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Involvement of α7 nAChR Signaling Cascade in Epigallocatechin Gallate Suppression of β-Amyloid-Induced Apoptotic Cortical Neuronal Insults

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Abstract Excessive generation and accumulation of the β-amyloid (Aβ) peptide in selectively vulnerable brain regions is a key pathogenic event in the Alzheimer's disease (AD), while epigallocatechin gallate (EGCG) is a very promising chemical to suppress a variety of Aβ-induced neurodegenerative disorders. However, the precise molecular mechanism of EGCG responsible for protection against neurotoxicity still remains elusive. To validate and further investigate the possible mechanism involved, we explored whether EGCG neuroprotection against neurotoxicity of Aβ is mediated through the α7 nicotinic acetylcholine receptor (α7 nAChR) signaling cascade. It was shown in rat primary cortical neurons that short-term treatment with EGCG significantly attenuated the neurotoxicity of $A\beta_{1–42}$, as demonstrated by increased cell viability, reduced number of apoptotic cells, decreased reactive oxygen species (ROS) generation, and downregulated caspase-3 levels after treatment with 25-μM $A\beta_{1–42}$. In addition, EGCG markedly strengthened activation of α7nAChR as well as its downstream pathway signaling molecules phosphatidylinositol 3-kinase (PI3K) and Akt, subsequently leading to suppression of Bcl-2 downregulation in Aβ-treated neurons. Conversely, administration of α7nAChR antagonist methyllycaconitine (MLA; 20 μM) to neuronal cultures significantly attenuated the neuroprotection of EGCG against Aβ-induced neurototoxicity, thus presenting new evidence that the α7nAChR activity together with PI3K/Akt transduction signaling may contribute to the molecular mechanism underlying the neuroprotective effects of EGCG against Aβ-induced cell death.

Keywords Epigallocatechin gallate · α7nAChR · β-Amyloid · Neuroprotection · Apoptosis · Phosphatidylinositol 3-kinase · Akt · Bcl-2

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative dementia pathologically characterized by cognitive and memory impairment [1, 2]. Compelling evidence shows that excessive production and deposition of β-amyloid peptides as senile plaques in the brain initiates pathological cascade in AD, leading to neuronal cell dysfunction and death [3–7]. By far, little is known regarding the precise molecular mechanisms in Aβ-mediated neurotoxicity. However, more and more evidence suggests that oxidative stress may play a key role in Aβ-mediated neurotoxicity [6]. Beyond the direct effect of Aβ-induced oxidative damage to the brain such as reactive oxygen species (ROS), nitric oxide synthase (NOS), and Ca^{2+} influx, Aβ leads to cell injury through cell membrane lipid destruction and cleavage of DNA [8–13]. In addition, Aβ causes a reduction in glutamate uptake in astrocytes, indicating that Aβ-induced cytotoxicity might mediate glutamate excitotoxicity to some extent [14]. Based on the aforementioned aspects, it is likely that other mechanisms must be involved in accumulation of Aβ in AD.

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Epigallocatechin gallate (EGCG), a major constituent in the green tea polyphenol extract, has shown protection against neuronal degeneration and injuries caused by a variety of interventions such as Aβ, 6-hydroxydopamine, hydrogen peroxide, *N*-methyl-4-phenyl-1,2,3,6-tetrahydroxynurenine (MPTP), and 3-hyoxykynurenine [15–17]. Furthermore, several lines of evidence suggest that the role of EGCG in enhancing cell resistance to oxidative stress may go beyond its simple radical scavenging and iron chelating activities [18, 19]. Meanwhile, accumulating studies revealed that a possible protective mechanism of EGCG also includes activation of protein kinase C [15, 17, 20, 21], regulation of PI3K [22], inhibition of GSK-3β [23], upregulation of some receptors related to antiapoptosis, and further modulation of cell survival genes [22], implying existence of a complementing molecular mechanism underlying EGCG neuroprotection. However, the precise mechanism that EGCG suppresses Aβ-induced cytotoxicity has been largely uncharacterized.

Neuronal nAChRs belong to a family of ligand ion and are members of the cys-loop receptor superfamily. In the mammalian brain, α4- and α7-nAChRs are the most abundant nAChRs. Both of nAChRs exert an important role in cognitive processes such as learning and memory [24, 25], in the modulation of neurotransmitter release [26], and in neuroprotection [27–29]. Particularly, an interesting finding is that α7nAChR activation protects against a great variety of cytotoxic insults, including Aβ, glutamate, oxygen and glucose deprivation, and kainic acid [22, 29–35]. In these studies, the mechanism of α7nAChR-mediated neuroprotection is likely to be associated with modulation of both survival and apoptotic signaling cascades. However, the neuroprotective action of EGCG through activation α7nAChR, resulting in a reduction of Aβ plaque burden in AD, has not yet been explored.

In the present study, we used an in vitro cultured neuron model to investigate α7nAChR-mediated neuroprotective actions of EGCG against Aβ-induced cytotoxicity. These data indicate that this neuroprotective effect of EGCG against Aβ-induced cytotoxicity may be mediated, at least in part, via activation of α7nAChR and its downstream PI3K/Akt signaling prosurvival cascades. The present study may lay a foundation for further investigation of EGCG molecular mechanism and its clinical application in the treatment of AD.

Material and Methods

Reagents

Neurobasal, B27 supplement, normal donkey serum, normal goat serum, and fetal calf serum (FCS) were purchased from Invitrogen (USA); EGCG, $A\beta_{1–42}$ penicillin G, streptomycin, glutamine, trypsin, poly-L-lysine (PLL), ethylenediaminetetraacetic acid (EDTA), HEPES, bovine serum albumin (BSA), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT), and MLA were purchased from Sigma-Aldrich (USA); anti-γ-tubulin and anti-Tuj1 antibody were purchased from Chemicon (USA); anti-α7nAChR antibody was purchased from Abcam (USA); caspase-3, PI3K, p-PI3K, Akt, and phosphorylated Akt were from Cell Signaling (USA); Bcl-2 antibody was from Oncogene Research (USA); the BCA kit was from Qiagen (Germany); chemiluminescence western blotting kit was purchased from Plus (Germany); and DCF-DA kit, DAPI Kit, Fluor488-conjugated donkey antimouse IgG, and Fluor594-conjugated goat antirabbit were purchased from Molecular Probes (USA). Cell culture plates, dishes, cover slips, and flasks were purchased from Nuncon (Denmark).

Primary Cortical Neuronal Cultures

Primary cultures were obtained from cerebral cortex of fetal rats (16–18 days of gestation) by procedures described previously [36]. Briefly, single cells dissociated from cerebral cortex of fetal rats were plated onto plastic cover slips in 35-mm dishes, 96-well plates, or six-well plates pretreated with 50 μg/ml PLL. The cell plating density is 2×10^5 cells/cm^2. Cultures were incubated in Neurobasal supplemented with 2 % B27, 0.5 mM glutamine and 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere of 5 % CO_2, and the culture medium was changed once every 3 days. After 7–8 days, the cells were harvested and prepared for further experiments.

Treatment of the Cultures

For drug treatment, primary neurons were treated with 25-μM $A\beta_{1–42}$ peptide for 24 h following pretreatment with EGCG (dissolved in saline) for 2 h at concentrations of 5, 10, 20, 40, 60, and 100 μM. Notably, prior to use, $A\beta_{1–42}$ was dissolved in DMSO at a concentration of 10^{-3} M and preaggregated for 16 h at 37 °C. As to the optimal concentration of EGCG for the subsequent experiments, MTT assay (next step neurotoxicity) was conducted. Meanwhile, in separate set of experiments to evaluate the mechanism of EGCG actions, a selective antagonist of α7nAChR (MLA, 20 μM) was coadministered with EGCG for a 48-h treatment. Notably, the antagonist concentrations were chosen on the basis of a previous study (Yu et al. 2011).

