Pretreatment of curcumin protects hippocampal neurons against excitotoxin-induced cell death

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Curcumin is a natural phenolic yellow curry spice, derived from the tumeric, which has been used for the treatment of diseases associated with oxidative stress and inflammation. Curcumin is known to have both anti-oxidative and anti-inflammatory properties. These properties can be beneficial to protect the brain from the neurodegenerative diseases. We now report the neuroprotective effects of curcumin pretreatment in primary hippocampal neurons to glutamate-induced excitotoxicity. Pretreatment of embryonic mouse hippocampal cell cultures with low does of curcumin protected neurons against glutamate-induced death, however, this neuroprotection was not correlated with the modulation of oxidative stress. Interestingly, high dose of curcumin showed the cytotoxicity in primary cultured hippocampal neurons. Immunoblot analyses showed that levels of stress response protein HSP70 were significantly elevated in neurons exposed to low dose of curcumin, whereas levels of cleaved PARP were increased in neurons exposed to high dose of curcumin. These findings show that curcumin can modulate neuronal responses to glutamate, and suggest possible use of curcumin and related compounds in the prevention and/or treatment of neurodegenerative disorders.

Key words – Apoptosis; curcumin; calcium; glutamate; hippocampal neuron; oxidative stress

Introduction

Curcumin (diferuloylmethane) is a naturally occurring phenolic yellow curry pigment, isolated from the rhizomes of the plant *Curcuma longa* Linn, that has traditionally been used in India for the treatment of diseases associated with injury and inflammation[15]. It has been reported that Alzheimer's disease in patients between 70 and 79 years old of India is much less than that of the United States[7]. Several other epidemiologic studies also indicated that there are a plenty of biological and pharmacological properties of curcumin, including anticarcinogenic[2], and anti-inflammatory[1], and anti-oxidative effects[5]. In particular, curcumin has been shown to reduce oxidative damage and amyloid pathology in Alzheimer's disease. Since the oxidative stress is one of major causes in the brain, the potential neuroprotective effects of curcumin might be due to its antioxidative property to protect the brain from free radical-induced damage. Moreover it has been reported that low dose of curcumin effectively disaggregates amyloid β-protein (Aβ) as well as prevents fibril and oligomer formation, supporting the preventative effects of curcumin in neurodegenerative diseases[6,22,27].

Glutamate, well known excitatory neurotransmitter in the mammalian central nervous system, is involved in most functions of the nervous system including the control of body movements, learning and memory, emotions, and sensory perception[8]. Although this excitatory neurotransmitter normally serves the fundamental functions, overactivation of glutamergic neurons can lead to the neuronal degeneration and death. Excitotoxic neuronal cell death is mediated by calcium influx which, if not rapidly removed or buffered, initiates a cascade of events involving oxyradical production, mitochondrial dysfunction and protease activation that ultimately kill the neuron. Thus, such excitotoxic cell death is considered to contribute to the pathogenesis of many different disorders including ischemic stroke, Alzheimer's and Parkinson's diseases, and amyotrophic lateral sclerosis[3,24].

Heat-shock protein 70 (HSP70) is molecular chaperone presented in the cytosol, which is one class of proteins involved in the stress response[10]. Previous studies have provided evidence that HSP70 can protect neurons against excitotoxic and oxidative insults. It has been reported that pre-induction of HSP70 protects cultured cerebellar granule neurons against excitotoxicity[16]. Moreover, HSP70 is induced in many neuronal populations following seizures or cerebral ischemia, in-

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cluding neurons that survive the insult[17].

The present study was aimed to induce excitotoxicity to primary cultured neurons and evaluate the neuroprotective effects of curcumin. We found that pretreatment of curcumin can protect neurons against excitotoxicity by a mechanism involving induction of stress-response protein.

Materials and Methods

Hippocampal Cell Cultures, Experimental Treatments and Quantification of Neuron Survival

Primary dissociated cell cultures of embryonic rat hippocampus and cortex were established from 18 day embryos of ICR mice (Dae Han Bio Link Co. Ltd, Korea) as described previously[23]. Briefly, hippocampi were removed and incubated for 15 min in Ca^{2+}- and Mg^{2+}-free Hank's Balanced Saline Solution (WelGENE Inc., Daegu, Korea) containing 0.2% trypsin. Cells were dissociated by trituration and plated into poly-L-Lysine-coated plastic culture dishes containing Neurobasal Medium supplemented with 10% B27, 2 mM L-glutamine, and 25 μM glutamate. Following cell attachment (24 hr post-plating), the culture medium was replaced with Neurobasal Medium without glutamate. Experiments were performed in 7-9 day-old cultures, a time period during which neurons are vulnerable to excitotoxic and oxidative insults[20].

