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Actinidia arguta Protects Cultured Cerebral Cortical Neurons against Glutamate-Induced Neurotoxicity via Inhibition of $[Ca^{2+}]_i$ Increase and ROS Generation

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Abstract – *Actinidia arguta* (Actinidiaceae) has been reported to have several pharmacological effects such as anti-inflammatory, anti-allergic, and anti-oxidant activities. The present study investigated the protective activity of an ethanol extract from the leaf and stem of *A. arguta* against glutamate-induced neurotoxicity using cultured rat cortical neurons. Exposure of cultured cortical neurons to 500 μM glutamate for 12 h triggered neuronal cell death. *A. arguta* inhibited glutamate-induced neuronal death and apoptosis, which were measured by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and Hoechst 33342 staining, respectively. The increase of pro-apoptotic proteins, Bax and c-caspase-3, in glutamate-treated neurons was significantly inhibited by treatment with *A. arguta*. *A. arguta* also inhibited 500 μM glutamate-induced elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) and reactive oxygen species (ROS) generation, which were measured by fluorescent dyes, Fluo-4 AM and H_2DCF-DA, respectively. These results suggest that *A. arguta* may prevent glutamate-induced apoptotic neuronal death by inhibiting $[Ca^{2+}]_i$ elevation and ROS generation and, therefore, may have a therapeutic role for the prevention of neurodegeneration in cerebral ischemic diseases.

Keywords – *Actinidia arguta*, Neuroprotection, Glutamate, Neurotoxicity, Cultured neurons

Introduction

L-glutamate, the major excitatory transmitter in the brain is associated with learning and memory (Herron *et al*., 1986; Zahr *et al*., 2008), whereas excessive amounts of glutamate are highly toxic to neurons causing glutamate excitotoxicity (Choi, 1987; Frandsen *et al*., 1989). Excitotoxicity mediated by glutamate receptors may underlay the pathology of a number of neurological abnormalities, including Alzheimer's disease, epilepsy, and stroke (Choi, 1988; McDonald *et al*., 1988; Weinberger, 2006). Glutamate excitotoxicity is triggered primarily by excessive Ca^{2+} influx due to overstimulation of the N-methyl-D-aspartate (NMDA) receptors, followed by disintegration of the endoplasmic reticulum, the generation of reactive oxygen species (ROS) as well as mitochondrial dysfunction, leading to neuronal apoptosis or necrosis (Nicholls, 2004; Schinder *et al*., 1996).

Actinidia arguta (Sieb. Et Zucc.) Panch (Actinidiaceae) is a smooth-skinned grape-sized kiwifruit native to northern China, Korea, Siberia and Japan. Extracts of the fruits, leaves, stems and barks have been traditionally used for the treatment of inflammatory diseases and gastrointestinal diseases in Korea (Bae, 2000). *A. arguta* possesses antioxidant, blocking atopic dermatitis, and anti-allergic properties (Choi *et al*., 2008; Kim *et al*., 2008; Latocha *et al*., 2010). It contains various kinds of anti-oxidant components including catechin, epicatechin, vitamin C, carotenoids, chlorophyll and anthocyanin (Latocha *et al*., 2010; Lim *et al*., 2005; Montefiori *et al*. 2009; Takano *et al*., 2003). Essential oil, linalool derivatives, which has potent antioxidant activity, was also isolated from the flowers of *A. arguta* (Matich *et al*., 2006). Various antioxidative products have been regarded as potential neuroprotective agents, which improve a number of pathological processes including ROS formation and inflammation (Pitchumoni and Doraiswamy, 1998). In this study, therefore, we attempted to investigate neuroprotective effect of an ethanol extract of the leaves and stems of *A. arguta* on glutamate-induced neurotoxicity in primarily cultured rat cortical neurons.

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Materials and Methods

Preparation of ethanol extract of *A. arguta* – The leaves and stems of *A. arguta* were gathered in Keryong Mountain in Daejeon, Korea, in July 2009 and identified by Professor KiHwan Bae of the College of Pharmacy, Chungnam National University, Korea. Dried leaves and stems of *A. arguta* (4 kg) were extracted 3 times with ethanol at room temperature for 3 days, filtered, and concentrated to yield an ethanol extract (300 g; yield: 7.5%), which was stored at room temperature until needed.

