Neurochem Res (2017) 42:468–480
DOI 10.1007/s11064-016-2093-8

ORIGINAL PAPER

Epigallocatechin Gallate Attenuates β-Amyloid Generation and Oxidative Stress Involvement of PPARγ in N2a/APP695 Cells

Zhao-Xu Zhang[1] · Yan-Bing Li[1] · Rui-Ping Zhao[2]

Received: 31 July 2016 / Revised: 24 September 2016 / Accepted: 27 October 2016 / Published online: 26 November 2016

Abstract The accumulation of β-amyloid (Aβ) peptide plaques is a major pathogenic event in Alzheimer's disease (AD). Aβ is a cleaved fragment of APP via BACE1, which is the rate-limiting enzyme in APP processing and Aβ generation. Nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) is considered to be a potential target for AD treatment, because of its potent antioxidant and inhibitory effects on Aβ production by negatively regulating BACE1. Epigallocatechin gallate (EGCG), a highly active catechin found in green tea, is known to enhance metabolic activity and cognitive ability in the mice model of AD. To investigate whether the therapeutic effect of EGCG is related to the PPARγ pathway, we analysed the alterations in the intracellular molecular expression of PPARγ after EGCG treatment in the N2a/APP695 cell line. In this study, we observed that EGCG attenuated Aβ generation in N2a/APP695 cells, such as the PPARγ agonist, pioglitazone, by suppressing the transcription and translation of BACE1 and that its effect was attenuated by the PPARγ inhibitor, GW9662. Intriguingly, EGCG significantly reinforced the activity of PPARγ by promoting its mRNA and protein expressions in N2a/APP695 cells. Moreover, EGCG also decreased the expression of pro-apoptotic proteins (Bax, caspase-3), reduced the activity of the anti-inflammatory agent NF-κB and inhibited the oxidative stress by decreasing the levels of ROS and MDA and increasing the expression of MnSOD. Co-administration of GW9662 also significantly decreased the EGCG-mediated neuroprotective effect evidenced by the increase in oxidative stress and inflammatory markers. The therapeutic efficacy of EGCG in AD may be derived from the up-regulation of PPARγ mRNA and protein expressions.

Keywords Alzheimer's disease (AD) · Epigallocatechin gallate (EGCG) · β-Amyloid · PPARγ · BACE1

Abbreviations

AD	Alzheimer's disease
Aβ	β-Amyloid peptides
APP	Amyloid precursor protein
BACE1	β-Site amyloid precursor protein-cleaving enzyme 1
PPARγ	Peroxisome proliferator activated receptor-γ
PIG	Pioglitazone

Introduction

Alzheimer's disease (AD) is the most common cause of dementia characterized by cognitive and memory impairment [1]. Increasing evidence indicates that excessive production and deposition of β-amyloid peptides (Aβ) as senile plaques, may initiate the process of neurodegeneration in AD brains [1, 2]. Aβ is generated by the sequential cleavage of amyloid precursor protein (APP) via APP cleaving enzyme 1 (BACE1) and γ-secretase [3, 4]. BACE1 is crucial for Aβ generation and is a major drug target for AD [5, 6]. According to the amyloid cascade hypothesis, aggregated Aβ in the forms of Aβ oligomers plays a pivotal role in the pathogenesis of AD [7]. This

✉ Zhao-Xu Zhang
hydzhangzhaoxu@163.com

✉ Rui-Ping Zhao
zhaozhehan33@163.com

[1] Department of Neurology, The Shandong Province Qianfoshan Hospital, Jinan 250000, China

[2] Division of Medical Quality Control, The Shandong Province Qianfoshan Hospital, Jinan 250000, China

hypothesis indicates that Aβ oligomers which more readily aggregates into amyloid plaques, can elicit a multistep cascade that disrupts neuronal homeostasis and causes the aberrant activation of kinases. These alterations in kinase activities ultimately result in neurofibrillary tangle formation and neuronal loss [8, 9]. Studies have shown that oxidative stress and inflammatory promote AD progression, and may play akey role in Aβ-mediated neurotoxicity [10–12].

The peroxisome proliferator activated receptor-γ (PPARγ) is a transcription factor with well-characterized functions in restoring insulin sensitivity in type 2 diabetes [3, 13]. It has been proved that PPARγ is able to protect cells from apoptosis through its strong anti-inflammatory and antioxidant effects [14, 15]. Accumulating evidence indicate that PPARγ agonists have been shown not only to repress oxidative stress and inflammation in AD model mice [16, 17] but also to decrease Aβ production through the negatively regulation of BACE1 both in vivo and in vitro [18, 19]. PPARγ deficiency has been shown to be involved in the pathological development of AD [20]. PPARγ-null mice showed cognitive impairment, along with increased BACE1level, NF-κB activity, oxidative stress and inflammation [21]. The pharmacological PPARγ agonist, pioglitazone (PIG), which has been approved for the treatment of type 2 diabetes patients, can enhance memory ability in Tg2576 APP mouse model, and suppress BACE1 expression and Aβ deposits [22]. These observations indicate that PPARγ is a promising therapeutic target for the treatment of AD.

Current drugs for AD treatment show limited benefits and are unable to arrest the progression of the disease. Epigallocatechin gallate (EGCG) is a highly active catechin found in green tea. Several lines of evidence shows that EGCG protects against Aβ and *N*-methyl-4-phenyl-1,2,3,6-tetrahydroxynurenine (MPTP) induced neuronal degeneration and injuries, exerting antioxidant, anti-inflammation, inhibition of tau phoshorylation and anti-apptopsis functions [23–25]. EGCG can increase the levels of enzymes related to oxidative stress, such as heme oxygenase-1 (HO-1) via PPARα activation in cultured cancer cells [26]. In addition, Dragicevic et al. [27] demonstrate that EGCG reduces amyloid-induced mitochondrial dysfunction and Aβ production both in vitro and in vivo. However, the role of EGCG on PPARγ is still unknown, and precise mechanism that EGCG suppresses Aβ production and its neuroprotective function need to be further explored.

To determine the therapeutic potential of EGCG to AD, we investigated the inhibition of Aβ generation, antioxidative stress and anti-inflammatory effects of EGCG in N2a-APP695 cells, a widely used in vitro model of Aβ production by amyloidogenesis pathway. The underlying mechanism involved in PPARγ was also studied.

Materials and Methods

Reagents

EGCG (E4143) purchased from Sigma-Aldrich (St. Louis, MO, USA) was of high purity (98.0%) as determined by HPLC analysis. PPARγ agonist, pioglitazone, was obtained from Ping Min Pharmaceutical Co., LTD (ShanDong, China), and GW9662, which is a specific PPARγ antagonist, was obtained from Sigma-Aldrich (St. Louis, MO, USA). DMEM/Opti-MEM (1:1, v/v) was obtained from Gibco Inc. (GrandIsland, NY, USA). DNA staining Hoechst 33258 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) were from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-BACE1, rabbit polyclonal anti-β-C-terminal fragment (β-CTF), and rabbit monoclonal anti-PPARγ antibody were obtained from Santa Cruz (Santa Cruz, CA). Rabbit polyclonal anti-cleaved Caspase-3, Bax, NF-κBp65, phospho-p65 antibodies were Cell Signaling (Shanghai, China). Rabbit polyclonal anti-manganese superoxide dismutase (anti-MnSOD) was obtained from Sigma-Aldrich (St. Louis, USA). β-Actin was obtained from Abcam (Cambridge, MA, USA). Secondary antibodies (HRP-conjugated goat anti-mouse, anti-rabbit) were obtained from Santa Cruz (Santa Cruz, CA).

Cell Culture and Treatments

Murine neuroblastoma N2a cells which were stably transfected with the human APP695 were obtained from Professor Huaxi Xu (Xiamen University, China). N2a-APP695 cells were maintained in DMEM/Opti-MEM (1:1, v/v; containing 200 μg/ml G418.5% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin) and kept at 37 °C in humidified 5% CO_2. N2a/wt cells were incubated in DMEM media, supplemented with 10% fetal bovine serumin 5% CO_2 at 37 °C. The cellswere passaged every 3 days when growing up to 80% confluence. Some of the cells were incubated with varied doses of EGCG (5–100 μM) or PPARγ agonist pioglitazone (10 μM) for 24 h to detect the effect of EGCG in N2a-APP695 cells. In some experiments, to evaluate the mechanism of EGCG action, a PPARγ antagonist GW9662 (30 μM) was co-administered with EGCG for the 24-h pretreatment.

Assessment of Cell Viability

MTT is absorbed into cells and transformed into formazan, which directly reflects the activity of mitochondria. Cells were plated in 96-well plates, cultured, and treated according to the methods described above. A total of 50 μl of 2 mg/ml MTT was added to 200 μl medium in each well. The final concentration of MTT was 0.5 mg/ml. Then the

medium containing MTT was removed from each well, and the formazancrystals were dissolved by addition of 150 µl dimethylsulfoxide (DMSO). Formazan absorbance was assessed at awavelength of 570 nm by a 550 Bio-rad microplate reader. The experiment was repeated five times.