Cell Toxicity and Viability

Overall neuronal viability was assessed by the lactate dehydrogenase (LDH) release from dead cells into the medium as assessed of LDH assay (LDH kit, Asan, Korea) performed in a 96 well assay format. The LDH activities were normalized as the percentage of control of DMSO-treated control groups (vehicle). The activity was monitored as the oxidation of NADH at 560 nm. The neuronal survival at the end stage was reconfirmed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl-tetrazolium bromide (MTT) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells into an insoluble purple formazan product. Because primary cultured neurons were mostly postmitotic and non-proliferating cells, the MTT analysis can be used for detecting neuronal viability. Cells were washed twice with PBS before 100 μl of 0.5 mg/ml MTT in PBS was added to each well. The plate was incubated at 37℃ for 4 hrs. Then, the cells were disrupted with solubilization solution (dimethly sulfoxide and ethanol, 1 to 1 ratio). The formazan dye produced by viable cells was quantified in an ELISA microplate reader at absorbance 560 nm.

Measurement of Cellular Reactive Oxygen Species Levels

ROS generation was measured as previously described, by utilizing a fluorescence probe[9]. Briefly, primary neuronal cultures were seeded onto a 96-well plate and pretreated with several concentrations of curcumin for 24 hrs, and then 30 μM of 2,7-dichlorofluorescein diacetate (DCF-DA) was added to neuronal culture for 30 min with 200 μl of final volume. 200 μM of glutamate was administered and changes in fluorescence intensity were measured at 30 min and 24 hrs after the glutamate administration on a Fluorescence Plate Reader (TECAN, Austria), with excitation and emission wavelengths set at 485 and 535 nm, respectively.

Immunoblot Analysis

The immunoblot methods were similar to those described previously[12]. Briefly, 50 μg of solubilized proteins were separated by electrophoresis in a polyacrylamide gel, transferred to a PVDF membrane, and immunoreacted with primary antibody overnight at 4°C. The PVDF membrane was further processed using HRP-conjugated anti-mouse secondary antibody and a chemiluminescence detection method (Amersham). The primary antibodies included a mouse monoclonal antibody against HSP70 (Sigma, St. Louis, MO; 1:5000 dilution), a mouse monoclonal antibody against PARP (1:2000) (Chemicon, Inc., Temecula, CA), and a mouse monoclonal antibody against β-actin (Santa Cruz; 1:10000 dilution).

Results and Discussion

Glutamate induces neuronal cell death.

To evaluate the excitotoxin-induced neuronal death, we have examined hippocampal cell cultures in the premarking microscope fields. After challenge of these cells with the excitotoxic agent, glutamate, we were able to monitor the number of surviving and dead cells in the same microscopic fields over time. Before the challenge with glutamate, neurons had intact neurites and a cell body that was smooth and round to oval in shape, and they were considered viable (Fig. 1A). However, many neurons with bead-

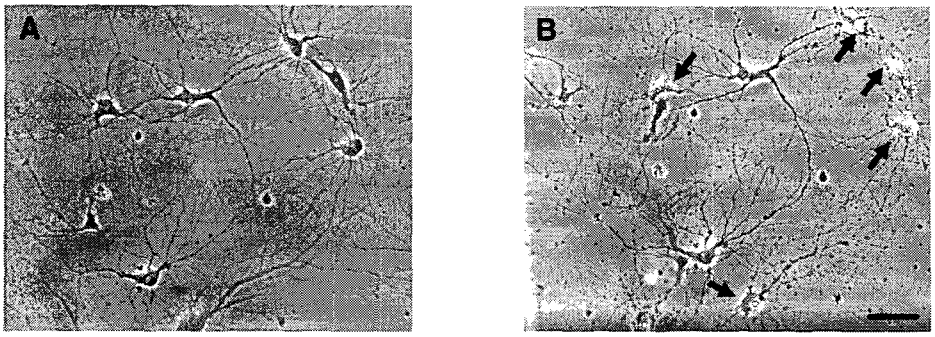

Fig. 1. Primary cultured hippocampal neurons were vulnerable to glutamate-induced excitotoxicity. Primary neurons at 8-10 days in culture showed intact neurites and healthy cell body, and they expressed the glutamate receptors making them vulnerable to excitotoxicity (Fig. 1A). After 400 μM of glutamate insult, many neurites were beaded or fragmented, and neuronal cell bodies were clearly destroyed primary hippocampal neurons in appearance (arrows), indicating the excitotoxicity of glutamate to the cultured neurons (Fig. 1B). Scale bar = 50 μm.

ed or fragmented neurites were detected at 48 hrs of post glutamate treatment and their cell body was clearly shrunken and rough in appearance, indicating the excitotoxicity of glutamate (Fig. 1B). These evaluation criteria correlate well with vital dye staining methods[19].

