Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 24 (2013) 298–310

Journal of Nutritional Biochemistry

Epigallocatechin-3-gallate prevents systemic inflammation-induced memory deficiency and amyloidogenesis via its anti-neuroinflammatory properties

Young-Jung Lee[a,b], Dong-Young Choi[a,c], Yeo-Pyo Yun[a], Sang Bae Han[a,b], Ki-Wan Oh[a], Jin Tae Hong[a,b,c,*]

[a]College of Pharmacy, Chungbuk National University, 12 Gaesin-dong, Heungduk-gu, Cheongju, Chungbuk 361-763, Korea
[b]Medical Research Center, Chungbuk National University, 12 Gaesin-dong, Heungduk-gu, Cheongju, Chungbuk 361-763, Korea
[c]CBITRC, Chungbuk National University, 12 Gaesin-dong, Heungduk-gu, Cheongju, Chungbuk 361-763, Korea

Received 21 December 2011; received in revised form 14 May 2012; accepted 12 June 2012

Abstract

Neuroinflammation has been known to play a critical role in the pathogenesis of Alzheimer's disease (AD) through amyloidogenesis. In a previous study, we found that systemic inflammation by intraperitoneal (ip) injection of lipopolysaccharide (LPS) induces neuroinflammation and triggers memory impairment. In this present study, we investigated the inhibitory effects of epigallocatechin-3-gallate (EGCG) on the systemic inflammation-induced neuroinflammation and amyloidogenesis as well as memory impairment. ICR mice were orally administered with EGCG (1.5 and 3 mg/kg) for 3 weeks, and then the mice were treated by ip injection of LPS (250 μg/kg) for 7 days. We found that treatment of LPS induced memory-deficiency-like behavior and that EGCG treatment prevented LPS-induced memory impairment and apoptotic neuronal cell death. EGCG also suppressed LPS-induced increase of the amyloid beta-peptide level and the expression of the amyloid precursor protein (APP), β-site APP cleaving enzyme 1 and its product C99. In addition, we found that EGCG prevented LPS-induced activation of astrocytes and elevation of cytokines including tumor necrosis factor-α, interleukin (IL)-1β, macrophage colony-stimulating factor, soluble intercellular adhesion molecule-1 and IL-16, and the increase of inflammatory proteins, such as inducible nitric oxide synthase and cyclooxygenase-2, which are known factors responsible for not only activation of astrocytes but also amyloidogenesis. In the cultured astrocytes, EGCG also inhibited LPS-induced cytokine release and amyloidogenesis. Thus, this study shows that EGCG prevents memory impairment as well as amyloidogenesis via inhibition of neuroinflammatory-related cytokines released from astrocytes and suggests that EGCG might be a useful intervention for neuroinflammation-associated AD.

Keywords: Amyloid-beta; Alzheimer's disease; EGCG; Green tea; Lipopolysaccharide

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia, accounting for 50% to 75% of all cases [1,2]. AD is pathologically characterized by senile plaques and neurofibrillary tangles in the brain. In particular, the senile plaques are extracellular aggregates of amyloid beta-peptide (Aβ) that are cleaved from the amyloid precursor protein (APP) [3]. Postmortem studies of AD brains also found a number of pathological abnormalities including a profound loss of synapses, microglial activation and inflammatory processes [4]. Previous studies with transgenic animals revealed that neuroinflammation also accelerates amyloidogenesis in the process of cerebral amyloid deposition [5–8]. In the process of neuroinflammation, various cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, etc.], chemokines [monocyte chemotactic protein (MCP)-1, macrophage-derived inflammatory mediator (MIP)-α, etc.], oxygen free radicals and reactive nitrogen species [9], and eicosanoids such as leukotriene B_4 and prostaglandins [10] are important signaling molecules of neuroinflammatory responses [11].

Intraperitoneal (ip) administration of lipopolysaccharide (LPS) can induce an immediate, strong and persistent up-regulation of proinflammatory cytokines IL-1β, IL-6 and TNF-α primarily from macrophages, and these proinflammatory cytokines exert neurobiological effects [12], suggesting that systemic inflammation can affect the neurobiological condition. Systemic administration of a single dose of LPS through ip injections induces neuroinflammation that persists for 10 months, which results in the progressive loss of dopaminergic neurons in the substantia nigra [13]. Mouton et al. [14] found that single ip injection of LPS induced elevation of several cytokines, such as IL-1β, IL-6 and TNF-α, in hippocampal tissue.

Abbreviations: Aβ, amyloid peptide; AD, Alzheimer's disease; APP, β-amyloid precursor protein; COX-2, cyclooxygenase-2; EGCG, (−)-epigallocatechin-3-gallate; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule-1; icv, intracerebroventricular; IL, interleukin; iNOS, inducible nitric oxide synthase; ip, intraperitoneal; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony-stimulating factor; MIP-α, macrophage-derived inflammatory mediator-α; RANTES, regulated on activation normal T cell expressed and secreted; TNF-α, tumor necrosis factor-α.

* Corresponding author. College of Pharmacy, Chungbuk National University, 12, Gaeshin-dong, Heungduk-gu, Cheongju, Chungbuk 361-763, Korea. Tel.: +82 43 261 2813; fax: +82 43 268 2732.
E-mail address: jinthong@chungbuk.ac.kr (J.T. Hong).

0955-2863/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2012.06.011

Erickson and Banks [15] reported that single and three repeated ip injections of LPS increased release of neuroinflammatory-related cytokines and chemokines granulocyte colony-stimulating factor, IL-1α, IL-6, MCP-1, MIP-1α and TNF-α, in mouse brain. Those inflammatory components accelerate amyloidogenesis via up-regulation of the β-secretase level and activity [16,17]. Recently, Jaeger et al. [18] reported that systemic injection of LPS increased brain influx of blood Aβ via alteration of low density lipoprotein receptor-related protein 1 (LRP-1) in mice brain. Our previous studies also showed that systemic administration of LPS could induce memory deficiency and Aβ accumulation through the elevation of β- and γ-secretase activities [19]. Moreover, administration of anti-inflammatory agents in AD patients could reduce amyloidogenesis, suggesting that neuroinflammation may cause the pathogenesis of AD via amyloidogenesis [20]. Thus, this animal model might be useful to study underlying mechanisms of neuroinflammation-associated development of AD.

Epigallocatechin-3-gallate (EGCG) is the most abundant biologically active compound in tea. Epidemiological studies have also suggested a positive relationship between consumption of EGCG and the prevention of AD [21]. Green tea extract or EGCG has been reported to attenuate Aβ-induced neurotoxicity in cultured human neuronal cell lines and to modulate both tau pathology and Aβ-mediated cognitive impairment in transgenic mice models of AD [22–26]. It was also reported that green tea has anti-β-secretase activity *in vitro* [27]. Moreover, Rezai-Zadeh et al. [22] reported that EGCG markedly elevated the α-secretase activity and promoted soluble APP-α production in the murine neuron-like cells transfected with the human Swedish mutant form of APP (SweAPP N2a cells), as well as in primary neurons derived from Swedish mutant APP-over-expressing mice (the Tg APPsw line 2576).

In our previous studies, we found that intracerebroventricular (icv) injection of LPS induced memory deficiency and Aβ accumulation through decreased α-secretase activity as well as elevation of the β- and γ-secretase activities, and these were all reduced by EGCG [28]. We previously also demonstrated that EGCG prevented amyloidogenesis via inhibition of β-secretase activity in Aβ-injected and presenilin2 mutant transgenic mice [28,29]. Thus, we investigated preventive effect of EGCG on the systemic neuroinflammation model via ip injection of LPS, and we investigated the possible mechanisms of EGCG effects to improve the memory deficiency in systemic LPS-injected AD mice models.

2. Materials and methods

2.1. EGCG

Green tea-derived flavonoid EGCG was purchased from Sigma-Aldrich (St. Louis, MO, USA). In our previous memory impairment animal model, 1.5 and 3 mg/kg EGCG treatment for 3 weeks showed a neuroprotective effect [28,29]. Therefore, a similar dose of EGCG (1.5 and 3 mg/kg) was used in the present study, which is about 1.5 times more than the dose of human consumption. The daily human consumption of green tea is about 2 mg/kg (12 g×1% yield from green tea leaf/70 kg) [30]. The average water consumption of mouse per day was about 3–4 ml; thus, the amount of EGCG intake was approximately 15 or 60 mg per mouse. The amount of EGCG that reached the brain was about 2–3 nmol/g tissue according to another research [31]. EGCG was added to drinking water, and mice were allowed access for 3 weeks *ad libitum* before induction of memory impairment.

2.2. LPS-induced memory impairment mouse model

Five-week-old male mice IcrTacSam:ICR (Samtako, Gyeonggi-do, Korea) was used as it was used for LPS-induced neurodegenerative mice model in our previous study and in another study [18,19]. These mice were maintained in accordance with the Institutional Animal Care and Use Committee of the Laboratory Animal Research Center at Chungbuk National University, Korea (CBNUA-144-1001-01). All mice were housed in a room that was automatically maintained at 21°C–25°C and relative humidity (45%–65%) with a controlled light–dark cycle. The LPS (serotype O55:B5, Sigma, St. Louis, MO, USA; final concentration of 0.1 mg/ml) was dissolved, and aliquots in saline were stored at −20°C until use. The ip injection (250 μg/kg) of LPS or vehicle (saline) was administered daily for 7 days after the EGCG treatment. Subsequently, the behavioral tests of learning and memory capacity were assessed using two separate tests (water maze, probe and passive avoidance tests). An 1-day interval was given between tests for adaptation of new circumstances (Fig. 1).

2.3. Water maze test

The water maze test is also a widely accepted method for memory test, and we performed this test as described by Morris [32]. Maze testing was performed by the SMART-CS (Panlab, Barcelona, Spain) program and equipment. A circular plastic pool (height: 35 cm, diameter: 100 cm) was filled with nontoxic black ink water kept at 22°C–25°C. An escape platform (height: 14.5 cm, diameter: 4.5 cm) was submerged 0.5–1 cm below the surface of the water in position. On training trials, the mice were placed in a pool of water and allowed to remain on the platform for 10 s and were then returned to the home cage during the second trial interval. Our water maze test employed a fixed platform and three start positions at the circumference of the pool randomly assigned to a different arm for each trial. The mice that did not find the platform within 60 s were placed on the platform for 10 s at the end of trial. They were allowed to swim until they sought the escape platform. Escape latency, escape distance, swimming speed and swimming pattern of each mouse were monitored by a camera above the center of the pool connected to a SMART-LD program (Panlab, Barcelona, Spain).

2.4. Probe test

In order to assess memory consolidation, a probe trial was performed 24 h after the 5-day acquisition tests. In this trial, the platform was removed from the tank, and the mice were allowed to swim freely. For these tests, the percentage time in the target quadrant and target site crossings within 60 s was recorded. The time spent in the target quadrant is taken to indicate the degree of memory consolidation that has taken place after learning. The time spent in the target quadrant was used as a measure of spatial memory. Swimming pattern of each mouse was monitored by a camera above the center of the pool connected to a SMART-LD program described above.