Chemicals – Glutamate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), trypsin, Dulbecco's modified Eagle's medium (DMEM), and Joklik-modified MEM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hoechst 33342 dye, Fluo-4 AM and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum was purchased from JRS Biosciences (Lenexa, KS, USA). (5R,10s)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) was purchased from RBI (Natick, MA, USA). Antibodies recognizing rabbit polyclonal antibody against Bax, cleaved-caspase-3 (c-caspase-3), and β-actin; and horseradish peroxidase-conjugated anti-rabbit secondary antibody were from KOMA Biotech Inc. (Seoul, Korea). All other chemicals used were of the highest grade available.

Experimental animals – Pregnant Sprague-Dawley (SD) rats were purchased from Daehan BioLink Co., Ltd (Chungbuk, Korea) and housed singly in environmentally controlled rooms at 22 ± 2 ºC, with a relative humidity of 55 ± 5%, a 12-h light/dark cycle, and food and water *ad libitum*. The procedures involving experimental animals complied with the regulations for the care and use of laboratory animals of the Animal Ethics Committee of Chungbuk National University.

Induction of glutamate toxicity in primary cultures of rat cerebral cortical neurons – Primary cerebral cortical neuronal cultures were prepared using SD rat fetuses at embryonic days 15 to 16, as previously described (Ban *et al*., 2006). Neurotoxicity experiments were performed on neurons after 5 - 6 days *in vitro*. Cultured neurons were treated with 500 μM glutamate in a HEPES buffer (incubation buffer, pH 7.4) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl, and 2.3 mM $CaCl_2$ at 37 ºC for 12 h (unless otherwise indicated) to produce neurotoxicity. Glutamate was solubilized and freshly diluted in the incubation buffer. *A. arguta* was dissolved in dimethylsulfoxide (DMSO) (100 mg/ml) and diluted in the incubation buffer. The final concentration of DMSO was less than 0.1%, which did not affect cell viability. The cells were treated with *A. arguta* 20 min prior to induction of toxicity in each experiment.

Measurements of neuronal viability, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and ROS – At the end of incubation with glutamate for 12 h, neuronal cell viability and apoptotic cell death were monitored using a colorimetric MTT assay and Hoechst 33342 staining, respectively, as previously described (Cho *et al*., 2009). Changes in $[Ca^{2+}]_i$ were monitored with Fluo-4 AM, a Ca^{2+}-sensitive fluorescent dye (Cho *et al*., 2009), using a laser scanning confocal microscope (TCS SP2 AOBS; Leica, Heidelberg, Germany). Microfluorescence assay of 2',7'-dichlorofluorescein (DCF), the fluorescent product of H_2DCF-DA, using a laser scanning confocal microscope (TCS SP2 AOBS; Leica, Heidelberg, Germany) was used to monitor the generation of ROS in neurons treated with 500 μM of glutamate for 4 h (Cho *et al*., 2009).

Western blots – At the end of the incubation, neurons were lysed in an RIPA buffer (mixture of 150 mM NaCl, 1 mM Na-EDTA, protease inhibitor cocktail, and 50 mM Tris-HCl; pH 7.4) and western blot analysis of Bax and c-caspase-3 was performed, as previously described (Ban *et al*., 2006; Kim *et al*., 2012). The level of protein was measured by the Bradford method (Bradford, 1976). Images were quantified using image analysis software (a freely available application in the public domain for image analysis and process, developed and maintained by Wayne Rasband at the Research Services Branch, National Institutes of Health, USA).

Statistical analysis – Data were expressed as means ± S.E.M., and statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey's test. *P* values of < 0.05 were considered significant.

Results

Inhibitory effect of *A. arguta* on glutamate-induced neuronal cell death – Based on a preliminary study, in which glutamate showed a concentration-dependent reduction of cell viability within a concentration range of 100 - 1000 μM, a glutamate concentration of 500 μM was used for determining glutamate-induced neuronal cell damage in the present experiments. When cerebral cortical neurons were exposed to 500 μM glutamate for 12 h, MTT absorbance was 57.9 ± 6.1% of untreated controls, indicating that glutamate induced neuronal cell death. In cultures treated with *A. arguta* (1, 10, and 50 μg/ml), glutamate-induced neuronal cell death was significantly reduced showing 62.9 ± 5.9%, 73.8 ± 4.3%, and 93.8 ±

