Note

Protective Effect of 2′,3′-Dihydroxy-4′,6′-dimethoxychalcone on Glutamate-Induced Neurotoxicity in Primary Cortical Cultures

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We have previously isolated 2′,3′-dihydroxy-4′,6′-dimethoxychalcone (DDC) from green perilla leaves as the activator of the nuclear factor erythroid 2-related factor 2 (Nrf2)–antioxidant response element (ARE) pathway. This study aims to evaluate the effects of DDC against glutamate neurotoxicity using rat primary cortical cultures. Treatment of cultures with DDC for 24h before glutamate exposure significantly inhibited glutamate neurotoxicity in a concentration-dependent manner. The involvement of hemeoxygenase-1 (HO-1) and reduced glutathione (GSH) in the protective effects of DDC on cortical cultures was also evaluated. While an HO-1 inhibitor did not have a significant effect on DDC-induced neuroprotection, a γ-glutamylcystein synthetase (γ-GCS) inhibitor significantly suppressed the protective effect of DDC. In an astrocyte culture, DDC induced a marked increase in the levels of intracellular reduced GSH. These results suggest that DDC mainly activates the Nrf2–ARE pathway of astrocytes, resulting in the increased extracellular release of reduced GSH, protecting neurons from glutamate neurotoxicity.

Key words glutamate; 2′,3′-dihydroxy-4′,6′-dimethoxychalcone; neurotoxicity; nucleus erythroid p45-related factor-2–antioxidant response element pathway; glutathione

INTRODUCTION

Oxidative stress is one of the risk factors of neuronal death in various central nervous system diseases, such as cerebral ischemia and Alzheimer’s disease.1–4) The nuclear erythroid p45-related factor-2 (Nrf2)–antioxidant response element (ARE) pathway is known as one of the defense mechanisms of living beings for adapting to oxidative stress. Antioxidant enzymes, such as hemeoxygenase-1 (HO-1) and γ-glutamylcystein synthetase (γ-GCS), are induced by Nrf2–ARE pathway activation.5,6) Therefore, substances that activate this pathway have been reported to have neuroprotective effects, and their effectiveness in preventing the onset and progression of various neurodegenerative diseases have been identified.7,8) Searching for food-derived components that could activate the Nrf2–ARE pathway, lead to the isolation of 2′,3′-dihydroxy-4′,6′-dimethoxychalcone (DDC) from green perilla.9) We have previously reported that DDC exerts a cytoprotective effect against oxidative stress by activating the Nrf2–ARE pathway and inducing antioxidant enzymes in PC12 cells.9) However, no study on its action on cerebral cortical cells has been conducted, and many points remain unclear. Therefore, the present study focuses on glutamate neurotoxicity, which is reported to be one of the factors causing oxidative stress to central neurons, leading to cell death,3,10,11) using rat primary cortical neuronal cultures and examines the protective effect of DDC as well as its mechanism of action.

MATERIALS AND METHODS

All animal experiments were conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee and Japanese Pharmacological Society. All procedures were approved by the Animal Research Committee of the Graduate School of Pharmaceutical Science, Kyoto University.

Primary Cortical Neuronal Cultures Cerebral cortex tissues were isolated from embryonic Wistar/ST rat fetuses on embryonic days 17–19 and seeded onto plates coated with 0.1% polyethylenimine. Primary cultures were maintained in Eagle’s minimum essential medium containing 10% fetal bovine serum (FBS) at 37°C in 5% CO2.

Primary Cortical Astrocytes Cerebral cortex tissues were isolated from 0- to 1-d-old Wistar/ST rats and cultured at 37°C in Eagle’s minimum essential medium containing 10% FBS in an atmosphere containing 5% CO2. Approximately 3 weeks after culturing, enrichment was performed using the shaking method, and cells were cultured for 7–14 d prior to the experiments.

Neurotoxicity Evaluation Neuron survival rates were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase release assays. The protective effects on neurons were expressed as the differences in survival rates between treatments with DDC and glutamate treatment groups.

Western Blot Cell lysates from DDC-cultured cells were
obtained using lysis buffer. The amount of protein was standardized for each sample before sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) was performed. The proteins were separated via SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.), incubated with anti-HO-1 antibody (Code #ADI-SPA-895, purchased from Enzo Biochem Inc., Stressgen, Victoria, Canada) and secondary antibody (anti-rabbit immunoglobulin G (IgG) horseradish peroxidase-linked whole antibody), and detected using an enhanced chemiluminescence detection system (GE Healthcare). Band intensities were measured using ImageJ software (National Institutes for Health, Bethesda, MD, U.S.A.).

