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Diallyl disulfide impairs hippocampal neurogenesis in the young adult brain

Seung Taek Ji[a,1], Min-Sun Kim[b,1], Hee Ra Park[a], Eunjin Lee[a], Yujeong Lee[a], Young Jung Jang[a], Hyung Sik Kim[a], Jaewon Lee[a,*]

[a] Department of Pharmacy, College of Pharmacy and Research Institute for Drug Development, Longevity Life Science and Technology Institutes, Pusan National University, Geumjeong-gu, Busan 609-735, Republic of Korea
[b] Department of Pharmacy, College of Pharmacy, Sunchon National University, Sunchon 540-742, Republic of Korea

HIGHLIGHTS

- DADS inhibited the proliferation of newly generated cells in the hippocampus.
- DADS decreased the levels of ERK and BDNF-CREB signaling in the hippocampus.
- DADS reduced memory retention observed in mice treated with high-dose.
- High-dose DADS has adverse effects on hippocampal neurogenesis and neurocognitive function.

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ABSTRACT

Garlic and garlic extracts are used as seasonings and are generally considered beneficial to human health, which include antioxidant and neuroprotective properties in neurological disorders. In the present study, we examined the effects of garlic sulfur components on the proliferation of neural progenitor cells (NPCs) and hippocampal neurogenesis. Of the sulfur compounds extracted, diallyl disulfide (DADS) significantly suppressed the proliferation of NPCs, whereas other sulfur containing components had no effect. In order to investigate the effect of DADS on adult hippocampal neurogenesis, DADS was administered orally to young (6 week-old) male C57BL/6 mice for 2 weeks. It was found that 10 mg/kg of DADS significantly decreased the proliferation of NPCs in the dentate gyrus without affecting the survival of newly generated cells. Furthermore, DADS decreased levels of hippocampal BDNF, phosphorylated CREB signaling, and phosphorylated ERKs, which are known to be related to hippocampal neurogenesis and NPCs proliferation. In addition, DADS induced significant memory defects as compared with controls. We report that DADS may have adverse effects on hippocampal neurogenesis and neurocognitive functions by modulating ERK and BDNF-CREB signaling, and suggest that the advisability of consuming large amounts of garlic products should be considered, particularly during the period of neural growth.

1. Introduction

Garlic is considered a health food, and its products are popular herbal supplements (Graham et al., 2008), and thus garlic essential oil, garlic oil macerate, garlic powder, and garlic extract are currently readily available to the consumer. Garlic has been shown to have antimicrobial and anticancer effects and to protect from cardiovascular disease (Lissiman et al., 2012). Actually, the sulfur compounds of garlic are responsible for its various effects. Allicin is a major component of crushed garlic, but is highly unstable to heat or light and is rapidly metabolized into various sulfur compounds. The bioactive compounds resulting from the degradation of allicin include diallyl disulfide (DADS), diallyl trisulfide (DATS), and diallyl sulfide (Amagase, 2006), and these diallyl sulfides are known to reduce reactive oxygen species levels and increasing glutathione-S-transferase expression (Iciek et al., 2012; Maldonado et al., 2011). Sulfur compounds can be conveniently classified as oil- or water-soluble. In dehydrated garlic powder oil-soluble sulfur compounds containing DADS and DATS predominate (Chauhan, 2006), whereas aged garlic extract (AGE; a product of fermented (10–20 months) garlic) contains mainly water-soluble compounds and has greater antioxidant activity than dehydrated

Abbreviations: CREB, cAMP-response element-binding protein; BDNF, brain-derived neurotropic factor; BrdU, 5′-bromo-2′-deoxyuridine; DG, dentate gyrus; GCL, granular cell layer; NPCs, neural progenitor cells.

* Corresponding author. Tel.: +82 51 510 2805; fax: +82 51 513 6754.
E-mail address: neuron@pusan.ac.kr (J. Lee).

[1] These authors contributed equally to this work.

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garlic powder (Borek, 2001; Brunetti et al., 2009). The main water soluble sulfur compounds in AGE are S-allyl cysteine (SAC) and S-allyl mercaptocysteine (SAMC) (Banerjee et al., 2003; Lawal and Ellis, 2011).