2.5. Passive avoidance performance test

The passive avoidance test is widely accepted as a simple and rapid method for memory test. The passive avoidance response was determined using a "step-through" apparatus (Med Associates Inc., St. Albans, VT, USA) that consisted of an illuminated and a dark compartment (each 20.3×15.9×21.3 cm) adjoining each other through a small gate with a grid floor, 3.175-mm stainless steel rod set 8 mm apart. Two days after the water maze test, the ICR mice were placed in the illuminated compartment facing away from the dark compartment for the training trial. When the mice moved

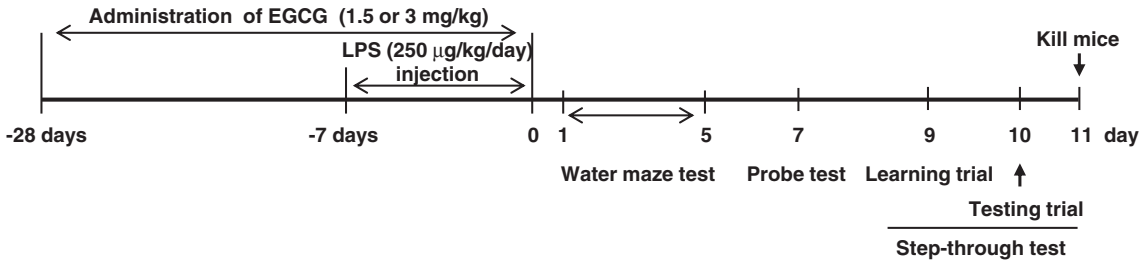

Fig. 1. Experimental scheme for effect of EGCG against LPS-induced memory impairment. Five-week-old mice were treated with EGCG (1.5 or 3 mg/kg) into drinking water for 3 weeks. Then, LPS (250 μg/kg) was administered intraperitoneally once daily for 7 days. The animals were then trained for three trials per day for 4 days. Test trials were performed once a day for 3 days. Subsequently, step-through test was performed.

completely into the dark compartment, it received an electric shock (2 mA, 3-s duration). Then, the mice were returned to their home case. Twenty-four hours later, the mice were placed in the illuminated compartment, and the latency period to enter the dark compartment defined as "retention" was measured. The time when the mice entered in the dark compartment was recorded and described as step-through latency. The retention trials were set at a limit of 180 s of cutoff time.

2.6. Astrocyte culture

As described elsewhere [33,34], 2-day-old rat pups were ice-anesthetized and decapitated. After the skull was cut and the skin was opened, the brain was released from the skull cavity. After washing with phosphate-buffered saline (PBS), the cerebrum was separated from the cerebellum and brain stem, and the cerebral hemispheres were separated from each other by gently teasing along the midline fissure with the sharp edge of forceps. The meninges were gently peeled from the individual cortical lobes, and the cortices were dissociated by mechanical digestion [using the cell strainer (BD Bioscience, Franklin Lakes, NJ, USA)] with Dulbecco's modified Eagle's medium (DMEM) containing F12 nutrient mixture (Invitrogen, Carlsbad, CA, USA). The resulting cells were centrifuged (1500 rpm, 5 min), resuspended in serum-supplemented culture media and plated into 100-mm dishes. Serum-supplemented culture medium was composed of DMEM supplemented with F12, fetal bovine serum (FBS) (5%), $NaHCO_3$ (40 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated in the culture medium in a humidified incubator at 37°C and in 5% CO_2 for 9 days. At confluence (9 days), the flask was subjected to shaking for 16–18 h at 37°C. The cultures were treated for 48 h with cytosine arabinoside, and the medium was replaced with DMEM/F12HAM containing 10% FBS. The monolayer was treated with 1.25% trypsin-EDTA for a short duration, after which the cells were dissociated and plated into uncoated glass coverslips. The astrocyte cultures formed a layer of process-bearing, glial fibrillary acidic protein (GFAP)-positive cells. The purity of astrocyte cultures was assessed by GFAP immunostaining. Under these conditions, we can assume that over 95% of the cells were astrocytes. The cultured cells were treated simultaneously with LPS (1 μg/ml) and several concentrations (10, 20, 50 μM) of EGCG dissolved in PBS, and the cells were harvested after 24 h. Reverse transcription polymerase chain reaction (PCR) analysis, Western blotting and cytokine array analysis were performed.

2.7. Brain collection and preservation

After the behavioral test, animals were perfused with PBS under inhaled ether anesthetization. The brains were immediately collected in the same manner and formalin stored at room temperature, and separated into cortical and hippocampal regions. All the brain regions were immediately stored at −80°C and used to measure biological assay of $A\beta_{1-42}$ and cell death detection.

2.8. Measurement of $A\beta_{1-42}$ level

Lysates of brain tissue or astrocytes were obtained through protein extraction buffer containing protease inhibitor. $A\beta_{1-42}$ levels were determined using a specific enzyme-linked immunosorbent assay (ELISA) kit (Immuno-Biological Laboratories Co., Ltd., Takasaki-Shi, Gunma, Japan). In brief, 100 μl of sample was added into the precoated plate and was incubated overnight at 4°C. After washing each well of the precoated plate with washing buffer, 100 μl of labeled antibody solution was added, and the mixture was incubated for 1 h at 4°C in the dark. After washing, chromogen was added, and the mixture was incubated for 30 min at room temperature in the dark. Finally, the resulting color was assayed at 450 nm using a microplate absorbance reader (Sunrise, TECAN, Switzerland) after adding stop solution.

2.9. β- and γ-secretase assay

The total activities of β- and γ-secretase in the brains and astrocytes were measured using a commercially available secretase activity kit, a β-secretase fluorescence resonance energy transfer (BACE 1 FRET) assay kit (PANVERA) and a γ-secretase activity kit (R&D Systems) according to the manufacturers' protocols and as described elsewhere. This formation of fluorescence was read using a Fluostar galaxy fluorometer (excitation at 355 nm and emission at 510 nm) with Felix software (BMG Labtechnologies). The level of γ-secretase enzymatic activity was proportional to the fluorometric reaction, and the β-secretase activity was expressed as units of fluorescence produced. β-Secretase enzyme activity was expressed as nmol/(mg protein·min).

2.10. Reverse transcription PCR analysis

Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen) and quantified spectrophotometrically. The cDNA was synthesized using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. The primers for TNF-α, IL-1β, macrophage colony-stimulating factor (M-CSF), soluble intercellular adhesion molecule-1 (ICAM-1), IL-16 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal PCR control were as follows: 5'-GTA GCC CAC GTC GTA GCA AA-3′ (sense) and 5′-CCC TTC TCC AGC TGG GAG AC-3′ (antisense) for TNF-α, 5′-TGA TGT TCC CAT TAG ACA GC-3′ (sense) and 5′-GAG GTG CTG ATG TAC CAG TT-3′ (antisense) for IL-1β, 5′-AGT GGT CTG TAA GCT CCA TC-3′ (sense) and 5′-GAG CTT CTT GCA ATG GGT TG-3′ (antisense) for M-CSF, 5′-AAA CGG GAG ATG AAT GGT ACC TAC-3′ (sense) and 5′-TGC ACG TCC CTG GTG ATA CTC-3′ (antisense) for ICAM-1, 5′-AAA TGG ACA CTG CCA ATG GTG CTC-3′ (sense) and 5′-AAA GGA GCT GAT TCT CTG CCG GAT-3′ (antisense) for IL-16, and 5′-TCC CTC AAG ATT GTC AGC AA-3′ (sense) and 5′-AGA TCC ACA ACG GAT ACA TT-3′ (antisense) for GAPDH [35]. All PCRs were run in a 7500 Real-Time PCR System (Applied Biosystems, Foster city, CA, USA). The PCR cycles consisted of denaturation at 94°C for 30 s; annealing at 55°C for 30 s (TNF-α, IL-1β and GAPDH), 50°C for 30 s (M-CSF and ICAM-1) or 60°C for 30 s (IL-16); and extension at 72°C for 90 s for 30 cycles. The PCR product was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and then detected under UV light.

2.11. Cytokine arrays

Mouse Cytokine Array Panels were used according to the manufacturer instructions (R&D Systems, Inc.). Briefly, brain or cultured astrocytes lysates were mixed with a cocktail of biotinylated detection antibodies prior to incubation at 4°C with the array membranes. Following washing, streptavidin–horseradish peroxidase was applied for 30 min at ambient temperature. Immunoreactivity was then visualized using enhanced chemiluminescence reagent (GE Healthcare). Densitometric analysis was then performed using MyImage (SLB, Seoul, Korea) and quantified by Labworks 4.0 software (UVP Inc., Upland, CA, USA).

2.12. Western blotting

An equal amount of total protein (40 μg) isolated from brain tissue or astrocytes was resolved on a sodium dodecyl sulfate/10% or 15% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (GE Water and Process Technologies). The membrane was incubated at room temperature with specific antibodies: anti-BACE (1/500, Sigma), anti-C99 (1/500, ABR-Affinity Bioreagents), anti-APP (1/500, Covance), anti-GFAP (1/500, Abcam), anti-caspase-3 (1/500, Cell Signaling) and anti-β-actin (1/2000, Santa Cruz Biotechnology) were used in this study. Rabbit polyclonal antibodies against the active form of inducible nitric oxide synthase (iNOS) (1/1000, Abcam) and cyclooxygenase (COX)-2 (1/1000, Cayman Chemical, Ann Arbor, MI, USA) were also used. The blot was then incubated with the corresponding conjugated anti-mouse or anti-rabbit IgG-horseradish peroxidase (1/4000, Santa Cruz Biotechnology). Immunoreactive proteins were detected with the ECL Western blotting detection system. The relative density of the protein bands was scanned by densitometry using MyImage and quantified by Labworks 4.0 software.

2.13. Immunohistochemistry and immunofluorescence

Brains were fixed in formalin and paraffin-enclosed for examination. Six-micrometer-thick tissue sections were used with immunohistochemistry. Paraffin-embedded sections were deparaffinized and rehydrated, washed in distilled water and then subjected to heat-mediated antigen retrieval treatment. Endogenous peroxidase activity was quenched by incubation in 2% hydrogen peroxide in methanol for 15 min and then cleared in PBS for 5 min. The sections were blocked for 30 min with 3% normal horse serum diluted in PBS. These sections were incubated overnight with appropriate antibodies: $A\beta_{1-42}$ (1/2000, 4G8, Covance, Berkeley, CA, USA), GFAP (1/5000, Abcam, Inc., Cambridge, MA, USA), iNOS (1/100, Abcam) and cleaved caspase-3 (1/200, Cell Signaling Technology, Inc.). After the incubation, sections were washed in PBS and incubated with the biotinylated secondary antibodies (ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 1 h. The sections were washed with PBS, incubated with the avidin–biotin complex (Vector Laboratories) for 30 min and visualized by chromogen 3,3' Diaminobenzidine (DAB;Vector Laboratories) reaction. It was then counterstained by hematoxylin. Finally, sections were dehydrated in ethanol, cleared in xylene and mounted with Permount (Fisher Scientific, Hampton, NH, USA).

To simultaneously determine level of GFAP and Aβ, we performed immunofluorescence assay in the paraffin section of the brain. The sections were then incubated to primary rabbit polyclonal antibody for GFAP (1/1000, Abcam, Inc., Cambridge, MA, USA) and mouse monoclonal antibody for $A\beta_{1-42}$ (1/2000, Covance, Berkeley, CA, USA) overnight at 4°C. After washes with ice-cold PBS followed by treatment with an anti-rabbit secondary antibody labeled with Alexa Fluor 568 and anti-mouse secondary antibody labeled with Alexa Flour 488 (1/100 dilution, Molecular Probes, Inc., Eugene, OR, USA) for 2 h at room temperature, immunofluorescence images were acquired using a confocal laser scanning microscope (TCS SP2, Leica Microsystems AG, Wetzlar, Germany).

2.14. Statistical analysis

Statistical analysis of the data was carried out using analysis of variance for repeated measures followed by Dunnett's post hoc analysis using GraphPad Prism 4 software (Version 4.03, GraphPad software, Inc.). "#" indicates significant difference from the vehicle group control ($P<.05$); "*" indicates significant difference from the LPS-treated group ($P<.05$).

