{-6}$ M)	E_{max} (%)[b]
Buergeriside A_1 (**1**)	0.044±0.003	76.4±2.7*** at 1 μM
Buergeriside B_1 (**2**)	0.050±0.003	70.3±1.8*** at 1 μM
Buergeriside B_2 (**3**)	1.3±0.01	48.5±3.3** at 1 μM
Buergeriside C_1 (**4**)	0.11±0.001	77.6±3.6*** at 1 μM
E-cinnamic acid (**5**)	N.D.	38.5±1.5* at 1 μM
E-*p*-MCA (**6**)	0.059±0.003	78.8±3.9*** at 1 μM
E-*p*-methoxycinnamoyl methyl ester (**7**)	2.0±0.1	45.2±3.1** at 1 μM
E-coumaric acid (**8**)	14±0.5	48.5±3.3** at 10 μM
E-caffeic acid (**9**)	52±1	48.1±0.5** at 50 μM
E-ferulic acid (**10**)	0.12±0.05	66.8±2.8*** at 1 μM
Angoroside C (**11**)	0.18±0.006	62.1±4.2*** at 1 μM
Isoangoroside C (**12**)	2.6±0.4	43.5±2.6** at 1 μM
2-(3-hydroxy-4-methoxyphenyl) ethanol (**13**)	N.D.	32.9±4.4* at 10 μM
E-*o*-methoxycinnamic acid (**14**)	0.17±0.06	58.0±4.1** at 1 μM
E-*m*-methoxycinnamic acid (**15**)	1.2±0.04	57.8±3.1** at 10 μM
E-isoferulic acid (**16**)	0.064±0.003	79.8±3.3*** at 10 μM
E-3,5-dimethoxy-4-hydroxy-cinnamic acid (**17**)	0.071±0.005	66.1±1.5*** at 1 μM
E-cinnamaldehyde (**18**)	N.D.	24.9±3.8 at 1 μM
4-hydroxyacetophenone (**19**)	N.D.	14.4±3.2 at 10 μM
3,5-dimethoxyacetophenone (**20**)	N.D.	20.8±3.1 at 1 μM
3,5-dimethoxy-4-hydroxy-acetophenone (**21**)	N.D.	15.0±1.8 at 1 μM
Acetovanillone (**22**)	N.D.	24.9±4.4 at 10 μM
Syringic acid (**23**)	N.D.	29.3±1.5 at 10 μM
Syringaldehyde (**24**)	N.D.	21.3±1.2 at 10 μM
MK-801[c]	0.36±0.006	83.6±4.2*** at 10 μM
APV[d]	32±0.5	63.6±3.2*** at 50 μM
CNQX[e]	1.4±0.06	61.6±2.7*** at 10 μM

[a]Cortical cell cultures were washed with DMEM and incubated with compounds for 1 h. The cultures were then exposed to 100 μM glutamate for 30 min and washed. After 24 h incubation in the presence of compounds, the cultures were assessed for the extent of neuronal damage (throughout treatment). The values shown are the mean±s.e.mean of three experiments (5–6 cultures per experiment). [b]LDH released from control and glutamate-injured cultures measured 111±8 and 198±10 mU ml^{-1}, respectively. Protection (%) was calculated as 100×(LDH released from glutamate-injured cultures minus LDH released from glutamate+test compound-treated cultures)/(LDH released from glutamate-injured cultures minus LDH released from control cultures). [c]MK-801: dizocilpine maleate, non-competitive antagonist of NMDA receptor. [d]APV: competitive antagonist of NMDA receptor. [e]CNQX: non-NMDA receptor antagonist. Glutamate-treated value differs significantly from the untreated, control cultures at a level of $P<0.001$. *$P<0.05$, **$P<0.01$, ***$P<0.001$ *vs* glutamate-intoxicated cells (ANOVA and Tukey).

E-p-*methoxycinnamic acid protected neurons more effectively against neurotoxicity induced by NMDA than KA*

In order to reveal how *E*-*p*-MCA protected against glutamate-mediated injury, two excitotoxins, NMDA and KA were used to induce selective receptor-mediated neurotoxicity in primary cultures of rat cortical cells. Therefore, we investigated the neuroprotective activity of *E*-*p*-MCA against NMDA- or KA-induced neuronal degeneration using our *in vitro* screening system (Figure 4), since phenylpropanoids containing the *E*-*p*-MCA structure had shown such a significant degree of neuroprotection against neurotoxicity induced by glutamate.

