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Ginkgo biloba L. extract protects against chronic cerebral hypoperfusion by modulating neuroinflammation and the cholinergic system

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ABSTRACT

Background: Ginkgo biloba extract (GBE)—a widely used nutraceutical—is reported to have diverse functions, including positive effects on memory and vasodilatory properties. Although numerous studies have assessed the neuroprotective properties of GBE in ischemia, only a few studies have investigated the neuro-pharmacological mechanisms of action of GBE in chronic cerebral hypoperfusion (CCH).
Purpose: In the present study, we sought to determine the effects of GBE on CCH-induced neuroinflammation and cholinergic dysfunction in a rat model of bilateral common carotid artery occlusion (BCCAo).
Methods: Chronic BCCAo was induced in adult male Wistar rats to reflect the CCH conditions. On day 21 after BCCAo, the animals were treated orally with saline or GBE (5, 10, 20, and 40 mg/kg) daily for 42 days. After the final treatment, brain tissues were isolated for the immunohistochemical analysis of glial markers and choline acetyltransferase (ChAT), as well as for the western blot analysis of proinflammatory cytokines, toll-like receptor (TLR)-related pathway, receptor for advanced glycation end products (RAGE), angiotensin-II (Ang-II), and phosphorylated mitogen-activated protein kinases (MAPKs).
Results: BCCAo increased glial proliferation in the hippocampus and white matter, whereas proliferation was significantly attenuated by GBE treatment. GBE also attenuated the BCCAo-related increases in the hippocampal expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6), TLR4, myeloid differentiation primary response gene 88, RAGE, Ang-II, and phosphorylated MAPKs (ERK, p38, and JNK). Furthermore, GBE treatment restored the ChAT expression in the basal forebrain following BCCAo.
Conclusions: These findings suggest that GBE has specific neuroprotective effects that may be useful for the treatment of CCH. The pharmacological mechanism of GBE partly involves the modulation of inflammatory mediators and the cholinergic system.

Introduction

Ginkgo biloba L. (Ginkgoaceae) leaf extract (GBE) is among the most widely used traditional herbal medicines, and has been used to treat various diseases including depression, anxiety, headache, and memory impairment in Asia and Europe (Shah et al., 2011; Zhang et al., 2012). With regard to composition, flavonoids—such as lipoxygenase and phospholipase A2—comprise 22–27% of GBE, whereas terpene lactones—including ginkgolides A, B, C, J, and bilobalide—comprise 5–7% of GBE (Kwak et al., 2012). To date, experimental evidence has indicated that GBE is a useful therapeutic agent for disorders of cerebral circulation (Zhang et al., 2012). Moreover, GBE treatment has been reported to alleviate hippocampal neuronal death following transient global ischemia via anti-inflammatory and anti-oxidative mechanisms (Tulsulkar and Shah, 2013), and has also been shown to improve memory deficits after transient ischemia in object location (Rocher et al., 2011) and aversive radial maze tasks (Paganelli et al., 2006).

Abbreviations: Ang-II, angiotensin II; BCCAo, Bilateral common carotid artery occlusion; CCH, chronic cerebral hypoperfusion; ChAT, choline acetyltransferase; DG, dentate gyrus; ERK, extracellular signal-regulated kinase; GBE, *Ginkgo biloba* extract; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; TLR, toll-like receptor; RAGE, receptor for advanced glycation end products; VaD, vascular dementia.

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The majority of evidence for the protective effects of GBE on vessel occlusion is derived from models of ischemia that mimic stroke. In addition to stroke, chronic cerebral hypoperfusion (CCH) is another serious disorder that is typically aging-related and can result in vascular dementia (VaD) (Lana et al., 2014). However, unlike stroke, which leads to severe focal brain damage, CCH results in mild global damage over time (Farkas et al., 2007). Hence, CCH models are typically used to investigate the long-term effects of vessel occlusion. By using a CCH model, previous studies have shown that GBE treatment resolved memory impairments, as measured by the Morris water maze (Li et al., 2013) and the eight-arm radial maze (Kwak et al., 2012); in fact, these effects were found to be associated with the inhibition of apoptosis and oxidative response.

The most commonly observed characteristics of CCH pathology are neuroinflammation and cholinergic deficiency (Choi et al., 2011); however, the effects of GBE on inflammatory responses and cholinergic dysfunction in CCH remain unclear. A recent study showed that GBE prevents the development of lung inflammatory responses, induced by lipopolysaccharide, by inhibiting NF-kB-p65 and Cox-2 (Xin et al., 2015). In addition, cholinergic neurotransmission in the rat medial prefrontal cortex was found to be enhanced by treatment with GBE and its flavonol derivatives (Kehr et al., 2012). Because GBE has protective effects against inflammation and cholinergic dysfunction, it is also expected to be able to treat related symptoms observed in CCH.

