European Journal of Pharmaceutical Sciences 88 (2016) 267–273

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Effects of epigallocatechin-3-gallate on proliferation and differentiation of mouse cochlear neural stem cells: Involvement of PI3K/Akt signaling pathway

Yubo Zhang, Qiang He, Jinhui Dong, Zhanwei Jia, Fang Hao, Chunguang Shan *

E.N.T. Department 1, The Second Hospital of Hebei Medical University, Shijiazhuang 50000, China

ARTICLE INFO

Article history:
Received 30 January 2016
Received in revised form 11 March 2016
Accepted 11 March 2016
Available online 22 March 2016

Keywords:
Cochlear neural stem cells
EGCG
Neural differentiation
PI3 kinase
Akt

ABSTRACT

Since the majority of hearing impaired patients suffer from the significant loss of sensory hair cells and associated neurons, stem cell-based approaches hold great promise by replacing the damaged tissues in the ears. For instance, stem cells from the spiral ganglion could be isolated and expanded to regenerate neural structures of the inner ear. It is thus necessary to explore the potential procedures that may promote the proliferation and differentiation of such cochlear neural stem cells. In the present study, we study the effects of epigallocatechin-3-gallate (EGCG), a known antioxidant, for potential therapeutic use in NSC regeneration. At a non-toxic concentration, EGCG stimulated both proliferation and neurosphere formation in isolated mouse cochlear neural stem cell (NSC) in vitro. Specifically, the neural differentiation of NSC was promoted by EGCG treatment. The up-regulated neural function by EGCG was also supported by the increased calcium spike frequencies and enhanced neurite complexity in NSC-differentiated neurons. Finally, the induced neuron differentiation and Akt activation of cochlear NSC by EGCG were blocked by PI3 kinase inhibition. These data suggested that EGCG acts through phosphoinositide 3-kinase (PI3K)/Akt signaling in cochlea NSC to promote cell growth and neuron differentiation, which may be exploited for the treatment of hearing loss.

1. Introduction

Deafness or other complications related to hearing impairment have become common illnesses that currently affect millions of people worldwide. Based on epidemiological studies, roughly 2 to 3 out of every one thousand children in the United States are born with a detectable level of hearing loss, and more than twenty five percent of elderly people (older than 65) have disabling hearing loss (Lin et al., 2011a, 2011b). In China, it is also estimated that 21 million people have hearing abnormalities and a number of genetic mutations associated with hearing loss have been found and characterized (Fu et al., 2010; Qing et al., 2015). Since the sensory epithelium tissues of human ear do not possess a self-regeneration property as lower vertebrates, the implantation of stem cells offers a feasible option in order to replace the degenerated auditory part, the cochlea (Ito et al., 2001; Izumikawa et al., 2005; Li et al., 2003). However, the detailed components of processes and fundamental knowledge are still not clearly defined. Some important questions remain open, such as the mechanisms underlying the stem cell survival, growth, and terminal cell differentiation. Until now, the cochlea neural stem cells (NSCs) have been an attractive option in ear tissue transplantation (Hu et al., 2005; Ito et al., 2001; Li et al., 2003). They not only could enhance neuronal differentiation and survival, also support the regeneration of cochlear hair cells with afferent innervation. The current focus of research is to find out approaches or drugs that can promote the proliferation and neural differentiation of NSC, which may greatly facilitate the development of stem cell-based therapy for hear loss.

An increased interest has been shown on epigallocatechin-3-gallate (EGCG), or catechins in green tea polyphenols, which has been successfully used for preventing the damages due to reactive oxygen species or other deleterious stresses (Higdon and Frei, 2003). EGCG may contribute beneficial effects as an antioxidative and anti-inflammatory agent to a variety of diseases such as cancer, metabolic disorders, and immune diseases (Katiyar et al., 1999; Shen et al., 2009; Yagi et al., 2013). In the neurological system, EGCG is known to play protective roles by blocking a wide array of inflammatory injuries on neuronal cells. For instances, there has been emerging evidence that EGCG may alleviate neurotoxicity or neuronal damages (Ramassamy, 2006) following brain ischemia, peripheral nerve damages, or diabetic neuropathy. It has also been reported that the association occurred between EGCG treatment and attenuated progression of degenerated neuronal diseases such as Alzheimer's and Parkinson's diseases (Weinreb et al., 2004). Importantly, the effects of EGCG on NSC have been implied. Consistent with the concept that antioxidants could protect cell damages by scavenging deleterious effects of free radicals, EGCG significantly increases