E-*p*-MCA showed neuroprotective activity on cortical neurons regardless of whether NMDA or KA was used as neurotoxicant (Figure 4). However, *E*-*p*-MCA protected neurons more selectively against NMDA-induced neurotoxicity of primary cultured cortical cells than from KA-induced neurotoxicity. This finding was supported by separate studies which determined the $[Ca^{2+}]_i$ (Figure 5) and the content of nitric oxide (Table 3). NMDA-receptor activation, elicited by a brief exposure to glutamate or NMDA, causes a significant change in Ca^{2+} influx, followed by the activation of nitric oxide synthase and subsequent overproduction of NO (McDonald & Johnston, 1990). Thus, we determined the effect of *E*-*p*-MCA on the incremental change in $[Ca^{2+}]_i$ by excess NMDA or KA. As shown in Figure 5, Ca^{2+} influx provoked by excess NMDA was effectively blocked by pre-

Table 2 Neuroprotective activity of *E*-*p*-methoxycinnamic acid on glutamate-induced neurotoxicity in primary cultures of rat cortical cells

	Concentration (μM)	*Protection* (%) *Pre-treatment*[a]	*Post-treatment*[b]
Control		100.0±2.4[c]	
Glutamate-injured		0.0±0.8[d]	
E-*p*-MCA	0.01	20.1±2.7**	13.2±1.8*
	0.10	62.3±1.3**,†	18.4±0.9**
	1.00	82.3±1.7**,†	20.3±2.4**
	10.00	73.1±1.0**,†	25.4±1.2**

[a]Pre-treatment: *E*-*p*-MCA treatment was conducted from 1 h prior to the glutamate insult to glutamate wash-out.[b]Post-treatment: *E*-*p*-MCA was added from glutamate wash-out to end of experiment. [c]LDH released from control and glutamate-injured cultures measured 111±8 and 198±10 mU ml^{-1}, respectively. Protection (%) was calculated as described in the footnote of Table 1. The values shown are the mean±s.e.mean of three experiments (5–6 cultures per experiment). [d]Glutamate-injured cells differ significantly from the control at a level of $P<0.001$. *$P<0.01$. **$P<0.001$ *vs* glutamate-intoxicated cells; †$P<0.001$ *vs* each post-treatment of *E*-*p*-MCA (ANOVA and Tukey).

