EGB 761 enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of Alzheimer’s disease

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ABSTRACT Standardized Ginkgo biloba extract EGB 761 exhibits beneficial effects to patients with Alzheimer’s disease (AD). It was previously demonstrated that EGB 761 inhibits amyloid beta (Aβ) oligomerization in vitro, protects neuronal cells against Aβ toxicity, and improves cognitive defects in a mouse model of AD (Tg 2576). In this study, the neurogenic potential of EGB 761 and its effect on cAMP response element binding protein (CREB) were examined in a double transgenic mouse model (TgAPP/PS1). EGB 761 significantly increases cell proliferation in the hippocampus of both young (6 months) and old (22 months) TgAPP/PS1 mice, and the total number of neuronal precursor cells in vitro in a dose-dependent manner. Furthermore, Aβ oligomers inhibit phosphorylation of CREB and cell proliferation in the hippocampus of TgAPP/PS1 mice. Administration of EGB 761 reduces Aβ oligomers and restores CREB phosphorylation in the hippocampus of these mice. The present findings suggest that 1) enhanced neurogenesis by EGB 761 may be mediated by activation of CREB, 2) stimulation of neurogenesis by EGB 761 may contribute to its beneficial effects in AD patients and improved cognitive functions in the mouse model of AD, and 3) EGB 761 has therapeutic potential for the prevention and improved treatment of AD.—Tchantchou, F., Xu, Y., Wu, Y., Christen, Y., Luo, Y. EGB 761 enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of Alzheimer’s disease. FASEB J. 21, 2400–2408 (2007)

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Alzheimer’s disease (AD) is the most common cause of dementia among elderly people. This neurodegenerative disorder is believed to start with synaptic dysfunction and subsequent loss of neuronal cells (1). Evidence of neurogenesis in the adult rodent’s brain (2–5) raised the hope that replacement of lost neurons could represent a therapeutic approach for management of AD (6). It is well accepted that neurogenesis occurs throughout adulthood and is confined to two different areas of the brain: the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus (7). As the epicenter of declarative memory in humans and cognition in many animal species (8, 9), the hippocampus is the principal focus of neuronal loss in AD, and thus is of special interest for the neuronal replacement therapeutic approach.

Neurogenesis can be regulated by several factors including age, stress, environmental enrichment, and pathological changes, as well as pharmacological agents (3, 10–13). A relationship between the status of neurogenesis in AD animal models and postmortem patient brains has not yet been defined (14–18). A better understanding of neurogenesis regulation by pharmacological agents in a defined animal model of AD would strengthen the idea that the normalization of hippocampal neurogenesis could be an important therapeutic intervention in the treatment of AD. The amyloid beta peptide (Aβ) and the transcription factor cyclic AMP response element binding protein (CREB) have important effects on the regulation of neurogenesis (19–25).

EGB 761 is one of the most commonly prescribed drugs for dementia in many countries and a leading dietary supplement in the United States for memory enhancement (26, 27). Several clinical trials have proved its efficacy as a symptomatic treatment for AD (28–31), and the clinical evaluation of EGB 761 as a preventive drug is currently under way (32). The neuroprotective effects of EGB 761 in vitro have been indicated by substantial experimental evidence (26, 33–38), including improvement in cognitive impairment of transgenic AD mice (Tg 2576) (39). The neuroprotective mechanism for EGB 761 in vitro has not yet been defined.

Considering the beneficial effects of EGB 761 on cognitive impairment in AD patients (28) and AD mice (39), and its ability to inhibit Aβ oligomerization in vitro (36, 40), the present study aimed to examine the neurogenic potential of EGB 761 in the hippocampus of a mouse model of AD (TgAPP/PS1) (41). The secondary goal was to determine the impact of Aβ deposition and/or CREB phosphorylation on neuro-
genesis after EGb 761 administration. Results from this study demonstrate that the reduction of neurogenesis in the hippocampus of the AD mice is normalized by EGb 761 administration, and that the neurogenic effect of EGb 761 is associated with Aβ oligomerization and phosphorylation of CREB.

