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Combined treatment with capsaicin and resveratrol enhances neuroprotection against glutamate-induced toxicity in mouse cerebral cortical neurons

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ABSTRACT

Capsaicin and resveratrol as natural products have a variety of beneficial effects. However, capsaicin is also a neurotoxic agent, rendering its effect on the nervous system confusing. The aim of this study was to investigate whether capsaicin and/or resveratrol have a protective effect against glutamate (Glu)-induced neurotoxicity. After exposure to glutamate for 15 min, cerebral cortical neurons of ICR mouse fetuses on embryonic days 15–16 were post-treated with capsaicin and/or resveratrol for 24 h. Glu induced a significant reduction in cell viability, but the cell viability increased significantly with capsaicin or resveratrol treatment and further was highest in the neurons co-treated with both phytochemicals. Glu-induced reactive oxygen species generation and apoptotic neuronal death also significantly decreased by a combined treatment with both phytochemicals. Due to Glu insults, the reduced mRNA levels of cytoplasmic glutathione peroxidase, copper/zinc and manganese superoxide dismutases, and Bcl-x_L and the overexpressed mRNA levels of interleukin-1β and tumor necrosis factor-α were significantly restored by post-treatment of capsaicin and/or resveratrol. These findings indicate that capsaicin and resveratrol are neuroprotective against Glu-induced toxicity and that the combined treatment of both phytochemicals can enhance the neuroprotection, suggesting a useful therapeutic application in the treatment of neurodegenerative disorders.

1. Introduction

Glutamate is a major excitatory amino acid neurotransmitter in the central nervous system (CNS) involved in fast synaptic transmission, neuronal plasticity, outgrowth and survival, as well as memory, learning and behavior (Sucher et al., 1996). However, glutamate plays a crucial role in pathological neuronal cell death within the CNS (Rossler et al., 2004). Glutamate toxicity induces neuronal apoptosis and cell loss associated with acute and chronic neurodegenerative diseases, including epilepsy, Parkinson's disease, Alzheimer's disease, and ischemia (Greenamyre et al., 1985; Siesjo, 1981). Therefore, neuroprotection against glutamate-induced toxicity has been a therapeutic strategy for treating both acute and chronic forms of neurodegeneration (Trist, 2000).

The mammalian brain has antioxidant enzyme defense mechanisms against oxidative stress such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Sampath et al., 1994; Spina et al., 1992). In neurodegenerative diseases, excessive accumulation of glutamate causes activation of glutamate receptors, generation of oxygen radicals, and inactivation of related antioxidant enzymes such as SOD and GPx, leading to neuronal oxidative injury, which contributes to the pathogenesis of neuronal degeneration in CNS diseases (Coyle and Puttfarcken, 1993; Satoh et al., 1998; Yasuda et al., 1980). Considerable evidence also suggests that cytokine overexpression, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), is important in the pathophysiology of neurodegenerative disorders (Chaparro-Huerta et al., 2008), and such cytokines participate in both excitotoxic and apoptotic neuronal death (Allan et al., 2005).

The polyphenolic compound resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a naturally occurring phytochemical found in more than 70 plant species and is enriched in grapes, mulberries, and red wine (Gong et al., 2007). Resveratrol possesses strong antioxidant and anti-inflammatory properties (Sakata et al., 2010) and its protective activity has been reported in different experimental models for neurodegenerative diseases both in vitro and in vivo (Robb and Stuart, 2010). Resveratrol appears to produce some of these benefits by activating silent mating type information regulation 2 homolog 1 (SIRT1) (Baur and Sinclair, 2006). SIRT1 is believed to

Abbreviations: ROS, reactive oxygen species; SOD1, copper/zinc superoxide dismutase; SOD2, manganese SOD; GPx1, cytoplasmic glutathione peroxidase; CNS, central nervous system; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; SIRT1, silent mating type information regulation 2 homolog 1; TRPV1, transient receptor potential vanilloid 1; DMEM, Dulbecco's modified Eagle's medium; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescin diacetate; MTT, 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl-tetrazolium bromide; Glu, glutamate; Cap, capsaicin; Res, resveratrol.