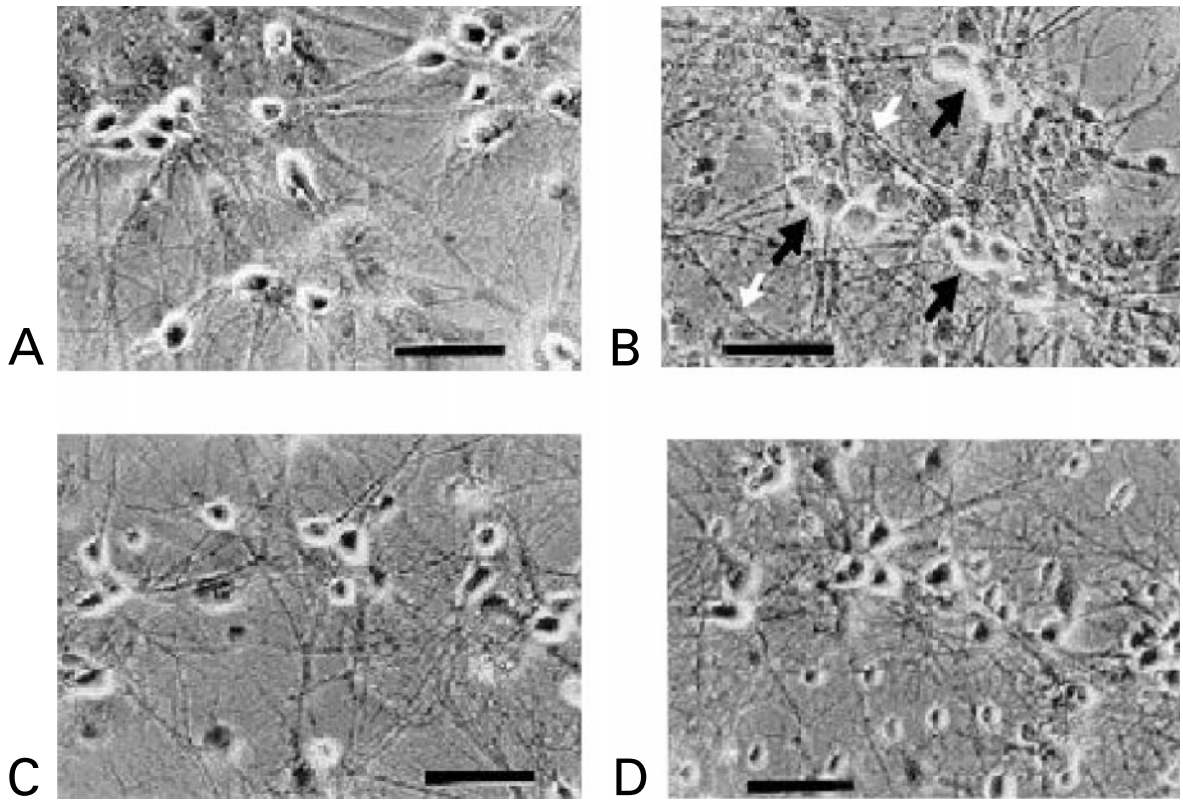

Figure 3 *E-p*-MCA protects primary cultures of rat hippocampal neurons against glutamate-induced neurotoxicity. Phase-contrast photographs of control neurons (A) and neurons exposed to 10 μM glutamate in the absence (B) or presence of 1 μM *E-p*-MCA (C) or 1 μM Mk-801 (D). Solid and empty arrows point to markedly swollen cell bodies and neurite fragmentation, respectively. Scale bar, 100 μm.

treatment with *E-p*-MCA. When *E-p*-MCA was used as a pre-treatment at a concentration of 1.0 μM, the $[Ca^{2+}]_i$ was 24.4$\pm$3.7% of that in NMDA-treated cells (Figure 5). However, *E-p*-MCA did not effectively block the increase of $[Ca^{2+}]_i$ induced by excess KA (Figure 5). The effect of *E-p*-MCA on preventing $[Ca^{2+}]_i$ influx induced by NMDA was comparable to that of MK-801, a non-competitive blocker of the NMDA receptor-channel. Furthermore, *E-p*-MCA significantly reduced over-production of NO that was attenuated by the NOS synthase inhibitor nitroarginine in cortical cells exposed to glutamate (Table 3).

E-p-*methoxycinnamic acid inhibited the binding of [propyl-2,3-^{3}H]-CGP39653 to NMDA receptor*

The effect of *E-p*-MCA on the binding of [propyl-2,3-^{3}H]-CGP39653 to NMDA receptors was studied using a cerebral cortical membrane preparation to investigate whether the neuroprotective activity of *E-p*-MCA was attributable to its binding to NMDA receptors. In our pilot study, *E-p*-MCA significantly inhibited [propyl-2,3-^{3}H]-CGP39653 binding to the receptors when cortical membranes were pre-incubated with the phenylpropanoid (Figure 6A). Using the pre-treatment paradigm, the inhibition of *E-p*-MCA on [propyl-2,3-^{3}H]-CGP 39653 (IC_{50}, 194$\pm$1.8 nM) was discovered to be less effective than that of APV, a competitive NMDA-