3. Results

3.1. Effect of EGCG on the LPS-induced memory impairment as determined by behavior tests

The memory-improving effect of EGCG was assessed in mice that were continuously administered with EGCG at a dose of 1.5 or 3 mg/kg/day daily for 3 weeks (from day 1 to day 28), and then they were ip injected with 250 μg/kg/day LPS for 1 weeks (from day 22 to day 28). The mice then performed the Morris water maze test after 15 training sessions (three times per day for 5 days) as shown in Fig. 1. Similar to previous findings [19,28], the LPS injection retarded arriving at the location of the platform without alteration of speed, demonstrating that LPS induced memory impairment. However, EGCG significantly ameliorated the memory impairment in the LPS-injected mice. The mice exhibited shorter escape latency by the end of the training trial, and the escape latency at the end of training to the platform was about 474.9±98.9 cm and 19.5±3.5 s after 15 training trials in the control (vehicle) group. The LPS-injected mice exhibited escape latencies to the platform of about 1073.3±252.2 cm ($F_{3,\ 30}$= 9.7565, P=.0074) and 45.0±7.5 s ($F_{3,\ 30}$=19.778, P=.0004) (Fig. 2A and B), respectively. However, those values for the mice given 1.5 and 3 mg/kg of EGCG at day 5 were significantly and dose-dependently decreased to 774.6±172.7 cm ($F_{3,\ 30}$=1.909, P= .0486) and 28.2±6.0 s ($F_{3,\ 30}$=6.6358, P=.020), and 402.8±123.9 cm ($F_{3,\ 30}$=11.385, P=.00454) and 21.4±5.2 s ($F_{3,\ 30}$=14.37, P= .0016). The swimming speed did not differ among the groups (data not shown). One day after the water maze test, we performed a probe trial to measure the maintenance of memory function. During the probe trial, the time spent on the target quadrant was decreased in the LPS-injected mice group (20.18%±1.93%) ($F_{3,\ 36}$= 13.912, P=.0016) as compared to that of the vehicle mice (32.77%±4.66%), but the time spent on the target quadrant by the EGCG-treated memory-impaired mice groups (1.5 or 3 mg/kg/day daily) was significantly increased to 27.53%±4.32% ($F_{3,\ 36}$= 5.348, P=.033) and 34.57%±5.18% ($F_{3,\ 36}$=15.140, P=.0011), respectively (Fig. 2C).

Two days after the probe test, a step-through test was performed. The vehicle group exhibited a step-through latency in the illuminated compartment of about 93.2±33.5 s, whereas the LPS-treated group's step-through latency was decreased to 20.8±8.2 s ($F_{3,\ 36}$=10.977, P= .0038). The EGCG-treated mice recovered from the LPS-scheme-induced step-through latency to 47.7±26.2 s ($F_{3,\ 36}$=2.412, P= .0478) and 84.0±36.2 s ($F_{3,\ 36}$=7.270, P=.0147), respectively, in a dose-dependent manner (Fig. 2D).

3.2. EGCG inhibits the LPS-induced iNOS and COX-2 expressions

To investigate the inhibitory effect of EGCG on memory impairment via the inhibition of neuroinflammation, the expressions of iNOS and COX-2 were determined by immunohistochemical analysis and Western blotting. The number of iNOS-positive cells in both the cortex and hippocampus of the LPS-injected mice was significantly greater than that in the vehicle mice, but EGCG treatment lowered the LPS-increased number of iNOS-positive cells (Fig. 3A and B). Similar to the expression level of iNOS detected by immunohistochemical analysis, Western blotting showed that systemic administration of LPS significantly increased the iNOS and COX-2 expressions in the mice's brains, and the LPS-induced elevation of the iNOS and COX-2 expressions was significantly inhibited by EGCG treatment (Fig. 3C).

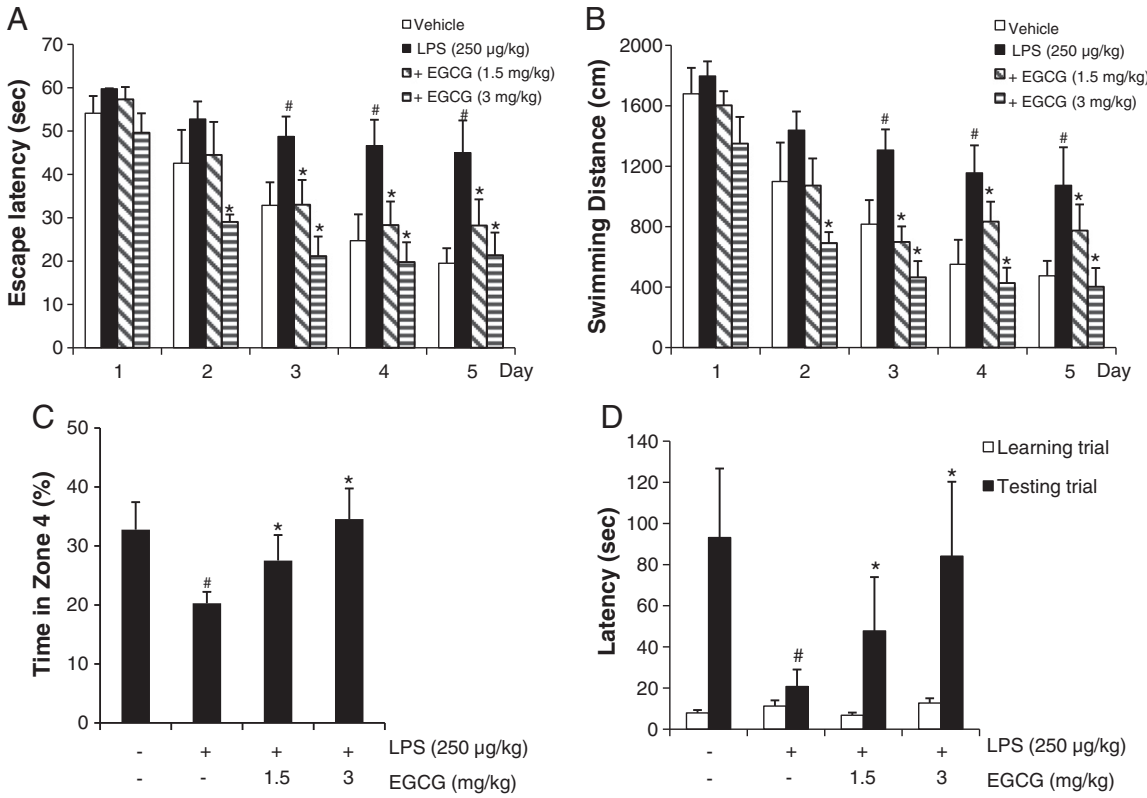

Fig. 2. Effect of EGCG on water maze test (A, B), probe test (C) and step-through-type passive avoidance test (D). Memory function was determined by the escape latencies (A, s) and distance (B, cm) for 5 days, and time to zone 4 (C, s) in probe test after administration of LPS. Each value is mean±S.E. from 10 mice. #Significantly different from vehicle group control (P<.05); *significantly different from LPS-treated group (P<.05).

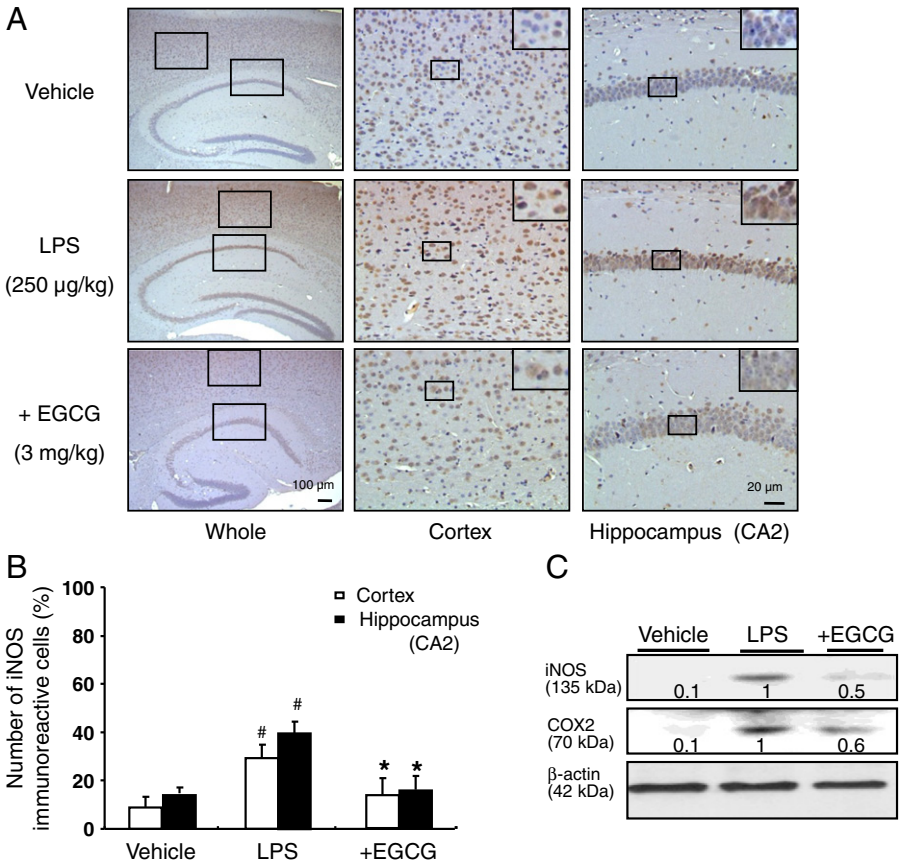

Fig. 3. Inhibitory effects of EGCG on the LPS-induced expression of inflammatory proteins. (A) Immunoreactive cells of iNOS antibody were detected in the cortex and hippocampus. Four-micrometer-thick sections of brains from mice were incubated with anti-iNOS antibodies and the biotinylated secondary antibody. It was then counterstained by hematoxylin. The resulting tissue was viewed with a microscope. (B) The present figure is representative of three different experiments with different animal brains. (C) The expression of iNOS and COX-2 was detected by Western blotting using specific antibodies. The Western blot bands were representative of three animals per group, and the values indicate average density over β-actin from three animals. #Significantly different from vehicle group control ($P<.05$); *significantly different from LPS-treated group ($P<.05$).

3.3. Effect of EGCG against the accumulation of $A\beta_{1-42}$ in the brains of the LPS-injected mice

We studied the effect of EGCG against the increased $A\beta_{1-42}$ level in the brains of the LPS-injected mice because the accumulation of $A\beta_{1-42}$ has been implicated in memory dysfunction. We found that, similar to the previous data [19], the number of $A\beta_{1-42}$ plaques in both the cortex and hippocampus of the LPS-injected mice was significantly greater than that in the vehicle mice, but EGCG treatment lowered this increased number of $A\beta_{1-42}$ labeled plaques (Fig. 4A). In agreement with the reduced number of $A\beta_{1-42}$ reactive plaques, the level of $A\beta_{1-42}$ and the activities of β- and γ-secretase were also significantly reduced in the EGCG-treated whole brains of the LPS-injected mice (Fig. 4B). Moreover, the expression of neuronal β-secretase (BACE1) and the number of BACE1-positive cells were significantly reduced by the EGCG treatment (Fig. 5A and B). We also investigated the levels of APP, C99 and BACE1 protein by Western blotting. The expression levels of APP, BACE1 and C99 were increased in the brains of the LPS-injected mice, and these elevated levels were reduced by EGCG treatment (Fig. 5C).