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play important roles in cellular differentiation, survival, and metabolism (Haigis and Guarente, 2006; Longo and Kennedy, 2006), and is highly expressed in the developing and adult brain (Iwahara et al., 2009; Sakamoto et al., 2004).

Capsaicin (8-methyl-*N*-vanillyl-6-nonemide), the major component in hot peppers of the plant genus *Capsicum*, is used in foods and as a traditional medicine worldwide to treat various disorders (Corson and Crews, 2007). Capsaicin exhibits excitatory properties through the transient receptor potential vanilloid 1 (TRPV1) receptor (Morita et al., 2006). TRPV1, which is involved in peripheral nociception, is widely expressed in the cerebellum, mesencephalon, hypothalamus, hippocampus, and cerebral cortex of the brain (Mezey et al., 2000; Roberts et al., 2004). Capsaicin exerts various biological and pharmacological properties, including antioxidative, anti-inflammatory, anti-apoptotic, and anti-epileptic effects (Joe and Lokesh, 1994; Kim et al., 2003; Lee et al., 2011; Pegorini et al., 2005). However, capsaicin efficiently induces apoptosis in some cells types, including glioma cells, hepatocarcinoma cells, rat thymocytes, and human B-cells (Amantini et al., 2004; Jeftinija et al., 1992; Qiao et al., 2005; Wolvetang et al., 1996). Furthermore, capsaicin induces apoptotic cell death in rat trigeminal primary neurons when administered during the neonatal period (Sugimoto et al., 1998) and causes neurotoxicity in a subpopulation of cultured dorsal root ganglion neurons (Chard et al., 1995). These findings suggest that capsaicin may have dual functions in cells as a beneficial and adverse agent in the CNS.

Based on the observed effects of capsaicin and resveratrol described above, we investigated the effect of capsaicin and/or resveratrol against glutamate-induced neurotoxicity in mouse cerebral cortical neurons in vitro using several parameters, including cell viability, reactive oxygen species (ROS) generation, apoptosis, and mRNA expression of antioxidant enzymes, the anti-apoptotic regulator Bcl-x_L, pro-inflammatory cytokines, TRPV1, and SIRT1.

2. Materials and methods

2.1. Animals

Male and female ICR mice (8–10 weeks old) were purchased from Samtako, a commercial breeder (Kyoungki, South Korea). One male and three female mice were housed in a cage for mating. The environmental conditions were controlled, with an ambient temperature of 21 ± 2 °C, relative humidity of 55 ± 10%, air ventilation rate of 10 cycles per hour, and a 12:12 h light: dark cycle. The animals were fed standard mouse chow (Samyang Ltd., Incheon, South Korea) and tap water *ad libitum* throughout the experimental period. All experiments were approved and carried out according to the Guide for Care and Use of Animals (Chungbuk National University Animal Care Committee, according to NIH #86-23).

2.2. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Thermo Scientific (Rockford, IL, USA). Neurobasal medium, B27 supplement, and trypsin were purchased from Gibco (Grand Island, NY, USA). 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA) was purchased from Molecular Probes (Eugene, OR, USA). Capsaicin, resveratrol, glutamate, 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl-tetrazolium bromide (MTT), poly-L-lysine, and Eagle's minimum essential medium (MEM) were purchased from Sigma (St. Louis, MO, USA).

2.3. Primary culture of cerebral cortical neurons

Primary cortical neuronal cultures were prepared using ICR mouse fetuses on embryonic days 15–16. Fetuses were isolated from a dam anaesthetized with ether. Cortical hemispheres were dissected under sterile conditions and placed in MEM containing 0.125% trypsin. After slightly triturating the cortices, the cells were incubated for 20 min at 37 °C. Dissociated cells were collected by centrifugation (1500 rpm, 5 min) and resuspended in DMEM with 10% fetal bovine serum to stop trypsin activity. Cells were plated onto poly-L-lysine (200 μg/ml)-coated 12-well plates to measure cell viability and to extract RNA at a density of about 1.5×10^5 cells/well and coverslips to measure ROS and apoptotic neuronal death at a density of about 0.7×10^5 cells/well. After cells attached to the substrate for 2 h, serum-free Neurobasal medium supplemented with B27 was added, and cultures were kept at 37 °C in a 5% CO_2 atmosphere. The medium was changed every 3 days. The following experiments were performed on neurons grown for 7 days in vitro. The glial content of cultures maintained in the Neurobasal medium was estimated to be about 0.5% of the total cell population (Brewer et al., 1993).