MATERIALS AND METHODS

Animals and EGb 761 treatment

Heterozygote APPsw/PSTgΔE9 transgenic (TgAPP/PS1) founder mice (42) were provided by Dr. Borchelt at Johns Hopkins University (Baltimore, MD, USA). These mice were maintained by cross-breeding with the wild-type (WT, C57BL/6J) breeders, and resulting offspring were genotyped and used at the age indicated. For in vivo studies, young (6 months) and old (22 months) TgAPP/PS1 and age-matched WT mice (3–4 in each treatment group) were fed for 1 month with a Purina “5001” basal diet (Dyets Inc., Bethlehem, PA, USA), supplemented with or without EGb 761 (Schwabe Pharmaceuticals, Karlsruhe, Germany) for an equivalent ad libitum daily intake of 100 mg/kg. For in vitro studies, day 14 WT mice embryonic brains were used. All animal handling and treatment were approved by the Institutional Animal Care and Use Committee.

BrdU injections and tissue preparation

The TgAPP/PS1 and the WT mice were intraperitoneally injected with 75 mg/kg 5-bromo-2-deoxyuridine solution (BrdU; Sigma, St. Louis, MO, USA) daily during the last 7 days of treatment. One day after the last injection, animals were anesthetized with phenobarbital and perfused transcardially with 4% paraformaldehyde in phosphate buffer (PF). The brains were removed, stored overnight in 4% PF, and transferred into 30% sucrose. Coronal sections (30 μm) were cut on a Bright OTF5000 Cryostat (Jencons Scientific Inc., San Antonio, TX, USA) overnight at 4°C, followed by secondary antibody anti-rat ALEXA FLUOR 488 (1:400, Invitrogen-Molecular Probes) for 2 h. Sections were rinsed, transferred on slides, and cover slipped in an anti-fading agent (Gel mount; Biomedica, Foster City, CA, USA). Fluorescent signals were detected with a confocal microscope (NIKON E-2000).

For phenotypic differentiation of BrdU incorporated cells, the sections were incubated with a primary antibody mixture containing antibodies against BrdU, glial fibrillary acidic protein (GFAP), and neuronal nuclei-specific protein (NeuN, 1:300, Chemicon International, Temecula, CA, USA) overnight at 4°C, followed by incubation with secondary antibodies. For double labeling of Aβ oligomers and BrdU incorporation, the sections were incubated with a primary antibody mixture containing antibodies against BrdU and Aβ oligomers (A11, 1:500, Biosource Inc., Camarillo, CA, USA), followed by incubation with secondary antibodies for 2 h. For double immunostaining for Aβ and phospho-CREB, the sections were incubated with primary antibodies to human amyloid beta (6E10) and to phosphorylated CREB (1:500, Cell Signaling Solutions, Lake Placid, NY, USA). The secondary antibodies are anti-mouse ALEXA FLUOR 488 for Aβ and anti-rabbit ALEXA FLUOR 594 for pCREB.

For immunostaining of cell proliferation in neuronal progenitor cells, cultured embryonic neuronal cells treated with or without EGb 761 in the presence of BrdU were costained for BrdU incorporation and for the expression of the neuron-specific marker (NeuN). Cells were fixed with 4% PF and blocked with normal serum. They were then incubated in a mixture of primary antibodies that contained antibodies against BrdU and NeuN overnight at 4°C, followed by the secondary antibodies at room temperature for 2 h. Cells were then rinsed and the coverslip from each Petri dish was transferred on a slide and examined under a fluorescence microscope.

Immunoblotting

Standard Western blotting analysis was performed. Hippocampus samples were homogenized in the lysis buffer containing: 50 mM HEPES, pH 7.5, 6 mM MgCl2, 1 mM EDTA, 75 mM sucrose, 2.5 mM benzamidine, 1 mM dithiothreitol, and 1% Triton X-100. Equal amounts of protein (20 μg) were resolved on a 12% SDS-AGE, then transferred to polyvinylidene difluoride membranes and blocked with 5% nonfat dry milk. This was followed by overnight incubation at 4°C with different primary antibodies, which included antibody against polysialic acid neural cell adhesion molecule (NCAM-180, Chemicon Inc.; 1:500); phosphorylated CREB (pCREB, 1:1000); CREB (Santa Cruz Inc., Santa Cruz, CA, USA; 1:500) or human Aβ (6E10, Biosource Inc.; 1:750). The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Inc.; 1:5000). Immunoreactivities were detected by an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Quantitative and statistical analyses