3.4. Effect of EGCG on the activation of astrocytes in the LPS-injected mice brains

Astrocytes are the source of released cytokine, which has been implicated in the amyloidogenesis during the development of AD [36]. So, we investigated the protective effect of EGCG against astrocyte activation. The significantly greater number of GFAP-reactive cells (an indication of astrocyte activation) was induced in the brains of the LPS-injected mice, whereas EGCG treatment reduced the number of GFAP-reactive cells in the cortex and hippocampus (Fig. 6A and B). Western blot analysis also showed that the level of GFAP was increased in the brains of the LPS-injected mice, and this was reduced by EGCG treatment (Fig. 6C). To further determine whether release of cytokines by activation of astrocytes could result in increased amyloidogenesis, the cells that were immunoreactive for both GFAP and $A\beta_{42}$ were detected by double immunofluorescence with GFAP and Aβ antibodies (Fig. 6D). The number of co-immunoreactive cells was significantly increased by LPS, but the number of co-immunoreactive cells was much lower in the brains of the EGCG-treated mice (Fig. 6D). We also investigated alterations of the cytokine levels in the brains of the LPS-injected mice since the activation of astrocytes releases cytokines in the mice brain. Systemic administration of LPS induced elevation of the levels of M-CSF, ICAM-1 and IL-16, whereas EGCG treatment reduced these cytokine levels in the mice brains (Fig. 6E).

3.5. Effect of EGCG on the release of cytokines in cultured astrocytes

To further define *in vivo* results, we investigated the level of cytokines in LPS-stimulated cultured astrocytes. As expected, administration of LPS induced up-regulation of M-CSF, ICAM-1 and IL-16 levels, and EGCG inhibited LPS-induced up-regulation of these cytokines (Fig. 7A and B). Besides these proteins, we also found

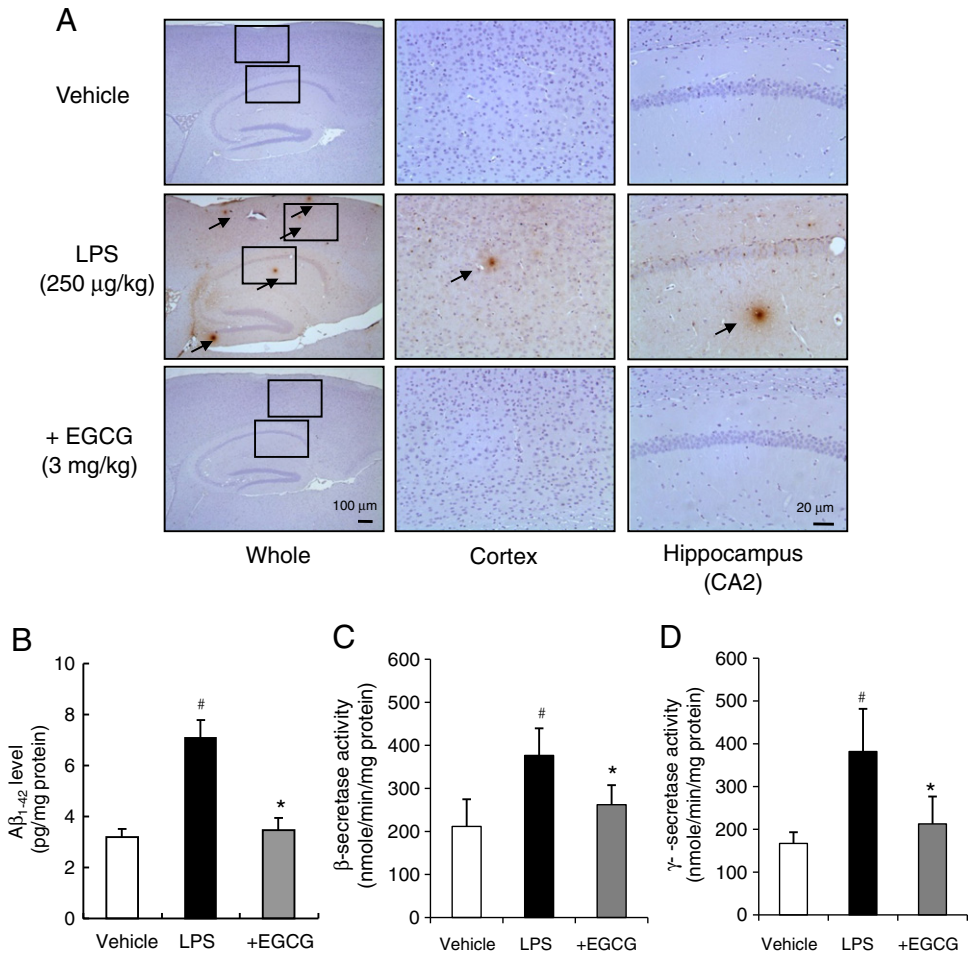

Fig. 4. Effect of EGCG on Aβ accumulation in the cortex and hippocampus. (A) Immunoreactive protein of anti-$A\beta_{42}$ antibody was investigated in the cortex and hippocampus. Four-micrometer-thick sections of brains from mice were incubated with anti-$A\beta_{42}$ antibody and counterstained with hematoxylin. Arrow indicates $A\beta_{42}$ accumulation which is clearly higher in the cerebral cortex and hippocampus of LPS-injected mouse. (B) The levels of $A\beta_{42}$ were assessed by using a specific Aβ ELISA as described in the Materials and Methods. The activity of β- and γ-secretase was investigated by using each assay kit as described in the Materials and Methods (C and D, respectively). Values measured from each group of mice were calibrated by amount of protein and expressed as mean±S.E. (n=3). #Significantly different from vehicle group control ($P<.05$); *significantly different from LPS-treated group ($P<.05$).

alternations of other cytokines and chemokines such as IL-1 receptor antagonist, regulated on activation normal T cell expressed and secreted (RANTES/CCL5), MIP-1α, MCP-1, chemokine ligand 1 and interferon-inducible protein 10 kDa (CCL10), which were elevated by LPS, but EGCG prevented these expressions in cultured astrocytes (Fig. 7A). Furthermore, we investigated through Western blotting whether EGCG prevented LPS-induced neuroinflammation and amyloidogenesis in cultured astrocytes. In the same manner with *in vivo* model, iNOS and COX-2 expression was markedly increased in response to LPS after 24 h. However, treatment with EGCG (10, 20, 50 μM) caused concentration-dependent decreases in LPS-induced iNOS and COX-2 expression in cultured astrocytes (Fig. 7C). The expression of APP, BACE and C99 proteins indicating amyloidogenesis was also increased in response to LPS (1 μg/ml) after 24 h; however, treatment with EGCG (10, 20, 50 μM) caused concentration-dependent decreases in LPS-induced BACE, C99 and APP expression in cultured astrocytes (Fig. 7C).

3.6. Effect of EGCG on the neuronal cell death

We investigated cell death to see the consequence of astrocytes activation and amyloidogenesis by LPS and the protective effect of EGCG. We investigated the expression of the proapoptotic protein cleaved caspase-3. LPS-injection increased the number of cleaved caspase-3-positive cells in the mice brain, but EGCG treatment decreased the number of cleaved caspase-3-positive cells in the mice brains (Fig. 8A and B). Moreover, the expression of cleaved caspase-3 was also significantly reduced by the EGCG treatment (Fig. 8C).

4. Discussion

The most important finding in this study is that systemic administration of LPS caused memory impairment and that EGCG suppressed the amyloidogenesis through its anti-neuroinflammatory property via modulation of cytokine release in systemic LPS-induced *in vivo* and *in vitro* models, and resulted in ameliorated memory impairment.

Many epidemiological and experimental animal studies have suggested that neuroinflammation may contribute to the occurrence and progress of AD [37–40]. Systemic administration of LPS can induce neurobiological effects through neuroinflammation [12,41–43], although the mechanisms are not fully clarified. Many studies have demonstrated that a neuroinflammatory condition caused by LPS induces AD-like pathology such as increased APP processing and Aβ accumulation by the concomitant increase of neuroinflammation

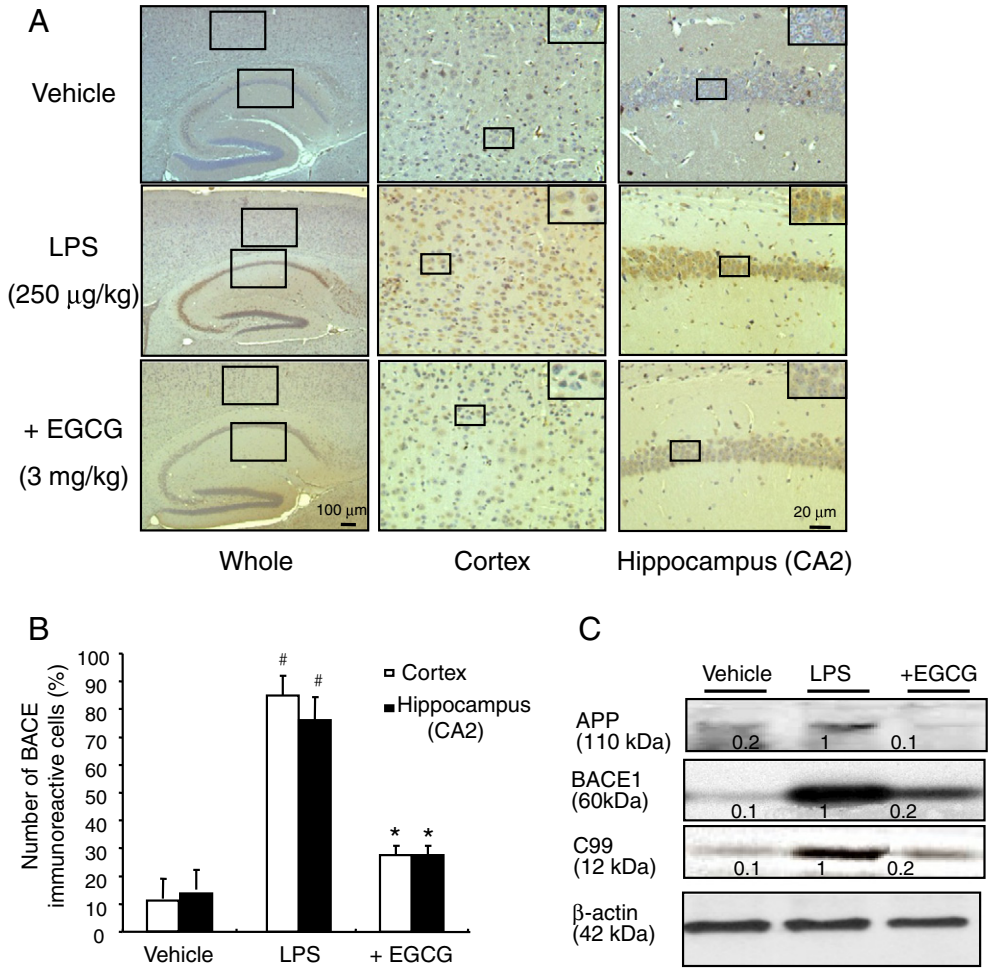

Fig. 5. Effect of EGCG on expression of amyloidogenic proteins. (A) Immunoreactive cells of anti-BACE1 antibody were investigated in the cortex and hippocampus. (B) The present figure is representative of three different experiments with different animal brains. (C) The expression of APP, C99 and BACE1 was detected by Western blotting using specific antibodies in mice brain. The Western blot bands were representative of three animals per group, and the values indicate average density over β-actin from three animals. #Significantly different from vehicle group control ($P<.05$); *significantly different from LPS-treated group ($P<.05$).