Assessment of Cell Viability

Aβ-induced neuronal cytotoxicity was assessed by using MTT assay, and the conversion rate of MTT represents cell viability in all groups. Briefly, MTT (Sigma-Aldrich) was

dissolved in saline (5 mg/ml) and diluted directly into the media at a final concentration of 0.2 mg/ml. Following incubation at 37 °C for 4 h to allow MTT conversion into insoluble purple formazan crystals, the medium was gently aspirated and 100 μl dimethyl sulfoxide was added to each well followed by incubation and mechanical shaking for 10 min to dissolve the formazan product. The absorbance, expressed as the optical density (OD), was determined on a Dynatech MR4000 microplate reader (Dynatech, USA) with 570 and 630 nm for the measurement and reference wavelengths, respectively.

Measurement of Intracellular ROS

Intracellular production of ROS was measured as described previously [37]. In brief, cells were incubated with 30-μM DCF-DA at 37 °C for 30 min and treated with experimental reagents at 37 °C for another 30 min. After chilling on ice, cells were washed with ice-cold PBS in darkness, detached from the dishes, and resuspended in 10-mM EDTA-containing PBS. The fluorescence intensity of DCF formed by the reaction of DCF-DA with peroxides of more than 10,000 viable cells from each sample was analyzed by fluorescence-activated cell sorting (FACS) Caliber flow cytometer (Becton, Dickinson and Company, USA). Similar results were obtained when experiments were repeated for at least three times.

Assessment of Hoechst 333258 DNA Staining of Apoptotic Nuclei

To unravel the nuclear morphological changes in cultured neurons, cells were stained with nuclear dye Hoechst 33258. Briefly, cultured neurons following different treatments were fixed in 4 % paraformaldehyde for 25 min at room temperature (RT) and incubated with 15 μg/ml Hoechst 33258 for 10 min at RT. After washing thrice with PBS, cells were cover slipped and further observed under an Olympus BX-51 fluorescence microscope (Olympus, Japan). All images were captured using a FV10-ASW 1.6 photo system (Olympus, Japan). Hoechst 33258-stained apoptotic cells, defined as those with bright blue fragmented nuclei containing one or more lobes of condensed chromatin, were counted and at least 15 fields per cover slip with at least 50 cells per field were examined, yielding >400 cells examined per cover slip.

For immunofluorescent staining, cells following various different treatments were fixed using 4 % (w/v) paraformaldehyde in 0.1 M PBS for 25 min. After blocking with medium containing 5 % normal goat or rabbit serum, 2 % bovine serum albumin, and 0.05 %Triton X-100 for 1 h, cells were incubated with antimonoclonal Tuj-1 antibody (1:1,000; Chemicon, Berkeley, CA, USA) and antipolyclonal α7nAChR at 4 °C overnight. Notably, primary antibodies were diluted in PBS containing 3 % (w/v) BSA. After washing thrice, cells were incubated with the corresponding secondary antibody of fluorescein-conjugated IgG. The secondary antibodies used were Alexa Flour 488 antimouse IgG (1:500 in PBS; Molecular Probes) for Tuj-1 and Alexa Flour 594 antirabbit IgG (1:500 in PBS; Molecular Probes) for α7nAChR. DIPA counterstain (1 μg/ml) was subsequently carried out. After washing, the cells were mounted with FluorSave and viewed using an Olympus BX-51 fluorescence microscope.

Western Blot

For analysis of protein changes after various treatments, cells were harvested and homogenized in a lysis buffer (50 mM tris–HCl, pH7.2; 10 mM EDTA; 0.1 % (w/v) SDS; 1 % (w/v) sodium deoxycholate; 1 % (v/v) Triton X-100; 0.6 % (w/v) PMSF; 100 U/ml Trasylol; 2 μg/ml leupetin; 100 μM sodium orthovanadate lysis buffer) on ice bath for 1 h. Cell lysates were then clarified and the total protein was determined using BCA assay [38]. Protein lysates were run on 4–20 % gradient SDS-PAGE, respectively, transferred onto nitrocellulose membrane and blocked with 5 % fat free milk. Following this step, the membranes were incubated with the primary antibodies (α7nAChR, caspase-3, PI3K, p-PI3K, p-Akt, Akt, and Bcl-2 diluted according to instructions) at 4 °C overnight, respectively. γ-Tubulin was used as loading control. Subsequently, the next blotting procedures and the digitized images of the immunoblotting bands were quantitated as described previously [39].

Statistics

All data are presented as mean±SEM. Differences between groups were determined by applying a one-way ANOVA followed by a Newman-Keuls test or Student's *t* test when appropriate. $P<0.05$ was considered to be statistically significant.

Results

Amelioration of the Decrease in Viability of Aβ-Treated Cortical Neurons by EGCG

To determine if EGCG can exert neuroprotective effects against Aβ-induced cell neurotoxicity, neuronal growth was first evaluated with an inverted phase-contrast microscope after pretreatment with gradually increasing concentrations of EGCG (5–100 μM) for 72 h in the presence of Aβ as described in the experimental procedures. As shown in Fig. 1, almost all primary cortical neurons exposed to EGCG exhibited intact cell bodies with elaborate networks of neurites (Fig. 1a), while those exposed to 25-μM $Aβ_{1–42}$ for 72 h resulted in apparent cell