* Corresponding author at: E.N.T. Department 1, The Second Hospital of Hebei Medical University, 215 Heping Road, Shijiazhuang 50000, China.
E-mail address: chunguangshan@126.com (C. Shan).

http://dx.doi.org/10.1016/j.ejps.2016.03.017
0928-0987/

hippocampus neural progenitor cell survival (Wang et al., 2012) and stimulates NSC growth following traumatic brain injury (Itoh et al., 2012). Additionally, the action of EGCG on neuron differentiation needs to be defined. While the effects of EGCG on some types of stem cells have emerged, there is limited information known about the effect of EGCG on neuronal cell differentiation derived from NSC. Whether or not EGCG could also extend its beneficial actions in promoting NSC proliferation and differentiation in the cochlea has not yet been investigated. In order to examine the potentially therapeutic impact of EGCG on hearing loss through stem cell replacement, we studied the in vitro effects of EGCG on cochlea neural stem cells and measured their proliferation, differentiation, and derived neural network. Our data have supported that EGCG can significantly enhance cochlea NSC-derived neuron numbers and functions, potentially through the activation of a phosphoinositide 3-kinase (PI3K)-dependent Akt signaling. These results suggest an important use of EGCG as a positive modulator for the NSC transplantation within the auditory part of the inner ear.

2. Methods & materials

2.1. Isolation and culture of cochlea neural stem cell

The sphere-forming stem cells were isolated from the neonatal mouse inner ear (postnatal day 1). In brief, the temporal bone was removed and cochlear organs were dissected, including otic capsule, spiral ligament and greater epithelial ridge from Reissner's membrane. The dissected tissues were dissociated by enzymatic digestion (0.25% Trypsin/EDTA) at 37 °C for 5 min, followed by pipetting up and down for 40 times. The cell suspensions were passed through 70 μm cell strainer and then transferred into a suspension culture plate in DMEM/F12 (Gibco, Grand Island, NY) with epidermal growth factor (EGF, 20 ng/ml, R&D Systems, Minneapolis, MN), basic fibroblast growth factor (bFGF, 10 ng/ml, R&D Systems), insulin-like growth factor 1 (IGF-1, 50 ng/ml, R&D Systems), and heparin sulfate (50 ng/ml) for five to seven days. All procedures performed in the study have been in accordance with the guidelines of Institutional Animal Care and Use Committee at The Second Affiliated Hospital of Hebei Medical University. For EGCG treatment, EGCG (Sigma, St Louis, MO) was added to the culture medium to make a final concentration of 0.05, 0.1, 1, 5, 10 or 20 μM, respectively. The culture medium was changed every 3 days and EGCG was supplemented in the new culture medium. EGCG existed in the entire period of culturing until examination. For inhibitor treatments, an inhibitor of PI3K, LY294002 (30 μM, Calbiochem, La Jolla, CA), or a selective inhibitor of MAP kinase, U0126 (10 μM, Sigma) was added in the culture medium for the last 2 days before examination.

2.2. MTT and LDH assay

The cell viability of NSC following EGCG treatments was assessed by MTT and lactate dehydrogenase (LDH) assay kits (Promega, Madison, WI). Briefly, cultured cochlear NSCs at various conditions as indicated were incubated with a premixed dye solution for four hours, and living cells were determined by the conversion of the tetrazolium component of the dye solution into a formazan product that was measured at 570 nm absorbance. On the other hand, cell death was evaluated by measuring LDH, a stable cytosolic enzyme released upon cell lysis. After the EGCG treatments, the released LDH in culture supernatants was measured by an enzymatic assay, which results in the conversion of a color change assayed by spectrometry at a wavelength of 490 nm.