[19,29,44–46]. We previously demonstrated that the neuroinflammatory condition by icv treatment of LPS induced accumulation of Aβ via increased β- and γ-secretase activities [28]. In addition, we also previously reported that the systemic administration of LPS induced an elevated expression of genes related to inflammation such as iNOS, COX-2 and GFAP as well as Aβ accumulation via increased β- and γ-secretase activities [19,47]. Recently, Jaeger et al. [18] was also reported that systemic injection of LPS increased brain influx of blood Aβ via alteration of LRP-1 in mice brain, although it is different with our mechanism of amyloid deposition. The result agreed with our data showing that systemic injection of LPS induced Aβ accumulation, thus causing memory impairment, although the mechanism for Aβ accumulation is different from ours. The present study confirmed that systemic LPS injection induced neuroinflammation as evidenced by the elevated expressions of inflammatory genes such as iNOS, COX-2 and GFAP as well as several cytokines including M-CSF, ICAM-1 and IL-16 in brain and in cultured astrocytes. These neuroinflammation responses were concomitantly correlated with the Aβ accumulation and increased β- and γ-secretase activities. However, EGCG inhibited the iNOS and COX-2 expressions and the increased inflammatory related cytokines in the mice's brains and in the cultured astrocytes. Consequently, these anti-inflammatory effects of EGCG decreased amyloidogenesis by decreasing expression of APP, BACE1 and C99 as well as Aβ level. The present data indicated that the anti-neuroinflammatory properties of EGCG could be associated with anti-amyloidogenesis.

The ways how anti-neuroinflammation inhibits amyloidogenesis have not been clearly demonstrated. However, it is noteworthy that several cytokines released from the activated astrocytes are involved in amyloidogenesis. In AD, inflammation and astrogliosis are primarily triggered by amyloid deposits in the extracellular space, and these amyloid deposits primarily consist of aggregated Aβ [48–50]. Reactive astrocytes have been shown to release TGF-β1, TGF-β3 and IL-10, as well as proinflammatory mediators such as MCP-1, RANTES, TNF-α and IL-1 [51]. Our present findings showed that systemic administration of LPS induced elevated levels of M-CSF, ICAM-1 and IL-16 in the mice's brains and in cultured astrocytes. These data indicated that LPS activates astrocytes, and activated astrocytes released these cytokines. Activation of astrocytes is also known to lead to elevated β-secretase activity [52–54] as well as γ-secretase activity [55], consequently increasing Aβ generation. In this present study, we also found that LPS induced an increase of co-located activated astrocytes and amyloid plaque. The increased BACE1 expression may locally increase the generation of amyloidogenic APP fragments, and this may potentially contribute to Aβ plaque formation [56]. These LPS-elevated cytokines and activation of astrocytes were prevented by EGCG. These data indicate that EGCG prevented LPS-induced amyloidogenesis via blocking of cytokines

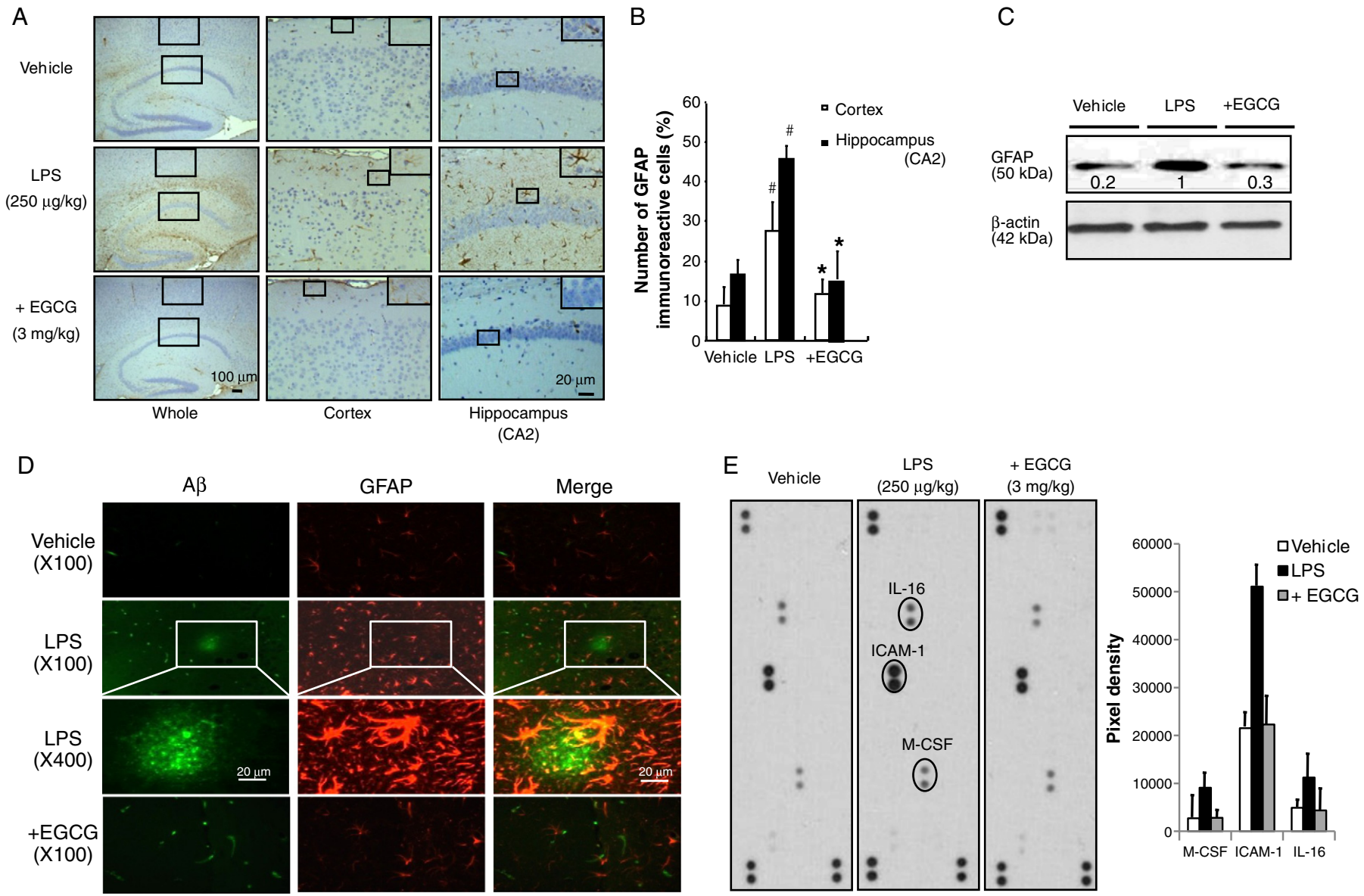

Fig. 6. Effect of EGCG on the LPS-induced neuroinflammation. (A) Immunoreactive cells of anti-GFAP antibody were investigated in the cortex and hippocampus. (B) The present figure is representative of three different experiments with different animal brains. (C) The level of GFAP was detected by Western blotting using specific antibodies in mice brain. The Western blot bands were representative of three animals per group, and the values indicate average density over β-actin from three animals. (D) Co-immunoreactivity against anti-GFAP antibody (red label) and anti-Aβ antibody (green label) was investigated in LPS-injected mice brain. The figure is representative of three experiments from different mice brains. (E) Cytokine profiles of saline-, LPS- or LPS+EGCG-treated brain. #Significantly different from vehicle group control ($P<.05$); *significantly different from LPS-treated group ($P<.05$).

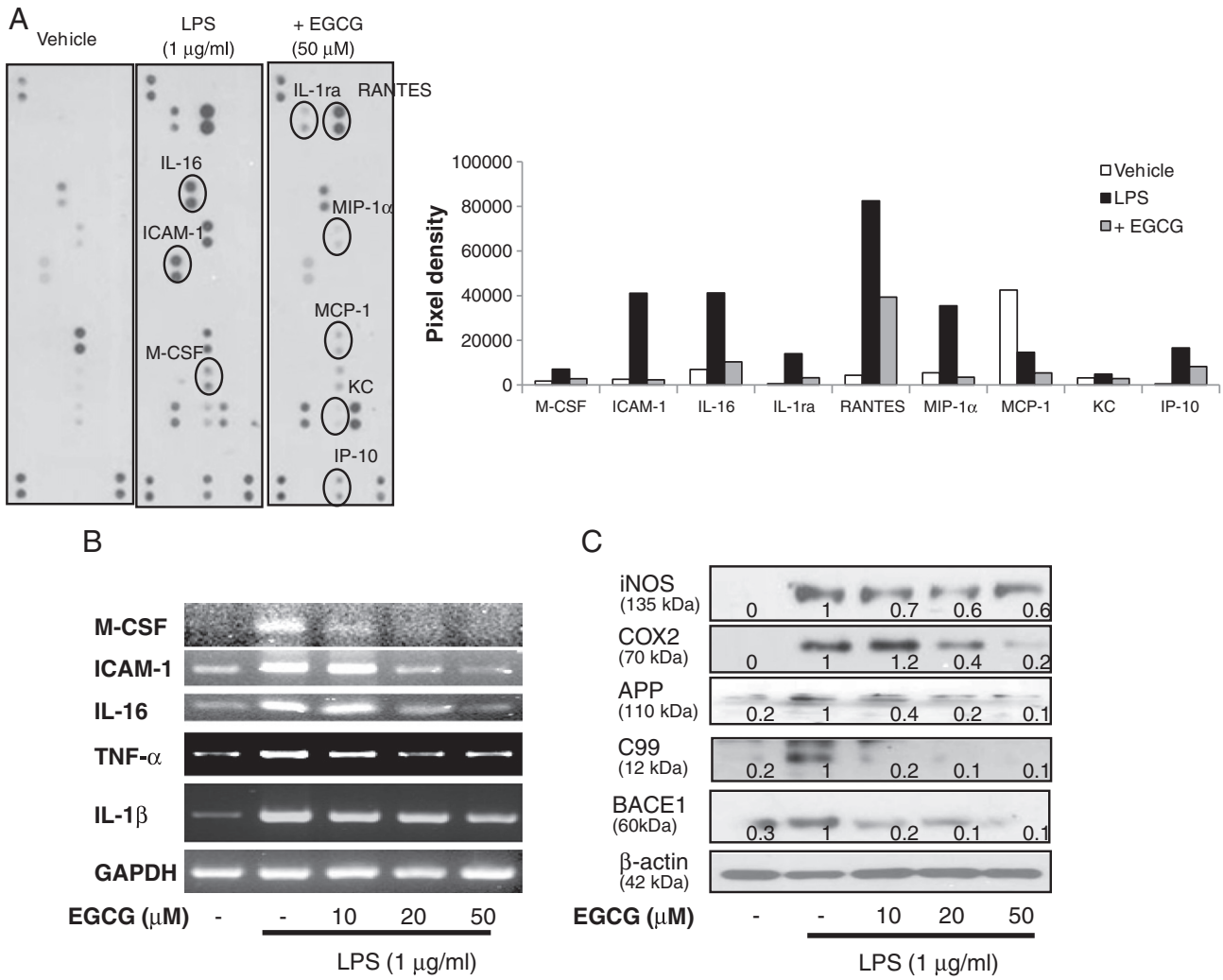

Fig. 7. EGCG inhibited LPS-induced up-regulation of cytokines in cultured astrocytes. (A) Cytokine profiles in cultured supernatant of saline-, LPS- or LPS+EGCG-treated astrocytes. (B) EGCG inhibited LPS-induced up-regulation of TNF-α, IL-1β, M-CSF, ICAM-1 and IL-16 in cultured astrocytes. Astrocytes were treated with LPS (1 μg/ml) and EGCG (10–50 μM). (C) Effect of EGCG on LPS-induced iNOS, COX-2, GFAP, APP, C99, Aβ and BACE generation. Astrocytes were treated with LPS (1 μg/ml) and EGCG (10–50 μM). Immunoblots of lysates from astrocytes were probed with iNOS, COX-2, APP, C99, Aβ and BACE antibodies, respectively. The Western blot bands were representative in three experiments, and the values indicate average density over β-actin from three experiments. #Significantly different from vehicle group control ($P<.05$); *significantly different from LPS-treated group ($P<.05$).