Pretreatment of curcumin protects primary cultured neurons against glutamate-induced excitotoxicity.

To determine the neuroprotective effects of curcumin, we pretreated hippocampal neuronal culture with several concentrations of curcumin including vehicle control. We have monitored the 96-well plate neuronal cultures with LDH release assay before and after treatment of curcumin to evaluate the equal distribution of neurons, and neuronal survival. There is no significant difference in basal LDH release before the pretreatment indicating that even numbers of neurons was seeded onto 96-well plate (data not shown). After 24 hrs of pretreatment of curcumin, the increased LDH release was observed in 20 μM of curcumin-treated group, suggesting the cytotoxicity of high dose of curcumin to primary cultured hippocampal neurons (Fig. 2A). Later, primary cultured neurons were insulted

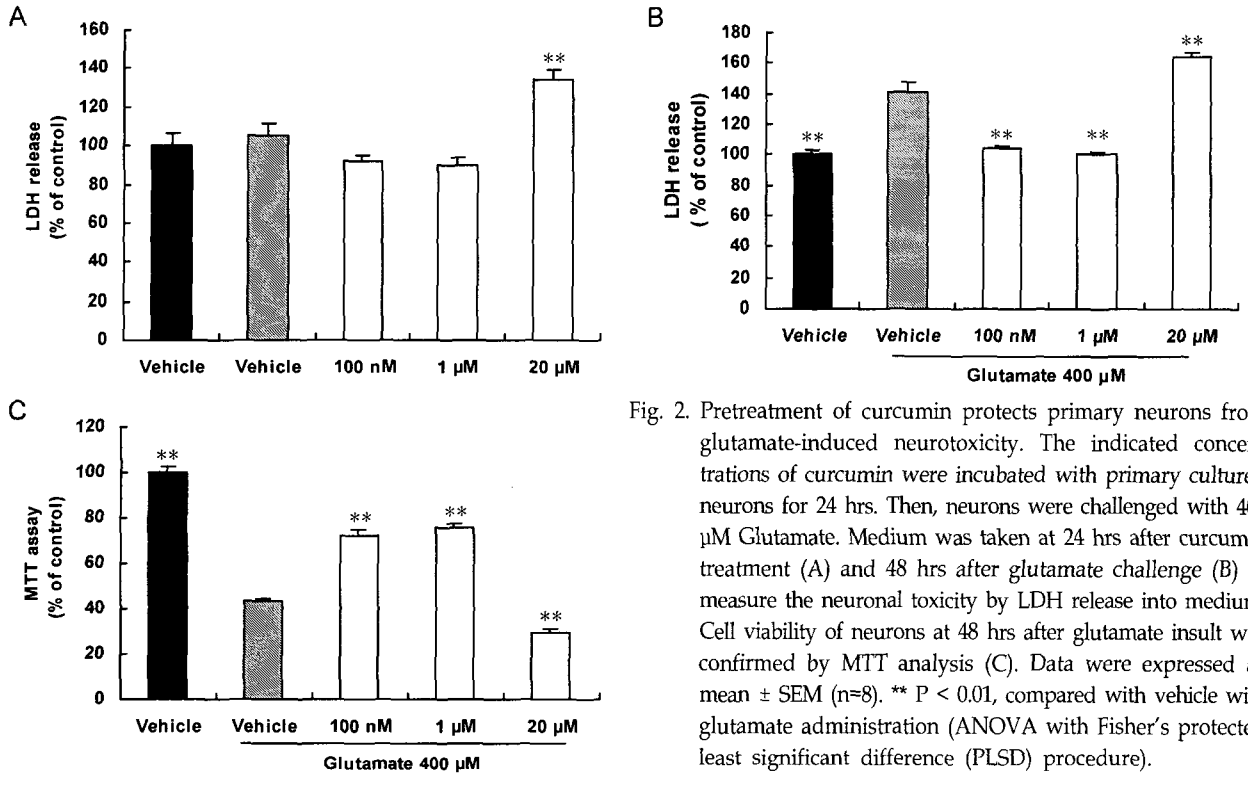

Fig. 2. Pretreatment of curcumin protects primary neurons from glutamate-induced neurotoxicity. The indicated concentrations of curcumin were incubated with primary cultured neurons for 24 hrs. Then, neurons were challenged with 400 μM Glutamate. Medium was taken at 24 hrs after curcumin treatment (A) and 48 hrs after glutamate challenge (B) to measure the neuronal toxicity by LDH release into medium. Cell viability of neurons at 48 hrs after glutamate insult was confirmed by MTT analysis (C). Data were expressed as mean ± SEM (n=8). ** $P < 0.01$, compared with vehicle with glutamate administration (ANOVA with Fisher's protected least significant difference (PLSD) procedure).

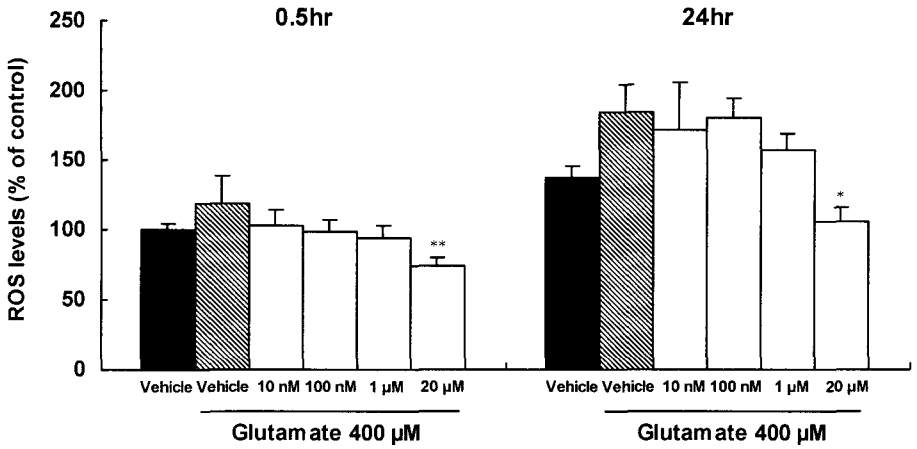

Fig. 3. The effects of curcumin on the intracellular reactive oxygen species generation. Glutamate-induced ROS was measured by DCF-DA incorporation. After 24-hrs pretreatment of curcumin, neurons were challenged with 400 μM of glutamate, and DCF-fluorescence was monitored at 0.5 hr and 24 hrs after glutamate administration. Data were expressed as mean ± SEM (n=6). * $P < 0.05$, ** $P < 0.01$, compared with vehicle with glutamate administration (ANOVA with Fisher's protected least significant difference (PLSD) procedure).

