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PROTECTIVE EFFECT OF NARINGENIN ON GLUTAMATE-INDUCED NEUROTOXICITY IN CULTURED HIPPOCAMPAL CELLS

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Abstract: Monosodium glutamate induces excitotoxicity in the central nervous system through hyperactivation of both ionotropic and metabotropic glutamate receptors, which leads to neuronal cell death. In this study, we investigated the neuroprotective effects of naringenin on excitotoxicity induced by glutamate in primary hippocampal neurons of neonatal mice. The expression levels of apoptosis-inducing proteins and as well as ischemic factors were observed by Western blot analysis. Immunocytochemistry and morphometric analysis of hippocampal cells with or without glutamate and naringenin treatment were performed. We observed that naringenin regulated Erk1/2 and Akt phosphorylation and reduced the demise of dendrites due to glutamate exposure in cultured hippocampal neurons. Furthermore, naringenin induced the brain-derived neurotrophic factor and other neuroprotective cytokines, and markedly improved the survival rates of the neurons 24 h following glutamate exposure. The observed results suggest that the naturally occurring bioflavonoid (naringenin) exerts neuroprotective effects via highly specific molecular targets in neurons.

Key words: excitotoxicity; glutamate; hippocampal neuronal cells; naringenin; neuroprotection

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INTRODUCTION

Oxygen/glucose deprivation (OGD), glutamate disturbance and inflammation comprise the multifactorial pathophysiological process of cerebral ischemia and stroke (Harukuni and Bhardwaj, 2006). Glutamate-induced neurotoxicity has been implicated in severe brain disorders, including the Alzheimer's disease, epilepsy, ischemic stroke and also in Parkinson's disease (Fukui et al., 2009; Xu et al., 2011). Previous studies have reported that glutamate-induced neuronal cell death occurs by receptor-mediated excitotoxicity and also through non-receptor-mediated oxidative stress (Stanciu et al., 2000). Monosodium glutamate (MSG) is extensively used as a flavoring agent in food. Glutamate causes excitotoxicity through the hyperactivation of glutamate receptors (both metabotropic and ionotropic) present in the central nervous system, leading to neuronal cell death (Pavlovic et al., 2009). MSG or sodium salt of glutamate exerts excitotoxicity by overactivation

of glutamate receptors, namely α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl D-aspartate (NMDA) and kainate receptors (KARs). The main toxicity of MSG includes neurotoxicity, disorders of the endocrine glands associated with neurological activity, learning difficulties, epileptic seizures, increases in glucose levels and increased incidences of metabolic diseases (Egbuonu et al., 2009). One of the ways to counteract these deleterious effects of neurotoxicity produced by excess MSG is to evaluate an effective neuroprotective compound of natural origin.

Naringenin, a naturally-occurring bioflavonoid that is found in fruit and vegetables such as grapefruit, orange and tomato, is reported to have anti-inflammatory as well as antioxidant properties (Shi et al., 2009; Baumann et al., 1980). The antioxidant effect of naringenin is mainly attributed to the presence of a 4' hydroxyl group in the B ring, which effectively quenches free radicals (van Acker et al., 2000). It exhibits beneficial effects in oxidative stress-mediated diseases, such as airway inflammation and neuroinflammation possibly due to its anti-oxidative and anti-inflammatory properties (Yang et al., 2011; Assini et al., 2013). Since glutamate-induced excitotoxicity and neuronal death are currently thought to contribute to the progression of cerebral ischemia and the occurrence of strokes, it is plausible to hypothesize that naringenin might exhibit neuroprotective potential. The validity of its potential neuroprotection has been evaluated in this study.

MATERIALS AND METHODS

Mouse hippocampal neuron isolation and culture

The primary cultures of hippocampal neurons were isolated from neonatal (C57Bl/6J) mice as described earlier by Berbari et al. (2007), with slight modifications. The study was approved by the Institutional Ethical Animal Care Committee of Zhengzhou University and was performed according to the institutional animal care committee guidelines. The tissues from hippocampal regions were isolated from neonatal mice on postnatal day (P) 1. The isolated tissues were treated with papain (0.4 mg/mL) and DNase (50 μg/mL) in Leibovitz L-15 medium containing bovine serum albumin (0.2 mg/mL) for around 15 min at 37°C. Treated tissue samples were washed with NEUROBASAL-A medium supplemented with B-27 thrice and gently ground. Following centrifugation at 4°C for 5 min at 200 g, the cells were resuspended in NEUROBASAL-A/B-27 medium containing DNase. Prior to plating, the cells were passed through a cell strainer (100 μm mesh) and plated (1.0 x10^5 cells/cm^2) onto poly-D-lysine precoated glass coverslips.