release by inhibition of astrocytes activation. Verdier et al. [57] reported that binding of Aβ to the neuronal receptor for advanced glycation end product induces M-CSF expression *in vitro*. The AD brain shows an increased neuronal expression of M-CSF in proximity to Aβ deposits and in the cerebrospinal fluid from AD patients. In addition, the postmortem brains of AD patients were found to have higher levels of M-CSF than those from the nondemented elderly control brains [58,59]. Furthermore, an increased M-CSF receptor expression by microglia has been reported in AD brains and in the APPv717f transgenic mouse model for AD [37,60]. Several *in vitro* studies have demonstrated that M-CSF can exhibit a synergistic effect on the fibrillar Aβ-induced release of proinflammatory cytokines and NO by a microglial cell line [60–62]. The administration of LPS was recently also demonstrated to induce elevated levels of the cell adhesion molecule ICAM-1 [63,64]. The expression of ICAM-1 in the AD brain is known to contribute to plaque formation, tissue remodeling and neurodegeneration [65,66]. Immunocytochemistry performed in the brains of Tg2576 mice at various ages has demonstrated that the ICAM-1 expression is correlated with the Aβ plaque load [67]. In addition, up-regulation of ICAM-1 on the surface of human brain microvascular endothelial cells is reported to be selectively induced by soluble $A\beta_{1\text{-}40}$ aggregates [68]. IL-16 has been reported to play a chemoattractant and immunomodulatory role in chronic inflammatory disorders in the brain [69,70]. IL-16 has been known to stimulate the production of the inflammatory cytokines IL-1β, IL-6 and TNF-α [71] and induces increases in intracellular Ca^{2+} or inositol-(1,4,5)-triphosphatase and translocation of protein kinase C [72]. In fact, each of these events causes Aβ accumulation and neuronal cell death [73,74]. In particular, elevation of IL-16 level was found in AD patient brain [75,76]. Thus, our study showed that the inhibitory effect of EGCG on the LPS-induced M-CSF, ICAM-1 and IL-16 release may be significant in the anti-amyloidogenic effect of EGCG.

In addition to astrocytes, microglia are also known to play a critical role in the neuroinflammation associated with AD because activated microglia are often a source of proinflammatory cytokines which could be related with amyloidogenesis [77,78]. In previous studies, we found that microglia were activated in brain of systemic inflammation inducing AD [79,80] and that several anti-inflammatory compounds inhibited the activation of microglia as well as prevented the development of AD inhibited the activation of microglia as well as prevented the development of AD [80,81]. The inhibitory effect of EGCG on microglial activation is also well known to be effective for

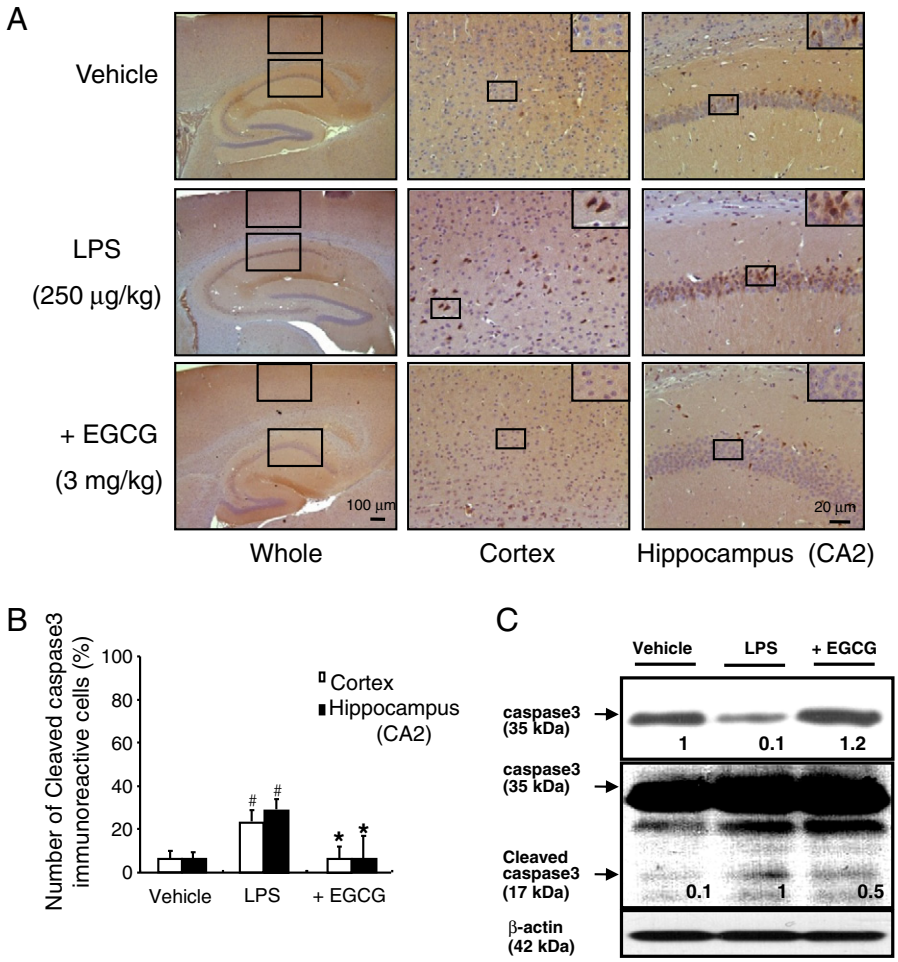

Fig. 8. Effect of EGCG on the LPS-induced neuronal cellular damage. (A) Immunoreactive cells of anti-cleaved caspase-3 antibody were investigated in the cortex and hippocampus. (B) The present figure is representative of three different experiments with different animal brains. (C) The level of cleaved caspase-3 was detected by Western blotting using specific antibodies in mice brain. The Western blot bands were representative of three animals per group, and the values indicate average density over β-actin from three animals. #Significantly different from vehicle group control ($P<.05$); *significantly different from LPS-treated group ($P<.05$).

other neuroinflammatory diseases such as ischemic stroke, Parkinson's disease (PD) and amyotrophic lateral sclerosis [82–84]. The anti-PD effect of EGCG shows neuroprotective effects in an animal model of PD by inhibiting microglial activation [85]. In addition, inactivation of microglia has been reported to ameliorate neuronal damage in an animal model of ischemia [86–88]. Thus, inhibition of microglial activation could also contribute to the inhibitory effect of EGCG on systemic LPS-induced neuroinflammation and amyloidogenesis, thus preventing the development of AD.

There have been reports that up to 12 g of green tea extract can be consumed with no adverse reactions [31,89]. As was reported previously [29], 3 mg/kg of EGCG is ~1.5 times more compared to the EGCG that a person receives when people have 300 ml of green tea drink, which generally contains 6 g of green tea. Therefore, the dose of EGCG used in the present study could be applicable for human trials without any side effects.

In many studies, EGCG has been used through oral or ip administration [22,23]. EGCG is normally absorbed through oral administration in human, but oral or ip administration is used for exact experimental animals. Each route should be required for the purpose of each study. Administration via an ip injection may have temporarily attained peak concentration after administration. Conversely, the oral consumption to drink over the course of a 24-h period may induce to maintain a steady-state concentration of EGCG over the whole period of treatment, although the oral consumption has a lower absorption rate. A daily dose of 800 mg EGCG for 4 weeks was shown to be safe in human subjects [90]. In animal safety studies for EGCG, a single administration of an oral dose of 2000 mg/kg or 10 days' administration of an oral dose of 1200 mg/kg/day to mice demonstrated an absence of genotoxic effects [91]. In acute toxicity, a single oral administration of 2000 mg/kg to rats showed signs of lethargy, calm behavior, hunched posture, labored respiration, piloerection and/or ptosis. [92]. An oral dose administration of EGCG preparation to rats and dogs for 13 weeks was not toxic at doses up to 500 mg/kg/day [92]. In reproductive toxicity studies in rats, the no observed adverse effect level was equivalent to 200 mg/kg/day [93]. EGCG is known to have potential side effects such as anxiolytic activity (30 mg/kg, ip in mice) [94] and hepatotoxicity (1500 mg/kg, intragastric, in mice) [95,96]. Thus, oral administration of 3 mg EGCG/kg/day for 3 weeks in our study is not expected to cause any negative side effects. Accumulated results of EGCG offer us the possibility of beneficial effects, but limited data from clinical trials of EGCG confuse us on its effects [97,98]. Bioavailability of EGCG is enhanced by several factors such as cool and dry storage conditions, albumin [99], ascorbic acid [100] and omega-3 fatty acids from salmon [101] and piperine [102]. However, EGCG has poor bioavailability contributed to by many factors such as air contact oxidation; gastrointestinal inactivation [103,104]; presence of calcium, magnesium and other metals [99]; catechol-*O*-

methyltransferase (COMT) polymorphisms [105]; sulfation and glucuronidation [106]. Enhancing bioavailability of EGCG is one of the important tasks for inducing ability of EGCG. Thus, for improving EGCG bioavailability, many researchers have developed methods such as the encapsulation of EGCG in chitosan nanoparticles [107,108], the design and semisynthesis *O*-acyl derivatives of EGCG [109], the solid-phase synthesis of EGCG derivatives [110] or even considering another application way, for instance, the transdermal delivery of EGCG [111], or co-administration of quercetin, a natural inhibitor of COMT [112].

In summary, our current study shows that EGCG has a recovery effect against LPS-induced memory deficiency, and the inhibition of Aβ generation through the inhibition of cytokine release may be another important mechanism of the anti-amyloidogenic effects of EGCG. Based on these findings, we suggest that EGCG may be a useful agent for the prevention of the neuroinflammation-associated development or progression of AD. In the future, we will investigate more detailed mechanisms on systemic inflammation-inducing neuroinflammation and anti-AD effect of EGCG on other AD model.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government [MEST] (MRC, 2010–0029480), by a grant (No. A101836) of the Korean Health Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea and by a grant of the Korea Ministry of Education, Science and Technology (The Regional Core Research Program/ Chungbuk BIT Research-Oriented University Consortium).