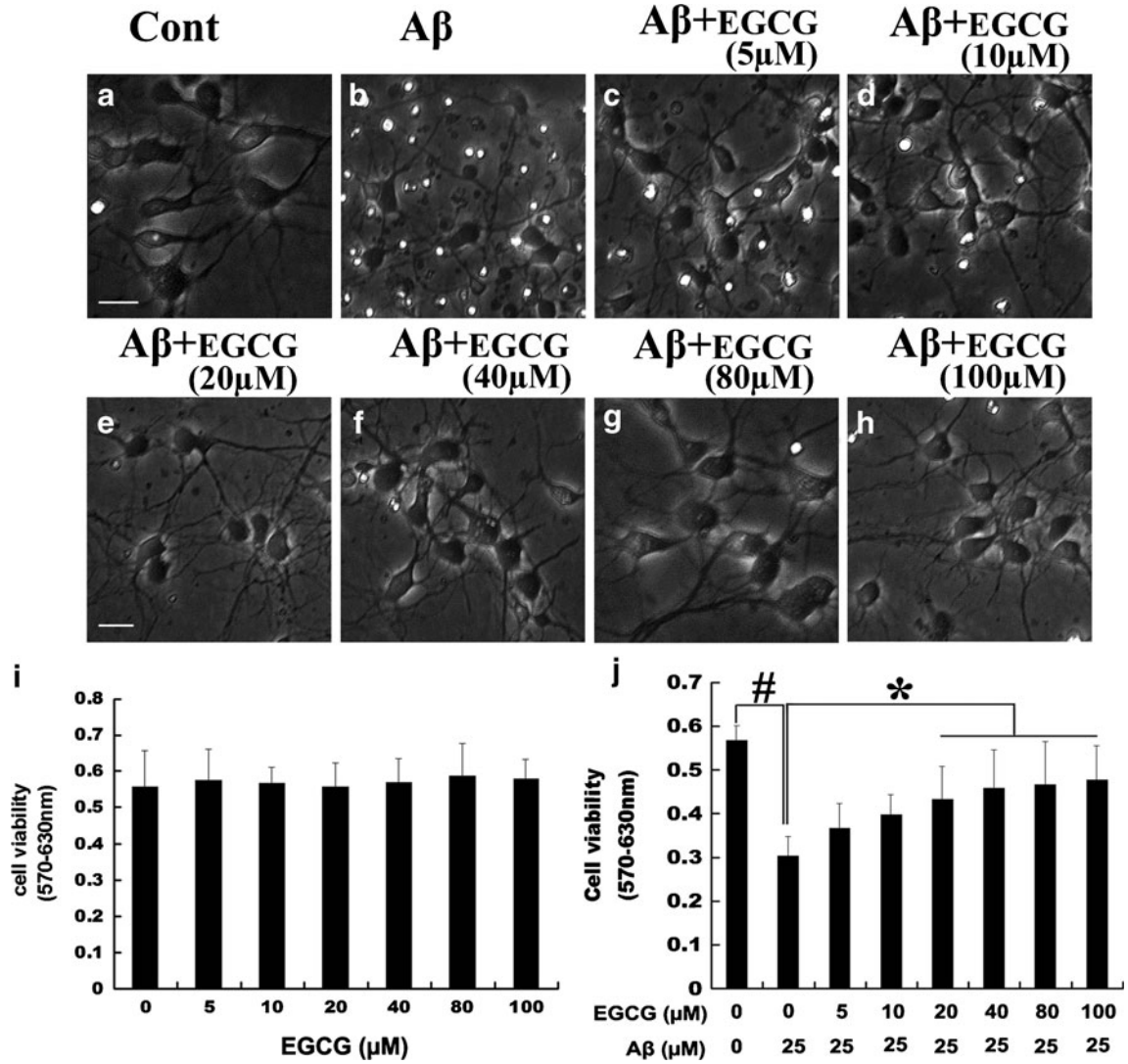

Fig. 1 Effect of EGCG on $A\beta_{1-42}$-induced neurotoxic insults. **a** Phase-contrast micrographs of primarily dissociated cortical neurons on 72 h. **b**–**h** Cultures were incubated with 25μ M $A\beta_{1-42}$ for 72 h in the presence and absence of EGCG pretreatment at indicated concentrations, respectively. **i** MTT was performed to examine cell viability after treatment with EGCG at indicated concentrations for 72 h. Notably, no significant changes were found among different groups. **j** The cells were pretreated with various concentrations (5–100 μM) prior to exposure to 25 μM $A\beta_{1-42}$. Pretreatment with EGCG strongly suppressed the decrease of cell viability caused by $A\beta_{1-42}$. *Scale bars* indicate 50 μm. Each data in **i** and **j** are expressed as the mean ± SEM ($n=5$) of a representative experiment that was repeated at least thrice. $^{*}P<0.05$ compared with 20, 40, 80, and 100 μM EGCG-pretreated cells; $^{\#}P<0.01$ compared with control

morphological distortion characterized by cell shrinkage, cytoplasmic blebbing, injury, and neurite fragmentation, mainly displaying extensive disintegration of neurites and scattered numerous cell debris in cultures (Fig. 1b). As to neurons pretreated with increased concentrations (5–100 μM) of EGCG prior to $A\beta_{1-42}$ exposure, their morphological distortion caused by $A\beta_{1-42}$ remarkably attenuated, and the EGCG neuroprotection against $A\beta_{1-42}$ neurotoxic insults displayed a dose-dependent fashion (Fig. 1c, d, e, f, g, and h). Comparatively, EGCG concentrations less than 20 μM resulted in slight suppression of $A\beta_{1-42}$ neurotoxic insults (Fig. 1c and d).

To investigate the neuroprotective effects of EGCG on primary cortical neurons, EGCG was applied in parallel to cells at various concentrations (5, 10, 20, 40, 80, and 100 μM), followed by assessment of cell viability by MTT assay. Strikingly, EGCG alone failed to cause a significant increase in cell viability. Although 80-μM EGCG increased it slightly,, there was no significant difference compared with the untreated control (Fig. 1i). To evaluate and figure out the optimal dose of EGCG against $A\beta_{1-42}$ neurotoxic insults in cortical neurons, we preincubated cells with the aforementioned concentrations of EGCG for 24 h prior to 25-μM $A\beta_{1-42}$ exposure. As shown in Fig. 1j, when subjected to the neurotoxic insult with $A\beta_{1-42}$, cell viability decreased significantly compared with control group (0.304±0.045 vs. 0.568±0.035, $p<0.05$). In contrast, the increased concentrations of EGCG ranging from 5 to 100 μM

for 24 h prior to $A\beta_{1-42}$ exposure resulted in a corresponding attenuation of cell viability decline. Similar to morphological assay, the EGCG concentration below 20 μM did not result in a significant inhibition of cell viability decline (0.304±0.045 vs. 0.368±0.056 and 0.398±0.046, $p>0.05$). In comparison, EGCG at 80 μM was the optimal concentration in suppression of $A\beta_{1-42}$-induced cell viability decrease because cell viability (0.478±0.098) was the highest. Then, 80 μM EGCG was selected as an optimal concentration for subsequent experiments.

Suppression of Aβ-Induced Decrease of Expression Levels of α7nAChR in Cultured Cortex Neurons by EGCG

To validate and investigate if possible involvement of α7nAChR signaling cascade contributed to EGCG suppression of Aβ-induced cell apoptotic insult, we determined the level of α7nAChR protein in cortical neurons treated with EGCG at the aforementioned concentrations (excluding 10-, 40-, and 100-μM groups because there was no significant change in α7nAChR levels in cells shown groups such as 5, 20, and 80 μM in our preliminary experiment) after exposure to $A\beta_{1-42}$. By means of double immunofluorescence, we found that almost all Tuj-1-positive cells treated with or without EGCG exhibited more pronounced α7nAChR immunoreactivity, whereas cells exposure to $A\beta_{1-42}$ only showed weak α7nAChR reactivity. However, preincubation of cells with EGCG prior to $A\beta_{1-42}$ remarkably attenuated the decrease of α7nAChR immunoreactivity in cells. In addition, EGCG dose-dependently suppressed the decrease of α7nAChR immunoreactivity (Fig. 2a). Strikingly, treatment with EGCG concentration above 20 μM showed more pronounced α7nAChR immunoreactivity. To further confirm if EGCG inhibited the decrease in intracellular α7nAChR protein level, parallel western blot analysis was carried out. Consistent with immunofluorescence, the bands for α7nAChR showed a similar result (Fig. 2b) that the most intense blot occurred in normal and EGCG alone groups, and there was no statistical significance ($P>0.05$) in the value of relative optical density (OD), while the amount of α7nAChR proteins in neuronal cells treated with $A\beta_{1-42}$ showed 1.2–3.3-fold decrease. When cells were pretreated with EGCG, the decrease of α7nAChR protein level caused by $A\beta_{1-42}$ was efficiently inhibited in a dose-dependent manner (Fig. 2b and c).

Attenuation of Aβ-Induced DNA Fragmentation by EGCG

DNA fragmentation is a vital biomarker of apoptosis. To further investigate in detail whether EGCG could attenuate Aβ-induced neuron apoptosis, DNA fragmentation was, thus, detected and quantified based on apoptotic morphology as revealed by Hoechst 33258 staining. In line with MTT assay results, a significant increase in the number of neurons displaying nuclear condensation or fragmentation was observed following 72-h exposure to 25-μM $A\beta_{1-42}$, while treatment with 80-μM EGCG significantly decreased the number of Aβ-induced DNA fragmentation in cells (1.85-fold vs. control, $^{**}P<0.01$). In addition, the administration of α7nAchR inhibitor MLA (20 μM) significantly aggravated Aβ-induced neuron apoptotic insults regardless of pretreatment with or without EGCG when compared to cells untreated with MLA ($^{\#\#}P<0.01$). Comparatively, pretreatment with EGCG resulted in a relatively lower DNA fragmentation in cells than that of unpretreatment group, but no significant difference was found. Strikingly, no remarkable condensed and DNA fragmentation nuclei were found between control and EGCG alone treatment groups, and there was no statistical difference in the number of cell DNA fragmentation nuclei (Fig. 3a and b). The results demonstrated that EGCG could prominently prevent neurons from $A\beta_{1-42}$-induced apoptosis via α7AchR-dependent pathways.

Suppression of Aβ-Induced Activity of Caspase-3 by EGCG

Caspase-3, a key downstream effector of the cysteine protease family, is involved in both mitochondrial apoptotic pathway and death receptor pathway. Although previous studies have shown that the EGCG-induced neuroprotection against $A\beta_{1-42}$ apoptotic neurotoxicity is likewise due to its regulation in caspse-3 level in neurons, it is still unknown whether the inhibition of caspase-3-dependent insult pathway is also mediated through α7nAchR. To address the issue, we investigated the ability of α7nAchR to affect caspse-3 activation in the presence of EGCG and its antagonist, MLA. As shown in Fig. 4a and b, immunohistochemistry revealed that exposure of neurons to $A\beta_{1-42}$ significantly increased the amount of cleaved caspase-3, displaying higher proportion of caspase-3-positive neurons (45.3 %) as compared to the control and EGCG groups (1.2 and 1.5 %; $P<0.05$). After administration of MLA into cultures in the presence of $A\beta_{1-42}$, the insult caused by $A\beta_{1-42}$ was further aggravated and the percentage of caspase-3-positive cells (51.8 %) was significantly increased ($P<0.05$ as compared to control and EGCG groups). In contrast, caspase-3-positive cells could hardly be found in the both normal group and EGCG treatment group. Notably, MLA alone could also cause excessive caspase-3 expression and the percentage of caspase-3-positive cells reached 40.4 and 42.1 %, respectively, implying possible involvement of α7AchR may contribute to EGCG-induced neuroprotection against $A\beta_{1-42}$-induced cell insult.