Experimental design

Isolated hippocampal neuronal cells were stabilized and cultured for about 8 days prior to naringenin exposure. The study was performed on separate groups of cells exposed to 50 μM of glutamate (1G 50-145-077, 97%; Fisher Scientific, Pittsburg, PA, USA). The control groups (regular cell culture conditions) were not exposed to either naringenin or glutamate. The treatment group cells (after 30 min of glutamate exposure) were treated with different concentrations (25, 50, 75 and 100 mg/L) of naringenin (529-55-5, 95%; Matrix Scientific Columbia, SC) for 30 min. The volume of DMSO/ethanol/H_2O solvent at 0.5% (v/v) of the culture medium was used. After cell treatment, they were rapidly stabilized with ice-cold TCA (10% v/v) and subjected to immunoassays and Western blot analysis. The optimized naringenin concentration was taken for the further analysis.

Western blot analysis

Equal concentrations of the isolated proteins were separated by SDS-PAGE and blotted to a nitrocellulose membrane. Phosphatase activity was inhibited by the addition of sodium fluoride (1 mM) to sample lysis buffer. The blots were blocked with blocking buffer (Tris-buffered saline with Tween 20 (TBST) containing 10% (w/v) non-fat dry milk) for 1 h at room temperature. Following treatment with blocking buffers, the blots were incubated overnight at 4°C with primary antibodies against Erk, p-Erk, Akt, p-Akt, caspase 3 and calpain 1 (1:500). After the incubation period, the blots were incubated with secondary antibody (1:500) conjugated with alkaline phosphatase, incubated at room temperature for 1 h and later washed with TBST, where NBT/BCIP was used to visualize the available immune reactive bands in the blots.

Morphometric analysis and immunocytochemistry study

The neurons were fixed on glass coverslips that were permeabilized with ice-cold methanol. The cells were then blocked with skimmed milk (10%) in phosphate-buffered saline (PBS) for around 30 min, followed by incubation with anti-MAP2 antibody (1:200 dilution) at 4°C for overnight. Samples were incubated with secondary antibody that was conjugated with Alexa Fluor 488 in a 1:200 dilution for 30 min at room temperature, after a washing with PBS. The cells were further stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted on a glass slide. Images of the stained cells were investigated using a microscope that was equipped with a CCD color camera and DP2-BSW software for recording the images. As cell compactness affects dendrite length, the cell density images tend to miscal-