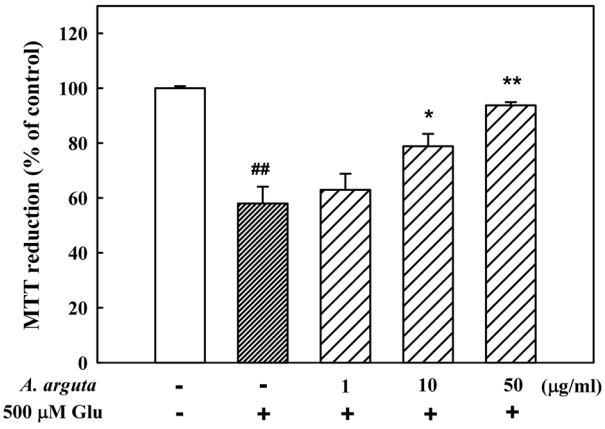

Fig. 1. Inhibitory effect of *A.arguta* on glutamate-induced neuronal cell death in cultured cortical neurons measured by MTT assay. Values are expressed as mean ± S.E.M of data obtained from at least three independent experiments performed in duplicate. $^{\#\#}p < 0.01$ *vs*. control, $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs*. 500 μM glutamate.

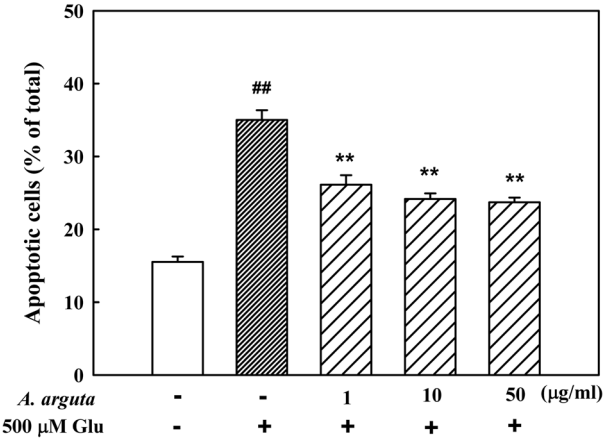

Fig. 2. Inhibitory effect of *A. arguta* on glutamate-induced apoptosis of cultured cortical neurons. Apoptotic cells measured by Hoechst33342 staining were counted in 5 to 6 fields per well. The values represent the apoptotic cells as a percentage of the total number of cells expressed as mean ± S.E.M of data obtained from 3 independent experiments. $^{\#\#}p < 0.01$ *vs*. control, $^{**}p < 0.01$ *vs*. 500 μM glutamate.

1.2%, respectively (Fig. 1).

An additional experiment was performed with Hoechst 33342 staining to detect condensed or fragmented DNA, which is indicative of glutamate-induced neuronal apoptotic death. Treatment of neurons with 500 μM glutamate induced apoptosis of 35.0 ± 1.3% of the total population of cultured cortical neurons compared with 15.5 ± 0.7% of apoptotic neurons in control cultures. The addition of *A. arguta* (1, 10, and 50 μg/ml) significantly decreased the glutamate-induced apoptotic cell death, showing apoptosis of 26.1 ± 1.3, 24.2 ± 0.8, and 23.7 ± 0.7%, respectively (Fig. 2).

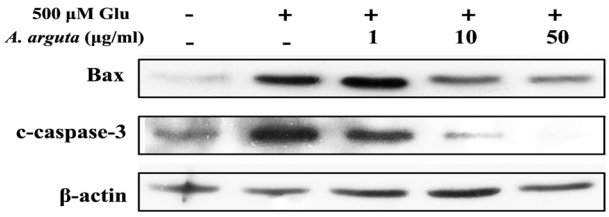

Fig. 3. Inhibitory effect of *A. arguta* on the expression of pro-apoptotic proteins in cultured cortical neurons. Representative western blotting bands of Bax and c-caspase-3 proteins are shown.

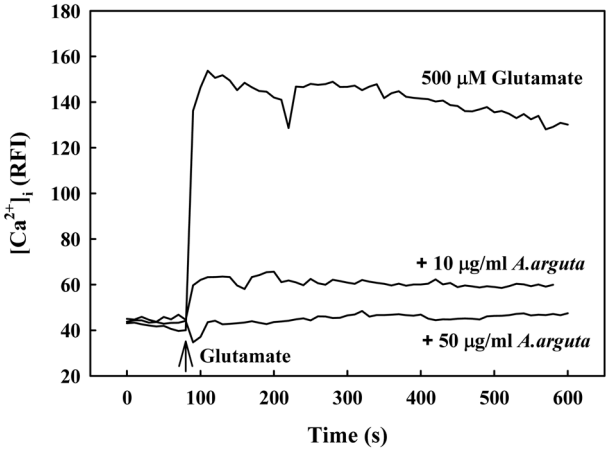

Fig. 4. Inhibitory effect of *A. arguta* on glutamate-induced elevation of $[Ca^{2+}]_i$ in cultured cortical neurons. $[Ca^{2+}]_i$ was monitored using a laser scanning confocal microscope. All images were processed to analyze changes in $[Ca^{2+}]_i$ at the single cell level. Values are expressed as mean relative fluorescence intensity (RFI). Each trace is a single cell that is representative of four independent experiments.