Stereology: BrdU-labeled cells in the dentate gyrus of one section out of every seven were counted under high power on an Nikon E2000 microscope with a Magnifier digital camera, and the image was displayed on a computer monitor. BrdU incorporated cells were quantified on dentate gyros (DG) using IPLab analysis software (Scanalytic, Inc., Fairfax, VA,
USA). The number of BrdU-positive cells in each subgroup of seven sections was obtained by multiplying the number of those cells found in the dentate gyrus of the section by seven, and the total number of BrdU-positive cells in whole brain’s dentate gyrus was expressed as the sum of those numbers in all subgroups of seven sections from the same brain (3, 23).

The immunoreactive bands from Western blots were scanned and the mean density was obtained with AlphaEase FC software (Alpha Innotech, San Leandro, CA, USA). The statistical significance of differences among mice genetic groups, age difference, and/or drug treatment was determined using either 3-way ANOVA or a Student’s t-test.

RESULTS

**EGB 761 enhances cell proliferation in the hippocampus of transgenic mice (TgAPP/PS1)**

To determine the neurogenic potential of EGB 761, cell proliferation levels were accessed in the dentate gyrus (DG) of the hippocampus of mice treated with or without EGB 761 by immunofluorescence of BrdU incorporated cells. As shown in images of BrdU incorporated cells in the DG of WT or Tg mice (Fig. 1A, a–h), the incorporation of BrdU was observed within the nuclei of dividing or divided cells (arrows). Statistically, Fig. 1B shows that EGB 761 treatment significantly enhanced cell proliferation in the DG of both young (6 months, filled column) and aged (22 months, open column) Tg mice compared with their untreated counterparts (young: Tg/EGb vs. Tg/Ctrl= 4427.5±224.33 vs. 2520±63.9, respectively; \( P<0.05 \); aged: Tg/EGb vs. Tg/Ctrl= 3091.66±274.35 vs. 1435±24.45; \( P<0.01 \)). Similar effects of EGB 761 administration were also observed in the aged WT mice (22 months, aged= 3721.66±294.45) compared with untreated age-matched controls (WT/Ctrl= 2298.33±30.86; \( P<0.01 \)). No significant difference was observed between levels of proliferating cells in the young (6 months) WT mice treated with or without EGB 761 (young WT/Ctrl vs. WT/EGb= 5215±225.01 vs. 5553.33±262.95; \( P=0.3 \)). These results highlight the fact that the EGB 761-enhanced cell proliferation is better manifested in older mice than in younger mice. In addition, it was also found that cell proliferation was significantly decreased with advanced age in WT (young WT/Ctrl vs. old WT/Ctrl= 5215±225.01 vs. 2298.33±30.86, \( P<0.01 \)) as well as in Tg mice (young Tg/Ctrl vs. old Tg/Ctrl= 2520±63.90 vs. 1435±24.45, \( P<0.01 \)). This phenomenon was greatly affected by the genotype. Cell proliferation levels were lower in untreated Tg mice (Tg/Ctrl) than WT age-matched controls (WT/Ctrl; Young: 5215±225.01 vs. Tg/Ctrl 2520±63.90; Old: 2298.33±30.86 vs. 1435±24.45, \( P<0.01 \)). Furthermore, the combination of age and genotype showed an augmented deleterious effect on cell proliferation (Young WT/Ctrl vs. young Tg/Ctrl: 5215±225.01 vs. 1435±24.45, \( P<0.01 \)) (Fig. 1A, B).