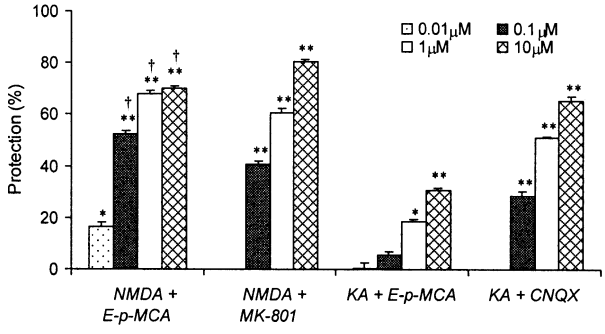

Figure 4 Neuroprotective activity of *E-p*-methoxycinnamic acid on NMDA or KA-injured rat cortical cells. Cortical cultures were pre-treated with *E-p*-MCA for 1 h. The cultures were then exposed to 50 μM NMDA and 10 μM glycine for 30 min or to 50 μM KA and 10 μM MK-801 for 3 h and then washed. The cultures were maintained for an additional 24 h in DMEM and assessed for the extent of neuronal damage by LDH assay. LDH release from control, NMDA or KA-injured cultures measured 111$\pm$8, 210$\pm$7 and 196$\pm$5 mU ml^{-1}, respectively. The values shown are the mean unit $\pm$s.e.mean of three experiments (5–6 cultures per experiment). Protection (%) was calculated as 100$\times$ (LDH released from NMDA-/KA-injured cultures minus LDH released from NMDA–/KA– + *E-p*-MCA-treated cultures)/(LDH released from NMDA–/KA-injured cultures minus LDH released from control cultures). NMDA- or KA-injured differs significantly from the control at a level of $P<0.001$. *$P<0.01$, **$P<0.001$ *vs* excitotoxin-intoxicated cells; †$P<0.001$ *vs* KA+ *E-p*-MCA (ANOVA and Tukey).

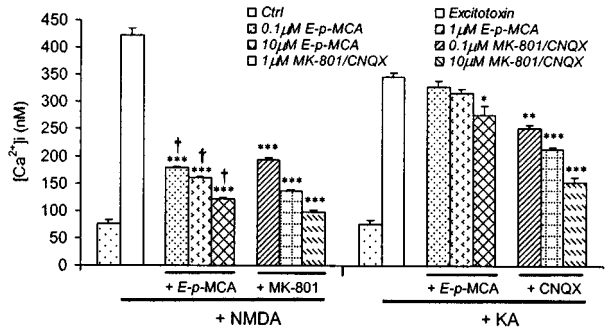

Figure 5 The effect of *E-p*-methoxycinnamic acid on intracellular $[Ca^{2+}]$ in injured rat cortical cells. One hour before exposure to 50 μM NMDA/10 μM glycine or 50 μM KA/10 μM MK-801, cultures grown on glass cover slides were treated with *E-p*-MCA and 5 μM Fura-2 AM in phosphate-buffered saline (PBS, pH 7.2) at 37°C in a humidified atmosphere of 95% air−5% CO_2. The change of $[Ca^{2+}]_i$ was measured 10 min after exposure to NMDA or KA. The values shown are the mean unit ±s.e.mean of three experiments (5–6 cultures per experiment). $^*P<0.05$, $^{**}P<0.01$ $^{***}P<0.001$ *vs* excitotoxin-intoxicated cells; †$P<0.001$ *vs* KA+*E-p*-MCA (ANOVA and Tukey).

Table 3 The effect of *E-p*-methoxycinnamic acid on nitrite content in glutamate-induced neurotoxicity

Compounds	*Concentration* (μM)	*Nitrite* (nM)
Control		262.5±10.8
Glutamate-injured cultures		1014.8±20.8
E-p-MCA	0.1	636.0±26.8**,††
	1.0	448.0±10.4**,†
	10.0	412.4±32.0**
N^{ω}-nitro-L-arginine[a]	0.1	815.2±15.2*
	1.0	569.2±14.0**
	10.0	444.4±16.4**

Cortical cultures were pre-treated with *E-p*-MCA 1 h before glutamate-induced neurotoxicity. The values shown are the mean unit±s.e.mean of three experiments (5–6 cultures per experiment). [a]N^{ω}-nitro-L-arginine is well-known NOS inhibitor. Glutamate-injured value differs significantly from control at a level of $P<0.001$. $^*P<0.01$, $^{**}P<0.001$ *vs* glutamate-intoxicated cells; †$P<0.01$, ††$P<0.001$ *vs* each value of N^{ω}-nitro-L-arginine-treated cells (ANOVA and Tukey).