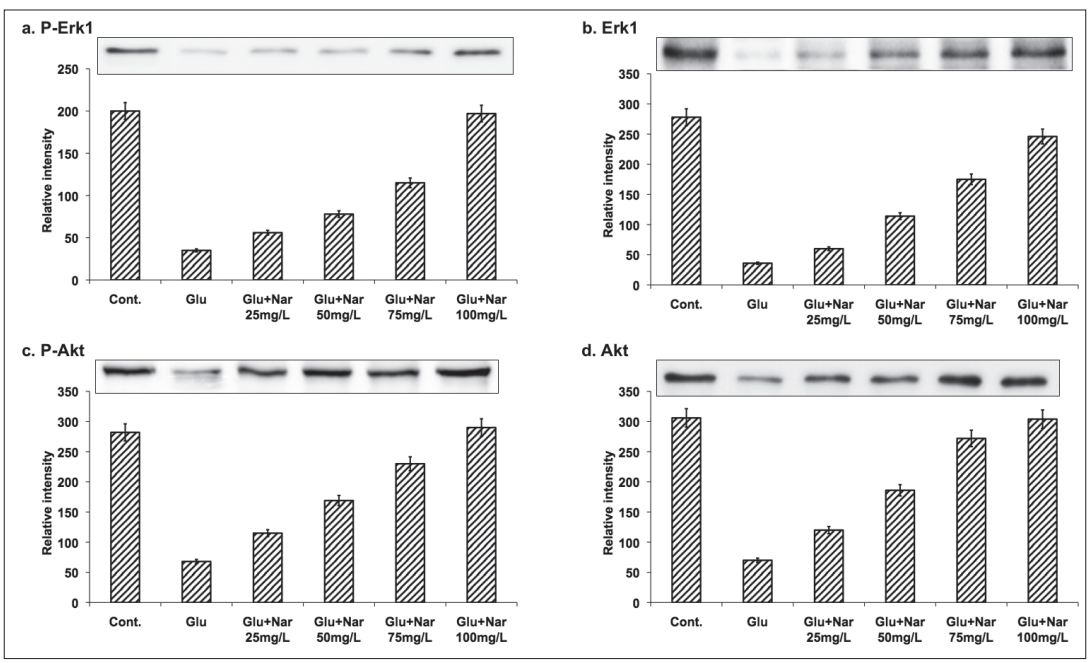

Fig. 1. Assessment of the maximum protective effect of naringenin (Nar) at different concentrations (25, 50, 75, 100mg/L) against glutamate (Glu) exposure in cultured Hippocampal cells (n=3). Western blot analysis of p-Erk1 (a), Erk (b), p-Akt (c) and Akt (d) with respective the graphical representations of band intensities. The analysis was performed in 3 samples from each experimental group.

culate the mean dendrite length due to overlap, random field selection was employed.

Real-time PCR analysis

Using TRIZOL reagent, total RNA was isolated from the experimental cell groups as per the manufacturer's instructions (Gibco-BRL, Grand Island, New York) and its purity was established by determining its absorbance at 260 and 280 nm. According to the manufacturer's instruction, the superscript preamplification system was used with Superscript II RNase H-reverse transcriptase for synthesizing the first cDNA from total RNA. Further amplification of the cDNA (1 µl) template was performed by PCR reaction that contained supermix (18 µl) and each specific primer (10 pmol) making up the total volume of 20 µl. For PCR reaction, primer sequences of caspase-3 (forward 5′-TGTCATCTCGCTCTGGTACG-3′ and reverse 5′-AA ATGACCCCTTCAT-CACCA-3′) and calpain 1 (forward, 5′-GT-GGTAGCCGCTGAAACTC C-3′; reverse, 5′-TGTTCCCGCTCTCATCTGC-3′) were used along with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was amplified and used (forward 5′-AACTTTGGCATTGTG-GAAGG-3′ and reverse 5′-GGAGACAACCTG-GTCCTCAG-3′) as an internal control. For the amplification of the cDNA, the PCR reaction for calpain 1 employed 30 cycles, caspase-3 35 cycles, and the internal control (GAPDH) 28 cycles. The obtained amplified products were separated using agarose gel (1.5%) electrophoresis and were analyzed using a Bio-Rad Imaging Densitometer (Bio-Rad, Hercules, California).

Statistical analysis

The obtained results were subjected to statistical analysis and values were expressed as the mean ± SEM. A two-tailed t-test was used to compare the differences between two groups and one-way analysis of variance (ANOVA) was used to compare differences that are seen among multiple groups. A *post hoc* test was also used to compare a few selected groups and $P<0.05$ was considered significant.

RESULTS

Naringenin protects glutamate-induced Erk1 phosphorylation

The neuroprotective effects of naringenin were assessed. Mouse hippocampal neurons were subjected to an excitotoxic concentration of glutamate in the presence of naringenin and the levels of Erk1 phosphorylation were assessed. The initial trial was carried out using different concentrations (25, 50, 75 and 100 mg/L) of naringenin against glutamate excitotoxicity. On exposure of cultured hippocampal neuron cells to glutamate (50 mM) for 30 min, a pronounced reduction of Erk1 phosphorylation was observed (Fig. 1). However, in glutamate-exposed cells treated with 100 mg/L naringenin, significant Erk1 phosphorylation (Fig.1) was observed, while lower concentrations of naringenin were ineffective. Further, naringenin also caused a similar increase in the phosphorylation of Akt (Fig. 1). These data collectively demonstrate that naringenin at a concentration of 100 mg/L showed the optimum protection of Erk1 during excitotoxic stress in cultured hippocampal neuronal cells.

Neuroprotective effects of naringenin

To determine cell survival, the hippocampal neurons were subjected to glutamate with or without naringenin (for 10 min) and were recovered using conditioned medium after 24 h. For the identifi-

cation of the surviving neurons and determination of dendrite length, the cells were immunostained (Fig. 2). Fig. 2 shows that 17% of neurons survived after glutamate exposure alone, whereas the survival rate increased to around 30% in those exposed to glutamate along with naringenin. Morphometric analysis (i.e., the total neuronal number and dendrite length) revealed that the mean dendrite length was longer for those treated with naringenin than for those treated with glutamate alone (Fig. 2) resulting in naringenin's dendritic protection and also cell survival after glutamate toxicity. The observations suggest that naringenin could protect the dendrites from glutamate toxicity and as well improve cell viability.

