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Short communication

Vitamin E isomer δ-tocopherol enhances the efficiency of neural stem cell differentiation via L-type calcium channel

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HIGHLIGHTS

- δ-Tocopherol enhances efficiency of neural stem cell differentiation.
- δ-Tocopherol promotes morphological maturation of the differentiated neurons.
- L-type Ca^{2+} channels are involved in δ-tocopherol induced neuronal differentiation.

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ABSTRACT

The effects of the vitamin E isomer δ-tocopherol on neural stem cell (NSC) differentiation have not been investigated until now. Here we investigated the effects of δ-tocopherol on NSC neural differentiation, maturation and its possible mechanisms. Neonatal rat NSCs were grown in suspended neurosphere cultures, and were identified by their expression of nestin protein and their capacity for self-renewal. Treatment with a low concentration of δ-tocopherol induced a significant increase in the percentage of β-III-tubulin-positive cells. δ-Tocopherol also stimulated morphological maturation of neurons in culture. We further observed that δ-tocopherol stimulation increased the expression of voltage-dependent Ca^{2+} channels. Moreover, a L-type specific Ca^{2+} channel blocker verapamil reduced the percentage of differentiated neurons after δ-tocopherol treatment, and blocked the effects of δ-tocopherol on NSC differentiation into neurons. Together, our study demonstrates that δ-tocopherol may act through elevation of L-type calcium channel activity to increase neuronal differentiation.

1. Introduction

Natural forms of vitamin E consist of (α-, β-, γ-, δ-) tocopherol and (α-, β-, γ-, δ-) tocotrienol isomers, which are hydrophobic fat-soluble compounds found in various food sources [1,2]. Recent studies have reported that vitamin E has many beneficial health effects such as antioxidant and anti-inflammatory properties [3,4]. Vitamin E can delay or prevent a clinical diagnosis of Alzheimer's disease in elderly persons with mild cognitive impairment. In particular, vitamin E supplementation protects cultured hippocampal neurons against the neurotoxic effects of oxidative damage [5] and also reverses melamine-induced deficits in spatial cognition and hippocampal synaptic plasticity in rats [6]. Dunn-Thomas reported that vitamin E could influence retinal precursor cell differentiation by reducing stress-related function [7]. These studies of vitamin E have primarily utilized α-tocopherol, as it exerts the highest biological activity [8]. However, recent studies have suggested that δ-tocopherol may be more effective. δ-tocopherol, but not α-tocopherol, showed promise as a chemopreventive agent due to its higher cancer preventive activity in animal models [9,10]. δ-Tocopherol can reportedly reduce cholesterol accumulation potentially through enhancement of lysosomal exocytosis, and it is a novel lead compound for drug development to treat lysosomal storage diseases [11]. However, the effects of δ-tocopherol on the differentiation of NSCs are less clear.

Neural stem cells (NSCs) cultured as neurospheres are capable of differentiating into neurons, astrocytes, and oligodendrocytes [12]. The grafting of NSCs improves neurological deficits in neurodegenerative diseases such as ischemic stroke and spinal cord

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injury [13]. However, the induction of new neuronal cells in the damaged brain is relatively low and might be nonfunctional [13,14]. An alternative is the transplantation of neural stem cells that could differentiate into neurons for tissue repair [15]. However, transplanted cells face the same challenge of neuronal differentiation, maturation, and integration into host neural networks [16]. Thus, determining how to promote NSC differentiation and maturation is a critical problem for establishing safe and practical cell therapies.

Voltage-gated Ca^{2+} channels (VGCCs) are classified as two main types, including high voltage-activated (HVA; L-, N-, and P/Q-type) and low voltage-activated (LVA; T-type) channels [17]. Recent studies showed that calcium influx through L-type Ca^{2+} channels might modulate activity-dependent processes involved in neuronal differentiation. L-type Ca^{2+} channel currents play a key role in orienting the in vitro differentiation of NSCs toward the neuronal phenotypes [18].

Thus, in the current study, we investigated the effects of δ-tocopherol on NSC differentiation and maturation, and found that L-type Ca^{2+} channels could play a role in δ-tocopherol-induced NSC differentiation.

2. Materials and methods

2.1. Cell culture and treatment

Primary NSCs were derived from the brains of postnatal day 1 wistar rats (Hunan slack scene of laboratory animal co.) and were prepared as neurospheres according to methods previously described [19]. Animal used was in accordance with the National Institutes of Health Guide for the care and use of laboratory animals. All procedures were approved by the ethics committee of Central South University for experimental usage of laboratory animals. Briefly, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the tissue surrounding the lateral ventricles was carefully removed and digested enzymatically with 2 mg/ml papain solution at 37 °C for 30 min. The digested tissue was subsequently triturated gently and passed through a mesh to make a single-cell suspension. Cells were centrifuged and resuspended in neurobasal-A medium (Gibco) containing 2% B27 supplements (Gibco), 20 ng/ml basic fibroblast growth factor (bFGF; Sigma), 20 ng/ml epidermal growth factor (EGF; Sigma), 2 mM L-glutamine (Gibco) and 5 μg/ml heparin (Sigma). Cells were then seeded at an initial cell density of 1×10^5 cells/ml and maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