antagonist (IC_{50}, 16±0.2 nM), or MK-801, a non-competitive NMDA-antagonist (IC_{50}, 46±0.9 nM). The mode of inhibition manifested itself as non-competitive as evidenced by having the same K_m value but an altered V_{max} value (Figure 6C). In a simultaneous incubation paradigm, the inhibitory effect of *E-p*-MCA on [propyl-2,3-^{3}H]-CGP 39653 binding to NMDA receptors was found to be decreased (*E-p*-MCA: IC_{50}, 6700±20.1 nM; APV: IC_{50}, 387±4.2 nM, MK-801: IC_{50}, 586±2.8 nM). In a separate study, we also investigated the effect of *E-p*-MCA on the binding of [^{3}H]-kainate to non-NMDA receptors. However, *E-p*-MCA did not show any significant effect on the binding of [^{3}H]-kainate to its receptors up to 10 μM ($IC_{50}>100$ μM, Figure 6B).

In previous reports, a non-competitive antagonist against glutamate was subsequently found to appear as a competitive antagonist against glycine (Corsi *et al*., 1996; Quartaroli *et al*., 1999). Therefore, the effect of *E-p*-MCA on [2-^{3}H]-glycine binding was investigated. In our preliminary study, *E-p*-MCA significantly inhibited [2-^{3}H]-glycine binding to its site when cortical membranes were pre-incubated with *E-p*-MCA (Figure 6B). Using a pre-incubation paradigm, the inhibition of *E-p*-MCA on [2-^{3}H]-glycine (IC_{50}, 260±4.1 nM) was discovered to be less effective than inhibition by glycine (IC_{50}, 132±1.7 nM). The mode of inhibition was competitive as evidenced by having the same V_{max} value but an altered K_m value (Figure 6D). However, the inhibitory effect of *E-p*-MCA on [2-^{3}H]-glycine to its binding sites was found to be slightly decreased when used in a simultaneous incubation paradigm (*E-p*-MCA: IC_{50}, 700±5.8 nM; GLYCINE: IC_{50}, 287±3.1 nM).

Cellular oxidation was decreased by E-p-*methoxycinnamic acid*

We measured the effect of *E-p*-MCA on the content of cellular peroxides using the specific fluorescent dye, 2,7-DCF-DA (Figure 7). Glutamate-induced toxicity is known to involve such free radicals as hydroxyl radicals and superoxide anions (Lafon-Cazal *et al*., 1993; Gilgun-Sherki *et al*., 2001). Indeed, *E-p*-MCA effectively reduced cellular peroxides in cultured rat cortical cells exposed to glutamate. When cultured cortical cells were affected by excess glutamate, the cellular peroxide content was increased 3 h after glutamate challenge. However, the pre-treatment with *E-p*-MCA significantly attenuated the formation of cellular peroxide up to 24 h after glutamate challenge. Therefore, we determined direct free radical scavenging activity of *E-p*-MCA using H_2O_2 and DPPH radicals. We found that *E-p*-MCA did not directly scavenge either of these free radicals in our studies ($IC_{50}>100$ μM).

E-p-*Methoxycinnamic acid increased intracellular* Ca^{2+} *and cellular peroxide in normal cultures*

In spite of the inhibitory effect of *E-p*-MCA on Ca^{2+} influx in the presence of excess glutamate, when primary cultures of rat cortical cells were pretreated with *E-p*-MCA at concentrations ranging from 0.1 to 10 μM, the $[Ca^{2+}]_i$ was significantly elevated (control: 75±15 nM; 0.1 μM *E-p*-MCA-treated: 166±4 nM; 1.0 μM *E-p*-MCA-treated: 269±8 nM; 10 μM *E-p*-MCA-treated: 305±2 nM). The increment of intracellular Ca^{2+} induced by *E-p*-MCA was completely blocked by pre-treatment of 10 μM MK-801 (data not shown). Further examination of cellular oxidation in primary cultures of rat cortical cells suggested that *E-p*-MCA might facilitate the production of peroxide followed by the increase of Ca^{2+} influx (control: 75±15; 10 μM *E-p*-MCA-treated: 367±7 arbitrary units). However, *E-p*-MCA did not affect cell viability.