To elucidate the mechanism of neuronal cell death, immunoreactivities of Bax and c-caspase-3, pro-apoptotic proteins, were measured after treatment with 500 μM glutamate for 12 h. In 500 μM glutamate-treated neurons, Bax and c-caspase-3 markedly increased compared to their activities in control cultures. *A. arguta* (1, 10, and 50 μg/ml) significantly blocked the glutamate-induced increases of Bax and c-caspase-3 expression (Fig. 3).

Inhibitory effect of *A. arguta* on glutamate-induced elevation of $[Ca^{2+}]_i$ – Glutamate-induced neuronal death is usually associated with the elevation of $[Ca^{2+}]_i$ following NMDA receptor activation. In response to treatment with 500 μM glutamate, $[Ca^{2+}]_i$ showed a steep increase followed by a gradual decrease in cultured cortical neurons. In contrast, pretreatment with *A. arguta* (10 and 50 μg/ml) significantly inhibited the increase of $[Ca^{2+}]_i$ induced by 500 μM glutamate throughout the measurement period (Fig. 4).

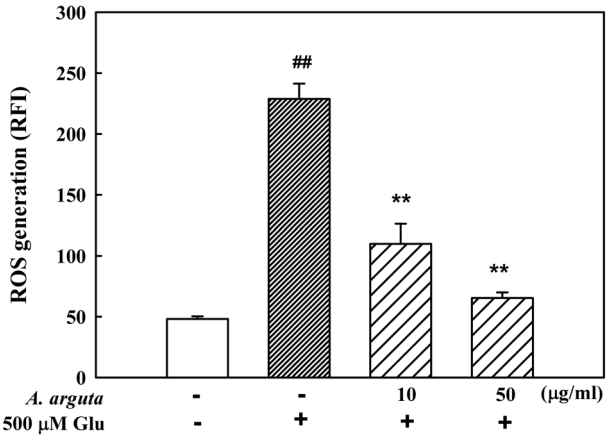

Fig. 5. Inhibitory effect of *A. arguta* on glutamate-induced ROS generation in cultured cortical neurons. RFI was monitored using a laser scanning confocal microscope. Values are expressed as mean ± S.E.M of data obtained from at least three independent experiments performed in duplicate. $^{\#\#}p < 0.01$ *vs*. control, $^{**}p < 0.01$ *vs*. 500 μM glutamate.

Inhibitory effect of *A. arguta* on glutamate-induced ROS generation – In H_2DCF-DA-loaded cerebral cortical neurons, the fluorescence intensity was increased by treatment with 500 μM glutamate for 4 h, indicating the generation of ROS. In 500 μM glutamate-treated neurons, the fluorescence intensity increased approximately five times to 237.7 ± 5.2 compared with control neurons of 48.11 ± 2.16. *A. arguta* (10 and 50 μg/ml) significantly blocked the glutamate-induced increase of fluorescence intensity, showing 53.9 ± 4.9 and 61.9 ± 1.7, respectively (Fig. 5).

Discussion

Many studies have demonstrated that excessive neuronal release of glutamate resulting in overstimulation of glutamate receptors, especially NMDA receptor subtype, is critically related to brain injury under neurodegenerative states such as stroke (Choi, 1988; McDonald *et al*., 1988; Weinberger, 2006). Since the NMDA receptor is highly permeable to Ca^{2+}, activation of the NMDA receptor elevates the influx of Ca^{2+} and triggers membrane depolarization. In turn, depolarization can activate plasma membrane voltage-dependent Ca^{2+} channels, resulting in additional Ca^{2+} influx (Mody and MacDonald, 1995). Although $[Ca^{2+}]_i$ promotes many normal intracellular signaling pathways, excessive increase in $[Ca^{2+}]_i$ causes mitochondrial Ca^{2+} overload, activation of the mitochondrial permeability transition, and excessive generation of ROS leading to lipid peroxidation and neuronal cell damage (Nicholls, 2009; Pereira and Oliveira, 2000). In the present study using cultured cortical neurons, glutamate increased $[Ca^{2+}]_i$ and ROS generation and produced neuronal cell death. In previous reports, we demonstrated that glutamate-induced neuronal cell death, increase of $[Ca^{2+}]_i$, and ROS generation were significantly inhibited by MK-801, indicating that the neurotoxicity was mediated by the activation of NMDA receptors (Ban *et al*., 2004; Kim *et al*., 2012). Treatment of neurons with *A. arguta* significantly suppressed glutamate-induced neuronal cell death, $[Ca^{2+}]_i$ increase, and ROS generation in cultured cortical neurons. It is suggested that *A. arguta* protected cultured cortical neurons against glutamate neurotoxicity via inhibiting $[Ca^{2+}]_i$ increase and ROS generation.