**Enhanced neurogenesis by EGB 761 in neural progenitor cell cultures is concentration dependent and is blocked by H89, an inhibitor of CREB phosphorylation**

Pharmacological evaluation of the neurogenic effects of EGB 761 in TgAPP/PS1 mice is time-consuming. To efficiently assess the dose-response effects of EGB 761 on neurogenesis, primary cultures of embryonic brain cells were treated with a range of EGB 761 concentrations (0, 60, 80, 100, and 120 μg/ml), followed by an evaluation of the number of proliferating cells. Representative images of costaining for BrdU and NeuN in cultures treated with or without EGB 761 are shown in Fig. 1C. The number of newly formed neuronal cells significantly increased in response to increasing concentrations of EGB 761 (Fig. 1D), starting from 80 μg/ml \( (P<0.05) \), \( (P<0.01 \) for 100 and 120 μg/ml EGB 761). Cultures treated with 60 μg/ml EGB 761 compared with untreated controls showed no significant neurogenic difference \( (P=0.07) \). To further validate these data, we conducted an experiment with a “positive control” using rolipram, which is known to stimulate CREB phosphorylation and subsequent neurogenesis in the animal model and in neuronal cells (44). Cells treated with rolipram (1 μM) for 48 h significantly enhanced neurogenesis compared with untreated controls. In contrast, EGB-761 (100 μg/ml) -induced neurogenesis was blocked in cells cotreated with H89 (50 μM for 48 h), which has been reported to block neurogenesis by blocking its upstream transcription factor CREB phosphorylation via inhibition of kinase PKA (43). These results further confirm the specific effects of EGB 761 on neurogenesis and implicate the involvement of CREB activation.

**The majority of the proliferating cells in adult hippocampus are neurons**

To determine the proportion of BrdU incorporated cells that were neurons vs. glial cells, coinunostaining was performed. Among the 230 BrdU immunopositive cells examined (from 16 brain sections, 8 for each genotype), >95% of the newly formed cells were found to differentiate into neurons, and <1% into glial cells (Fig. 2A). The differentiation of newly formed cells into neurons or glial cells was not affected by EGB 761 treatment, genotype, or age (data not shown).

Next, the expression pattern of the major isomer of the immature neuronal marker NCAM-180 was examined by Western blotting (Fig. 2B). Quantitative analysis shows a similar pattern (Fig. 2C), as observed with neurogenesis (Fig. 1B). A significant increase of NCAM-180 was observed in EGB 761-treated young (6 months, open columns) and aged (22 months, filled columns) Tg mice (Tg/EGb) compared with untreated age-matched controls (Tg/Ctrl, \( P<0.01 \)). This increase in the expression of NCAM was also
Figure 1. EGB 761 enhances cell proliferation in the DG of TgAPP/PS1 mice and in primary cultures of embryonic brain cells. A) Representative confocal images showing BrdU-positive cells (green) in the DG of young (6 months, a–d) and old (22 months, e–h), WT (a, b and e, f) and Tg (c, d and g, h) mice maintained on a dietary regimen supplemented with (a, c and e, g) or without (b, d and f, h) 100 mg/kg of EGB 761 for 1 month and intraperitonially injected with BrdU (75 mg/kg/daily) for 7 consecutive days. B) Quantification of BrdU incorporated cells from fluorescence images presented in panel A. BrdU-positive cells were counted in both DG of one brain section taken out of every consecutive seven, and were expressed as the mean ± se of total number of BrdU-positive cells/DG of 3–4 WT or Tg mice per treatment category (see Materials and Methods for serology). **P < 0.01 (3-way ANOVA). C) Representative immuno-fluorescence images of BrdU (green) and neuronal marker NeuN (red) from the primary culture of WT embryonic brain cells. A range of EGB 761 concentrations (0, 60, 80, 100, or 120 μg/ml) and 20 μM BrdU were coincubated with isolated WT mice embryonic brain cells for 48 h, followed by immunostaining. As controls, the cells were treated in triplicate with Rolipram (1 μM), which is known to stimulate CREB phosphorylation, or a protein kinase A inhibitor (PKAI or H89) that blocks CREB phosphorylation (50 μM) for 48 h intermitted by a change of culture medium containing the treatment. D) Quantification of BrdU-positive cells in primary culture of embryonic brain cells untreated (control) or treated with EGB 761. Values are mean ± se. *P < 0.05, **P < 0.01 (3-way ANOVA) compared with values from control (three cultures per condition and five field captured per culture). Scale bars = 200 μm.
present in the aged WT mice maintained on an EGb 761-supplemented diet (open columns, WT/Ctrl vs. WT/EGb, \( P < 0.01 \)). However, no significant difference was found in young (6 months) WT mice treated with or without EGb 761 (filled columns, WT/Ctrl vs. WT/EGb, \( P = 0.5 \)). Moreover, there was a significant decrease of NCAM in the Tg mice (Tg/Ctrl) compared with wild type (WT/Ctrl), and this decrease was augmented with advanced age (22 months, \( P < 0.01 \)), suggesting that expression of the transgenes affects the expression of the immature neuron marker NCAM-180 (Fig. 2B, C).