Discussion

Numerous natural products have been used by Asian societies in treating neurodegenerative disorders such as stroke (Shanghai Acad Sci & Shogaku Kan, 1985). However, there is no scientific evidence for their effectiveness, nor has a systematic screen of the components of these products been undertaken. This study has been performed in an attempt to

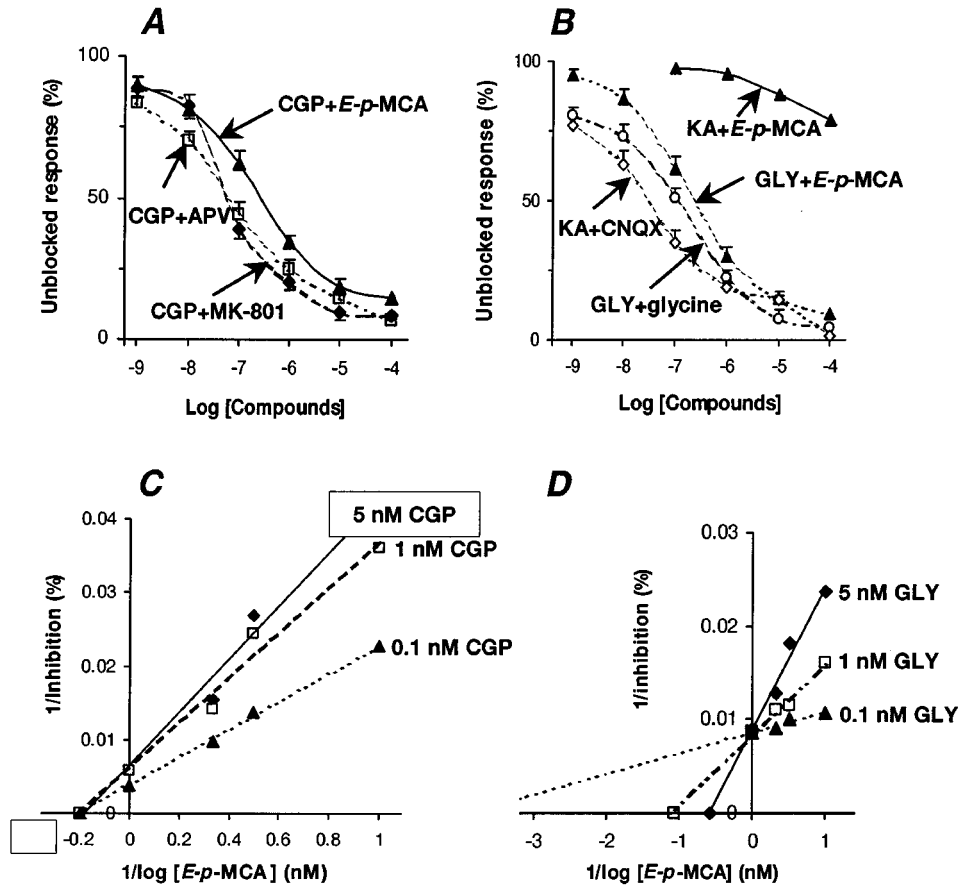

Figure 6 *E-p*-MCA is a glutamatergic antagonist. Concentration-inhibition curves for [propyl-2,3-^{3}H]-CGP39653 (CGP) [^{3}H]-kainate (KA) or [2-^{3}H]-glycine (GLY) binding by *E-p*-MCA, APV or MK-801 (A), CNQX or glycine (B). Lineweaver-Burk plot of 1/inhibition *vs* 1/log [*E-p*-MCA] in the presence of 5, 1, or 0.1 nM [propyl-2,3-^{3}H] CGP39653 (C) or [2-^{3}H]-glycine (D).