by excitotoxin after the pretreatment of curcumin and vehicle. Low doses of curcumin pretreatment effectively protect the primary cultured neurons from glutamate-induced excitotoxicity, while high dose of curcumin exacerbates the neuronal death (Fig. 2B). These LDH release data were confirmed by MTT analysis, clearly indicating that significantly more neurons survived exposure to glutamate in cultures that had been pretreated with low doses of curcumin. However, high dose of curcumin pretreatment seems to be toxic to neurons and exacerbate the glutamate-induced neuronal toxicity (Fig. 2C). The cytotoxicity of high dose of curcumin has been reported in primary retinal cultures[18], and several cancer cell lines including colon cancer and neuroblastoma[4,13,26].

ROS levels were not modulated by low dose of curcumin pretreatment.

Since most studies have already shown the direct antioxidative properties of curcumin[25], we examined the pretreatment effects of curcumin on reactive oxygen species (ROS) to explain the neuroprotective effects of curcumin. Neurons were pretreated with several concentrations of curcumin for 24 hrs, and ROS levels were analyzed at 0.5 hr and 24 hrs after glutamate challenge by utilizing DCF-DA fluorescence probe. Intracellular ROS generation in primary cultured neurons was slightly elevated by glutamate insult, and levels of ROS were significantly increased at 24 hrs of incubation with glutamate. Although there are a decreasing trend of ROS levels in curcumin-treated group, levels of ROS were not significantly different from vehicle and low dose of curcumin at which neurons were protected from glutamate-induced toxicity (Fig. 3). We found that 20 μM of curcumin treated group showed a significant decrease in intracellular ROS level compared to other group. However, the decreased ROS levels might be caused by the less availability of metabolically active neurons to generate ROS because there is a significant neuronal loss in 20 μM curcumin-pretreated group.