To further substantiate this possibility, western blot assessment was performed. As expected, treatment of $A\beta_{1-42}$ resulted in a significant increase of caspase-3 in cells, and pretreatment with 20-μM MLA remarkably exacerbated the increase of caspase-3 level. In agreement with immunocytochemistry, MLA alone also resulted in a relatively high caspase-3 level (Fig. 4c and d), further suggesting α7AchR

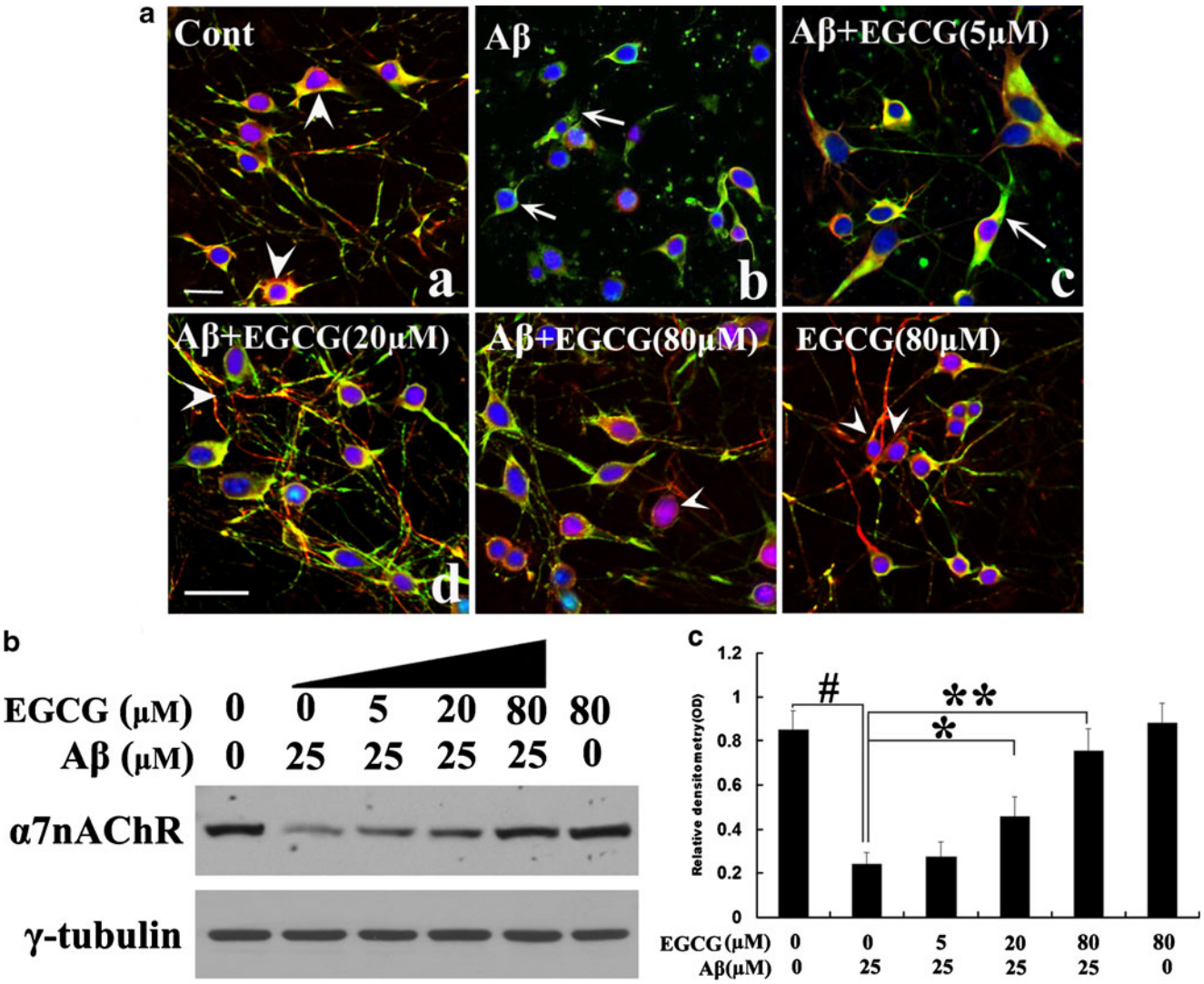

Fig. 2 Changes of α7AchR expression in EGCG-pretreated neurons after exposure to $A\beta_{1–42}$. **a** Cortical neurons were doubly immunostained for Tuj-1 (*green*) and α7AchR (*red*) after pretreatment with various concentrations of EGCG prior to $A\beta_{1–42}$ stimulation. Of note, untreated cells and EGCG alone cells exhibited the strongest α7AchR immunoreactivity, and $A\beta_{1–42}$ remarkably decreased α7AchR immunoreactivity. EGCG effectively suppressed the decrease caused by $A\beta_{1–42}$ in a dose-dependent manner. In agreement with immunocytochemistry, western blot analyses showed similar changes in α7AchR expression in each corresponding treatment groups (**b**). *Scale bars* indicate 50 μm. **c** Quantitative assessment of immunoblot of three independent experiments. Data are reported as mean ± SEM the pretreatment induced from spinal cord under different conditions of three independent experiments. *$P<0.05$ and **$P<0.01$ compared with 20 and 80 μM EGCG-pretreated cells, respectively; $^{\#}P<0.001$ compared with control

signaling may be involved in EGCG-induced neuroprotection against $A\beta_{1–42}$-induced cell insult.

Decrease of Aβ-Induced Accumulation of ROS by EGCG

Oxidative stress is a mechanism that drives cells into apoptosis through excessive production of ROS. To test whether α7nAchR-mediated signaling is involved in the suppressive effect of EGCG on ROS production in neurons after exposure to $A\beta_{1–42}$, we measured changes of intracellular ROS production by DCF formation rate. As shown in Fig. 5, similar mean DCF fluorescence intensity was found in control and EGCG groups (100.0±1.5 for EGCG group; 102.0±5.3, $P>0.05$). Increased DCF intensity was 1.8-fold in Aβ group in the absence of EGCG (179.9±18.4) compared with the control group. Such increased DCF intensity was reduced by about 35 % in Aβ plus EGCG group (128.3±3.3), and no statistical difference was found when compared to the control group. Strikingly, when MLA was administrated to cultures exposed to $A\beta_{1–42}$, ROS production was remarkably exacerbated in the presence or absence of EGCG, and the mean fluorescent intensity of cellular DCF was approximately 1.5- to twofold as compared to control groups. Comparatively, no significant difference in cellular DCF intensities was found between cells pretreated with or without