elucidate the action mechanism of an active compound isolated from natural products, *E-p*-MCA, *in vitro*. On the basis of the results shown in Table 1, we concluded that the presence of the α,β-unsaturated carboxyl moiety in phenylpropanoids played a very important role in neuroprotective activity. Indeed, cinnamaldehyde (**18**) and acetophenone derivatives (**19**–**24**) which have no α,β-unsaturated carboxyl moiety in the structure showed less neuroprotective activity as compared to the compounds containing the moiety. Furthermore, the neuroprotective indices of phenylpropanoids with a *trans*-double bond are more potent than those with a *cis* form (**3** and **12**) or with the phenylalcohol, α,β-saturated form (**13**). Moreover, where the aromatic *para*-methoxy group is linked to an α,β-unsaturated carboxyl moiety, the compound exerted stronger neuroprotective activity than either unsubstituted or *para*-hydroxy substituted structures which showed weaker neuroprotective indices as in the cases of *E*-cinnamic acid (**5**), *E-p*-coumaric acid (**8**) and *E*-caffeic acid (**9**). However, aromatic substitution with methoxy groups at C-2 or C-3 showed little or no effect on potentiation of neuroprotective activity. As such, *E-p*-MCA (**6**) showed stronger neuroprotective activity than either *E-m*-methoxycinnamic acid (**15**) or *E-o*-methoxycinnamic acid (**14**). Thus, it appears that *E-p*-MCA (**6**) is a crucial structure for providing a neuroprotective effect.

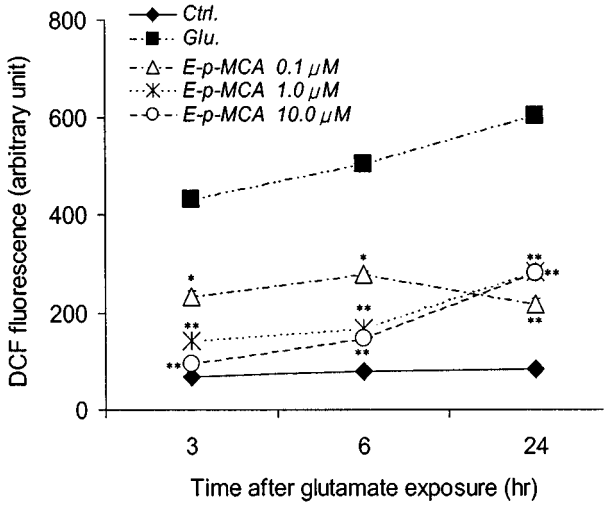

Figure 7 *E-p*-MCA reduces cellular oxidation in primary cultures of rat cortical cells. *E-p*-MCA was pretreated 1 h prior to glutamate-insult. The relative content of intracellular peroxide was determined using fluorescent dye, 2,7-DCF-DA. Glutamate-injured values significantly differ from control, $P<0.001$, $^*P<0.01$, $^{**}P<0.001$ *vs* glutamate-intoxicated cells (ANOVA and Tukey).

Glutamate produces its effects by acting mainly on NMDA receptors and, to a minor extent, on other receptor subtypes such as non-NMDA receptors (Lipton & Rosenberg, 1994). NMDA receptor activation elicited by a brief exposure to glutamate or NMDA causes a significant change in Ca^{2+} influx, the activation of nitric oxide synthase and subsequent overproduction of NO (McDonald & Johnston, 1990). The neuroprotective activity of *E-p*-MCA in the pre-treatment paradigm shows that *E-p*-MCA-treatment can alleviate toxicity in cells provided that a considerable amount of time has not passed after the excitotoxic challenge. In the case of non-NMDA mediated excitotoxicity, even though the initial sodium-dependent phase of the damage is past, and while intracellular calcium levels have already become raised, neuroprotective activity of CNQX, a non-NMDA antagonist, can still be observed in the post-treatment paradigm (Prehn *et al*., 1995). Since *E-p*-MCA did not show significant neuroprotective activity in the post-treatment paradigm, *E-p*-MCA might not be an inhibitor of non-NMDA receptor-mediated cellular responses. Indeed, *E-p*-MCA protected neurons more selectively against NMDA-induced than KA-induced neurotoxicity in primary cultured cortical cells. Therefore, it is likely that the neuroprotective activity via *E-p*-MCA might be attributed primarily to the protection of NMDA receptors. Such view was further confirmed by results showing that *E-p*-MCA had affinity for NMDA receptors and inhibited excess Ca^{2+} influx and NO overproduction. In recent papers, substituted cinnamides showed high affinity for NMDA receptors (Tamiz *et al*., 1998; Moloney, 1999). The structural similarity between *E-p*-MCA and cinnamides further supports the possibility that *E-p*-MCA binds to NMDA receptors.