References

[1] Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet 2006;368: 387-403.
[2] Ferri CP, Sousa R, Albanese E, Ribeiro WS, Honyashiki M. In: Prince M, Jackson J, editors. World alzheimer report 2009 — executive summary. London: Alzheimer's disease international; 2009. p. 1–22.
[3] Mattson MP, Maudsley S, Martin B. A neural signaling triumvirate that influences ageing and age-related disease: insulin/IGF-1, BDNF and serotonin. Ageing Res Rev 2004;3:445-64.
[4] Pratico D, Trojanowski JQ. Inflammatory hypotheses: novel mechanisms of Alzheimer's neurodegeneration and new therapeutic targets? Neurobiol Aging 2000;21:441-5 [discussion 51–3].
[5] Maeda J, Ji B, Irie T, Tomiyama T, Maruyama M, Okauchi T, et al. Longitudinal, quantitative assessment of amyloid, neuroinflammation, and anti-amyloid treatment in a living mouse model of Alzheimer's disease enabled by positron emission tomography. J Neurosci 2007;27:10957-68.
[6] Xu F, Grande AM, Robinson JK, Previti ML, Vasek M, Davis J, et al. Early-onset subicular microvascular amyloid and neuroinflammation correlate with behavioral deficits in vasculotropic mutant amyloid beta-protein precursor transgenic mice. Neuroscience 2007;146:98–107.
[7] Nichol KE, Poon WW, Parachikova AI, Cribbs DH, Glabe CG, Cotman CW. Exercise alters the immune profile in Tg2576 Alzheimer mice toward a response coincident with improved cognitive performance and decreased amyloid. J Neuroinflammation 2008;5:13.
[8] Fang F, Lue LF, Yan S, Xu H, Luddy JS, Chen D, et al. RAGE-dependent signaling in microglia contributes to neuroinflammation, Abeta accumulation, and impaired learning/memory in a mouse model of Alzheimer's disease. FASEB J 2010;24: 1043-55.
[9] McGeer EG, McGeer PL. Inflammatory processes in Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 2003;27:741-9.
[10] Guo JT, Yu J, Grass D, de Beer FC, Kindy MS. Inflammation-dependent cerebral deposition of serum amyloid a protein in a mouse model of amyloidosis. J Neurosci 2002;22:5900-9.
[11] Simic G, Lucassen PJ, Krsnik Z, Kruslin B, Kostovic I, Winblad B, et al. nNOS expression in reactive astrocytes correlates with increased cell death related DNA damage in the hippocampus and entorhinal cortex in Alzheimer's disease. Exp Neurol 2000;165:12-26.
[12] Bluthe RM, Laye S, Michaud B, Combe C, Dantzer R, Parnet P. Role of interleukin-1beta and tumour necrosis factor-alpha in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice. Eur J Neurosci 2000;12:4447-56.
[13] Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 2007;55: 453-62.
[14] Mouton PR, Kelley-Bell B, Tweedie D, Spangler EL, Perez E, Carlson OD, et al. The effects of age and lipopolysaccharide (LPS)-mediated peripheral inflammation on numbers of central catecholaminergic neurons. Neurobiol Aging 2012;33: 423.e27-36.
[15] Erickson MA, Banks WA. Cytokine and chemokine responses in serum and brain after single and repeated injections of lipopolysaccharide: multiplex quantification with path analysis. Brain Behav Immun 2011;25:1637-48.
[16] Sastre M, Dewachter I, Landreth GE, Willson TM, Klockgether T, van Leuven F, et al. Nonsteroidal anti-inflammatory drugs and peroxisome proliferator-activated receptor-gamma agonists modulate immunostimulated processing of amyloid precursor protein through regulation of beta-secretase. J Neurosci 2003;23:9796-804.
[17] Vassar R. The beta-secretase, BACE: a prime drug target for Alzheimer's disease. J Mol Neurosci 2001;17:157-70.
[18] Jaeger LB, Dohgu S, Sultana R, Lynch JL, Owen JB, Erickson MA, et al. Lipopolysaccharide alters the blood–brain barrier transport of amyloid beta protein: a mechanism for inflammation in the progression of Alzheimer's disease. Brain Behav Immun 2009;23:507-17.
[19] Lee JW, Lee YK, Yuk DY, Choi DY, Ban SB, Oh KW, et al. Neuro-inflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. J Neuroinflammation 2008;5:37.
[20] Breitner JC, Baker LD, Montine TJ, Meinert CL, Lyketsos CG, Ashe KH, et al. Extended results of the Alzheimer's disease anti-inflammatory prevention trial. Alzheimers Dement 2011;7:402-11.
[21] Singh M, Arseneault M, Sanderson T, Murthy V, Ramassamy C. Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. J Agric Food Chem 2008;56:4855-73.
[22] Rezai-Zadeh K, Shytle D, Sun N, Mori T, Hou H, Jeanniton D, et al. Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. J Neurosci 2005;25:8807-14.
[23] Rezai-Zadeh K, Arendash GW, Hou H, Fernandez F, Jensen M, Runfeldt M, et al. Green tea epigallocatechin-3-gallate (EGCG) reduces beta-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice. Brain Res 2008;1214:177-87.
[24] Mandel S, Weinreb O, Amit T, Youdim MB. Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (−)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. J Neurochem 2004;88: 1555-69.
[25] Antonio AM, Druse MJ. Antioxidants prevent ethanol-associated apoptosis in fetal rhombencephalic neurons. Brain Res 2008;1204:16-23.
[26] Cho HS, Kim S, Lee SY, Park JA, Kim SJ, Chun HS. Protective effect of the green tea component, L-theanine on environmental toxins-induced neuronal cell death. Neurotoxicology 2008;29:656-62.
[27] Okello EJ, Savelev SU, Perry EK. vitro anti-beta-secretase and dual anti-cholinesterase activities of Camellia sinensis L. (tea) relevant to treatment of dementia. Phytother Res 2004;18:624-7.
[28] Lee YK, Yuk DY, Lee JW, Lee SY, Ha TY, Oh KW, et al. (−)-Epigallocatechin-3-gallate prevents lipopolysaccharide-induced elevation of beta-amyloid generation and memory deficiency. Brain Res 2009;1250:164-74.
[29] Lee JW, Lee YK, Ban JO, Ha TY, Yun YP, Han SB, et al. Green tea (−)-epigallocatechin-3-gallate inhibits beta-amyloid-induced cognitive dysfunction through modification of secretase activity via inhibition of ERK and NF-kappaB pathways in mice. J Nutr 2009;139:1987-93.
[30] Muramatsu K, Fukuyo M, Hara Y. Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats. J Nutr Sci Vitaminol (Tokyo) 1986;32: 613-22.
[31] Rietveld A, Wiseman S. Antioxidant effects of tea: evidence from human clinical trials. J Nutr 2003;133:3285S-92S.
[32] Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47-60.
[33] Lee SM, Nguyen TH, Park MH, Kim KS, Cho KJ, Moon DC, et al. EPO receptor-mediated ERK kinase and NF-kappaB activation in erythropoietin-promoted differentiation of astrocytes. Biochem Biophys Res Commun 2004;320: 1087-95.
[34] Park KS, Lee RD, Kang SK, Han SY, Park KL, Yang KH, et al. Neuronal differentiation of embryonic midbrain cells by upregulation of peroxisome proliferator-activated receptor-gamma via the JNK-dependent pathway. Exp Cell Res 2004;297:424-33.
[35] Choi SH, Joe EH, Kim SU, Jin BK. Thrombin-induced microglial activation produces degeneration of nigral dopaminergic neurons in vivo. J Neurosci 2003;23:5877-86.
[36] Nguyen HN, Lee MS, Hwang DY, Kim YK, Yoon do Y, Lee JW, et al. Mutant presenilin 2 increased oxidative stress and p53 expression in neuronal cells. Biochem Biophys Res Commun 2007;357:174-80.
[37] Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, et al. Inflammation and Alzheimer's disease. Neurobiol Aging 2000;21:383-421.
[38] McGeer PL, McGeer EG. NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. Neurobiol Aging 2006;28:639-47.
[39] Veerhuis R. Histological and direct evidence for the role of complement in the neuroinflammation of AD. Curr Alzheimer Res 2011;8:34-58.