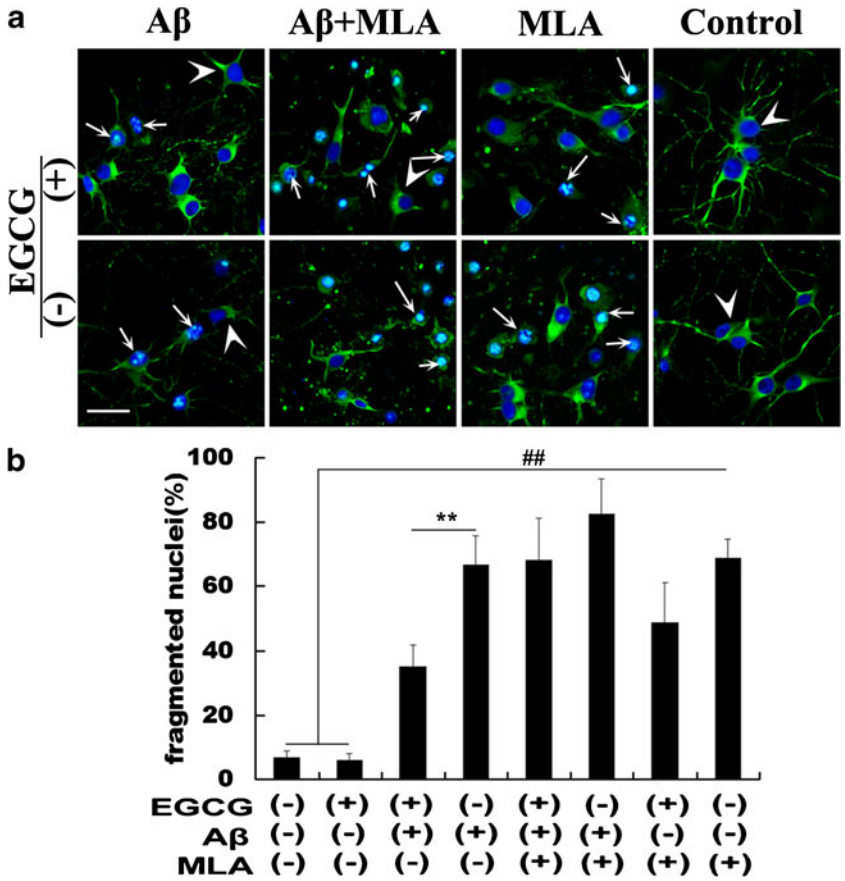

Fig. 3 Effects of EGCG and α7AchR antagonist, MLA on Aβ-induced nuclei DNA fragmentation. **a** Photomicrographs of Tuj-1 (*green*) immunofluorescently and Hoechst 33258 (*blue*)-labeled counter (*blue*)-stained neurons with indicative treatments, respectively, depicting the changes of nuclei DNA. Notably, normal cells and apoptotic cells (with fragmented or condensed nuclei) are indicated by the *triangle arrowheads* and *arrows*, respectively. *Scale bars* indicate 50 μm. The *histogram* illustrating the percentages of apoptotic neurons with indicative treatments is presented in **b**. EGCG significantly reduced the percentages of $A\beta_{1-42}$-induced apoptotic neurons, but the presence of MLA aggravated $A\beta_{1-42}$-induced cell apoptosis regardless of pretreatment with or without EGCG. $^{\#\#}P<0.001$ represents significant differences between normal/EGCG groups and indicative groups. $^{**}P<0.01$ represents significant difference between pretreatment with and without EGCG after exposure to $A\beta_{1-42}$

EGCG, suggesting that α7nAchR-mediated signaling may play a crucial role in the neuroprotective effect of EGCG against $A\beta_{1-42}$-induced neurotoxic insult.

Effects of EGCG on PI3K/Akt Activation and Its Downstream Molecule Bcl-2 in Cortical Neurons

Numerous studies have demonstrated that α7nAchR activation can be against various cytotoxic insults by downstream signaling such as PI3K/Akt and Bcl-2 [22, 30, 33]; therefore, we next ascertained the potential intracellular signaling mechanisms responsible for the protective effects of EGCG against $A\beta_{1-42}$-induced neurotoxicity. As shown in Fig. 6a and b, exposure of cells to $A\beta_{1-42}$ (25 μM) alone for 72 h markedly resulted in a decrease in PI3K levels in cells, while pretreatment with EGCG for 24 h prior to $A\beta_{1-42}$ exposure significantly inhibited the decrease of PI3K level. Moreover, administration of MLA could exacerbate the decrease of PI3K level caused by $A\beta_{1-42}$ and the pretreatment of EGCG failed to inhibit the decrease of expression of PI3K even though a slightly elevated PI3K level occurred (Fig. 6a). In comparison, no significant effect was observed following application of 80-μM EGCG alone (Fig. 6b). These data suggested that EGCG-mediated neuroprotection against $A\beta_{1-42}$ neurotoxic insult via α7nAchR stimulation involves the activation of the PI3K prosurvival pathway in neurons.

Akt is a putative effector of PI3K. When PI3K is activated, it phosphorylates Akt. To further confirm the above-mentioned possibility, we examined the level of phosphorylated Akt using western blot. As we expected, EGCG-induced Akt phosphorylation via α7nAchR downstream signaling molecule PI3K showed a similar change in tendency with PI3K expression even though there was slight inconsistency under certain treatments (Fig. 6a and b), indicating that EGCG indeed restored Akt phosphorylation in $A\beta_{1-42}$-treated neurons via α7AchR signaling cascade.

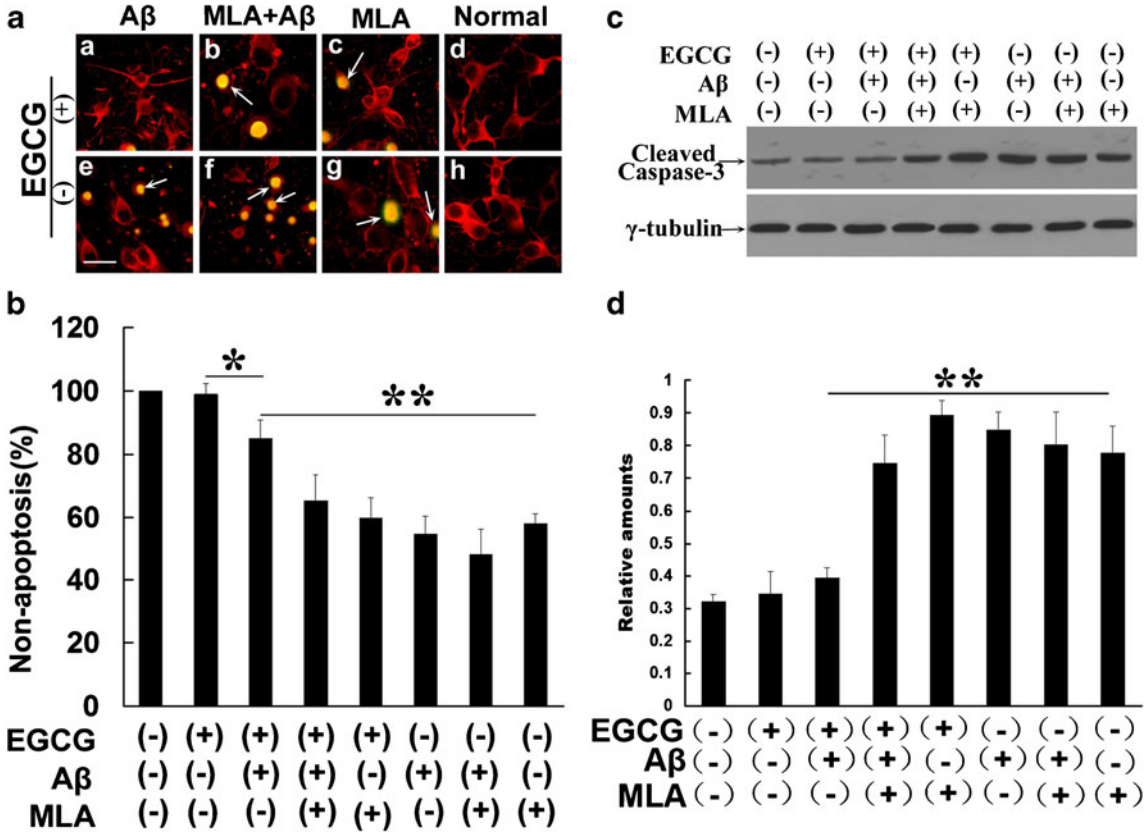

Fig. 4 Effects of EGCG on activation of caspase-3 in $A\beta_{1–42}$-induced cortical neurons in the presence or absence of MLA. **a** Immunocytochemistry showing caspase-3 expression (*yellow*) in cortical neurons (Tuj-1, *red*) after indicative treatments. EGCG-reduced $A\beta_{1–42}$ induced the upregulation of caspase-3 in neurons, and MLA remarkably aggravated caspase-3 expression. *Scale bars* indicate 50 μm. **b** Quantitative analyses of non-caspase-3-positive cells under the above-mentioned treatments. Data correspond to means and SEM of three experiments from three different cell cultures. **c** A representative immunoblot of caspase-3 in $A\beta_{1–42}$-treated neurons after EGCG pretreatment in the presence or the absence of MLA. **d** Quantitative analysis of western blotting results. The intensity of blots of MLA + $A\beta_{1–42}$ and MLA in the presence or the absence of EGCG pretreatment groups were significantly higher than that of EGCG alone and control groups, respectively. $^{**}P<0.01$, whereas with $A\beta_{1–42}$ in the presence of EGCG group, there was no statistical significance in comparison with EGCG alone and the control groups