Aβ oligomers impair cell proliferation and inhibit phosphorylation of CREB in the hippocampus of the transgenic mice

Aβ deposition has been shown to impair neurogenesis in cortical neurons and in AD mice (15, 18). Next we examined the impact of Aβ oligomers on cell proliferation by double labeling BrdU and Aβ oligomers using specific antibody A11 (45). Apparently, Aβ oligomers impair cell proliferation, as illustrated in Fig. 3A: the disruption of the paired division pattern of cells located in close proximity to Aβ suggests that the division of cells associated with Aβ oligomers was impaired. Those away from the Aβ oligomers show a paired or complete division pattern. Subsequently, the effect of Aβ aggregation on pCREB expression in the hippocampus of Tg mice was assessed. Figure 3B demonstrates that the aggregation of Aβ completely blocks pCREB in the same regions of the DG of the hippocampus (arrows). Furthermore, levels of pCREB and CREB expression in proliferating cells were evaluated by double labeling. Quantitative analysis shows that compared with nonproliferating cells, BrdU-positive cells exhibit a 3-fold increase in pCREB (Fig. 3C, open bars, \( P < 0.01 \)), but not CREB (Fig. 3C, \( P = 0.2 \), filled bars). This suggests that activity of CREB rather than CREB protein synthesis plays a role in mediating cell proliferation.

EGb 761 reduces Aβ oligomers and restores pCREB levels in the hippocampus of transgenic mice

Given the impact of Aβ aggregation on cell proliferation and pCREB expression, the effect of EGb 761 treatment on these two proteins was assessed in the brains of Tg mice (12 months old). As demonstrated in Fig. 4A, B, EGb 761 treatment for 1 month (Tg/EGb) significantly decreases Aβ oligomers at a molecular size of ~21 kDa compared with untreated controls (Tg/Ctrl, \( P = 0.02 \)). The reduced pCREB levels in the Tg mice (Tg/Ctrl) compared with that of WT control mice (WT/Ctrl) was partially restored by EGb 761 treatment.
vs. Egb 761 untreated WT mice of the same age ($P<0.01$, Fig. 4C, D). In WT mice, Egb 761 treatment only induced a moderate increase in pCREB levels when compared with untreated control mice of the same age ($P>0.05$; Fig. 4C, D). There was no significant difference in levels of CREB expression in WT and Tg mice treated with or without Egb 761 ($P>0.05$, Fig. 4D). These results suggest that the neurogenic effects of Egb 761 include a possible association between pCREB expression and beta amyloid aggregation.

**DISCUSSION**

Neurogenesis is considered to be a neuronal replacement therapeutic approach for neurodegenerative disorders, including AD. In this study, we tested the hypothesis that the beneficial effect of Egb 761 on dementia in humans (28) and on cognitive impairment of AD mice (39) is mediated by enhanced adult neurogenesis in their hippocampus. The present results provide evidence to support this hypothesis by demonstrating that 1) Egb 761 enhances adult neurogenesis in hippocampal neuronal cells of the AD mice (TgAPP/PS1); and 2) neurogenesis by Egb 761 is associated with enhanced phosphorylation of CREB as well as reduced Aβ oligomers in the AD mice.