Damage to neurons caused by excessive ROS generation is associated with mitochondrial impairment, which leads to activation of an apoptosis cascade under ischemic neuronal damage (Chan, 2004, Matsuda *et al*., 2009). Members of the BcL-2 family, such as BcL-2, BcL-xs, BAK, and Bax, regulate the progress of apoptosis (Adams and Cory, 1998). BcL-2, a well-known anti-apoptotic protein, prevents apoptosis by preserving mitochondrial integrity and inhibiting the release of cytochrome c into cytosol (Graham and Chen, 2001; St Clair *et al*., 1997). In contrast, pro-apoptotic proteins, such as Bax and BAK, are inserted into the outer mitochondrial membrane and trigger the release of cytochrome c (Chao and Korsmeyer, 1998). Released cytochrome c into cytosol forms the apoptosome to activate caspase-9 and caspase-3 (Hetz *et al*., 2005). Activation of caspase-3 cleaves numerous proteins, triggering biochemical cascades that lead to cell death (Chan and Mattson, 1999). In the present study, glutamate caused overexpression of Bax and c-caspase-3, and in result, apoptotic neuronal death in cultured cortical neurons, which was confirmed by Hoech33342 staining. These changes were significantly suppressed by treatment with *A. arguta* in the present study. These results suggest that *A. arguta* inhibited glutamate-induced apoptosis cascade to protect cultured neurons.

Many studies suggested that Ca^{2+} signals activate enzymes associated in ROS generation leading to lipid peroxidation and neuronal damage, whereas ROS increase is required for the intracellular Ca^{2+} rise derived from intracellular Ca^{2+} stores or the increase of Ca^{2+} entry can be related to a facilitatory role of ROS (Espinosa *et al*., 2009; Giambelluca and Gende, 2008). It was not elucidated whether *A. arguta* inhibited ROS generation through the inhibition of $[Ca^{2+}]_i$ increase, or vice versa, in

the present study. *A. arguta* is abundant in anti-oxidant phenolic compounds (Latocha *et al*., 2010; Lim *et al*., 2005; Montefiori *et al*. 2009; Takano *et al*., 2003). It, thus, was presumed that the suppression of glutamate-induced apoptotic neuronal death by *A. arguta* might be due to its inhibition of ROS generation. However, the significant increase of ROS generation took more than 1 h, while the elevation of $[Ca^{2+}]_i$ occurred within seconds after the treatment with glutamate. This result suggests that *A. arguta* could inhibit the glutamate-induced ROS generation via the blockade of $[Ca^{2+}]_i$ increase, and in result, inhibit neuronal apoptosis.

Phytochemically, various anti-oxidant compounds such as catechin and epicatechin have been isolated from *A. arguta* (Lim *et al*., 2005; Takano *et al*., 2003). Many studies have demonstrated that these compounds showed significant neuroprotective activities against β amyloid protein- and hydrogen peroxide-induced neurotoxicity in cultured neurons (Ban *et al*., 2006; Crispo *et al*., 2010). Therefore, these compounds are probably involved in the mechanisms of neuroprotective effects of *A. arguta* against glutamate-induced cultured neuronal death. However, further studies should determine the specific components in *A. arguta* that are responsible for preventing glutamate-induced neurotoxicity.

In summary, we demonstrated in the present study a novel pharmacological activity of *A. arguta* and its mechanism. *A. arguta* effectively prevented glutamate-induced neurotoxicity in primarily cultured neurons via the inhibition of $[Ca^{2+}]_i$ increase and ROS generation. This result may help to explain a beneficial effect of the leaf and stem of *A. arguta* on neurodegenerative effect of glutamate in pathophysiological conditions such as stroke, epilepsy and other central disease.

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