Hoechst 33258 Staining to Assess Cell Apoptosis

Cell apoptosis was assessed by nuclear DNA staining with Hoechst 33258. Neurons plated on coverslips were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, washed twice in PBS, and stained with Hoechst 33258 (1 µg/ml) for 5 min. Stained cells were then washed twice with PBS and mounted under glass coverslips with Mowiol (slides). Nuclei were visualized using a fluorescent microscope. The percentage of Hoechst-positive cells was estimated in five randomly selected fields covering at least 50 cells.

Aβ ELISA Assay

Conditioned media from cultured cells were collected. The concentrations of Aβ1–40 and Aβ1–42 were measured using a sandwich ELISA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Aβ levels were normalized to total protein content in the samples. Aβ1–40 and Aβ1–42 levels are expressed in pg/mg of protein. The optical densities in each well were measured using a plate reader at 450 nm.

Western Blot Analysis

The treated cells were washed with ice-cold PBS and thenwere lysed in cell lysis buffer containing (50 mM Tris-HCl, pH 6.8, 1 mM PMSF, 1 mM EDTA, 150 mM NaCl, 1% NP–40, 1 mM Na_3VO_4, 10 mM NaF, 1 µg/ml aprotinin, 1 µg/ml pepstain, 1 µg/ml leupeptin). Then the cell lysates were centrifuged at 14,000*g* for 10 min at temperature 4 °C. The protein concentrations in the supernatants were determined by the BCA kit (Pierce, Rockford, IL, USA). 20 µg protein lysate were loaded on a SDS polyacrylamide gel (10%) and transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking with TTBS (TBS with 0.1% Tween-20) containing a 5% nonfat dry milk for 2 h. The membranes were incubated overnight at 4 °C with the appropriate primary antibodies, including anti-cleaved Caspase-3 (1:500), anti-PPARγ (1:500), anti-MnSOD (1:500), anti-NF-κBp65 (1:500), anti-phospho-p65 (1:500) anti-Bax (1:500), anti-BACE1 (1:500), anti-β-CTF (1:500), and β-actin (1:1000).

After washing three times for 10 min in TBS, membranes were incubated for 1 h with HRP-conjugated goat anti-rabbit secondary antibody (Invitrogen, Eugene, OR) at room temperature. Following the post-secondary washes, immunolabeled proteins were detected using an ECL+ detection kit (Amersham Pharmacia Biotech). The blot was visualized by exposures to Kodak film and the densities of the bands were determined using Bandscan 5.0 software (ProZyme Inc, USA).

Real-Time Quantitative PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). RNA concentration was measured using a spectrophomometer. RNA samples were then reverse-transcripted into cDNA using Revert Aid™ First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany). Specific primers were designed: BACE1: [forward, 5′-GCATGATCATTGGTGGTATC-3′, reverse, 5′-CCATCTTGAGATCTTGACCA-3′]; PPARγ: [forward, 5′-CCACCAACTTCGGAATCA-3′, reverse, 5′-TTTGTG GATCCGGCAGTTA-3′]; GAPDH: [forward, 5′-ACA GCCGCATCTTCTTGTGC-3′, reverse, 5′-CACTTTGC CACTGCAAATGG-3′]. The relative mRNA levels of the individual samples were calculated using the $2^{-\Delta\Delta CT}$ method.

Detection of Reactive Oxygen Species (ROS)

Intracellular production of ROS was measured as described previously Sun et al [31]. In brief, cells were incubated with 30 µMDCF-DA at 37 °C for 30 min and treated with modified Krebs solution (135 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM glucose, 11.6 mM HEPES, pH 7.4) 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 re-suspended in 10 mM EDTA containing PBS. The intracellular oxidant productions were measured. Mean intensity of DCF fluorescence for randomly selected 3 fields were measured and analyzed by Image J 1.41o software (NIH, USA). The experiment was repeated five times.

Malondialdehyde Determination

Malondialdehyde (MDA) level was a marker of lipid peroxidation and was assessed by high-performance liquid chromatography. Cells were homogenized and centrifuged, and the supernatants collected and stored on ice. Samples were hydrolyzed by boiling in diluted phosphoric acid. MDA, one of the low-molecular weight end products formed by lipid-peroxidation, reacted with thiobarbituricacid (TBA) to form MDA-TBA adducts which were eluted with methanol-phosphate buffer to be quantified by spectrophotometry at 532 nm.

Statistical Analysis

The data from all procedures were expressed as the means ± SEM (n = 5 in every independent experiment). Statistical differences between values were estimated by analysis of variance (ANOVA) followed by Duncan's multiple range test. For all statistical tests $p < 0.05$ was considered significant.

Results

Effect of EGCG on the Survival of N2a Cells

N2a/wt and N2a-APP695 cells were incubated with EGCG at different doses (5–100 μM) for 24 h. Cell viability was measured using the MTT assay. For the N2a/wt cells, EGCG (5–100 μM) appeared not to alter cell viability (Fig. 1a). For the N2a/APP695 cells, cell survival increased in a dose-dependent manner following exposure to EGCG, from 10 μM onwards ($p < 0.05$), reaching a plateau at the concentration of 40 μM ($p < 0.001$, Fig. 1b). Therefore, 40 μ MEGCG was selected as the optimal concentration for subsequent experiments.

It has been reported that pioglitazone (PIG), a special pharmacological PPARγ agonist, used to treat patients with type 2 diabetes, plays an important role in cell survival [28]. To better elucidate the neuroprotective mechanism of EGCG, PIG was used as a positive control. As shown in Fig. 1c, the cell viability of N2a/APP695 cells incubated with10 μM PIG was also obviously increased. To investigate the influence of EGCG on apoptosis, Hoechst 33258 staining was used to assess cell apoptosis. The apoptosis rate ofN2a/APP695 cells was higher than N2a/wt cells, however, EGCG treatment decreased the apoptosis of N2a/APP695 cells (Fig. 1d).

EGCG Decreased Bax and Cleaved Caspase-3 Expressions

To further determine the effect of EGCG on apoptosis, the expressions of apoptosis-related proteins (Bax and cleaved-caspase-3) were assessed by western blot analysis. In comparison to N2a/WT cells, N2a/APP695 cells showed a significant elevation in Bax ($p < 0.001$) and cleaved caspase-3 ($p < 0.001$) expressions. Interestingly, both EGCG (40 μM) and PIG (10 μM) significantly decreased the levels of Bax and cleaved caspase-3 (Fig. 2).

EGCG Reduced Aβ Level in N2a/APP695 Cells

The extracellular accumulation of Aβ in the form of plaques is a hallmark pathological feature of AD [1]. ELISA assay was used to determine the effect of EGCG treatment on Aβ level in N2a/APP695 cells. We found that Aβ1–40 ($p < 0.001$, Fig. 3a) and Aβ1–42 ($p < 0.001$, Fig. 3b) levels were significantly increased in N2a/APP695 cells compared with N2a/wt cells. Interestingly, a lower levels of Aβ1–40 and Aβ1–42 were found in N2a/APP695 cells incubated with both EGCG ($p < 0.001$ for Aβ1–40 and Aβ1–42) and PIG ($p < 0.01$, $p < 0.001$ for Aβ1–40 and Aβ1–42 respectively) compared with the control-treated group (Fig. 3). These data indicate that EGCG may suppress the secretion of Aβ1–42 and Aβ1–40.

EGCG Suppressed the mRNA and Protein Expressions of BACE1 in N2a/APP695 Cells

BACE1 is the rate limiting enzyme for Aβ peptide generation and is a major drug target for AD. Real-time PCR (Fig. 4a) and western blot analysis (Fig. 4b, c) respectively revealed increased levels of BACE1 mRNA and protein in N2a/APP695 cells, in comparison with those in N2a/wt cells. In addition, pretreatment of N2a/APP695 cells with EGCG or PIG markedly attenuated the mRNA and protein expressions of BACE1 (Fig. 4).

EGCG Reduced β-CTF Protein Level in N2a/APP695 Cells

β-CTF is the soluble C-terminal fragment generated by cleavage of APP at the BACE-1 cleavage site, and is the direct precursor of Aβ. Western blot was used to determine the protein level of β-CTF and APP. As shown in Fig. 5a, b, the expressions of β-CTF ($p < 0.001$) and APP ($p < 0.001$) in N2a/APP695 cells were increased than that of N2a/WT cells. However, both EGCG (40 μM) and PIG (10 μM) significantly decreased the protein levels of β-CTF and APP in N2a/APP695 cells. Moreover, neither EGCG nor PIG treatment changed the level of APP protein in N2a/APP695 cells (Fig. 5a, c). We also used western blot to further explore the effect of EGCG on native APP production and procession in N2a/WT cells. As shown in (Fig. 5c–e) EGCG had no influence on the expressions of APP and β-CTF in N2a/WT cells. These results indicated that EGCG may inhibit the activity of BACE1 to reduce Aβ production in N2a/APP695 cells.