Neuroprotective effects of curcumin pretreatment might be involved in the preconditioning mechanism of mild stress.

Because the neuroprotective effects of curcumin is not derived from the reduced oxidative stress in primary cultured neurons in the present study, we determined the effects of curcumin on "preconditioning" effects of mild stress have been reported in studies of cultured neurons[11,14,28]. There was an increase in levels of HSP70 in neurons exposed to low dose of curcumin for 24 hrs, but not to high dose (Fig. 4), suggesting that the excitoprotective mechanism of action of curcumin might involve a mild stress response. Since a similar preconditioning mechanism could account for the neuroprotective effect of curcumin we observed, this appears to be the case because curcumin causes an increase in levels of the stress protein, HSP70. PARP is a highly conserved nuclear enzyme and is shown that PARP cleavage is mediated by caspase-3 activity occurs at the onset of apop-

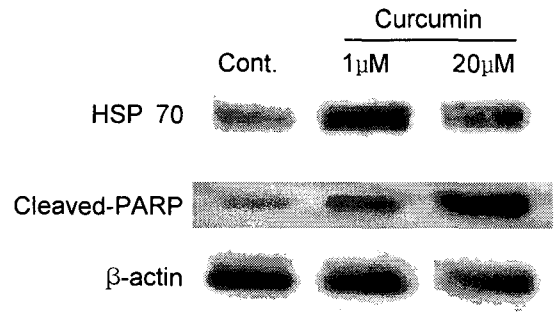

Fig. 4. Levels of HSP70 protein increased in primary cultured neurons following expose to low dose of curcumin. Whole cell extracts from pretreatment with the indicated concentration of curcumin, were prepared for western blot analysis. Cell proteins (50 μg/lane) were separated by SDS-PAGE, transferred to a PVDF membrane and immunoreacted with antibodies against HSP70 and PARP. The PVDF membrane was stripped and re-probed with anti β-actin antibody to evaluate the equal loading.

tosis[21]. Interestingly, levels of 85 kD-cleaved product of PARP were significantly increased in neurons pretreated with 20 μM of curcumin, indicating that high dose of curcumin induces apoptosis in primary neurons.

In conclusion, our findings show the neuroprotective effects of curcumin pretreatment and propose the curcumin-mediated important signaling in primary cultured neurons because low doses of curcumin pretreatment effectively protected neurons against glutamate-induced excitotoxicity. The present demonstration of the dose-dependant actions of curcumin suggests not only the potential efficacy in the neurology clinic, but also the harmful toxicity to neurons with high dose treatment.

Acknowledgments

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초록 : Curcumin의 전처리는 excitotoxin에 의한 세포사멸로부터 해마신경세포를 보호

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Curcumin은 자연에 존재하는 황색의 페놀성분의 커리 향신료이며 항산화 및 항염증의 성질을 가지고 있어서 산화 스트레스와 면역염증과 관련한 여러 질병의 치료로 사용되어져 왔다. 이러한 curcumin의 항산화 및 항염증 효과는 여러 퇴행성 신경질환으로부터 뇌를 보호하는데 유용하게 적용될 수 있다. 본 연구에서는 glutamate에 의한 excitotoxicity로부터 해마신경세포를 보호하는 curcumin의 신경보호효과에 대하여 보고한다. 태아 생쥐의 해마로부터 얻어진 신경세포를 저농도의 curcumin으로 전처리한 경우, 신경세포는 glutamate에 의한 세포사멸로부터 보호되었다. 그러나 이러한 신경보호효과는 산화스트레스의 조절과는 무관하였다. 흥미롭게도 고농도의 curcumin 전처리는 오히려 초대배양 신경세포의 세포사멸을 유도하였다. 해마신경세포에서 스트레스 반응 단백질인 HSP70이 저농도의 curcumin을 처리하였을 때 현저하게 증가하였으며 반면 세포사멸의 마커인 절단된 PARP의 양은 고농도의 curcumin을 처리하였을 때 급증함이 immunoblot 분석을 통하여 관찰되었다. 이러한 발견은 curcumin이 excitotoxin인 glutamate에 대한 신경세포의 반응을 조절할 수 있음을 보여주고 curcumin과 관련 화합물들의 퇴행성 신경질환에서의 예방 및 치료법으로의 가능성을 제시하고 있다.