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Chlorogenic acid, a polyphenol in coffee, protects neurons against glutamate neurotoxicity

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ABSTRACT

Aims: The present study has been designed to explore the molecular mechanism of chlorogenic acid (CGA) in the protective effect against glutamate-induced neuronal cell death.
Main methods: Cortical neurons in primary culture were exposed to 300 μM L-glutamic acid or vehicle, with or without 10 μM CGA or 10 μM MK-801. After 16 h, primary cultures were stained with propidium iodide (PI)/Hoechst or calcein. Double-staining with PI and Hoechst was performed to confirm whether cell death induced by glutamate was apoptotic. In addition, intracellular concentrations of Ca^{2+} were observed using Ca^{2+} indicator fura-2.
Key findings: We investigated the protective effects of CGA on glutamate-induced neuronal cell death using primary cultures of mouse cerebral cortex because the release of glutamate during brain ischemia triggers death of neurons. Glutamate-induced neuronal cell death was inhibited by treatment with CGA. In addition, CGA prevented the increase in intracellular concentrations of Ca^{2+} caused by the addition of glutamate to cultured neurons. On the other hand, there was little effect of CGA on cell death induced by nitric oxide, which is downstream of the ischemic neuronal cell death. Our results suggested that the polyphenol CGA in coffee protects neurons from glutamate neurotoxicity by regulating Ca^{2+} entry into neurons.
Significance: CGA in coffee may have clinical benefits for neurodegenerative diseases such as ischemic stroke.

1. Introduction

Ischemic stroke is a leading cause of human death and disability around the world. There is considerable interest in neuroprotection for ischemic stroke, as indicated by the number of experimental papers and clinical articles published in recent years [9].

Previous epidemiological studies have reported that coffee consumption reduces the risk of stroke [7,24,29]. Since coffee is a complex chemical mixture [13], the molecular mechanisms of its anti-ischemic properties are poorly understood. Chlorogenic acid (CGA) is one of the most abundant polyphenol compounds in coffee [48]. CGA has a number of beneficial biological effects such as anti-inflammatory, anti-cancer, and anti-oxidative activities [13,48]. CGA is one of the candidate components in coffee to reduce the risk of ischemic damage because of its neuroprotective effect in PC12 cells [1,28,38].

In a rat model of transient middle cerebral artery occlusion (MCAO), intraperitoneal administration of CGA reduced infarct volume and sensory-motor functional deficits [26]. To understand the molecular mechanism of CGA in the protective effect against ischemic injury, further investigations were necessary.

Aberrant Ca^{2+} dynamics have a well-established role in ischemic cell death. A coupling has been demonstrated among glutamate release, Ca^{2+} influx, and enhanced production of nitric oxide (NO) [21,23]. The release of glutamate during brain anoxia or ischemia triggers the death of neurons [3,37]. Glutamate-induced neurotoxicity is Ca^{2+}-dependent and it is mainly mediated through the activation of *N*-methyl D-aspartate (NMDA) receptor [2]. NMDA receptors amplify Ca^{2+} influx into neurons resulting in Ca^{2+}-dependent activation of death-signaling proteins which in turn trigger signaling cascades of neuronal death [40]. Indeed, brain injury after MCAO is significantly milder in mice treated with neuronal nitric oxide synthase (nNOS) inhibitors and in nNOS-deficient mice [14]. In addition, NO releases Ca^{2+} from endoplasmic reticulum through an *S*-nitrosylation of the type 1 ryanodine receptor (RyR1) channel [17,18]. NO-induced cell death was attenuated by dantrolene, an inhibitor of RyR1, and dantrolene had a protective effect on ischemic brain injury in the MCAO model [17].

To elucidate the mechanism of the anti-ischemic injury effect of CGA, we evaluated the effects of CGA on glutamate- or NO-induced cell death. CGA protected neurons from glutamate-induced neuronal cell death. On the other hand, CGA had little effect on NO-induced cell

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death, which is downstream of glutamate signaling in ischemia. CGA protects neurons by inhibiting excess increase of Ca^{2+} influx through glutamate receptors. Our results suggest that CGA could protect neurons from glutamate neurotoxicity by regulating the concentrations of Ca^{2+}. CGA may be a potent therapeutic agent for the prevention of neuronal cell death caused by ischemic stroke.

2. Materials and methods

2.1. Chemicals

CGA hemihydrate, D (+)-glucose, HEPES, magnesium chloride ($MgCl_2$) and sodium chloride (NaCl) were purchased from Nacalai Tesque (Kyoto, Japan). Calcium chloride dehydrates and L-Glutamic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl) and trypsin were purchased from Wako Pure Chemicals (Osaka, Japan). Calcein-AM, Hoechst 33342, NOC12 and propidium iodide (PI) were purchased from Dojindo (Kumamoto, Japan). MK-801 was purchased from Tocris Bioscience (Minneapolis, MN, USA).

2.2. Cerebral neuronal culture

All animal-related procedures were in accordance with the guidelines of The University of Tokyo. Neurons were prepared from the cerebral cortices of C57BL6/NCr mouse fetuses (embryonic day 16; Japan SLC, Shizuoka, Japan) based on a modification of a previously described procedure [17,19]. Briefly, dispersed cells were plated at 1.0×10^5 cells/cm^2 on 48 well plates coated with poly-L-lysine and laminin (Sigma-Aldrich). Cells cultured for 9 days were used for experiments.