EGCG Increased PPARγ mRNA and Protein Expressions in N2a/APP695 Cells

To explore the mechanism(s) underlying the inhibitory effect of EGCG on Aβ production, we investigated the expression PPARγ, an important transcription factor presented in the BACE1 promoter, reduces the activity of BACE1. As shown in Fig. 6, there was a distinct

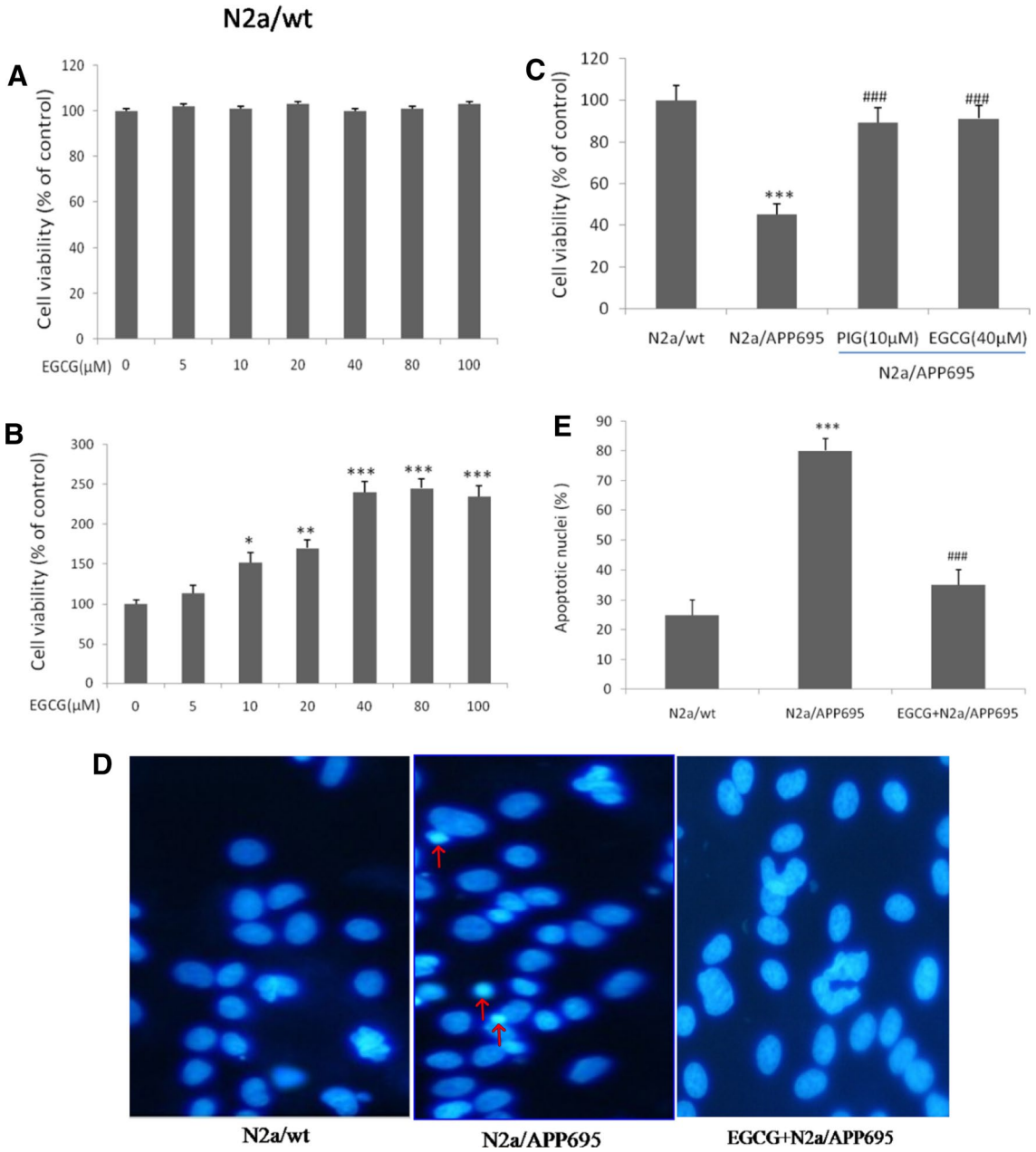


Fig. 1 Effects of epigallocatechin gallate (EGCG) on the cell viability and apoptosis of N2a cells. **a** The cell viability was assessed by MTT reduction assay. N2a/wt cells were incubated with different concentrations of EGCG (5–100 μM), and the cell viability was not changed. **b** EGCG increased N2a/APP695 cell viability in a dose-dependent manner, from 10 μM onwards, reaching a plateau at the concentration of 40 μM. **c** Both pioglitazone (PIG 10 μM) and EGCG (40 μM) significantly increased the cell viability of N2a/APP695. **d** Hoechst 33258 Staining (×200) was used to assess cell apoptosis. The *red arrow heads* show nuclear fragmentation, indicating that cell apoptosis. **e** The percent of apoptosis was increased in N2a/APP695 cells in comparison to N2a/wt cells, however, EGCG (40 μM) treatment inhibited apoptosis in N2a/APP695 cells. The data are expressed as the mean ± SEM $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with the control group; $^{\#\#\#}p<0.001$ compared with N2a/APP695 control group

reduction of PPARγ mRNA ($p<0.001$, Fig. 6a) and protein ($p<0.001$, Fig. 6b, c) levels in N2a/APP695 cells compared with N2a/WT cells and that of EGCGtreated-N2a/APP695 cells was markedly increased in comparison with the levels of the control-treated group.

EGCG Inhibited Expression of BACE1 via PPARγ

In the result above, we found that 40 μM EGCG for24 h significantly regulated expressions of BACE1 and PPARγ, as well as level of secreted Aβ. To determine whether

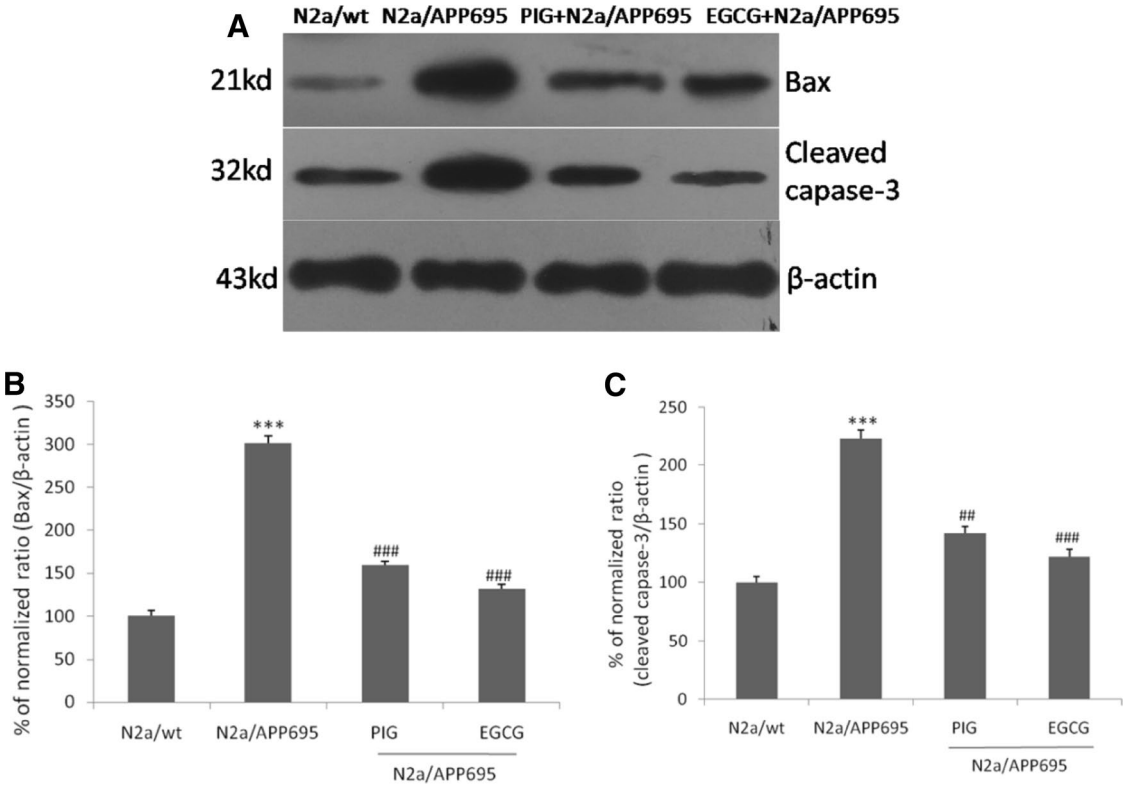


Fig. 2 EGCG decreased Bax and cleaved caspase-3 protein expressions. **a** Western blot was used to detect the Bax and cleaved caspase-3 protein expressions. **b** The corresponding histogram showed that both PIG and EGCG decreased the normalized level of Bax. **c** The corresponding histogram showed that both PIG and EGCG decreased the normalized level of cleaved caspase-3. The data are expressed as the mean ± SEM. $^{***}p < 0.001$ compared with the N2a/wt control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the N2a/APP695 control group

PPARγ is involved in the effect of EGCG on the expression level of BACE1 protein in N2a/APP695 cells, the cells were incubated with the PPARγ antagonist GW9662 (30 μM) 1 h prior to EGCG treatment for 24 h. As shown in Fig. 7, GW9662 pretreatment markedly reversed the EGCG-induced suppression of BACE1 mRNA ($p < 0.05$, Fig. 7a) and protein ($p < 0.01$, Fig. 7b, c) expressions compared with the EGCG only treatment group. These results indicate that, EGCG may be a PPARγ agonist to enhance the transcription and translation of PPARγ, by suppressing the activity of BACE1 and inhibiting Aβ production.