[40] Heneka MT, Kummer MP, Weggen S, Bulic B, Multhaup G, Munter L, et al. Molecular mechanisms and therapeutic application of NSAIDs and derived compounds in Alzheimer's disease. Curr Alzheimer Res 2011;8:115-31.
[41] Mastronardi CA, Licinio J, Wong ML. Candidate biomarkers for systemic inflammatory response syndrome and inflammation: a pathway for novel translational therapeutics. Neuroimmunomodulation 2010;17:359-68.
[42] Hughes MM, Field RH, Perry VH, Murray CL, Cunningham C. Microglia in the degenerating brain are capable of phagocytosis of beads and of apoptotic cells, but do not efficiently remove PrPSc, even upon LPS stimulation. Glia 2010;58: 2017-30.
[43] Bay-Richter C, Janelidze S, Hallberg L, Brundin L. Changes in behaviour and cytokine expression upon a peripheral immune challenge. Behav Brain Res 2011;222:193-9.
[44] Sheng JG, Bora SH, Xu G, Borchelt DR, Price DL, Koliatsos VE. Lipopolysaccharide-induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid beta peptide in APPswe transgenic mice. Neurobiol Dis 2003;14:133-45.
[45] Michelucci A, Heurtaux T, Grandbarbe L, Morga E, Heuschling P. Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: effects of oligomeric and fibrillar amyloid-beta. J Neuroimmunol 2009;210:3–12.
[46] McAlpine FE, Lee JK, Harms AS, Ruhn KA, Blurton-Jones M, Hong J, et al. Inhibition of soluble TNF signaling in a mouse model of Alzheimer's disease prevents pre-plaque amyloid-associated neuropathology. Neurobiol Dis 2009; 34:163-77.
[47] Scholtzova H, Kascsak RJ, Bates KA, Boutajangout A, Kerr DJ, Meeker HC, et al. Induction of toll-like receptor 9 signaling as a method for ameliorating Alzheimer's disease-related pathology. J Neurosci 2009;29:1846-54.
[48] Hensley K, Maidt ML, Yu Z, Sang H, Markesbery WR, Floyd RA. Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. J Neurosci 1998;18:8126-32.
[49] Paris D, Townsend KP, Obregon DF, Humphrey J, Mullan M. Pro-inflammatory effect of freshly solubilized beta-amyloid peptides in the brain. Prostaglandins Other Lipid Mediat 2002;70:1–12.
[50] Rodriguez JJ, Olabarria M, Chvatal A, Verkhratsky A. Astroglia in dementia and Alzheimer's disease. Cell Death Differ 2009;16:378-85.
[51] Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B. How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. Aging Cell 2004;3:169-76.
[52] Cho HJ, Kim SK, Jin SM, Hwang EM, Kim YS, Huh K, et al. IFN-gamma-induced BACE1 expression is mediated by activation of JAK2 and ERK1/2 signaling pathways and direct binding of STAT1 to BACE1 promoter in astrocytes. Glia 2007;55:253-62.
[53] Gunasingh MJ, Philip JE, Ashok BS, Kirubagaran R, Jebaraj WC, Davis GD, et al. Melatonin prevents amyloid protofibrillar induced oxidative imbalance and biogenic amine catabolism. Life Sci 2008;83:96–102.
[54] Schubert D, Soucek T, Blouw B. The induction of HIF-1 reduces astrocyte activation by amyloid beta peptide. Eur J Neurosci 2009;29:1323-34.
[55] Nadler Y, Alexandrovich A, Grigoriadis N, Hartmann T, Rao KS, Shohami E, et al. Increased expression of the gamma-secretase components presenilin-1 and nicastrin in activated astrocytes and microglia following traumatic brain injury. Glia 2008;56:552-67.
[56] Hartlage-Rubsamen M, Zeitschel U, Apelt J, Gartner U, Franke H, Stahl T, et al. Astrocytic expression of the Alzheimer's disease beta-secretase (BACE1) is stimulus-dependent. Glia 2003;41:169-79.
[57] Verdier Y, Zarandi M, Penke B. Amyloid beta-peptide interactions with neuronal and glial cell plasma membrane: binding sites and implications for Alzheimer's disease. J Pept Sci 2004;10:229-48.
[58] Lue LF, Walker DG, Rogers J. Modeling microglial activation in Alzheimer's disease with human postmortem microglial cultures. Neurobiol Aging 2001;22: 945-56.
[59] Laske C, Stransky E, Hoffmann N, Maetzler W, Straten G, Eschweiler GW, et al. Macrophage colony-stimulating factor (M-CSF) in plasma and CSF of patients with mild cognitive impairment and Alzheimer's disease. Curr Alzheimer Res 2010;7:409-14.
[60] Murphy Jr GM, Zhao F, Yang L, Cordell B. Expression of macrophage colony-stimulating factor receptor is increased in the AbetaPP(V717F) transgenic mouse model of Alzheimer's disease. Am J Pathol 2000;157:895-904.
[61] Murphy Jr GM, Yang L, Cordell B. Macrophage colony-stimulating factor augments beta-amyloid-induced interleukin-1, interleukin-6, and nitric oxide production by microglial cells. J Biol Chem 1998;273:20967-71.
[62] Mitrasinovic OM, Perez GV, Zhao F, Lee YL, Poon C, Murphy Jr GM. Over-expression of macrophage colony-stimulating factor receptor on microglial cells induces an inflammatory response. J Biol Chem 2001;276:30142-9.
[63] O'Sullivan JB, Ryan KM, Harkin A, Connor TJ. Noradrenaline reuptake inhibitors inhibit expression of chemokines IP-10 and RANTES and cell adhesion molecules VCAM-1 and ICAM-1 in the CNS following a systemic inflammatory challenge. J Neuroimmunol 2010;220:34-42.
[64] Wang G, Namura S. Effects of chronic systemic treatment with peroxisome proliferator-activated receptor alpha activators on neuroinflammation induced by intracerebral injection of lipopolysaccharide in adult mice. Neurosci Res 2011; 70:230-7.
[65] Verbeek MM, Otte-Holler I, Wesseling P, Ruiter DJ, de Waal RM. Differential expression of intercellular adhesion molecule-1 (ICAM-1) in the A beta-containing lesions in brains of patients with dementia of the Alzheimer type. Acta Neuropathol 1996;91:608-15.
[66] Eikelenboom P, Zhan SS, Kamphorst W, van der Valk P, Rozemuller JM. Cellular and substrate adhesion molecules (integrins) and their ligands in cerebral amyloid plaques in Alzheimer's disease. Virchows Arch 1994;424:421-7.
[67] Apelt J, Leßig J, Schliebs R. [beta]-Amyloid-associated expression of intercellular adhesion molecule-1 in brain cortical tissue of transgenic Tg2576 mice. Neurosci Lett 2002;329:111-5.
[68] Gonzalez-Velasquez FJ, Kotarek JA, Moss MA. Soluble aggregates of the amyloid-beta protein selectively stimulate permeability in human brain microvascular endothelial monolayers. J Neurochem 2008;107:466-77.
[69] Cruikshank WW, Long A, Tarpy RE, Kornfeld H, Carroll MP, Teran L, et al. Early identification of interleukin-16 (lymphocyte chemoattractant factor) and macrophage inflammatory protein 1 alpha (MIP1 alpha) in bronchoalveolar lavage fluid of antigen-challenged asthmatics. Am J Respir Cell Mol Biol 1995;13: 738-47.
[70] Schluesener HJ, Seid K, Kretzschmar J, Meyermann R. Leukocyte chemotactic factor, a natural ligand to CD4, is expressed by lymphocytes and microglial cells of the MS plaque. J Neurosci Res 1996;44:606-11.
[71] Mathy NL, Scheuer W, Lanzendorfer M, Honold K, Ambrosius D, Norley S, et al. Interleukin-16 stimulates the expression and production of pro-inflammatory cytokines by human monocytes. Immunology 2000;100:63-9.
[72] Lipton P. Ischemic cell death in brain neurons. Physiol Rev 1999;79:1431-568.
[73] Mattson MP. ER calcium and Alzheimer's disease: in a state of flux. Sci Signal 2010;3:10.
[74] Zara S, Rapino M, Sozio P, Di Stefano A, Nasuti C, Cataldi A. Ibuprofen and lipoic acid codrug 1 control Alzheimer's disease progression by down-regulating protein kinase C epsilon-mediated metalloproteinase 2 and 9 levels in beta-amyloid infused Alzheimer's disease rat model. Brain Res 2011;1412:79-87.
[75] Motta M, Imbesi R, Di Rosa M, Stivala F, Malaguarnera L. Altered plasma cytokine levels in Alzheimer's disease: correlation with the disease progression. Immunol Lett 2007;114:46-51.
[76] Di Rosa M, Dell'Ombra N, Zambito AM, Malaguarnera M, Nicoletti F, Malaguarnera L. Chitotriosidase and inflammatory mediator levels in Alzheimer's disease and cerebrovascular dementia. Eur J Neurosci 2006;23:2648-56.
[77] Morales I, Farias G, Maccioni RB. Neuroimmunomodulation in the pathogenesis of Alzheimer's disease. Neuroimmunomodulation 2010;17:202-4.
[78] Halleskog C, Mulder J, Dahlstrom J, Mackie K, Hortobagyi T, Tanila H, et al. WNT signaling in activated microglia is proinflammatory. Glia 2011;59:119-31.
[79] Lee YJ, Choi DY, Choi IS, Kim KH, Kim YH, Kim HM, et al. Inhibitory effect of 4-*O*-methylhonokiol on lipopolysaccharide-induced neuroinflammation, amyloidogenesis and memory impairment via inhibition of nuclear factor-kappaB in vitro and in vivo models. J Neuroinflammation 2012;9:35.
[80] Lin GH, Lee YJ, Choi DY, Han SB, Jung JK, Hwang BY, et al. Anti-amyloidogenic effect of thiacremonone through anti-inflamation in vitro and in vivo models. J Alzheimers Dis 2012;29:659-76.
[81] Lee YJ, Choi DY, Lee YK, Lee YM, Han SB, Kim YH, et al. 4-*O*-methylhonokiol prevents memory impairment in the Tg2576 transgenic mice model of Alzheimer's disease via regulation of beta-secretase activity. J Alzheimers Dis 2012;29:677-90.
[82] Li R, Huang YG, Fang D, Le WD. (−)-Epigallocatechin gallate inhibits lipopolysaccharide-induced microglial activation and protects against inflammation-mediated dopaminergic neuronal injury. J Neurosci Res 2004;78: 723-31.
[83] Xu Z, Chen S, Li X, Luo G, Li L, Le W. Neuroprotective effects of (−)-epigallocatechin-3-gallate in a transgenic mouse model of amyotrophic lateral sclerosis. Neurochem Res 2006;31:1263-9.
[84] Jang S, Jeong HS, Park JS, Kim YS, Jin CY, Seol MB, et al. Neuroprotective effects of (−)-epigallocatechin-3-gallate against quinolinic acid-induced excitotoxicity via PI3K pathway and NO inhibition. Brain Res 2010;1313:25-33.
[85] Li R, Peng N, Du F, Li XP, Le WD. Epigallocatechin gallate protects dopaminergic neurons against 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity by inhibiting microglial cell activation. Nan Fang Yi Ke Da Xue Xue Bao 2006;26:376-80.
[86] Franco ECS, Cardoso MM, Gouvêia A, Pereira A, Gomes-Leal W. Modulation of microglial activation enhances neuroprotection and functional recovery derived from bone marrow mononuclear cell transplantation after cortical ischemia. Neurosci Res 2012;73:122-32.
[87] Ortega FJ, Gimeno-Bayon J, Espinosa-Parrilla JF, Carrasco JL, Batlle M, Pugliese M, et al. ATP-dependent potassium channel blockade strengthens microglial neuroprotection after hypoxia–ischemia in rats. Exp Neurol 2012;235:282-96.
[88] Yenari MA, Kauppinen TM, Swanson RA. Microglial activation in stroke: therapeutic targets. Neurotherapeutics 2010;7:378-91.
[89] Lambert JD, Elias RJ. The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. Arch Biochem Biophys 2010;501:65-72.
[90] Chow HH, Cai Y, Hakim IA, Crowell JA, Shahi F, Brooks CA, et al. Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. Clin Cancer Res 2003;9:3312-9.
[91] Isbrucker RA, Bausch J, Edwards JA, Wolz E. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 1: genotoxicity. Food Chem Toxicol 2006;44: 626-35.
[92] Isbrucker RA, Edwards JA, Wolz E, Davidovich A, Bausch J. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: dermal, acute and short-term toxicity studies. Food Chem Toxicol 2006;44:636-50.

[93] Isbrucker RA, Edwards JA, Wolz E, Davidovich A, Bausch J. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 3: teratogenicity and reproductive toxicity studies in rats. Food Chem Toxicol 2006;44: 651-61.
[94] Vignes M, Maurice T, Lante F, Nedjar M, Thethi K, Guiramand J, et al. Anxiolytic properties of green tea polyphenol (−)-epigallocatechin gallate (EGCG). Brain Res 2006;1110:102-15.
[95] Lambert JD, Kennett MJ, Sang S, Reuhl KR, Ju J, Yang CS. Hepatotoxicity of high oral dose (−)-epigallocatechin-3-gallate in mice. Food Chem Toxicol 2010;48: 409-16.
[96] Mereles D, Hunstein W. Epigallocatechin-3-gallate (EGCG) for clinical trials: more pitfalls than promises? Int J Mol Sci 2011;12:5592-603.
[97] Fujiki H, Suganuma M, Okabe S, Sueoka E, Suga K, Imai K, et al. Mechanistic findings of green tea as cancer preventive for humans. Proc Soc Exp Biol Med 1999;220:225-8.
[98] Hsu CH, Liao YL, Lin SC, Tsai TH, Huang CJ, Chou P. Does supplementation with green tea extract improve insulin resistance in obese type 2 diabetics? A randomized, double-blind, and placebo-controlled clinical trial. Altern Med Rev 2011;16:157-63.
[99] Ishii T, Ichikawa T, Minoda K, Kusaka K, Ito S, Suzuki Y, et al. Human serum albumin as an antioxidant in the oxidation of (−)-epigallocatechin gallate: participation of reversible covalent binding for interaction and stabilization. Biosci Biotechnol Biochem 2011;75:100-6.
[100 Peters CM, Green RJ, Janle EM, Ferruzzi MG. Formulation with ascorbic acid and sucrose modulates catechin bioavailability from green tea. Food Res Int 2010;43: 95–102.
[101] Giunta B, Hou H, Zhu Y, Salemi J, Ruscin A, Shytle RD, et al. Fish oil enhances anti-amyloidogenic properties of green tea EGCG in Tg2576 mice. Neurosci Lett 2010;471:134-8.
[102] Lambert JD, Hong J, Kim DH, Mishin VM, Yang CS. Piperine enhances the bioavailability of the tea polyphenol (−)-epigallocatechin-3-gallate in mice. J Nutr 2004;134:1948-52.
[103] Huo C, Wan SB, Lam WH, Li L, Wang Z, Landis-Piwowar KR, et al. The challenge of developing green tea polyphenols as therapeutic agents. Inflammopharmacology 2008;16:248-52.
[104] Lambert JD, Sang S, Hong J, Kwon SJ, Lee MJ, Ho CT, et al. Peracetylation as a means of enhancing in vitro bioactivity and bioavailability of epigallocatechin-3-gallate. Drug Metab Dispos 2006;34:2111-6.
[105] Lee MJ, Maliakal P, Chen L, Meng X, Bondoc FY, Prabhu S, et al. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. Cancer Epidemiol Biomarkers Prev 2002;11:1025-32.
[106] Lu H, Meng X, Li C, Sang S, Patten C, Sheng S, et al. Glucuronides of tea catechins: enzymology of biosynthesis and biological activities. Drug Metab Dispos 2003;31:452-61.
[107] Dube A, Nicolazzo JA, Larson I. Chitosan nanoparticles enhance the intestinal absorption of the green tea catechins (+)-catechin and (−)-epigallocatechin gallate. Eur J Pharm Sci 2010;41:219-25.
[108] Hu B, Ting Y, Yang X, Tang W, Zeng X, Huang Q. Nanochemoprevention by encapsulation of (−)-epigallocatechin-3-gallate with bioactive peptides/chitosan nanoparticles for enhancement of its bioavailability. Chem Commun (Camb) 2012;48:2421-3.
[109] Vyas S, Sharma M, Sharma PD, Singh TV. Design, semisynthesis, and evaluation of O-acyl derivatives of (−)-epigallocatechin-3-gallate as antitumor agents. J Agric Food Chem 2007;55:6319-24.
[110] Tanaka H, Miyoshi H, Chuang YC, Ando Y, Takahashi T. Solid-phase synthesis of epigallocatechin gallate derivatives. Angew Chem Int Ed Engl 2007;46: 5934-7.
[111] Lambert JD, Kim DH, Zheng R, Yang CS. Transdermal delivery of (−)-epigallocatechin-3-gallate, a green tea polyphenol, in mice. J Pharm Pharmacol 2006;58:599-604.
[112] Wang P, Heber D, Henning SM. Quercetin increased bioavailability and decreased methylation of green tea polyphenols in vitro and in vivo. Food Funct 2012:635-42.