Actinidia arguta Protects Cultured Cerebral Cortical Neurons against Glutamate-Induced Neurotoxicity via Inhibition of $[\text{Ca}^{2+}]_i$ 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 $\mu$M 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 $\mu$M glutamate-induced elevation of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) and reactive oxygen species (ROS) generation, which were measured by fluorescent dyes, Fluo-4 AM and H$_2$DCF-DA, respectively. These results suggest that A. arguta may prevent glutamate-induced apoptotic neuronal death by inhibiting $[\text{Ca}^{2+}]_i$ 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 $\text{Ca}^{2+}$ 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; Schindler 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$_2$DCF-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). Fetal bovine serum was purchased from JRS Biosciences (Lenexa, KS, 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 °C, 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 °C 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$_2$DCF-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 ±