For the differentiation assay, neurospheres were dissociated and plated at 50,000 cells/ml on Matrigel (BD Biosciences)-coated glass coverslips. The cells were maintained for 7 days in neurobasal-A medium supplemented with 2% B27, 1 μM retinoic acid (RA, Sigma) and 10 ng/ml recombinant Human brain derived neurotrophic factor (BDNF, PeproTech). These NSCs were assigned to 6 treatment groups: control, δ-tocopherol (10 μM), verapamil (10 μM, Sigma), ω-conotoxin (10 μM, Alomone labs), δ-tocopherol (10 μM) plus verapamil (10 μM), δ-tocopherol (10 μM) plus ω-conotoxin (10 μM). The duration of the treatment was 6 days. The medium was changed every 3 days.

2.2. Immunofluorescence

Cells were fixed in 4% paraformaldehyde (PF) for 20 min and pre-incubated in PBS containing 5% donkey serum for 2 h. Subsequently, cells were incubated in PBS containing appropriate mixture of primary antibodies overnight at 4 °C. We used rabbit anti-nestin (1:500, Sigma), mouse anti-β-III-tubulin (1:500, Millipore) and rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, Sigma). This was followed by a 2 h reaction with Alexa Fluor_488 and/or Alexa Fluor_594 conjugated donkey anti-mouse, rabbit, or goat IgGs (1:400, Jackson ImmunoResearch) in the dark. Cells were then counterstained with bisbenzimide (Hoechst 33,258, 1:50,000), washed and mounted before microscopic examination. Samples were then viewed and photographed by using Olympus (BX60) fluorescent microscopes equipped with image analysis systems (cellSens Standard, Olympus). For quantitative analysis, the number of β-III-tubulin-positive or GFAP-positive cells on three coverslips was quantified in twenty randomly selected microscopic visual fields per coverslip, and results were expressed as the percentages of bisbenzimide-positive cells that were also β-III-tubulin-positive or GFAP-positive cells. Cell counting was performed under a fluorescence microscope with a 10× objective.

2.3. Morphometric measurements of neurites

Neurite branching was analyzed by counting the number of neurites for each neuron. Only neurons whose soma and neurites were completely contained inside the image border were analyzed. Neurite length measurements were taken for the longest neurite present on 150 β-III-tubulin-positive cells from control and δ-tocopherol treated groups, using the measurement tool in ImageJ. Fields were sampled randomly, and the person executing the measurements was blinded as to the treatment condition. Briefly, images of β-III-tubulin-positive cells were captured at 20× magnification and neurites were measured for each neuron, however, only one longest neurite per cell was used for comparative purposes. The longest neurite on each cell was drawn using the pencil tool in the ImageJ program. Lengths were determined as the distance between the edge of the cell body and the tip of the growth cone. Lengths of outlined neurites were then computed using the associated macro.

2.4. Immunocytochemistry

For immunocytochemistry, cells were fixed with 4% PF in PBS for 20 min and permeabilized with PBS/tween20 (0.1%) for 15 min. Permeabilization was followed by 30 min incubation in 3% H_2O_2. Cells were then blocked with 10% normal horse serum in PBS for 1 h. Subsequently, cells were incubated in PBS containing polyclonal rabbit anti-$Ca_v\beta 3$ antibody (1:100, Alomone Labs) overnight. In the following day, the cells were reacted with a pan-specific secondary antibody (biotinylated goat anti-rabbit IgG) at 1:400 for 2 h, and then with the ABC reagents (1:400, Vector Laboratories) for another hour. Immunoreactivity (IR) was visualized with 0.05% diaminobenzidine (DAB) and 0.003% H_2O_2. Three 10-min washes with PBS were used between incubations.

2.5. Electrophysiological recording

Matrigel coated coverslips with differentiated NSCs at day 14 were used for electrophysiological recordings. The coverslip was transferred to the recording chamber of an upright microscope (DMLFS, Leica). Cells were perfused with an extracellular solution containing (in mM): 127 NaCl, 3 KCl, 1 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1 D-glucose, and 2 $CaCl_2$ (pH 7.4). The pipette solution contained (in mM): 5 NaCl, 1 $CaCl_2$, 10 HEPES, 0.2 EGTA, 3 ATP, and 0.4 GTP (pH 7.4). Whole-cell patch-clamp recordings were carried out with patch-clamp amplifiers (Axopatch 200B, Axon instruments) and Clampfit 10.2 for data acquisition and analyses. To examine the excitability of neurons, transient membrane currents were induced by stepping the holding membrane potential from −80 mV to +80 mV (500 ms) with activities recorded. Then the cell was switched to current-clamp and currents (−0.02 nA to 0.05 nA, 300 ms) were injected through the patch pipette to examine whether action potentials could be induced. Spontaneous action

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