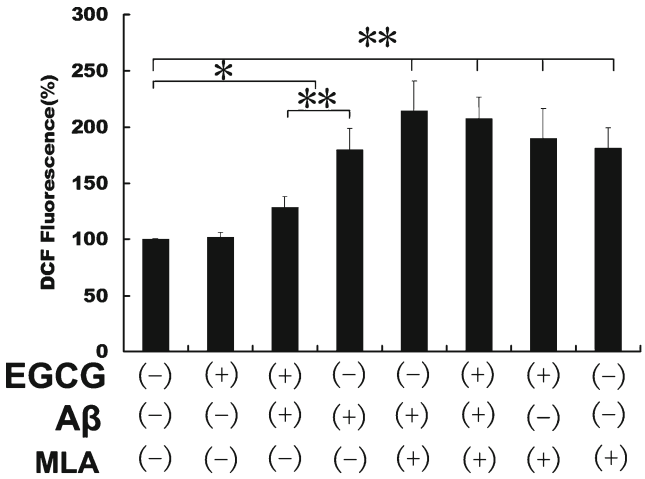

Fig. 5 Effect of EGCG on ROS generation in cultured cortical neurons exposed to $A\beta_{1–42}$. The fluorescence intensity of DCF was measured after neurons were treated with indicated conditions, respectively. All data are presented as means ± SEM for three independent experiments with triplicate determinations. $^{*}P<0.05$ and $^{**}P<0.01$ represent significant differences, respectively, compared with its controls

Bcl-2 protein, a type of antiapoptotic protein, can exert a pivotal role in preventing cell death caused by a variety of toxic insults. It has been documented that Akt activation leads to the overexpression of Bcl-2; thus, we next determined the Bcl-2 protein levels. Intriguingly, exposure to $A\beta_{1–42}$ significantly suppressed the level of Bcl-2, and the downregulation of Bcl-2 caused by $A\beta_{1–42}$ was reduced by pretreatment with EGCG for 24 h. More importantly, the addition of MLA to cultures remarkably exacerbated downregulation of Bcl-2 regardless of the presence or absence of EGCG. In comparison, EGCG alone inhibited the decrease of Bcl-2 induced by MLA, but no a significant difference was found as compared with the control group (Fig. 6a and b).

Discussion

EGCG, a major component of green tea, exerts an important role in endogenous antioxidative defense against oxidative

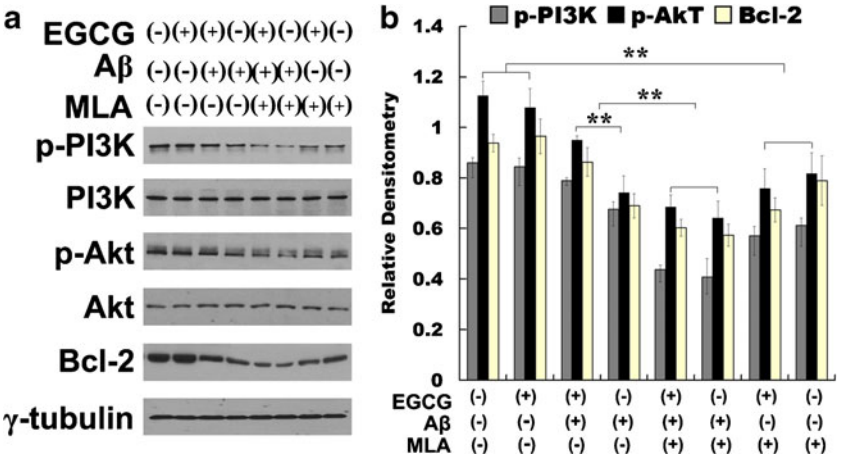

Fig. 6 Involvement of α7nAchR signaling cascade in EGCG-mediated neuroprotection against Aβ-induced neurotoxic insult. **a** EGCG (80 μM) was added to primary cortical neuron cultures for 2 h prior to $A\beta_{1-42}$ treatment. MLA was simultaneously administrated to cultures to block α7nAchR biofunctions. Cell lysates were immunoblotted for phosphor-PI3K, phosphor-Akt, and Bcl-2, respectively. **b** The densitometric analysis of the representative bolts was shown. All data are presented as means ± SEM for three independent experiments with triplicate determinations. $^{**}P<0.01$ represents significant differences, compared with its controls

injury and suppression of several harmful molecule-triggered neurotoxic insults [35, 40]. Increasing evidence has shown that EGCG-neuroprotective properties and a possible mechanism responsible for the effects are intimately relevant to its attenuating cell apoptotic insult caused by accumulation of free radical [41], excessive ROS generation [23], Ca^{2+} overload and destruction of cell membrane ion homeostasis, etc. [35, 40, 42]. EGCG simultaneously promotes antiapoptotic molecule (Bcl-2) production and, subsequently, activates downstream neuroprotective signaling involved in the suppressive apoptotic event [16, 17], suggesting this neuroprotective compound with potential therapeutic interest might be used clinically after neurons already have become vulnerable to damage or other insults in various neurodegenerative diseases such as AD or PD. However, there is limited agreement in the literature concerning the pathways involved in EGCG protection against $A\beta_{1-42}$-induced toxicity, and a clear and precise cellular/molecular mechanism underlying these effects hitherto remains elusive. We, therefore, sought to elucidate the detailed underlying mechanism using in vitro cultured cell model. Our present results revealed that these neuroprotective effects of EGCG on $A\beta_{1-42}$-induced insult are likely to be mediated by α7AchR and its downstream signal PI3K/Akt and Bcl-2.

Our in vitro experiment provided the first morphological evidence that EGCG treatment reduced damage caused by $A\beta_{1-42}$, displaying a significant attenuation of decrease of cell survival and growth. Meanwhile, cell apoptotic death and mitochondrial damage were both reduced after treatment with EGCG, as demonstrated by suppression of proapoptotic protein activation including caspase-3, further resulting in a significant decease of the accumulation and production of ROS, and attenuation of cell nuclear condensation or fragmentation. Moreover, the protective effect of EGCG on injured neurons was dose dependent. These findings strongly suggest that EGCG can attenuate damage and cell death caused by $A\beta_{1-42}$ toxicity, which is consistent with numerous previous reports [23, 43, 44]. Furthermore, we found $A\beta_{1-42}$ significantly decreased the expression of α7AchR protein levels, but the decrease of α7AchR protein was efficiently suppressed in the presence of EGCG. Notably, $A\beta_{1-42}$-induced neurotoxic insult is tightly linked to α7AchR protein levels, namely lower protein levels of α7AchR result in a more serious cell injury or death. In addition, MLA, α7nAchR antagonist, remarkably exacerbated cell injury or death caused by $A\beta_{1-42}$, as demonstrated by 33258 nuclear staining assay, ROS production, and caspase-3 activation. These results revealed that the EGCG protection against $A\beta_{1-42}$-inuduced cell insult may be associated with α7nAchR signaling cascade. Of course, there may be other underlying mechanisms involved in EGCG neuroprotection because MLA also resulted in neuronal insult with or without EGCG pretreatment. Therefore, with these results, a new question arises whether α7nAchR is also responsible for neuronal survival. From a previous study that the activation of α7nAchR mediates specifically and selectively the neuroprotective effects of nicotine along the caspase-independent apoptosis via inhibition of Aβ actions [22], we could hardly eliminate the possibility of protection of α7nAchR itself; in other words, α7nAchR prosurvival signaling cascade itself mediates neuroprotection. Based on our present results in combination with several previous reports, we can speculate that the protective effect of EGCG on $A\beta_{1-42}$-induced neurotoxicity and involvement of α7AchR signaling could be, at least in part, causally related.