**Immunocytochemistry** After fixation of treated cells with 4% paraformaldehyde, cell membrane permeabilization was performed using 1% Triton-X-100-containing phosphate buffered saline (PBS). It was then incubated with each antibody and examined under a confocal microscope.

**Measurement of Intracellular Reduced Glutathione (GSH) Content** Cells were incubated with 50 µM monochlorobimane and lysed using a buffer containing 1% Triton-X-100. Fluorescence intensity was measured ($E_c/E_m = 355/460$ nm).

**Statistical Analysis** All data are expressed as the mean ± standard error. Statistically significant differences were evaluated using the Tukey test in a one-way ANOVA. Statistical significance was inferred when the risk factor was lower than 5%.

**RESULTS**

**Protective Effect of DDC against Glutamate Neurotoxicity** In a rat primary cortical neuronal culture, pretreatment with DDC (3–30 µM) for 24 h showed a significant protective effect against glutamate neurotoxicity at concentrations of 10 µM and higher (Fig. 1A). When DDC (30 µM) treatment time was changed, DDC was subjected to 24 h of glutamate treatment, and a significant protective effect was observed after glutamate treatment in addition to 24-h pretreatment. The protective effects of both were comparable. On the other hand, simultaneous treatment with DDC and glutamate did not show a protective effect (Fig. 1B).

**Increased Expression of HO-1 by DDC** It has been reported that the activation of the Nrf2–ARE pathway increases the expression of the antioxidant enzyme HO-1.12) To elucidate the neuroprotective mechanism of DDC against glutamate toxicity, the effects of DDC on HO-1 expression were examined. When cells were treated with DDC (10–30 µM) for 24 h, HO-1 expression increased in a concentration-dependent manner (Fig. 2).

**Changes in Intracellular GSH Levels by DDC and Protective Effect of Reduced GSH against Glutamate Neurotoxicity** It has been reported that the activation of the
Nrf2–ARE pathway induces γ-GCS, a rate-limiting enzyme for intracellular GSH synthesis, and increases the amount of GSH.\(^{13}\) We examined the effect of DDC on the amount of intracellular GSH. In cortical neuronal cultures, DDC (3–30 µM) tended to increase intracellular GSH content (Fig. 3A). It also markedly increased the amount of intracellular GSH in cortical astrocyte cultures (Fig. 3B). Next, we examined the effect of GSH added to the medium, extracellularly. A significant protective effect was observed by the addition of GSH (Fig. 3C).

**DISCUSSION**

The present study reveals that DDC, which activates the Nrf2–ARE pathway, has a marked protective effect against glutamate neurotoxicity in rat primary cortical neuronal cultures. Glutamate neurotoxicity is mainly triggered by excessive intracellular calcium influx via the N-methyl-D-aspartate receptor and is caused by downstream activation of neuronal nitric oxide synthase (nNOS).\(^{14}\) It has been pointed out that NO causes oxidative stress. The protective effect of DDC against glutamate neurotoxicity in neuronal cultures required pretreatment; however, no protective effect was observed after simultaneous treatment, suggesting that changes in the intracellular environment, such as the induction of antioxidant enzymes via the Nrf2–ARE pathway, are essential for the expression of the protective effect of DDC.

Our data suggest that astrocytes play an important role in the expression of the protective effects of DDC. When the amount of reduced GSH was measured, DDC markedly increased in cortical astrocyte cultures. These results suggest that the activation of the antioxidant mechanism via the Nrf2–ARE pathway by DDC mainly occurs in the astrocytes.
of DDC against glutamate neurotoxicity in cerebral cortical cells was not suppressed by ZnPP IX, an HO-1 inhibitor. However, buthionine sulfoximine (BSO), a γ-GCS inhibitor, significantly suppressed the protective effect of DDC. In addition, extracellularly-applied reduced GSH prevented glutamate neurotoxicity. These results suggest that an increase in reduced GSH greatly contributes to the protective effect of DDC on glutamate neurotoxicity. This is consistent with our previous reports that increased intracellular GSH levels via the NrF2–ARE pathway protects against oxidative stress in cultured striatal cells.10)

In conclusion, DDC mainly activates the NrF2–ARE pathway of astrocytes in primary cortical neuronal cultures, resulting in the increased extracellular release of reduced GSH, thus protecting neurons from glutamate neurotoxicity.

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Conflict of Interest The authors declare no conflict of interest.

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