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Effects of [6]-shogaol on cholinergic signaling in HT22 cells following neuronal damage induced by hydrogen peroxide

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ABSTRACT

Cholinergic neurons play a major role in memory and attention. The dysfunction and death of these neurons, especially in the hippocampus, are thought to contribute to the pathophysiology of memory deficits associated with Alzheimer's disease (AD). Therefore, studying the cholinergic properties and cell survival may help in treating this disease. We investigated the possible effects of [6]-shogaol on cholinergic signaling in HT22 hippocampal neuronal cells. HT22 cells express essential cholinergic markers, including choline acetyltransferase (ChAT) and choline transporter (ChTp). HT22 cells treated with H_2O_2 for 3 h showed an increase in ROS production (35%). These features were partly recovered by [6]-shogaol. Treating H_2O_2-treated HT22 cells with [6]-shogaol markedly increased the expression of ChAT and ChTp, an effect similar to that of brain-derived neurotrophic factor (BDNF). Furthermore, K-252a, an inhibitor of the BDNF receptor Trk B, attenuated the effects of both [6]-shogaol and BDNF. These data suggest that [6]-shogaol protects neurons by increasing ChAT and ChTp expression through a BDNF increase and thus may be useful for treating neurodegenerative diseases.

1. Introduction

Alzheimer's disease (AD) is the most devastating form of dementia leading to progressive memory loss and other cognitive decline. Of several brain regions affected by AD, the hippocampus, a key player in memory and learning, is most remarkably affected. Decreased hippocampal function and loss of cholinergic neurons have been implicated in progressive memory deficits in both normal aging and AD (Counts et al., 2010; Elvander-Tottie et al., 2009; Hartig et al., 2010; Schliebs and Arendt, 2011).

The cholinergic neurotransmitter system requires the expression of proteins involved in acetylcholine synthesis, storage, and release. Choline acetyltransferase (ChAT) and Choline transporter (ChTp) play important roles in cholinergic neurons. ChAT, a specific marker for cholinergic neurons and functions, catalyzes acetylcholine synthesis from acetyl-coenzyme A and choline in the cytoplasm at the nerve terminals (Aizawa and Yamamuro, 2010; Gallagher et al., 1990; Lee et al., 1994). AD patients have reduced levels of ChAT mRNA and its activity, along with a selective loss of cholinergic neurons associated with cognitive decline (Aizawa and Yamamuro, 2010; Hartig et al., 2002; Schliebs and Arendt, 2006). ChTp is a transmembrane protein also localized at the nerve terminals that bind to extracellular acetylcholine with high affinity and transports it into the cell (Bales et al., 2006; Perry et al., 1993). Reduced expression of these two proteins is common in the pathogenesis of AD and indicates cholinergic dysfunction (Aizawa and Yamamuro, 2010; Bales et al., 2006; Desmarais and Gauthier, 2010). Therefore, increasing the expression of cholinergic signaling proteins may result in new treatments for neurodegenerative diseases.

A recent study determined that HT22 cells exhibit characteristics of cholinergic neurons and is a useful as an *in vitro* model of hippocampal cholinergic neurons (Liu et al., 2009). Ginger, the rhizome of *Zingiber officinale,* is widely used throughout the world as an important spice and traditional herb (White, 2007). Ginger extract and its biologically active compounds, gingerols and [6]-shogaol, exhibit certain pharmacological activities, including anti-inflammation (Grzanna et al., 2005; Lantz et al., 2007), anti-emesis (Sharma et al., 1997), anti-tumor (Katiyar et al., 1996; Surh, 2002), antioxidation (Eguchi et al., 2005; Masuda et al., 2004), and analgesic effects (Aktan et al., 2006; Lantz et al., 2007) in numerous diseases. Recent studies have shown that treating cells with [6]-shogaol blocks neuronal cell-death *in vitro* and enhances motor neuronal

Abbreviations: AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; ChAT, choline acetyltransferase; ChTp, choline transporter; DCF-DA, 2′,7′-dichlordihydrofluorescin diacetate; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; Trk, tyrosine kinase.