EGCG Inhibited NF-κB Activity in N2a/APP695 Cells

NF-κB is reported as a downstream molecule of PPARγ [29], and is a transcription factor known as a central regulator of inflammation. NF-κB activity was studied by western blot analysis, using antibodies against active subunitp-p65. As shown in Fig. 8, compared with that of N2a/wt cells, the protein level of p-p65 in N2a/APP695 cells was higher, and that of EGCG-treated N2a/APP695 cells was markedly decreased when in comparison with the level of the control-treated group, without affecting total expression of p65. The effect of EGCG was obviously blocked by GW9662, indicating the involvement of PPARγ signal transduction.

EGCG Attenuated Oxidative Stress in N2a/APP695 Cells

Oxidative stress has been implicated in pathological progress of AD, and is associated with Aβ mediated neurotoxicity. To evaluate the antioxidant activity of EGCG, several biochemical and protein expression of oxidative stress markers were estimated. We detected an increase of ROS and MDA levels compared to N2a/wt cells in N2a/APP695 cells (Fig. 9a, b). Moreover, we also observed notable decrease of MnSOD protein expression (Fig. 9c, d) in N2a/APP695 Cells, demonstrating an oxidative stress induction. As expected, EGCG treatment significantly reduced ROS and MDA levels, and increased MnSOD protein expression, however, GW9662prevented EGCG's effect (Fig. 9).

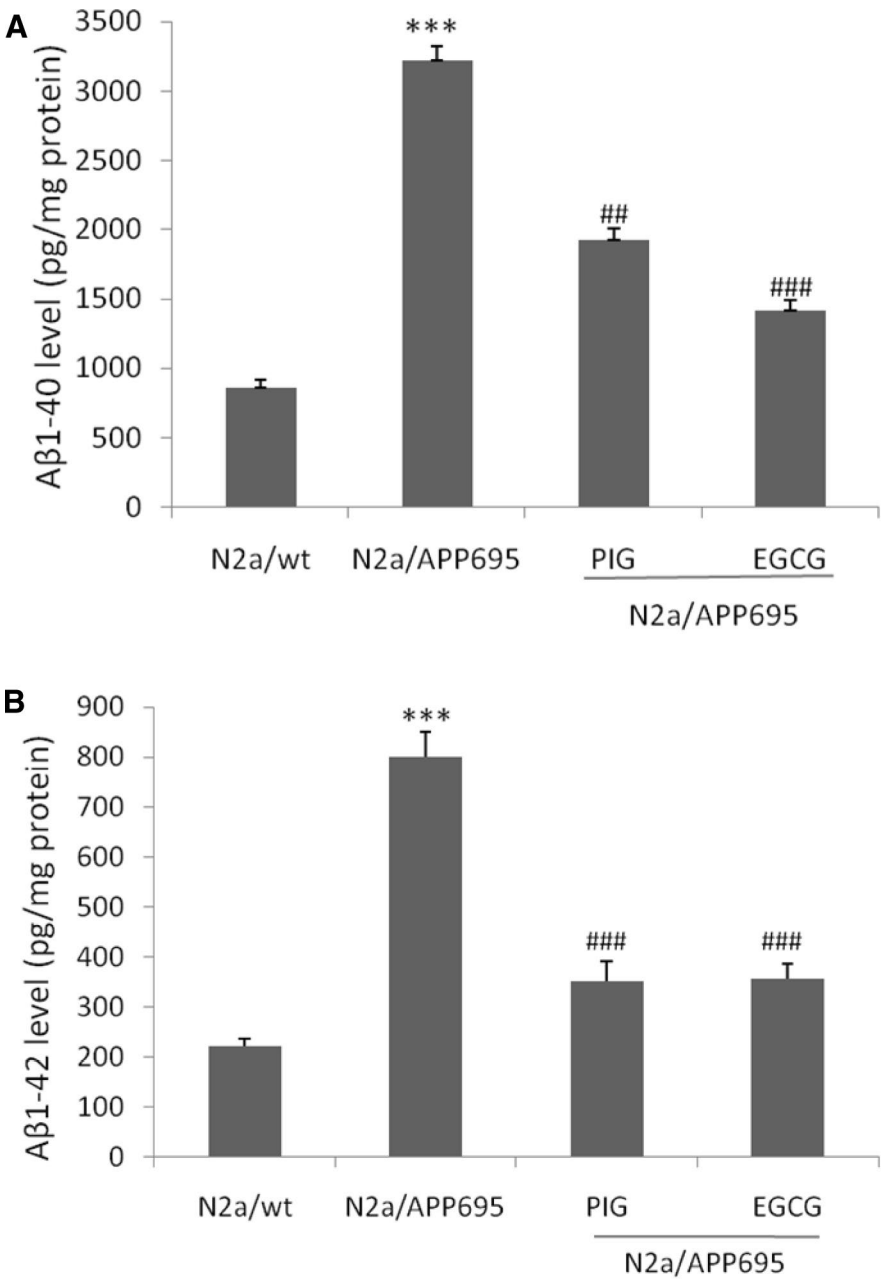


Fig. 3 EGCG reduced Aβ level in N2a/APP695 cells. **a** Both PIG and EGCG decreased Aβ1–40 level; **b** both PIG and EGCG decreased Aβ1-42 level. The data are expressed as the mean ± SEM. ***p < 0.001 compared with the N2a/wt control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the N2a/APP695 control group

Discussion

In our present experiment, we first demonstrated that EGCG attenuated Aβ generation in N2a/APP695 cells like the PPARγ agonist, pioglitazone, via suppressing transcription and translation of BACE1, and that its effect was attenuated by PPARγ inhibitor GW9662. Intriguingly, EGCG significantly reinforced the activity of PPARγ by promoting its mRNA and protein expressions in N2a/APP695 cells. Moreover, EGCG also decreased pro-apoptotic protein (Bax, caspase-3) expressions, reduced anti-inflammatory agent NF-κB activity, and inhibited oxidative stress, and that its effect is likely mediated by PPARγ activation.

EGCG, derived from green tea, exerts a crucial role in protecting neuronal cells from β-amyloid induced neurotoxicity and oxidative injury [30, 31]. EGCG can significantly promote anti-apoptotic molecule (Bcl-2) production, increase cell survival, and attenuate Aβ1–42-induced tau phosphorylation [32]. In the present study, we employed APP-transfected N2a cells to investigate the neuroprotective mechanisms of EGCG in vitro. Consistent with previous studies [30, 32], herein, we revealed that EGCG increased cell viability in a concentration-dependent manner and inhibited cell apoptosis in N2a/APP695 cells. Caspase-3 cascade and Bax family members are key mediators for the apoptotic signaling transduction [33]. Our present