Previous studies by Stackman and colleagues demonstrated that chronic administration of Egb 761 to the Tg2576 mice enhanced spatial learning and cognition (39). Since Tg2576 mice share the same transgene and cognitive deficits as the TgAPP/PS1 mice used in the present study, Egb 761-induced neurogenesis (Fig. 1)
may provide a mechanism through which EGb 761 enhances cognitive performance (39). Along with observations of increased Aβ deposition with advanced age and its association with an increased loss of neuronal cells (18), these findings underscore the ability of EGb 761 to induce neurogenesis as compensation for the cell loss seen in AD. This notion is further supported by the observation of a similar increase in the pattern of NCAM found in the hippocampus of Tg mice treated with EGb 761 (Fig. 2B, C). The dose dependency of EGb 761 on neurogenesis assessed by double labeling of embryonic stem cells validates its specificity and provides a tool for initial screening. A good correlation has been reported between neurogenesis in rodent embryonic culture and in the adult hippocampus by Aβ and neurogenesis agents (15, 46).

The physical association between proliferating cells and toxic Aβ oligomers obtained by double labeling for BrdU and Aβ oligomers is striking. EGb 761 has been reported to inhibit Aβ oligomerization both in solution (36, 40) and in a transgenic C. elegans model of Alzheimer’s disease (47) in contrast to observations showing no effect of EGb 761 on soluble and fibril Aβ levels in the mouse model of AD (39). An explanation could be that the toxic Aβ oligomers and nontoxic monomers are both present in the soluble fraction (48) and that there is equilibrium between the oligomers and the monomers (49). EGb 761 may prevent oligomerization or shift the equilibrium from the toxic oligomeric conformation to the nontoxic monomers, which would not affect the total level of soluble Aβ as determined by ELISA (39).

The immuno-histofluorescence double labeling for Aβ and pCREB presented in Fig. 3B demonstrates that cells of the GLC, SGZ, and the hilus zone, which are immuno-negative to Aβ, are immuno-positive to pCREB antibodies, suggesting that Aβ inhibits CREB activation. These observations are consistent with those reported by Vitolo and colleagues (43), who demonstrated that treatment of hippocampal neurons with Aβ (1 μM) inhibited CREB phosphorylation vis a the inactivation of protein kinase A, which is an upstream kinase for CREB. Furthermore, they showed that downregulation of protein kinase A and of CREB phosphorylation by Aβ could be reversed by rolipram, which enhances the cAMP signaling pathway. Alternatively, PS-1 mutant present in these mice could directly or indirectly down-regulate CREB-CBP signaling as well (50). To this end, EGb 761 has been reported to increase a small αAPP release, a nontoxic, nonamyloidogenic metabolite of APP, through a PKC-independent manner in hippocampi and cortices of EGB761-treated rats (51). They proposed that the benefit of EGb761 in clinical studies is underscored by directly affecting the release of the nonamyloidogenic metabolite (51).

The statistically significant increase in pCREB expression in Tg, but not WT, mice treated with EGb 761 observed in this study is consistent with our previous findings in WT mice (52). Most important, we observed by double labeling for BrdU incorporation and pCREB expression a 3-fold increase in pCREB levels in BrdU-positive cells compared with BrdU negative ones, indicating that the increased neurogenesis due to EGb 761 treatment would be mediated by an increase in pCREB expression. Taking into account the neurogenic regulating properties of pCREB and its association with Aβ and long-term potentiation (53), it is possible that the enhanced neurogenesis by EGb 761 is associated with its inhibitory effect on Aβ oligomerization (Fig. 4A, B). This effect may be augmented by some of EGb 761’s constituents directly regulating the CREB-mediated signaling pathway (38).

Although other downstream effector of CREB activation by EGb 761 is unknown, it could be multiple (54, 55). pCREB has been known to also mediate important neurosurvival signaling pathway by increasing the expression of Bcl-2 and spinophilin (56, 57). We previously reported, by microarray assay, an up-regulation of Bcl-2 in the neuronal cells treated with EGb 761 (37) that would be mediated via CREB activation. Studies using specific pharmacological modulators and/or inhibitors would allow revealing specific mechanism of CREB activation by EGb 761. Primary hippocampal cell culture might provide a better means for pharmacological manipulation and mechanistic studies.

Considering the deleterious effect of the association between aging and Aβ in the DG and the role of newly generated neurons in hippocampal-dependent learning and memory (58), the neurogenic enhancing property of EGb 761 and the potential mechanisms suggested by this study qualify this top-selling dietary supplement to be a therapeutic candidate in the management of Alzheimer’s dementia.

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