2.3. WST-based proliferation assay

NSC cell proliferation was assessed by water soluble tetrazolium-1 (WST-1) assay kit (Cayman chemical, Ann Arbor, MI) that measure the purple formazan by extracellular NADH from the reduction of tetrazolium salt WST-1 from dividing cells. WST-1 mixtures were added into the culture wells and incubated for two hours at 37 °C in a CO_2 incubator, and the absorbance at a wavelength of 450 nm was then measured.

2.4. Immunofluorescence staining

The cochlea NSCs were fixed with paraformaldehyde (PFA) followed by permeabilization with 0.05% Triton-X in PBS, and then blocked with PBS with 5% goat serum for 1 h. The primary antibodies used for nestin (rat polyclonal antibody, 1:400), Tuj-1 (neuron-specific class III beta-tubulin, rabbit polyclonal antibody, 1:250), GFAP (glial fibrillary acidic protein, mouse polyclonal antibody, 1:400), and MAP-2 (microtubule-associated protein 2, mouse polyclonal antibody, 1:200) are all from Abcam (Cambridge, MA). The secondary goat anti-rat/rabbit/mouse antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 were used (1:400, Invitrogen, Pleasanton, CA). For a negative control, cells were exposed to the secondary antibody alone. Before imaging, the cell nuclei were counter stained by DAPI (4′,6-diamidino-2-phenylindole) in Vectashield mounting medium (Vector Labs, Cambridgeshire, UK). Immunofluorescence signals were then imaged by Olympus inverted fluorescent microscope, and then processed by Image J (NIH, Bethesda, MD).

2.5. Western blotting

For immunoblotting, cells were lysed in buffer (50 mM Tris HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.25% Na deoxycholate, 2 mM $NaVO_3$ and protease inhibitor). The 50 μg of total protein was run on SDS-PAGE and subsequently transferred to PVDF membranes. The blots after blocking were incubated with primary antibody overnight at 4 °C, followed by HRP conjugated secondary antibody incubation and detected by ECL-based imaging system. The antibodies used in western blot included Tuj-1 and GFAP (Abcam, 1:2000), GAPDH (glyceraldehyde 3-phosphate dehydrogenase, Sigma, 1:5000), Akt (1:5000) and phosphorylated Akt at Ser473 or Thr308 (1:1000, Cell signaling, Danvers, MA).

2.6. Calcium imaging

The calcium spikes were monitored by Fluo-3 based fluorescent imaging. Briefly, treated NSCs were maintained in a temperature-controlled solution containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM D-glucose at pH = 7.3 that was continuously bubbled with 95% O_2 and 5% CO_2. 2.5 μM Fluo-3-AM (Invitrogen) and pluronic F-127 [Sigma, 0.004% (*w*/*v*) final] were added for 45 min and removed, and the cells were then incubated for an additional 25 min. Fluorescent signals of samples (at excitation 488 nm/emission 510 nm) were imaged with a Carl Zeiss scanning confocal microscope (Carl Zeiss Company, Oberkochen, Germany). All settings of the canning system and the complete data acquisition were controlled and collected by the LSM 510 software (Carl Zeiss Company), in order to keep the baseline maximal fluorescence levels of 40–100 (arbitrary units) in eight-bit images (with the maximal signal output at 256). The cell fluorescence during the 5 min baseline period was denoted as F. Fluorescence measurements for each cell were normalized to the average fluorescence intensity. Region of indexes (ROIs) were defined in the first image, and the normalized fluorescence changes ΔF/F were calculated.

2.7. Statistics

Data are expressed as mean ± SD as indicated. One-way or two-way ANOVA analysis followed by a Tukey's post hoc test was used. Significances were indicated at $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control, or $\#p < 0.05$ and $\#\#p < 0.01$ versus EGCG alone group, respectively.