Evaluation of apoptotic signaling protein/mRNA level

The mRNA levels of caspase-3 and calpain 1 were assessed following exposure to glutamate and naringenin. A 30-min exposure of cells to glutamate caused a robust increase in the levels of caspase-3 and calpain mRNA. (Fig. 3a). In neurons treated with 100 mg/L naringenin, the expression levels of caspase and calpain 1 decreased (Fig. 3b) following 6 h of glutamate exposure. These results suggest that naringenin could be used effectively to target the caspase-mediated apoptosis cascade.

DISCUSSION

Preventing hippocampal neuronal death could be a beneficial therapeutic approach against cognitive and memory impairments accompanying brain disorders. The bioflavonoid, naringenin could be a potential therapeutic candidate. Glutamate-induced oxidative stress causes apoptotic and necrotic neuronal death in HT22 cells (Fukui et al., 2009). Glutamate-mediated

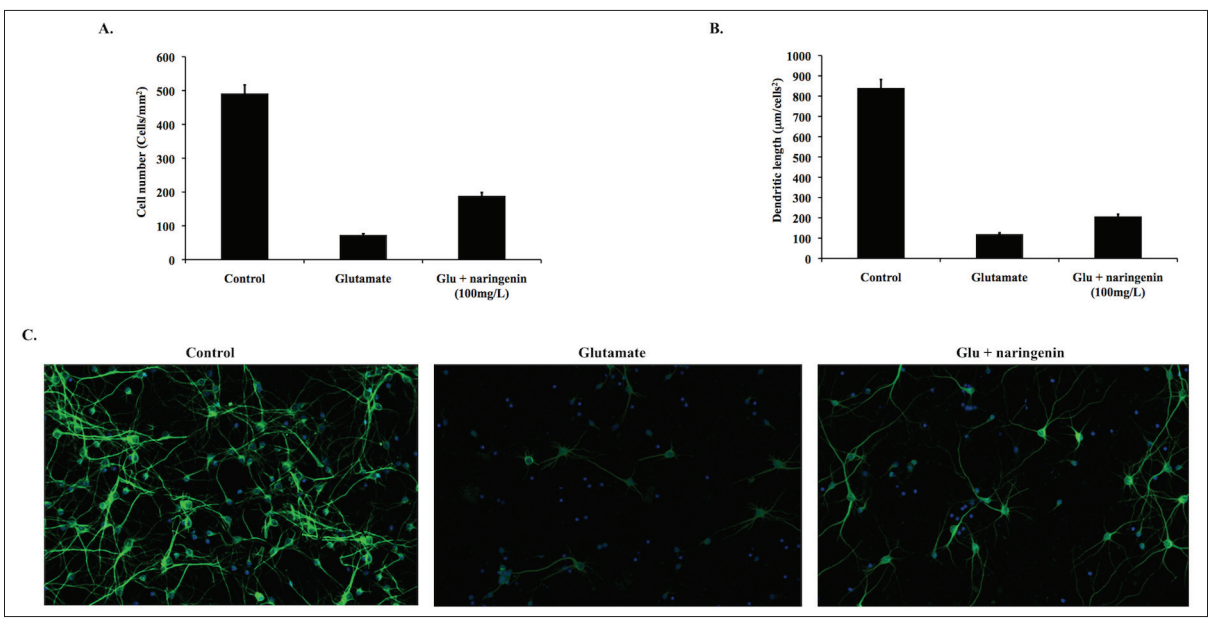

Fig. 2. A. Mean density of MAP2-positive neurons surviving 24 h after glutamate exposure. B. Quantification of the mean dendrite length by morphometric analysis. C. Experiment performed to determine the survival of hippocampal neurons after a 30 min exposure to glutamate and treated with 100 mg/L of naringenin. Control cell without any exposure. Scale bar 100 mm.

toxicity is mainly induced via excitotoxicity mediated by glutamate receptors and through ROS-mediated oxidative stress (Fukui et al., 2010; Poddar et al., 2010).