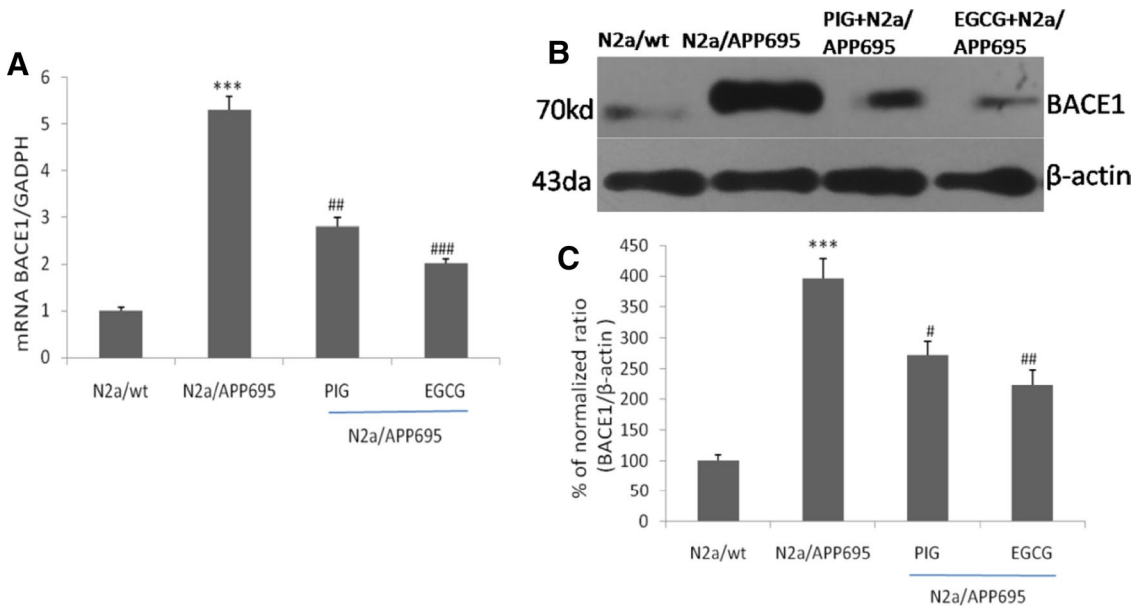


Fig. 4 EGCG suppressed the mRNA and protein expressions of BACE1 in N2a/APP695 Cells. **a** Real-time PCR revealed the BACE1mRNA level; **b** western blot revealed the BACE1protein level; **c** the corresponding histogram of BACE1normalized protein level. The data are expressed as the mean ± S.E.M. $^{***}p < 0.001$ compared with the N2a/wt control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with theN2a/APP695 control group

study demonstrated that incubation with EGCG for 24 h resulted in an apparently reduction of Bax and caspase-3 expressions in N2a/APP695 cells.

Deposition of β-amyloid peptides as senile plaques, which closely correlates with cognitive function of AD patients, is one of the pathological hallmarks of AD [2]. The leading candidate explanation for the molecular basis of AD pathology is the amyloid cascade hypothesis [34]. This states that the Aβ protein initiates the disease process, activating downstream neurotoxic mechanisms including oxidative stress, inflammation, dysregulation of tau and eventually cell apoptosis [35]. In this study, consistent with prior research conclusions [13, 36], N2a/APP695 cells show decreased cell viability, increased cell apoptosis as well as pro-apoptotic protein expressions, and higher level of secreted Aβ in comparison with N2a/wt.

Aβ peptides, existing as two main species, Aβ1–40 and Aβ1–42, derive from the sequential proteolysis of APP via BACE1, the canonical β-secretase, and γ-secretase [4]. BACE1initiates the amyloidogenic pathway by secreting the soluble APPβ fragment (sAPPβ) and generating the membrane bound C-terminal fragment β (β-CTF or C99) [6]. β-CTF is then cleaved by γ-secretase, generating the Aβ peptide. BACE1 appears to be the key enzyme for Aβ production, as BACE1-deficient mice do not generate Aβ [37]. Thus, BACE1 is an important drug target for AD [37, 38]. Our current study reveals that the levels of BACE1 and β-CTF in N2a-APP695 cells are higher than those in N2a-WTcells. EGCG treatment of N2a/APP695 cells decreased the generation of Aβ1–42 and Aβ1–40 by inhibiting transcription and translation of BACE1, but did not change APP protein level. Interestingly, a previous study has been reported that EGCG can negatively regulated BACE1 activity [39], however the underlying mechanism needs to be explored.

During the process of BACE1 transcriptional regulation, a number of transcription factor binding sites, including various inflammation-related transcription factors such as PPARγ, NF-κB and PGC-1are involved [40–42]. Regulating of Aβ production and metabolism by PPARγ has been explored in many recent studies [22]. The nuclear receptor PPARγ is a newly recognized therapeutic target for the treatment of AD [20]. In AD models, the PPARγ agonists were demonstrated to improve impaired memory and reduce BACE1 activity and Aβ generation [43]. In PPARγ-null mice, the expression of the BACE1 increased, paralleling the cognitive impairment as well as the development of inflammation in the cortex [21]. Some studies have shown that the binding site of PPARγ in the BACE1 promoter region is functional and that PPARγ has direct effects on BACE1 transcription and Aβ generation [3, 44]. Collectively, these studies supported that PPARγ agonist may represent new drugs to treat AD by reducing Aβ generation.

PPARγ is a type II nuclear receptor whose primary action is to regulate lipid and energy metabolism in restoring insulin sensitivity in type 2 diabetes. Clinical and epidemiological evidence suggest that type 2 diabetes is now a known risk factor for AD. Pioglitazone (PIG), the

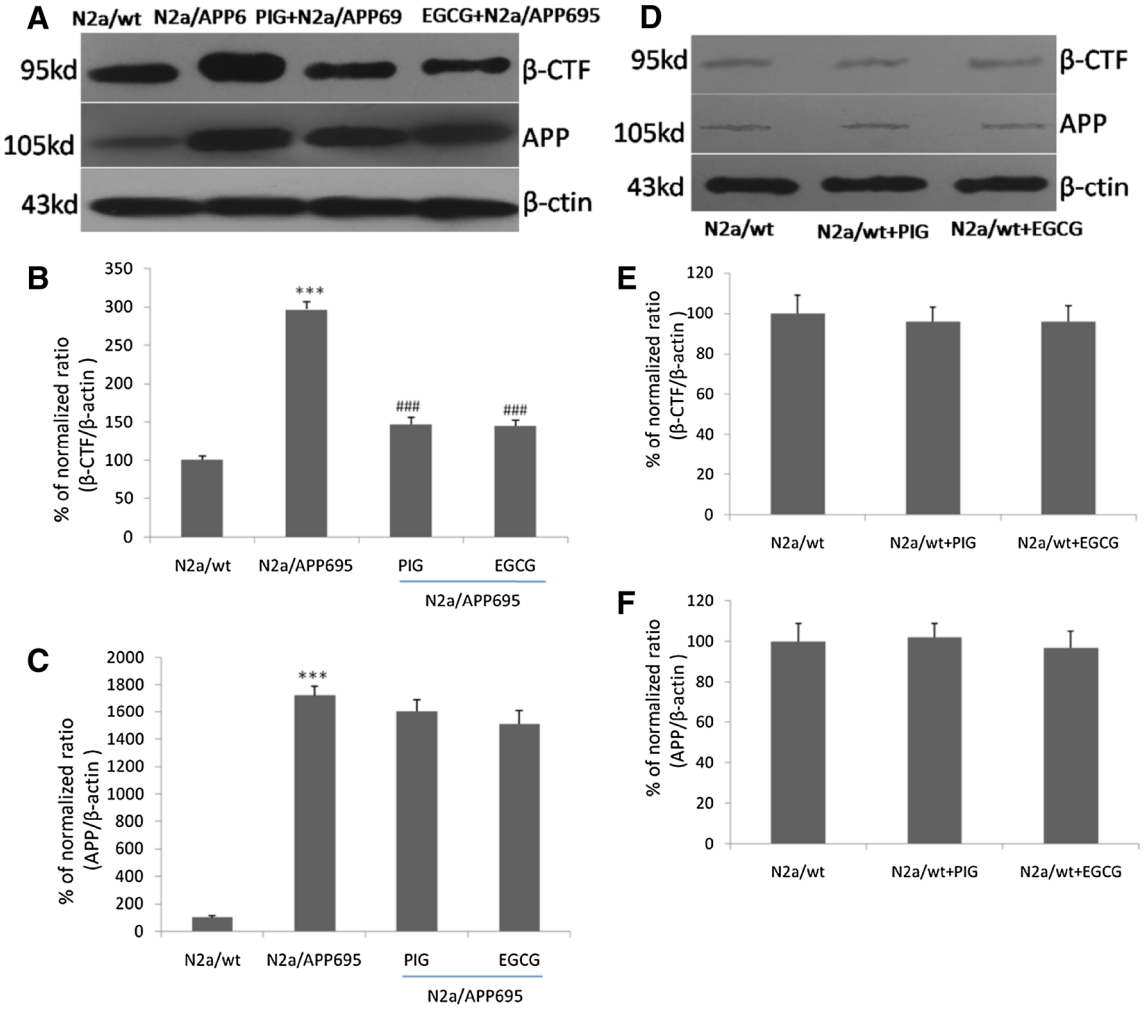
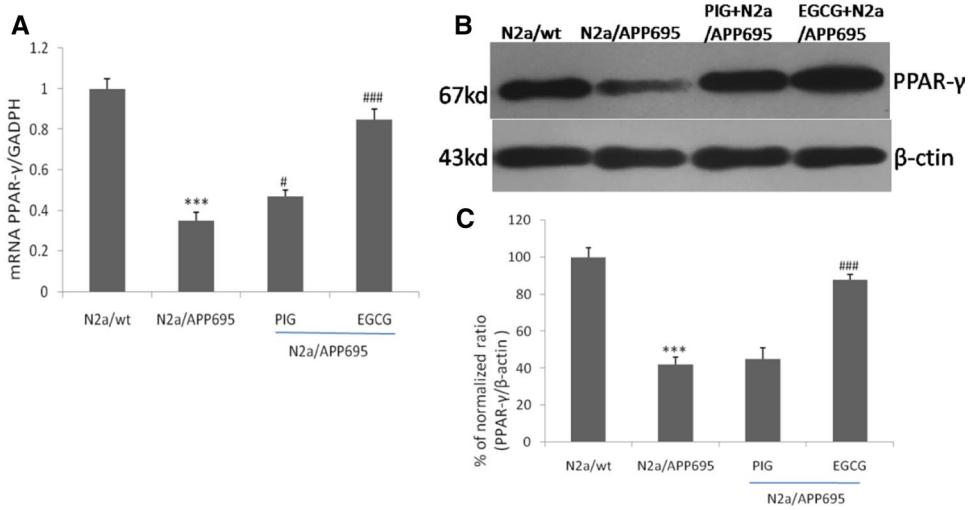