2.3. Analysis of neuronal cell death

Neurons in primary culture were exposed to 300 μM L-glutamic acid or vehicle, with or without 10 μM CGA or 10 μM MK-801. After 16 h, primary cultures were stained with calcein-AM or PI and Hoechst 33342 and observed by fluorescent microscopy as described previously [17,19]. Cell death was expressed as the number of PI-positive cells divided by that of Hoechst 33342-positive cells.

2.4. Ca^{2+} imaging

Ca^{2+} imaging was carried out based on a modification of a previously described procedure [17,35]. Image analyses were carried out using ImageJ (National Institutes of Health, Bethesda, MD, USA). We used the F345/F380 ratio (R; the value of F at an excitation wavelength of 345 nm divided by the value of F at an excitation wavelength of 380 nm).

2.5. Statistical analysis

All statistical analyses of the data were carried out based on a modification of a previously described procedure [17,33]. Comparisons between two groups were made with Student's *t* test. Differences among multiple groups were analyzed with one-way analysis of variance (ANOVA). *Post hoc* multiple comparisons were made using the Bonferroni/Dunn test.

3. Results

3.1. CGA inhibits glutamate-induced neuronal cell death, but does not affect NO-induced neuronal cell death

To examine the neuroprotective effect of CGA against glutamate-induced cell death, we first observed the morphological changes upon exposure to glutamate measured by differential interference contrast images. After 9 days *in vitro* (DIV) growth, neurons in primary culture showed normal healthy morphology (Fig. 1A). The concentration dependence of glutamate toxicity has been studied in neuronal cultures [2,11]. Five minutes exposure to only 100 μM glutamate can produce neuronal injury [2], and EC_{50} was found to be approximately 300 μM [8]. Higher concentrations of glutamate can induce complete neuronal cell death. However, we sought to assess milder forms of excitotoxicity and therefore applied 300 μM glutamate, which induced swelling of treated neurons within 16 h of the exposure and degradation of the neurite network (Fig. 1A).

Morphometric analysis of developing primary culture neurons revealed that 10 μM of CGA is effective in promoting neurite growth [16]. The morphological changes of neurons treated with glutamate in the presence of CGA were weaker than that of vehicle-treated and MK-801-treated neurons (Fig. 1A). Furthermore, double-staining with PI, which indicates condensed chromatin damaged cells, and Hoechst 33342 was also performed to confirm whether cell death induced by glutamate was apoptotic (Fig. 1B). After application of glutamate, there was a significant increase in PI positive cells. However, in the presence of CGA, glutamate-induced neuronal cell death was attenuated. Treatment with MK-801 also greatly inhibited glutamate-induced cell death (Fig. 1C). These results indicated that CGA protects neurons from glutamate-induced neuronal cell death.

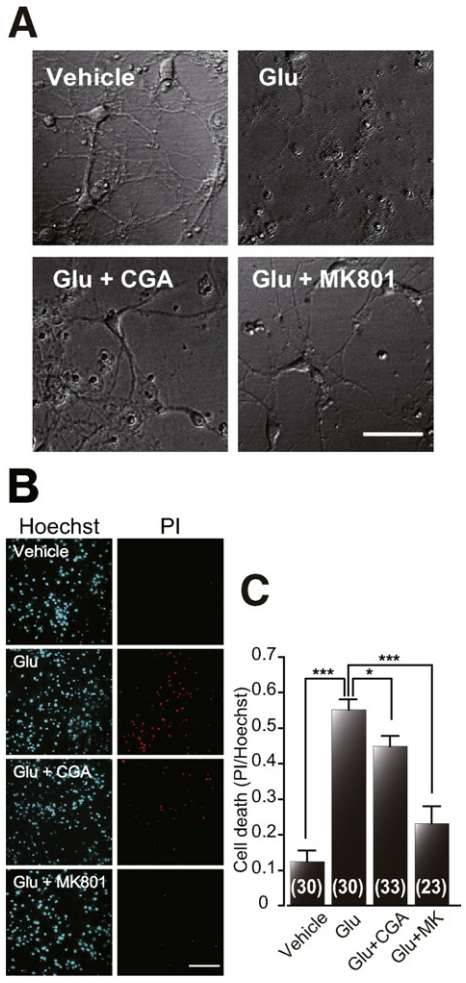

Fig. 1. Effects of CGA on glutamate-induced neuronal cell death. (A) Neuronal cell death assayed 16 h after treatment with 300 μM glutamate (Glu) without or with 10 μM CGA or MK-801 in cultured cerebral neurons of mice. Scale bar, 20 μm. (B) The extent of cell death was expressed as a ratio of the number of PI-positive cells to that of Hoechst-positive cells. Scale bar, 50 μm. (C) Numbers in parentheses (23–33) indicate the number of determinations in each condition using different cultures. Data are expressed as mean ± s.e.m. *, $p < 0.05$; ***, $p < 0.0001$, *t*-test compared with treatment of 300 μM glutamate.