NMDA receptors are encoded by three gene families which give rise to at least six types of subunit; NR1, NR2A, NR2B, NR2C, NR2D and NR3A that have four different drug binding sites (Nakanishi *et al*., 1990; Ciabarra *et al*., 1995). It is known that NMDA receptors carry two glutamate binding sites whose simultaneous occupation results in receptor activation and the opening of ion channels (Doble, 1999). NMDA receptor activation by glutamate is only possible when the glycine site is activated (Corsi *et al*., 1996). The binding site for glycine has been identified on the NR1 subunit (Williams *et al*., 1996), whereas the recognition site for glutamate and NMDA has been shown to be on the NR2 subunit (Laube *et al*., 1997). The competitive NMDA antagonist CGP 39653 used in our NMDA receptor binding studies has selective affinity for NR1/NR2A > NR1/NR2B > NR1/NR2C > NR1/NR2D (Sucher *et al*., 1996). *E-p*-MCA inhibited [propyl-2,3-^{3}H]-CGP39653 binding to NMDA receptors in a non-competitive manner (see Figure 6C). This result suggested that *E-p*-MCA might act as a competitive antagonist to glycine (Quartaroli *et al*., 1999). Indeed, *E-p*-MCA inhibited [2-^{3}H]-glycine binding to its binding site in a competitive manner despite its relatively low affinity for glycine binding sites (see Figure 6D). Therefore, the significant neuroprotective activity of *E-p*-MCA against NMDA-induced toxicity suggests that *E-p*-MCA might interfere with co-agonist binding to glycine sites as well as with agonist binding of the NMDA receptor. However, *E-p*-MCA was expected to be a partial NMDA/glycine antagonist since the inhibitory effect of *E-p*-MCA on [propyl-2,3-^{3}H]-CGP 39653 binding or [2-^{3}H]-glycine to its receptors was decreased when used in a simultaneous incubation paradigm. Even high concentrations (up to 1 mM) of *E-p*-MCA failed to inhibit completely [propyl-2,3-^{3}H]-CGP39653 and [2-^{3}H]-glycine binding (data not shown). These findings differ from those reported for 7-chlorokynurenic acid which has been shown to be a full antagonist of glycine receptors (Kemp *et al*., 1988). These results were similar to those previously reported for HA-966 which appeared to be a low-efficacy, partial agonist at glycine sites (Fletcher & Lodge, 1988; Foster & Kemp, 1989; Singh *et al*., 1990). Accordingly, *E-p*-MCA therefore should be considered a low-efficacy, partial antagonist rather than a full antagonist.

In spite of the inhibitory effect of *E-p*-MCA on Ca^{2+} influx in the presence of excess glutamate, when primary cultures of rat cortical cells were pre-treated with only *E-p*-MCA, the $[Ca^{2+}]_i$ and the production of cellular peroxide were significantly elevated in our separate preliminary studies. It is assumed that *E-p*-MCA might act as a glutamatergic agonist in the absence of glutamate and an antagonist in the presence of glutamate. Alternatively, these effects of *E-p*-MCA might arise through actions on cellular calcium and/or redox homeostasis.

The major potential therapeutic applications of agonists and antagonists acting on glutamate receptors are acute stroke, Alzheimer's disease, depression, drug dependency, epilepsy, pain, Parkinson's disease and schizophrenia (Trist, 2000). All three types of NMDA antagonists, viz. channel blocker, competitive NMDA antagonist, and competitive glycine antagonist, show marked efficacy in animal models and humans (Meldrum & Garthwaite, 1990). NMDA receptor antagonists and channel blockers have a number of side effects, most of which are predictable from the physiological roles of NMDA receptors. However, despite the ability of glycine antagonists to completely block glutamate responses, the therapeutic window of these agents is considerably wider than that of the first two types of antagonists (Danysz & Parsons, 1998). These results encourage the view that a glycine antagonist will be of use in acute stroke (Doble, 1999). In conclusion, neuroprotection by partial glycine antagonism as observed with *E-p*-MCA may prove useful in prevention and/or treatment of neurodegenerative diseases such as stroke and Alzherimer's disease.

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