Fig. 5 EGCG reduced β-CTF protein level in N2a/APP695 cells. **a** Western blot was used to detect the β-CTF and APP protein expressions; **b** the corresponding histogram indicted that both PIG and EGCG decreased β-CTF protein level; **c** neither EGCG nor PIG changed the level of APP protein in N2a/APP695 cells; **d, e**, and **f** western blot was used to further explore the effect of EGCG on native APP production and procession in N2a/WT cells. EGCG had no influence on the expressions of β-CTF (**e**) and APP (**f**) in N2a/WT cells. The data are expressed as the mean ± S.E.M. $^{***}p < 0.001$ compared with the N2a/wt control group; $^{\#\#\#}p < 0.001$ compared with the N2a/APP695 control group

Fig. 6 EGCG increased the mRNA and protein expressions of PPARγin N2a/APP695 cells. **a** Real-time PCR revealed the PPARγ mRNA level; **b** western blot revealed the PPARγ protein level; **c** the corresponding histogram of PPARγ normalized protein level. The data are expressed as the mean ± S.E.M. $^{***}p < 0.001$ compared with the N2a/wt control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with the N2a/APP695 control group



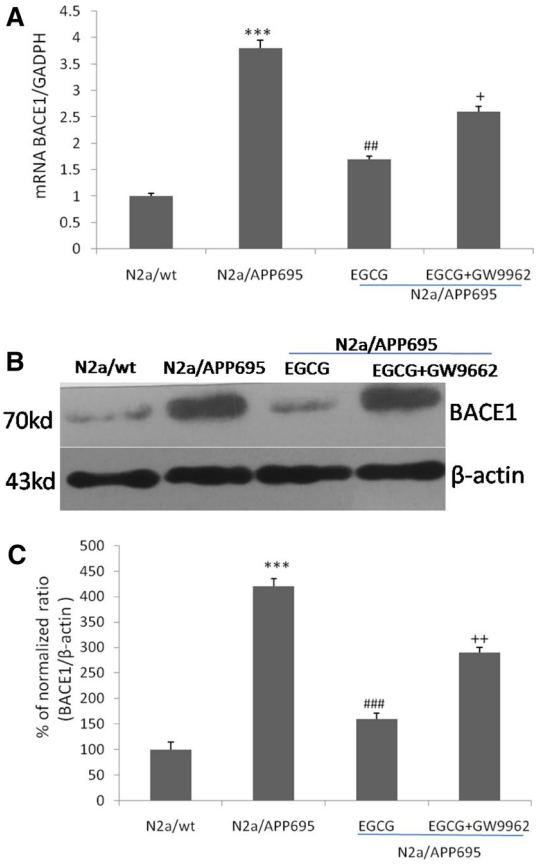


Fig. 7 PPARγ antagonist GW9662 blocked the inhibitive role of EGCG on BACE1mRNA and protein expressions. **a** Real-time PCR revealed that GW9662 prevented EGCG induced the reduction of BACE1 mRNA level; **b** western blot and **c** the corresponding histogram revealed that the decreased protein level of BACE1 by EGCG was reversed by GW9662. The data are expressed as the mean ± S.E.M. $^{***}p < 0.001$ compared with the N2a/wt control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with theN2a/APP695 control group; $^{+}p < 0.05$, $^{++}p < 0.01$ compared with EGCG treated group

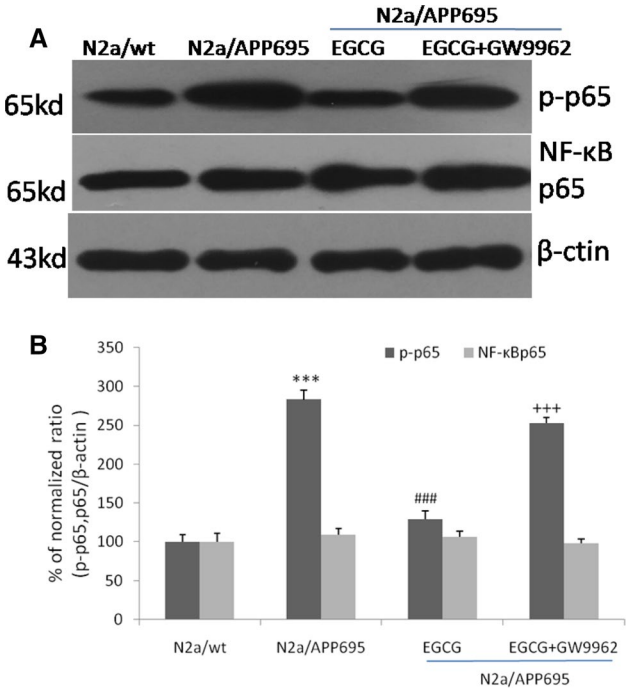


Fig. 8 EGCG inhibited NF-κB activity in N2a/APP695 cells. **a** Western blot and **b** the corresponding histogram revealed that the level of p-p65 was significantly reduced in EGCG treated group compared to N2a/APP695 control group, without affecting total level of p65. The effect of EGCG was obviously blocked by GW9662. The data are expressed as the mean ± S.E.M. $^{***}p < 0.001$ compared with the N2a/wt control group; $^{\#\#\#}p < 0.001$ compared with theN2a/APP695 control group; $^{+++}p < 0.001$ compared with EGCG treated group

pharmacological PPARγ agonists, has been shown not only to suppress chronic cerebral inflammation in AD, but also to decrease Aβ1–42 level by inhibiting BACE1 expression [44, 45]. Consistently, our experiment indicated that PIG, used as a positive control, reduced BACE1 mRNA and protein expressions, paralleling with the decreased Aβ generation in N2a/APP695 cells. Notably, EGCG treatment of N2a/APP695 cells dramaticlly resulted in up-regulation of PPARγ mRNA and protein expressions. As expected, GW9662, an inhibitor of PPARγ, significantly blocked the beneficial role of EGCG on BACE1 expression. Thus, we can conclude that EGCG's inhibitory effect on BACE1 may be mediated by PPARγ activation, which resulted in reduction of BACE1 expression and Aβ levels.

In addition to repressing Aβ production, due to its anti-inflammation and antioxidant functions, PPARγ may also regulate different aspects of AD [46–48]. For example, PPARγ acts as a key regulator of a broad array of anti-inflammatory factors, i.e., IL-6, IL-2, iNOS, NF-κ Band Cox-2 [48]. Of these factors, NF-κB is known as a central regulator of inflammation, to stimulate apoptotic signaling, and its activity is tightly regulated by PPARγ [47, 49]. Pascual et al. [49] revealed that PPARγ might exert anti-inflammatory effects by interfering with NF-κB activity innerve cells. Interestingly, a previous study indicates that the transcription factor NF-κB can also positively regulate BACE1 transcription, as a downstream molecule of PPARγ [12]. As mentioned above, EGCG can enhance the expression of PPARγ, therefore, we hypothesize that EGCG protects against inflammation by decreasing NF-κB activity. As expected, EGCG attenuates phosphorylation of NF-κB p65 and this effect is prevented by PPARγ antagonist GW9662. Moreover, as illustrated in the previous data, EGCG has also been shown to suppress inflammatory mediators including IL-6, IL-2 and NF-κB [50]. This indicates that, not only PPARγ but also NF-κB may be involved in EGCG's regulation on BACE1.