Therefore, in the present study, we aimed to examine the impact of GBE on inflammation and cholinergic deficits related to CCH. We induced permanent bilateral common carotid artery occlusion (BCCAo) in rats to establish a CCH model, which is a widely used preclinical model for VaD therapeutic screening (Kwon et al., 2014). BCCAo rats were orally dosed with GBE extracted from *Ginkgo biloba* leaves (commercial name: Tanasol solution [Yuyu Pharmaceutical company, Seoul, Korea]), and the glial populations were evaluated in the white matter and hippocampus, which are regions vulnerable to CCH (Ueno et al., 2015). Thereafter, we investigated the protective effects of GBE in BCCAo by studying the expression of toll-like receptor (TLR) pathways and choline acetyltransferase (ChAT). To our knowledge, the present study is the first such evaluation on this topic and provides important mechanistic insights into the therapeutic benefits of GBE in CCH.

Materials and methods

Animals

All the procedures described in this study were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Oriental Medicine (KIOM) (permit number: 15-018). Male Wistar rats (280 ± 10 g, 12 weeks) were obtained commercially (Charles River Co., Gapyung, South Korea). For 2 weeks prior to the experiment, rats were housed in a vivarium at the KIOM, in an environment with controlled temperature (22 ± 1 °C) and humidity (55 ± 10%), on a 12-h light/dark cycle, with water and food available *ad libitum*.

Surgery

Rats were anesthetized with 5% isoflurane in a mixture of 30% oxygen/70% nitrogen and a modified BCCAo surgery was performed (Choi et al., 2011). Briefly, a skin incision was made to expose both the common carotid arteries, which were then separated from the vagus nerve and its sheath. Both arteries were tightly double-ligated with 4-0 silk sutures and the incision was closed. The sham group underwent the same procedure without carotid artery ligation. During the surgical procedure, the body temperature was monitored and maintained at 37.0 ± 0.5 °C and all efforts were made to minimize pain and distress.

Pharmacological treatment

Ginkgo biloba L. is an accepted plant name, and is listed in The Plant List (www.theplantlist.org). Tanasol Solution (40 mg/20 ml liquid bottle, batch number 150253, Yuyu Pharmaceutical Company, Seoul, Korea)—a commercial drug containing GBE—was used in this study. The drug:genuine extract ratio (DER) was between 35:1 and 67:1. As stated by the manufacturer, the drug contains 3 major components: quercetin (229.11 ± 5.06 μg/ml), kaempferol (224.01 ± 1.37 μg/ml), and isorhamnetin (52.88 ± 4.55 μg/ml); these components are the principal flavonoids in *Ginkgo biloba* and are listed as marker compounds for quality control in the Korean pharmacopeia. All the rats were randomly assigned to 1 of 6 treatment groups: (1) sham-operated + saline ($n = 12$), (2) BCCAo + saline ($n = 12$), (3) BCCAo + GBE 5 mg/kg ($n = 12$), (4) BCCAo + GBE 10 mg/kg ($n = 12$), (5) BCCAo + GBE 20 mg/kg ($n = 12$), or (6) BCCAo + GBE 40 mg/kg ($n = 12$). GBE and vehicle were administered daily via oral gavage from 21 days after surgery to 63 days after surgery (treatment period, 42 days). The dose range of GBE was determined based on previous reports (Kwak et al., 2012; Nooshinfar et al., 2008). Animals were sacrificed at 63 days after surgery and tissues were collected for analysis.

Immunohistochemical analysis

The immunohistochemical analysis of glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule-1 (Iba-1), and ChAT was performed to determine the number of astrocytes, microglia, and cholinergic neurons, respectively. Whole brains were immersed in 4% paraformaldehyde for 3 days, cryoprotected in 30% sucrose, and then stored at −70 °C until use. Brain cryosections (40 μm) were prepared and incubated with GFAP (Abcam, CA, USA), Iba-1 (Wako, Tokyo, Japan), or ChAT (Millipore, MA, USA) primary antibodies in phosphate buffered saline (PBS) containing 3% casein and 0.1% Triton-X 100 overnight at 4 °C. After washing with PBS, the tissues were incubated with anti-rabbit IgG secondary antibody (Cell Signaling, MA, USA). Tissues were subsequently exposed to a Vector SG substrate kit and a Vector DAB kit (Vector Laboratories, CA, USA) for peroxidase staining, and then mounted onto resin-coated slides by using Permount reagent (Fisher Scientific, PA, USA). All immunoreactions were examined using light microscopy (Bx 51; Olympus, Japan), and the number of cells stained positive for Iba-1 and GFAP was assessed in specific regions (0.03 mm^2) of white matter (the corpus callosum, fimbria, and optic tract) and the hippocampus (CA1, CA3, and dentate gyrus [DG]). The number of ChAT-positive neurons was quantified in regions (0.03 mm^2) of the basal forebrain. A minimum of 3 sections were selected per rat and averaged for quantification.