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recovery in rats (Kim and Kim, 2004; Kyung et al., 2006). The effects of [6]-shogaol on the cholinergic neuronal damages, however, is still unknown. Herein, we investigated how [6]-shogaol changes the expression of cholinergic proteins and may protect hippocampal neuronal cells using HT22 cells.

2. Materials and methods

2.1. Cell culture

HT22 cells were maintained in DMEM (Hyclone, Canada) containing 5% FBS (Hyclone, Canada) and 1% antibiotic solution (Sigma, USA) in a humidified incubator with 5% CO_2 in air at 37 °C. The medium was changed every 2–3 days. For neuronal differentiation experiments, cells were seeded in 60-mm cell culture dishes (Nunc, Tokyo, Japan) 24 h before treatment. Cells were treated with hydrogen peroxide (H_2O_2, 1 mM), [6]-shogaol (10 μM), brain-derived neurotrophic factor (BDNF, 10 μM), and the specific tyrosine kinase (Trk) B inhibitor, K-252a (300 nM). [6]-Shogaol was purchased from Wako chemicals. Recombinant human BDNF was purchased from Peprotech and K-252a from ENZO Life Sciences.

2.2. Measurement of intracellular reactive oxygen species (ROS) production by DCF-DA assay

HT22 plated in a 48-well dish were untreated or treated with 1 mM H_2O_2 (in DMEM medium) in the absence or presence of [6]-shogaol and incubated for 3 h. After washing with PBS, cells were stained with 10 μM 2′,7′-dichlordihydrofluorescin diacetate (DCF-DA) in PBS for 30 min in the dark, washed twice with PBS, and extracted with 1% Triton X-100 in PBS for 10 min at 37 °C. Fluorescence was recorded with an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

2.3. Chromatin condensation assay; Hoechst 33258

Hoechst 33258, purchased from Invitrogen, is a noncytotoxic DNA dye that permits the determination of the total chromatin quantity variations and the degree of chromatin condensation (Ezoulin et al., 2008). It preferentially binds to triplet adenine and thymidine base pairs. HT22 plated in a 48-well dish were untreated or treated with [6]-shogaol and 1 mM H_2O_2 in the absence or presence of [6]-shogaol. Hoechst 33258 was used on cells at a final concentration of 5 μg/ml (in PBS) (Ex. 360 nm/Em. 450 nm). Propidium iodide (Sigma–Aldrich) at 0.5 μl/ml (in PBS) was added into the cells, as previously validated (Debbasch et al., 2001).

2.4. Western blotting

Cell pellets were treated with lysis buffer (140 mM NaCl, 25 mM Tris–HCl (pH 7.4) and 1% NP-40) with freshly added protease inhibitor cocktail (BD Biosciences, San Jose, CA). The supernatants were then collected after rigorous agitation and protein concentration was measured. The proteins were subjected to SDS–polyacrylamide gel electrophoresis using 10% gels, transferred to PVDF membranes (Bio-Rad, Hercules, CA), and blocked with 5% non-fat milk in TBS (50 mM Tris base, pH 7.5, 150 mM NaCl). The membranes were incubated with primary antibody raised against BDNF, Bax, or Bcl-2 for cell survival (Santa Cruz, 1:1000 dilutions). ChAT and ChTp were used as cholinergic neuron markers (Millipore, 1:1000 dilutions). The blots were further incubated with peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG (1:20,000 dilution; Millipore). The immunoreactions were visualized using a Super Signal West Dura Extended Duration Substrate (Pierce) and analyzed using a ChemiImager (Alpha Innotech, San Leandro, CA).