Evidence suggests that oxidative stress is a prominent early feature of AD and plays an important role in

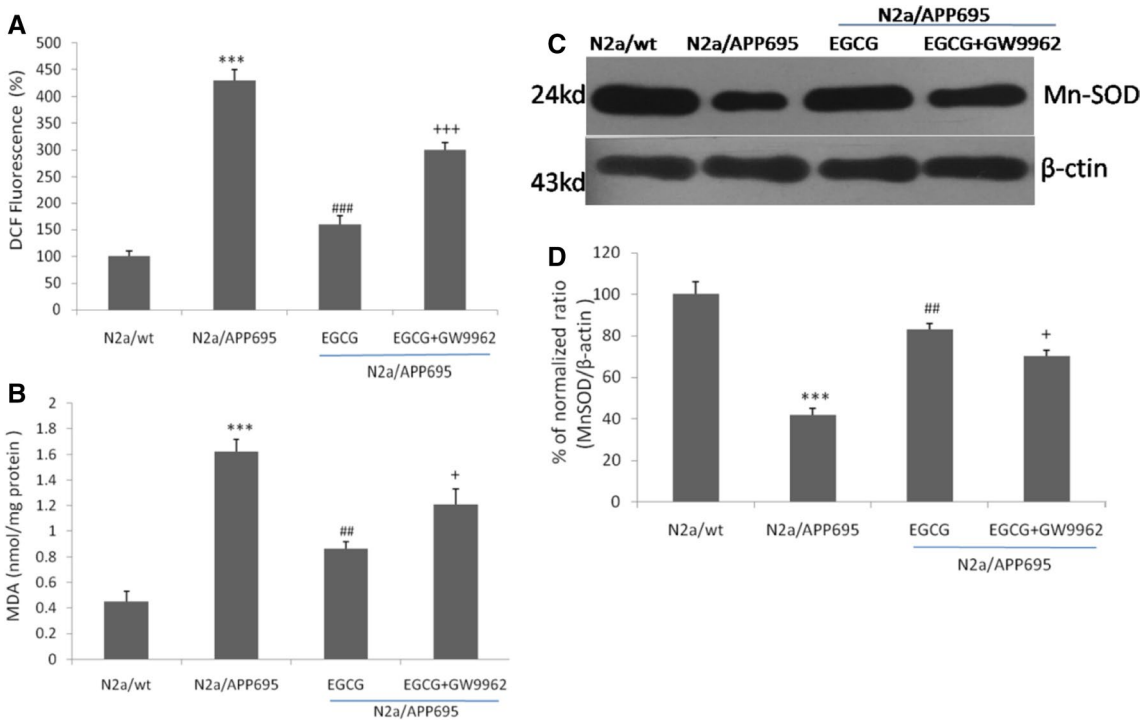


Fig. 9 EGCG attenuated oxidative stress in N2a/APP695 Cells. **a** The fluorescence intensity of DCF was measured indicating level of ROS; **b** MDA level was assessed by high-performance liquid chromatography; **c** MnSOD protein expression was detected by western blot; **d** the corresponding histogram of normalized MnSOD protein expression. EGCG treatment significantly reduced ROS and MDA levels, and increased MnSOD protein, however, GW9662 prevented the EGCG's effect. The data are expressed as the mean ± S.E.M. $^{***}p < 0.001$ compared with the N2a/wt control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the N2a/APP695 control group; $^{+}p < 0.05$, $^{+++}p < 0.001$ compared with EGCG treated group

amyloidogenesis and Aβ deposition [51]. Aβ induced neurotoxicity is mediated by oxidative stress and keep down oxidative stress processes can improve neurodegeneration in AD [7, 9]. Oxidative stress is due to the imbalance between ROS and anti-oxidative system. MnSOD is a possible antioxidant defense system that can scavenge ROS to prevent cell damage, and MDA is the marker of lipid peroxidation [10]. Interestingly, a previous study has shown that EGCG can inhibit oxidative stress via increased HO-1 expression involvement of PPARα activation in cultured cancer cells [26]. Herein, we investigated the role of EGCG on oxidative stress and found that EGCG evidently enhance MnSOD protein expression, decreased the level of MDA production and the accumulation of ROS. However, the PPARγ antagonist, GW9662 blunts the EGCG's effect above. Increasing evidence has shown PPARγ activation diminished the damage of oxidative stress [17, 28]. A previous data indicate that some inflammatory mediators such as NF-κB, IL-1β and IL-6 may involve in PPARγ-mediated antioxidant effect [28]. Additionally, it has been reported that EGCG could protect cardiomyocytes from doxorubicin-induced oxidative stress by attenuating ROS production [30, 31]. Consistently, our present study reveals that EGCG inhibit oxidative stress in N2a/APP695 cells, which was obviously blocked by GW9662, indicating the involvement of PPARγ signal transduction.

In conclusion, in this study, we investigated the neuroprotective effects and potential molecular mechanisms of EGCG in cultured N2a/APP695. For the first time, we present the evidence that EGCG significantly suppresses BACE1 mRNA and protein expressions as well as the subsequent Aβ production, and reduces inflammation, oxidative stress and eventually cell apoptosis. These findings provide new insights into the neuroprotective role of EGCG and its possible beneficial therapeutic value for AD pathology.

Acknowledgements This work was supported by Grants from the National Natural Science Foundation of China (No. 81303013).

References

1. Song MS, Rauw G, Baker GB, Kar S (2008) Memantine protects rat cortical cultured neurons against beta-amyloid-induced toxicity by attenuating tau phosphorylation. Eur J Neurosci 28:1989–2002