In this study, we also found an interesting phenomenon that the level of α7nAchR was tightly associated with neuroprotective action of EGCG against $A\beta_{1-42}$-induced

toxic injury or death, suggesting that α7nAchR is likely to be involved in mediating specifically the actions of EGCG on $A\beta_{1-42}$ toxicity. Consistent with this observation, additional evidence has demonstrated that α7nAchR shares a downstream signaling mechanism against $A\beta_{1-42}$-induced neurotoxicity via activating various signaling pathways related to neuroprotection such as Akt and further modulating calcium homeostasis, the expression of antiapoptotic proteins, and release of the neurotransmitters [45–47]. This finding led us to postulate that there may be a possible mechanism underlying the involvement of α7nAchR signaling cascade in neuroprotection of EGCG against $A\beta_{1-42}$-induced neurotoxicity. Nevertheless, it is still unknown whether change of α7nAchR levels can result in a corresponding change in its downstream signaling such as PI3K/Akt and Bcl-2, an effector against cell apoptosis. We, consequently, conducted the examination of the above-mentioned signaling pathway components. Intriguingly, western blot analyses showed p-PI3K/p-Akt and Bcl-2 expression patterns are similar to that of α7nAchR. That is, p-PI3K/p-Akt levels in cells showed a consistent change of α7nAchR levels. We, thus, surmised that involvement of α7nAchR signaling cascade is likely to participate actively in EGCG-mediated neuroprotection against $A\beta_{1-42}$-induced toxic injury or death. Also, it has been documented that PI3K is one of the main intracellular factors responsible for the transmission of antiapoptotic signals and PI3K signaling pathway plays a central role in neuronal survival and death [18, 48–51]. Additionally, activated PI3K phosphorylates Akt, which can further foster cell survival against several toxic insults such as oxidative stress, excitatory neurotoxin, and matrix detachment [47, 52, 53]. Based on these reports, we subsequently tested the α7nAchR downstream signaling cascade. Strikingly, the administration of MLA to the neurons pretreated with or without EGCG failed to result in any significant increase of p-PI3K/p-Akt, suggesting α7nAchR is likely to mediate the neuroprotection via PI3K/Akt signaling. Besides, we monitored the Bcl-2 levels in cultured neurons subjected to $A\beta_{1-42}$. Coincidentally, the administration of EGCG to cultures exposed to $A\beta_{1-42}$ also caused remarkable alterations of Bcl-2 levels, similar to α7nAchR levels. Of note, MLA also abrogated the increase of Bcl-2 caused by EGCG in the presence of $A\beta_{1-42}$. These data imply that α7nAchR signaling cascade may partly contribute to the mechanism underlying the neuroprotective effect of EGCG against $A\beta_{1-42}$-induced neurotoxic insults.

Taken together, our in vitro study in combination with previous studies indicates that in addition to other signaling pathways, α7nAchR signaling cascade must be involved in the EGCG neuroprotection against $A\beta_{1-42}$-induced neurotoxicity, implying that there may be a variety of mechanisms of neuroprotective actions of EGCG against $A\beta_{1-42}$ toxicity and that these effects are not fully attributable to the antiapoptotic properties of EGCG and may depend upon its effect on α7nAchR. Therefore, activation of this receptor could potentially prevent apoptotic death in therapy for neurodegenerative diseases.

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Conflict of interest None.

References

1. Selkoe DJ, Selkoe MD (2004) Alzheimer disease: mechanistic understanding predicts novel therapies. Ann Intern Med 140(8):627–638
2. Binder LI, Guillozet-Bongaarts AL, Garcia-Sierra F, Berry RW (2005) Tau, tangles, and Alzheimer's disease. Biochim Biophys Acta 1739(2–3):216–223
3. Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. Neuron 6(4):487–498
4. Roher AE, Lowenson JD, Clarke S, Woods AS, Cotter RJ, Gowing E, Ball MJ (1993) Beta-amyloid-(1–42) is a major component of cerebrovascular amyloid deposits: implications for the pathology of Alzheimer disease. Proc Natl Acad Sci USA 90(22):10836–10840
5. Harris ME, Hensley K, Butterfield DA, Leedle RA, Carney JM (1995) Direct evidence of oxidative injury produced by the Alzheimer's beta-amyloid peptide (1–40) in cultured hippocampal neurons. Exp Neurol 131(2):193–202
6. Butterfield DA (2002) Amyloid beta-peptide (1–42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. Free Radic Res 36(12):1307–1313
7. Drachman DA (2006) Aging of the brain, entropy, and Alzheimer disease. Neurology 67(8):1340–1352
8. Landfield PW, Thibault O, Mazzanti ML, Porter NM, Kerr DS (1992) Mechanisms of neuronal death in brain aging and Alzheimer's disease: role of endocrine-mediated calcium dyshomeostasis. J Neurobiol 23(9):1247–1260
9. Tamura Y, Sato Y, Akaike A, Shiomi H (1992) Mechanisms of cholecystokinin-induced protection of cultured cortical neurons against *N*-methyl-D-aspartate receptor-mediated glutamate cytotoxicity. Brain Res 592(1–2):317–325
10. Jiang Q, Gu Z, Zhang G, Jing G (2000) Diphosphorylation and involvement of extracellular signal-regulated kinases (ERK1/2) in glutamate-induced apoptotic-like death in cultured rat cortical neurons. Brain Res 857(1–2):71–77
11. LaFerla FM (2002) Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. Nat Rev Neurosci 3(11):862–872
12. Blass JP (2002) Alzheimer's disease and Alzheimer's dementia: distinct but overlapping entities. Neurobiol Aging 23(6):1077–1084
13. Ill-Raga G, Ramos-Fernández E, Guix FX, Tajes M, Bosch-Morató M, Palomer E, Palomer E, Godoy J, Belmar S, Cerpa W, Simpkins JW, Inestrosa And NC, Muñoz FJ (2012) Amyloid-β peptide fibrils induce nitro-oxidative stress in neuronal cells. J Alzheimers Dis 22(2):641–652
14. Matos M, Augusto E, Oliveira CR, Agostinho P (2008) Amyloid-beta peptide decreases glutamate uptake in cultured astrocytes: involvement of oxidative stress and mitogen-activated protein kinase cascades. Neurosci 156(4):898–910
15. Levites Y, Amit T, Youdim MB, Mandel S (2002) Involvement of protein kinase C activation and cell survival/cell cycle genes in