Usually neurogenesis occurs only during embryonic development, although it is generally accepted that newly generated cells in the hippocampus can be integrated into neural networks throughout adulthood (Zhao et al., 2008). This process of hippocampal neurogenesis plays important roles in new memory formation and in the maintenance of old memories (Kim and Sun, 2012). Adult hippocampal neurogenesis can be regulated by various signals that modulate the effects of neurotransmitters, growth factors, and neurotrophic factors, and by various environmental stimuli (Park and Lee, 2011). Interestingly, it was reported herbal supplementation can positively and negatively modulate hippocampal neurogenesis even in adulthood (Jiang et al., 2005; Kim et al., 2008; Kong et al., 2010; Oh et al., 2012; Park et al., 2012; Si et al., 2011; Wang et al., 2012). Several reports have been issued on the neuroprotective effects of garlic or AGE in Alzheimer's disease and cerebral ischemia, and on apoptotic cell death (Chauhan, 2005; Colin-Gonzalez et al., 2011; Jackson et al., 2002). However, few reports are available on the effects of AGE or of its bioactive components on the regulation of neural stem cells and neurogenesis (Nam et al., 2011).

In the present study, we evaluated the effects of the bioactive components of garlic and AGE on the regulation of neural progenitor cells (NPCs) in culture and on hippocampal neurogenesis and neurocognitive functions in young adult mice.

2. Materials and methods

2.1. Reagents

SAC, SAMC, and DADS were purchased from Sigma Chemical (St. Louis, MO). 5′-Bromo-2′-deoxyuridine (BrdU) was obtained from Acros Organics (Morris Plains, NJ).

2.2. Cell proliferation and viability

C17.2 NPCs were originally isolated from neonatal mouse cerebellum and immortalized (Snyder et al., 1992). This neural progenitor cell line can differentiate into three brain cell types, namely, neurons, astroglia, and oligodendrocytes. The C17.2 NPCs used in the present study were generously provided by Dr. Cepko at Harvard University. Cells were maintained in plastic culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 5% horse serum, and 2 mM glutamine in humidified 5% CO_2/95% air atmosphere at 37 °C. Cell viability and proliferation were measured using MTT assays. Briefly, cell proliferation assays were conducted by seeding cells (1×10^4 cells/ml) in 96-well plates containing DMEM containing 10% FBS, and after 24 h, treated with different concentrations of garlic compounds (0.1–10 μM). After treatment, media was removed, cells were washed twice with PBS, and 200 μl of a 0.5 mg/ml MTT solution in PBS was added to each well. Plates were incubated at 37 °C for 4 h, MTT solution was removed, and cells were lysed using a solubilization solution (1:1 DMSO:ethanol). The formazan dye product was quantified using an ELISA microplate reader at an absorbance of 560 nm.

2.3. Animals and DADS administration

Male C57BL/6 mice (5 weeks old) were obtained from Daehan Biolink Co. Ltd. (Chungbuk, Korea) and maintained under temperature- and light-controlled conditions (20–23 °C, 12 h light/12 h dark cycle) with food and water provided *ad libitum*. Mice were divided randomly into three groups (proliferation group, survival group, and biochemical analysis group), and each group contained control and DADS administrations (15 mice/group). Mice were housed 5–6 animals per cage. The mice were acclimatized for one week prior to oral administration of DADS at 1 or 10 mg/kg daily for 14 days. To evaluate new cell generation, mice in each group were administered six intraperitoneal (i.p.) injections of BrdU (100 mg/kg body weight, twice a day) on the last three days of DADS administration, and to evaluate new cell survival, mice were administered BrdU for three consecutive days prior to DADS administration. The animal protocol used in this study has been reviewed by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC) on their ethical procedures and scientific care, and it has been approved (PNU-2011-000331).

2.4. Tissue preparation

Mice were sacrificed by ether exposure and cerebral cortices and hippocampi were excised for biochemical analysis. Tissues were stored at −80 °C and homogenized in a buffer containing 10 mM Tris buffer (pH 8.0), 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 2 μg/ml aprotinin, and 10 μg/ml pepstatin A. For histologic studies, mice were sacrificed 24 h after the last BrdU injection and then intracardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains were then removed, placed in the same fixative solution at 4 °C overnight, transferred to 30% sucrose solution, left at 4 °C to impregnate fully, and rapidly frozen in liquid nitrogen cooled 2-methylbutane. Brains were then serially sectioned at 40 μm in the coronal plane using a freezing microtome (MICROM, Walldorf, Germany). Sections were collected in Dulbecco's phosphate buffered saline (DPBS) solution containing 0.1% sodium azide and stored at 4 °C. Sections containing a portion of the hippocampal formation were saved.