2. Lashuel HA, Hartley D, Petre BM, Walz T, Lansbury PT Jr (2002) Neurodegenerative disease: amyloid pores from pathogenic mutations. Nature 418:291
3. Wang X, Wang Y, Hu JP, Yu S, Li BK, Cui Y et al (2016) Astragaloside IV, a natural PPARgamma agonist, reduces abeta production in Alzheimer's disease through inhibition of BACE1. Mol Neurobiol. doi:10.1007/s12035-016-9874-6
4. Zhou W, Cai F, Li Y, Yang GS, O'Connor KD, Holt RA et al (2010) BACE1 gene promoter single-nucleotide polymorphisms in Alzheimer's disease. J Mol Neurosci 42:127–133
5. Zheng K, Dai X, Xiao N, Wu X, Wei Z, Fang W et al (2016) Curcumin ameliorates memory decline via inhibiting bace1 expression and beta-amyloid pathology in 5×FAD transgenic mice. Mol Neurobiol. doi:10.1007/s12035-016-9802-9
6. Zhao Y, Wang Y, Hu J, Zhang X, Zhang YW (2012) CutA divalent cation tolerance homolog (*Escherichia coli*) (CUTA) regulates beta-cleavage of beta-amyloid precursor protein (APP) through interacting with beta-site APP cleaving protein 1 (BACE1). J Biol Chem 287:11141–11150
7. Karran E, Mercken M, De Strooper B (2011) The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. Nat Rev Drug Discov 10:698–712
8. Guo LL, Guan ZZ, Huang Y, Wang YL, Shi JS (2013) The neurotoxicity of beta-amyloid peptide toward rat brain is associated with enhanced oxidative stress, inflammation and apoptosis, all of which can be attenuated by scutellarin. Exp Toxicol Pathol 65:579–584
9. Cai Z, Zhao B, Ratka A (2011) Oxidative stress and beta-amyloid protein in Alzheimer's disease. Neuromolecular Med 13:223–250
10. Chen Z, Zhong C (2014) Oxidative stress in Alzheimer's disease. Neurosci Bull 30:271–281
11. Schwalm MT, Pasquali M, Miguel SP, Dos Santos JP, Vuolo F, Comim CM et al (2014) Acute brain inflammation and oxidative damage are related to long-term cognitive deficits and markers of neurodegeneration in sepsis-survivor rats. Mol Neurobiol 49:380–385
12. Chen CH, Zhou W, Liu S, Deng Y, Cai F, Tone M et al (2012) Increased NF-kappaB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. Int J Neuropsychopharmacol 15:77–90
13. Lin N, Chen LM, Pan XD, Zhu YG, Zhang J, Shi YQ et al (2015) Tripchlorolide attenuates beta-amyloid generation via suppressing PPARgamma-regulated BACE1 activity in N2a/APP695 Cells. Mol Neurobiol 53:6397–6406
14. Barroso E, del Valle J, Porquet D, Vieira Santos AM, Salvado L, Rodriguez-Rodriguez R et al (2013) Tau hyperphosphorylation and increased BACE1 and RAGE levels in the cortex of PPAR-beta/delta-null mice. Biochim Biophys Acta 1832:1241–1248
15. Abdallah DM (2010) Anticonvulsant potential of the peroxisome proliferator-activated receptor gamma agonist pioglitazone in pentylenetetrazole-induced acute seizures and kindling in mice. Brain Res 1351:246–253
16. Nicolakakis N, Hamel E (2010) The nuclear receptor PPAR-gamma as a therapeutic target for cerebrovascular and brain dysfunction in Alzheimer's disease. Front Aging Neurosci 2. doi:10.3389/fnagi.2010.00021
17. Rani N, Bharti S, Bhatia J, Nag TC, Ray R, Arya DS (2016) Chrysin, a PPAR-gamma agonist improves myocardial injury in diabetic rats through inhibiting AGE-RAGE mediated oxidative stress and inflammation. Chem Biol Interact 250:59–67
18. Quan Q, Wang J, Li X, Wang Y (2013) Ginsenoside Rg1 decreases Abeta(1–42) level by upregulating PPARgamma and IDE expression in the hippocampus of a rat model of Alzheimer's disease. PLoS One 8:e59155
19. Xiong H, Callaghan D, Jones A, Walker DG, Lue LF, Beach TG et al (2008) Cholesterol retention in Alzheimer's brain is responsible for high beta- and gamma-secretase activities and Abeta production. Neurobiol Dis 29:422–437
20. Kaundal RK, Sharma SS (2010) Peroxisome proliferator-activated receptor gamma agonists as neuroprotective agents. Drug News Perspect 23:241–256
21. Jiang M, Jerome WG, Hayward SW (2010) Autophagy in nuclear receptor PPARgamma-deficient mouse prostatic carcinogenesis. Autophagy 6:175–176
22. Nenov MN, Laezza F, Haidacher SJ, Zhao Y, Sadygov RG, Starkey JM et al (2014) Cognitive enhancing treatment with a PPARgamma agonist normalizes dentate granule cell presynaptic function in Tg2576 APP mice. J Neurosci 34:1028–1036
23. Xicota L, Rodriguez-Morato J, Dierssen M, de la Torre R (2015) Potential role of (–)-epigallocatechin-3-gallate (EGCG) in the secondary prevention of Alzheimer disease. Curr Drug Targets 29:665–672
24. He Y, Cui J, Lee JC, Ding S, Chalimoniuk M, Simonyi A et al (2011) Prolonged exposure of cortical neurons to oligomeric amyloid-beta impairs NMDA receptor function via NADPH oxidase-mediated ROS production: protective effect of green tea (–)-epigallocatechin-3-gallate. ASN Neuro 3:e00050
25. Gao Z, Han Y, Hu Y, Wu X, Wang Y, Zhang X et al (2016) Targeting HO-1 by epigallocatechin-3-gallate reduces contrast-induced renal injury via anti-oxidative stress and anti-inflammation pathways. PLoS One 11:e0149032
26. Zhang S, Yang X, Luo J, Ge X, Sun W, Zhu H et al (2014) PPA-Ralpha activation sensitizes cancer cells to epigallocatechin-3-gallate (EGCG) treatment via suppressing heme oxygenase-1. Nutr Cancer 66:315–324
27. Dragicevic N, Smith A, Lin X, Yuan F, Copes N, Delic V et al (2011) Green tea epigallocatechin-3-gallate (EGCG) and other flavonoids reduce Alzheimer's amyloid-induced mitochondrial dysfunction. J Alzheimers Dis 26:507–521
28. El-Sahar AE, Safar MM, Zaki HF, Attia AS, Ain-Shoka AA (2015) Neuroprotective effects of pioglitazone against transient cerebral ischemic reperfusion injury in diabetic rats: modulation of antioxidant, anti-inflammatory, and anti-apoptotic biomarkers. Pharmacol Rep 67:901–906
29. Mao JW, Tang HY, Wang YD (2012) Influence of rosiglitazone on the expression of PPARgamma, NF-kappaB, and TNF-alpha in rat model of ulcerative colitis. Gastroenterol Res Pract 2012:845672
30. Zhang X, Wu M, Lu F, Luo N, He ZP, Yang H (2014) Involvement of alpha7 nAChR signaling cascade in epigallocatechin gallate suppression of beta-amyloid-induced apoptotic cortical neuronal insults. Mol Neurobiol 49:66–77
31. Sun TL, Liu Z, Qi ZJ, Huang YP, Gao XQ, Zhang YY (2016) (–)-Epigallocatechin-3-gallate (EGCG) attenuates arsenic-induced cardiotoxicity in rats. Food Chem Toxicol 93:102–110
32. Jang S, Jeong HS, Park JS, Kim YS, Jin CY, Seol MB et al (2010) Neuroprotective effects of (–)-epigallocatechin-3-gallate against quinolinic acid-induced excitotoxicity via PI3K pathway and NO inhibition. Brain Res 1313:25–33
33. Karmakar I, Haldar S, Chakraborty M, Chaudhury K, Dewanjee S, Haldar PK (2016) Regulation of apoptosis through bcl-2/bax proteins expression and DNA damage by Zanthoxylum alatum. Pharm Biol 54:503–508
34. Golde TE (2005) The Abeta hypothesis: leading us to rationally-designed therapeutic strategies for the treatment or prevention of Alzheimer disease. Brain Pathol 15:84–87
35. Kowalska A (2004) The beta-amyloid cascade hypothesis: a sequence of events leading to neurodegeneration in Alzheimer's disease. Neurol Neurochir Pol 38:405–411

36. Gao R, Wang Y, Pan Q, Huang G, Li N, Mou J et al (2015) Fuzhisan, a chinese herbal medicine, suppresses beta-secretase gene transcription via upregulation of SIRT1 expression in N2a-APP695 cells. Int J Clin Exp Med 8:7231–7240
37. Hitt B, Riordan SM, Kukreja L, Eimer WA, Rajapaksha TW, Vassar R (2012) beta-Site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1)-deficient mice exhibit a close homolog of L1 (CHL1) loss-of-function phenotype involving axon guidance defects. J Biol Chem 287:38408–38425
38. Wong PC (2008) Translational control of BACE1 may go awry in Alzheimer's disease. Neuron 60:941–943
39. Shimmyo Y, Kihara T, Akaike A, Niidome T, Sugimoto H (2008) Epigallocatechin-3-gallate and curcumin suppress amyloid beta-induced beta-site APP cleaving enzyme-1 upregulation. Neuroreport 19:1329–1333
40. Wang R, Chen S, Liu Y, Diao S, Xue Y, You X et al (2015) All-trans-retinoic acid reduces BACE1 expression under inflammatory conditions via modulation of nuclear factor kappaB (NFkappaB) signaling. J Biol Chem 290:22532–22542
41. Zheng N, Yuan P, Li C, Wu J, Huang J (2015) Luteolin reduces BACE1 expression through NF-kappaB and through estrogen receptor mediated pathways in HEK293 and SH-SY5Y cells. J Alzheimers Dis 45:659–671
42. Wang R, Li JJ, Diao S, Kwak YD, Liu L, Zhi L et al (2013) Metabolic stress modulates Alzheimer's beta-secretase gene transcription via SIRT1-PPARgamma-PGC-1 in neurons. Cell Metab 17:685–694
43. Heneka MT, Sastre M, Dumitrescu-Ozimek L, Hanke A, Dewachter I, Kuiperi C et al (2005) Acute treatment with the PPARgamma agonist pioglitazone and ibuprofen reduces glial inflammation and Abeta1-42 levels in APPV717I transgenic mice. Brain 128:1442–1453
44. Jiang LY, Tang SS, Wang XY, Liu LP, Long Y, Hu M et al (2012) PPARgamma agonist pioglitazone reverses memory impairment and biochemical changes in a mouse model of type 2 diabetes mellitus. CNS Neurosci Ther 18:659–666
45. Liu LP, Yan TH, Jiang LY, Hu W, Hu M, Wang C et al (2013) Pioglitazone ameliorates memory deficits in streptozotocin-induced diabetic mice by reducing brain beta-amyloid through PPARgamma activation. Acta Pharmacol Sin 34:455–463
46. Wang F, Liu Y, Bi Z (2016) Pioglitazone inhibits growth of human retinoblastoma cells via regulation of NF-kappaB inflammation signals. J Recept Signal Transduct Res 1:1–6
47. Valles SL, Dolz-Gaiton P, Gambini J, Borras C, Lloret A, Pallardo FV et al (2010) Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPAR gamma expression in cultured astrocytes. Brain Res 1312:138–144
48. Kalinin S, Richardson JC, Feinstein DL (2009) A PPARdelta agonist reduces amyloid burden and brain inflammation in a transgenic mouse model of Alzheimer's disease. Curr Alzheimer Res 6:431–437
49. Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V et al (2005) A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. Nature 437:759–763
50. Lee JY, Paik JS, Yun M, Lee SB, Yang SW (2016) The effect of (–)-epigallocatechin-3-gallate on il-1beta induced il-8 expression in orbital fibroblast from patients with thyroid-associated ophthalmopathy. PLoS One 11:e0148645
51. Bhat AH, Dar KB, Anees S, Zargar MA, Masood A, Sofi MA et al (2015) Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. Biomed Pharmacother 74:101–110