green tea polyphenol (−)-epigallocatechin 3-gallate neuroprotective action. J Biol Chem 277(34):30574–30580
16. Levites Y, Youdim MB, Maor G, Mandel S (2002) Attenuation of 6-hydroxydopamine (6-OHDA)-induced nuclear factor-kappaB (NF-kappaB) activation and cell death by tea extracts in neuronal cultures. Biochem Pharmacol 63(1):21–29
17. Levites Y, Amit T, Mandel S, Youdim MB (2003) Neuroprotection and neurorescue against Abeta toxicity and PKC-dependent release of nonamyloidogenic soluble precursor protein by green tea polyphenol (−)-epigallocatechin-3-gallate. FASEB J 17(8):952–954
18. Koh SH, Kim SH, Kwon H, Kim JG, Kim JH, Yang KH, Kim J, Kim SU, Yu HJ, Do BR, Kim KS, Jung HK (2004) Phosphatidylinositol-3 kinase/Akt and GSK-3 mediated cytoprotective effect of epigallocatechin gallate on oxidative stress-injured neuronal-differentiated N18D3 cells. Neurotoxicology 25(5):793–802
19. Lee H, Bae JH, Lee SR (2004) Protective effect of green tea polyphenol EGCG against neuronal damage and brain edema after unilateral cerebral ischemia in gerbils. J Neurosci Res 77(6):892–900
20. Li R, Huang YG, Fang D, Le WD (2004) (−)-Epigallocatechin gallate inhibits lipopolysaccharide-induced microglial activation and protects against inflammation-mediated dopaminergic neuronal injury. J Neurosci Res 78(5):723–731
21. Mandel SA, Avramovich-Tirosh Y, Reznichenko L, Zheng H, Weinreb O, Amit T, Youdim MB (2005) Multifunctional activities of green tea catechins in neuroprotection. Modulation of cell survival genes, iron-dependent oxidative stress and PKC signaling pathway. Neurosignals 14(1–2):46–60
22. Kihara T, Shimohama S, Sawada H, Honda K, Nakamizo T, Shibasaki H, Kume T, Akaike A (2001) Alpha 7 nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block A beta-amyloid-induced neurotoxicity. J Biol Chem 276(17):13541–13546
23. Lin CL, Chen TF, Chiu MJ, Way TD, Lin JK (2009) Epigallocatechin gallate (EGCG) suppresses beta-amyloid-induced neurotoxicity through inhibiting c-Abl/FE65 nuclear translocation and GSK3 beta activation. Neurobiol Aging 30(1):81–92
24. Gauthier M (2010) State of the art on insect nicotinic acetylcholine receptor function in learning and memory. Adv Exp Med Biol 683:97–115
25. Srivareerat M, Tran TT, Salim S, Aleisa AM, Alkadhi KA (2011) Chronic nicotine restores normal Aβ levels and prevents short-term memory and E-LTP impairment in Aβ rat model of Alzheimer's disease. Neurobiol Aging 32(5):834–44
26. Barik J, Wonnacott S (2006) Indirect modulation by alpha7 nicotinic acetylcholine receptors of noradrenaline release in rat hippocampal slices: interaction with glutamate and GABA systems and effect of nicotine withdrawal. Mol Pharmacol 69(2):618–628
27. Picciotto MR, Zoli M (2008) Neuroprotection via nAChRs: the role of nAChRs in neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Front Biosci 13:492–504
28. Akaike A, Takada-Takatori Y, Kume T, Izumi Y (2010) Mechanisms of neuroprotective effects of nicotine and acetylcholinesterase inhibitors: role of alpha4 and alpha7 receptors in neuroprotection. J Mol Neurosci 40(1–2):211–216
29. Yu W, Mechawar N, Krantic S, Quirion R (2011) α7 Nicotinic receptor activation reduces β-amyloid-induced apoptosis by inhibiting caspase-independent death through phosphatidylinositol 3-kinase signaling. J Neurochem 119(4):848–858
30. Shaw S, Bencherif M, Marrero MB (2002) Janus kinase 2, an early target of alpha 7 nicotinic acetylcholine receptor-mediated neuroprotection against Abeta-(1–42) amyloid. J Biol Chem 277(47):44920–44924
31. Stevens TR, Krueger SR, Fitzsimonds RM, Picciotto MR (2003) Neuroprotection by nicotine in mouse primary cortical cultures involves activation of calcineurin and L-type calcium channel inactivation. J Neurosci 23(31):10093–10099
32. Arias E, Gallego-Sandín S, Villarroya M, García AG, López MG (2005) Unequal neuroprotection afforded by the acetylcholinesterase inhibitors galantamine, donepezil, and rivastigmine in SH-SY5Y neuroblastoma cells: role of nicotinic receptors. J Pharmacol Exp Ther 315(3):1346–1353
33. Egea J, Rosa AO, Sobrado M, Gandía L, López MG, García AG (2007) Neuroprotection afforded by nicotine against oxygen and glucose deprivation in hippocampal slices is lost in alpha7 nicotinic receptor knockout mice. Neurosci 145(3):866–872
34. Shin EJ, Chae JS, Jung ME, Bing G, Ko KH, Kim WK, Wie MB, Cheon MA, Nah SY, Kim HC (2007) Repeated intracerebroventricular infusion of nicotine prevents kainate-induced neurotoxicity by activating the alpha7 nicotinic acetylcholine receptor. Epilepsy Res 73(3):292–298
35. Weinreb O, Amit T, Mandel S, Youdim MB (2009) Neuroprotective molecular mechanisms of (−)-epigallocatechin-3-gallate: a reflective outcome of its antioxidant, iron chelating and neuritogenic properties. Genes Nutr 4(4):283–296
36. Noh MY, Koh SH, Kim Y, Kim HY, Cho GW, Kim SH (2009) Neuroprotective effects of donepezil through inhibition of GSK-3 activity in amyloid-beta-induced neuronal cell death. J Neurochem 108(5):1116–1125
37. Yang H, Cui GB, Jiao XY, Wang J, Ju G, You SW (2010) Thymosin-beta4 attenuates ethanol-induced neurotoxicity in cultured cerebral cortical astrocytes by inhibiting apoptosis. Cell Mol Neurobiol 30(1):149–160
38. Stoscheck CM (1990) Quantitation of protein. Methods Enzymol 182:50–68
39. Yang H, Cheng X, Yao Q, Li J, Ju G (2008) The promotive effects of thymosin beta4 on neuronal survival and neurite outgrowth by upregulating L1 expression. Neurochem Res 33(11):2269–2280
40. Wei IH, Tu, HC, Huang CC, Tsai MH, Tseng CY, Shieh JY (2011) (−)-Epigallocatechin gallate attenuates NADPH-d/nNOS expression in motor neurons of rats following peripheral nerve injury. BMC Neurosci 12:52.
41. Levites Y, Weinreb O, Maor G, Youdim MB, Mandel S (2001) Green tea polyphenol (−)-epigallocatechin-3-gallate prevents *N*-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine-induced dopaminergic neurodegeneration. J Neurochem 78(5):1073–1082
42. Mattson MP, Chan SL (2001) Dysregulation of cellular calcium homeostasis in Alzheimer's disease: bad genes and bad habits. J Mol Neurosci 17(2):205–224
43. Choi YT, Jung CH, Lee SR, Bae JH, Baek WK, Suh MH, Park J, Park CW, Suh SI (2001) The green tea polyphenol (−)-epigallocatechin gallate attenuates beta-amyloid-induced neurotoxicity in cultured hippocampal neurons. Life Sci 70(5):603–614
44. Mandel S, Reznichenko L, Amit T, Youdim MB (2003) Green tea polyphenol (−)-epigallocatechin-3-gallate protects rat PC12 cells from apoptosis induced by serum withdrawal independent of P13-Akt pathway. Neurotox Res 5(6):419–424
45. Wang HY, Lee DH, D'Andrea MR, Peterson PA, Shank RP, Reitz AB (2000) Beta-amyloid(1–42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. J Biol Chem 275(8):5626–5632
46. Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C (2003) PI3K/Akt and apoptosis: size matters. Oncogene 22(56):8983–8998
47. Jo H, Mondal S, Tan D, Nagata E, Takizawa S, Sharma AK, Hou Q, Shanmugasundaram K, Prasad A, Tung JK, Tejeda AO, Man H, Rigby AC, Luo HR (2012) Small molecule-induced cytosolic activation of protein kinase Akt rescues ischemia-elicited neuronal death. Proc Natl Acad Sci USA 109(26):10581–10586
48. Koh SH, Noh MY, Kim SH (2008) Amyloid-beta-induced neurotoxicity is reduced by inhibition of glycogen synthase kinase-3. Brain Res 1188:254–262
49. Lee KY, Koh SH, Noh MY, Kim SH, Lee YJ (2008) Phosphatidylinositol-3-kinase activation blocks amyloid beta-induced neurotoxicity. Toxicology 243(1–2):43–50

50. Lee YJ, Park KH, Park HH, Kim YJ, Lee KY, Kim SH, Koh SH (2009) Cilnidipine mediates a neuroprotective effect by scavenging free radicals and activating the phosphatidylinositol 3-kinase pathway. J Neurochem 111(1):90–100
51. Lou H, Fan P, Perez RG, Lou H (2011) Neuroprotective effects of linarin through activation of the PI3K/Akt pathway in amyloid-β-induced neuronal cell death. Bioorg Med Chem 19(13):4021–4027
52. Rytömaa M, Lehmann K, Downward J (2000) Matrix detachment induces caspase-dependent cytochrome c release from mitochondria: inhibition by PKB/Akt but not Raf signalling. Oncogene 19(39):4461–4468
53. Brazil DP, Yang ZZ, Hemmings BA (2004) Advances in protein kinase B signalling: AKTion on multiple fronts. Trends Biochem Sci 29(5):233–242