2.5. Western blot analysis

After experimental treatments, tissue homogenates were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer and protein concentrations in samples were determined using a Bio-Rad protein assay kit using bovine serum albumin as the standard. Total protein equivalents for each sample (50 μg protein per lane) were then separated in SDS-polyacrylamide gels and transferred electrophoretically to Immobilon-P^{SQ} transfer membranes (Millipore Corporation, Billerica, MA). Membranes were placed immediately in a blocking solution (5% nonfat milk) at room temperature for 30 min, and then incubated with a diluted primary antibodies of phospho-ERK (Santa Cruz Biotechnology, CA), β-actin (Sigma), and ERK (Cell Signaling, MA) in TBS-T buffer (Tris–HCl based buffer with 0.2% Tween 20, pH 7.5) at 4 °C overnight. After washing (4 × 10 min), membranes were incubated with secondary antibody, that is, monoclonal anti-mouse antibody or polyclonal anti-rabbit antibody (Santa Cruz Biotechnology) in TBS-T buffer at room temperature for 1 h. Horseradish-conjugated secondary antibody labeling was detected by enhanced chemiluminescence and blots were then exposed to radiographic film. Pre-stained blue markers were used to determine molecular weights.

2.6. Quantification of newly generated cells

For immunostaining, free-floating brain sections were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS; pH 7.5), incubated in sodium citrate buffer containing 50% formamide, and then in 2 M HCl at 37 °C for 30 min. After denaturation, sections were neutralized with 0.1 M borate buffer, incubated in TBS containing 0.1% Triton X-100 and 3% goat serum (TBS-TS) at 37 °C for 30 min, and then with primary anti-BrdU antibody (Abcam, Cambridge, UK) in TBS-TS overnight at 4 °C, with biotinylated secondary goat anti-rat IgG antibody (1:200, Vector Laboratories, CA) at room temperature for 3 hr, and with ABC solution (avidin–peroxidase complex, Vectastain ABC reagent Elite Kit, Vector Laboratories) at room temperature for 1 h. They were then stained with diaminobenzidine (DAB) solution for 3 min, placed on slides, dried, mounted with permanent mounting medium (Fisher Scientific, Fair Lawn, NJ), and cover slipped. Images were acquired using a Nikon ECLIPSE TE 200-U microscope. BrdU-labeled cells were counted in 8 sections per brain (every sixth section); sections were taken at the rostro-caudal extent of the hippocampus in 5 mice per group. The granular cell layer (GCL) of the DG served as a reference for BrdU quantification. All cell counts were performed by one investigator unaware of group identities.

2.7. Immunostaining

Brain sections were co-immunostained with BrdU, NeuN, and phospho-CREB (Cell Signaling Technology, Danvers, MA), or with two cell markers, namely, GFAP (an astrocyte marker; Sigma) and Iba-1 (a microglia marker; Wako, Tokyo). Briefly, sections were blocked with TBS-TS at 37 °C for 30 min, incubated with primary antibodies in TBS-TS at 4 °C overnight, washed with TBS, incubated with secondary antibody labeled with Alexa Fluor-488, 568, or 633 (Invitrogen, Carlsbad, CA), and mounted onto slides using aqueous/dry mounting medium (Biomeda Corp., CA). Confocal fluorescence images were acquired using a FV10i Fluoview confocal microscope (Olympus, Tokyo).

2.8. BDNF assay

BDNF protein levels were measured using an ELISA kit (Millipore Corporation). Briefly, 100 μl of hippocampal homogenate samples and standards were added to wells pre-coated with rabbit anti-human BDNF polyclonal antibody. Plates were sealed with a plate sealer, incubated at 4 °C overnight, and then the plate sealer was gently removed and plates were washed with washing buffer at least 4 times. Diluted biotinylated mouse anti-BDNF monoclonal antibody (100 μl) was then added to each well and plates were then incubated at room temperature for 2–3 h and washed four times with washing buffer. Diluted streptavidin–HRP conjugate solution (100 μl) was then added to each well, and plates were incubated at room temperature for 1 h on a shaker. TMB/E substrate (100 μl) was then added to each well and plates were incubated at room temperature for 15 min. The reaction was stopped by adding