2.5. Reverse-transcription PCR analysis

Total RNA was extracted from HT22 cells using a Rivoex extraction kit (Geneall, Korea) following the manufacturer's instruction. First-strand cDNA was synthesized with 0.5–1 μg of total RNA using an RT-PCR kit (Invitrogen, CA, USA). The cDNA (1 μL) was then amplified in a 20 μL reaction using a PCR kit (Invitrogen, CA, USA) and the appropriate forward and reverse primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The PCR products (10 μL) were analyzed and visualized on 1% agarose gels.

2.6. Statistical analysis

Data are expressed as mean ± SD. The data were analyzed using a Student's *t* test and repeated-measures ANOVA followed by a Bonferroni test. A *P* value <0.05 was considered significant.

3. Results

3.1. Expression of cholinergic markers in HT22 cells

To verify that HT22 cells possess properties of cholinergic neurons, we extracted proteins from undifferentiated and differentiated HT22 cells and performed Western blots for the cholinergic markers ChAT and ChTp. Western blots revealed that both undifferentiated and differentiated HT22 cells expressed both molecules. However, differentiated cells expressed significantly more of both molecules than undifferentiated cells (Fig. 1).

3.2. [6]-Shogaol attenuates the increase of DNA condensation

After 3 h of H_2O_2 (1 mM in DMEM medium) incubation of HT22 cells showed a significant overproduction of ROS (35%) (Fig 2) and significant increased DNA condensation (21%) (Fig 3). ROS production was significantly lower in cells co-treated with [6]-shogaol (10 μM) than in H_2O_2-treated cells (Fig. 2). There was also slightly but statistically noteworthy reduction in DNA condensation when cells were co-treated with [6]-shogaol as compared to H_2O_2 only treated cells (Fig3).

3.3. Influence of [6]-shogaol on the expression of cholinergic proteins

In order to determine whether [6]-shogaol affects the expression of ChAT and ChTp in cultured HT22 cells, we performed Western blots to identify the expression profiles of ChAT and ChTp in response to [6]-shogaol treatment and oxidative stress (Fig. 4). Cells were treated with H_2O_2 showed decreased expression of cholinergic markers. Cells treated with both H_2O_2 and [6]-shogaol expressed significantly more cholinergic proteins than treated cells with H_2O_2 alone (Fig 4). Treating cells with both H_2O_2 and [6]-shogaol (10 μM) increased ChAT and ChTp expression by ~158.2% and ~158.7%, respectively ($P < 0.05$) (Fig. 4).

3.4. [6]-Shogaol increases BDNF expression

Because BDNF is an important neurotrophic factor in regulating neuronal function (Aizawa and Yamamuro, 2010; Jean et al., 2008; Navakkode and Korte, 2011; Ward and Hagg, 2000), we assayed BDNF expression in HT22 cells by RT-PCR analysis and Western blot. The results revealed that HT22 cells expressed BDNF

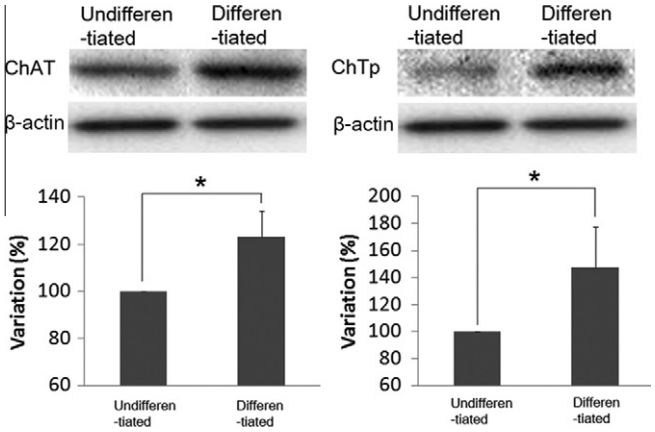

Fig. 1. Expression of cholinergic proteins in undifferentiated and differentiated HT22 cells. HT22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and differentiated in NeuroBasal medium supplemented with 1× N2 for 24 h. The data are reported as the mean ± SD of five different preparations. $^*P < 0.05$ vs. control.