2.4. Cell treatment

At 7 days in vitro, the culture medium was removed and cells were stimulated with 1–100 μM glutamate for 15 min at 37 °C. After washing with HEPES-buffered solution (incubation buffer), containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl, and 2.3 mM $CaCl_2$ at pH 7.4, to remove residual glutamate, neurons were incubated in Neurobasal medium supplemented with B27 in the presence or absence of 25–75 μM capsaicin and/or 20–100 μM resveratrol for 24 h at 37 °C.

2.5. MTT colorimetric assay

This method is based on reduction of the tetrazolium salt MTT into a crystalline blue formazan product by cellular oxidoreductase (Berridge and Tan, 1993). Therefore, the amount of formazan produced is proportional to the number of viable cells. After the incubation was completed, culture medium was replaced with a solution of MTT (0.5 mg/ml) in serum-free DMEM. After a 4 h incubation at 37 °C, this solution was removed, the resulting blue formazan was solubilized in 0.4 ml of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Serum-free DMEM was used as the blank solution. Cell viability data are expressed as percentages relative to that of control.

2.6. Measurement of ROS generation

The H_2DCF-DA fluorescent product from the microfluorescence assay was used to monitor ROS generation. When taken up by cell, H_2DCF-DA is converted to nonfluorescent H_2DCF by esterases, and rapidly oxidized to the highly fluorescent DCF by intracellular H_2O_2 and other peroxides (Zhu et al., 1994). Neurons grown on coverslips were washed twice with incubation buffer, and the uptake of H_2DCF-DA (final concentration, 10 μM) dissolved in DMSO was carried out for 20 min. After washing, coverslips containing cortical neurons loaded with H_2DCF-DA were mounted on microscope slides, and fluorescence images were visualized under a fluorescence microscope (Zeiss, Oberkochen, Germany) using 490 nm excitation and 520 nm emission filters. The H_2DCF-DA challenge and fluorescence intensity measurements were performed in the dark. Quantitative analysis of the immunofluorescence data was conducted using ImageJ (http://rsbweb.nih.gov/ij/). ROS data are expressed as percentages relative to those of the control.

2.7. Measurement of apoptotic neuronal death

The bis-benzimidazole dye, Hoechst 33342, penetrates the plasma membrane and stains DNA in cells without permeabilization (Ishikawa et al., 1999). In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained with Hoechst 33342. These morphological changes in the nuclei of apoptotic cells can be visualized by fluorescence microscopy. Neurons on coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min, and then stained with Hoechst 33342 dye (final concentration, 1 μg/ml) dissolved in incubation buffer for 10 min. The morphological changes were examined under UV illumination using a fluorescence microscope (Zeiss). The dye was excited at 360 nm, and emission was filtered with a 460 nm barrier filter. Neurons with condensed or fragmented DNA and normal DNA were counted to quantify the apoptotic process. Data are shown as the number of apoptotic neurons as a percentage of total neurons.

2.8. Quantitative real-time reverse transcription polymerase chain reaction (RT–PCR)

Total RNA was isolated from cortical neurons using a Trizol Reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Four micrograms of total RNA was utilized for reverse transcription to generate cDNA using a high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), and the generated cDNA was used as a template for subsequent PCR reactions. Quantitative real-time PCR reactions were conducted using the Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sets were used to amplify copper/zinc superoxide dismutase (SOD1), manganese SOD (SOD2), cytoplasmic GPx (GPx1), IL-1β, TNF-α, TRPV1, SIRT1, and an internal standard GAPDH (Table 1). Reactions were carried out in a 7500 Real-Time PCR System (Applied Biosystems).

2.9. Statistical analysis

Statistical differences between groups were analyzed by one-way analysis of variance with subsequent Tukey's multiple comparison tests. Statistical significance was established at $P < 0.05$. All data are expressed as mean ± SEM.