Overactivation of NMDA, along with other glutamate/glycine receptors, disturbs calcium homeostasis, which is the key mediator of glutamate-induced excitotoxic neuronal damage. Oxidative stress and excitotoxicity are the foremost activities induced by brain ischemia, leading to the damage of neuronal cells (Coyle and Puttfarcken, 1993). Namura et al. (2001) reported earlier a reduction in the phosphorylation of Erk1/2 in the hippocampus of the mice during a forebrain ischemia experiment. However, the underlying mechanism contributing to the cytotoxic action of glutamate has not been clearly established. This present research demonstrates that naringenin eased the glutamate-induced reduction in the level of Erk1/2 phosphorylation in hippocampal neuronal cells and protected the dendrites from refutation during a 30-min glutamate exposure. Almeida et al. (2005) previously reported that the brain-derived neurotrophic factor increased the phosphorylation of Erk1/2 in a hippocampal neuronal culture by preventing glutamate-induced apoptosis. Our study results are consistent with those of Almeida et al. (2005). Our results also exhibited the phosphorylation of the Akt-signaling pathway induced by naringenin hippocampal neurons.

Further, the cell survival rate and dendrite length of the cultured hippocampal neuronal cells after 24 h of glutamate exposure was examined for confirmation of neuroprotective effect induced by naringenin, though the molecular mechanism is still to be understood. It can be inferred that naringenin inhibits glutamate receptors or the direct modulators, thereby reducing the excitotoxicity in the hippocampal neuronal cells. Gao et al. (2006) demonstrated that concentrations (10-100 mM) of trans-resveratrol can inhibit postsynaptic glutamate receptors in hippocampal neurons, with NMDA receptors being more sensitive than AMPA receptors. Inhibition of Erk phosphatases could also lead to an increase in Erk phosphorylation in cells stimulated with glutamate and exposed to naringenin (Levinthal and Defranco, 2005; Choi et al., 2006). From the analysis of the mRNA levels of

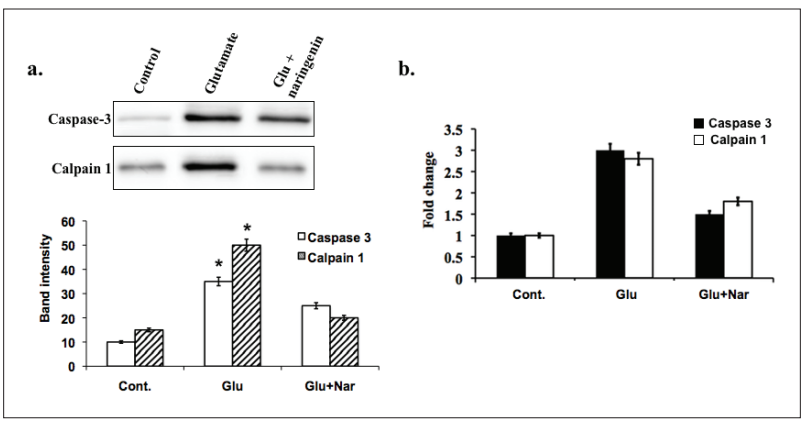

Fig. 3. Naringenin protects from apoptotic protein expression. A – Western blot analysis of caspase-3 and calpain 1 protein levels and the graphical representation of quantification of band intensities. B – Real-time PCR analysis showing the fold changes of caspase-3 and calpain 1 mRNA levels between groups; * indicates the significant ($p<0.01$) differences from the gluatamate (Glu)-exposed and naringenin (Nar)-treated groups; # indicates the significant ($p<0.05$) difference from the control group.

caspase-3, it could be concluded that naringenin inhibits glutamate-induced caspase-3 activation at an initial stage. Activation of caspase occurs in the execution phase, as it is an effector caspase that can destroy cells extensively.

The glutamate-induced oxidative toxicity in HT22 cells was reported to be mediated via a caspase-independent pathway involving calpain (Elphick et al., 2008; Zhang and Bhavnani, 2006). Calpains (Ca^{2+}-dependent proteases) require an increase in the cytosolic concentration of calcium via extracellular influx and an increase of this level has been reported in glutamate-treated HT22 cells (Herrera et al., 2007). Elevated levels of ROS and the activation of calpains through the induction of caspase-independent cell death have also been reported in association with some environmental pollutants (Tofighi et al., 2011). Our data suggest that naringenin protects neurons by preventing apoptosis. These beneficial effects of naringenin could be possibly attributed its antioxidant and anti-inflammatory properties (Yang et al., 2011; Assini et al., 2013).

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

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