Western blot analysis

Western blot was performed as previously described with some modifications (Kim et al., 2015). Briefly, hippocampal tissue was dissected and homogenized in cold lysis buffer containing 25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% nonyl phenoxypolyethoxylethanol, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, (Thermo Scientific, MA, USA), and protease and phosphatase inhibitor cocktail solutions (GenDEPOT, TX, USA). Homogenates were centrifuged at 20,000 × g for 30 min at 4 °C, and the supernatants were collected and stored at −70 °C until use. Protein concentrations were determined by using Bradford's reagent (Bio-Rad, CA, USA), and equivalent amounts of protein (40 μg) were electrophoresed by using SDS-PAGE. The proteins

were transferred to a PVDF membrane and blocked in 5% dry non-fat milk for 1 h at room temperature, followed by overnight incubation at 4 °C with the following primary antibodies: tumor necrosis factor alpha (TNF-α, Santa Cruz, CA, USA); interleukin-1 beta (IL-1β, Millipore); IL-6 (Abcam); TLR2 (Santa Cruz); TLR4 (Santa Cruz); myeloid differentiation factor 88 (MyD88, Santa Cruz); Toll/IL-1R domain–containing adaptor-inducing IFN-β (TRIF, Santa Cruz); receptor for advanced glycation end products (RAGE, Abcam); angiotensin-II (Ang-II, Abcam); extracellular signal-regulated kinase (ERK, Cell signaling); p38 MAPK (Cell Signaling); and c-Jun N-terminal kinases (JNK, Cell Signaling). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Santa Cruz) was used as the internal loading control. After primary incubation, the membranes were washed and incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling). Western blots were visualized by using an ECL system (Thermo Scientific) with a Lumino Image Analyzer (Las-4000; Fujifilm, Tokyo, Japan). The densitometric values of the bands of interest were normalized to the GAPDH values by using Multi Gauge software (Fujifilm).

Statistical analysis

All data are expressed as mean ± standard deviation. The data shown are the average of 2 independent replicate experiments. For statistical analysis, the homogeneity of variances was tested by Levene's test. Between-group comparisons were performed by using a one-way ANOVA with a post hoc test (the Least Significant Difference test), and p-values ≤ 0.05 were considered to be statistically significant. If the variances were not homogeneous, a Tamhane test was applied as a post-hoc test. To detect a dose-related effect, a regression with linear trend estimation was used. All the statistical analyses were performed using SPSS 20.0 software (IBM, IL, USA).

Results

Effects of GBE on the glia in the hippocampus and white matter

A common feature of the BCCAo rat model is glial (microglia and astrocytes) proliferation (Bang et al., 2013; Cai et al., 2010; Cho et al., 2006). Therefore, we quantified the Iba-1- and GFAP-positive cells in the hippocampal subregions (including the CA1, CA3, and DG cells) as well as the major white matter regions of the brain (including the corpus callosum, fimbria, and optic tract). The number of Iba-1-positive cells was estimated to indicate the number of microglia (Fig. 1). A one-way ANOVA revealed significant between-group effects in all the regions investigated ($F_{(5,30)} \geq 10.446$, $p < 0.001$). The number of microglia in the hippocampus and white matter was significantly greater in the BCCAo group than in the sham group (###$p < 0.001$; Fig. 1A-D). However, the administration of GBE attenuated the BCCAo-induced microglial proliferation in the hippocampus and white matter, particularly at a dose of 40 mg/kg (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Fig. 1A-D). The number of microglia in the BCCAo + GBE 40 mg/kg group was not significantly different from that in the sham group. GBE exhibited the dose-related inhibition of microglial proliferation.

The number of GFAP-positive cells was also estimated to reflect the number of astrocytes in the hippocampus and white matter regions (Fig. 2). A one-way ANOVA showed that the effects between groups were significant in all the regions investigated ($F_{(5,30)} \geq 5.192$, $p < 0.002$). Post-hoc analyses revealed that the number of astrocytes was significantly greater in the BCCAo group than in the sham group, and decreased following GBE treatment after BCCAo, particularly at a dose of 40 mg/kg (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Fig. 2A-D). GBE exhibited the dose-related inhibition of astrocytic proliferation. These results indicate that GBE inhibits the BCCAo-induced glial proliferation in the hippocampus and white matter.

Effects of GBE on the levels of hippocampal proinflammatory cytokines

Previous studies have reported that glia secrete proinflammatory cytokines in pathological conditions (Smith et al., 2012; Yasuda et al., 2011). To investigate the effects of GBE on the increased levels of proinflammatory cytokines, the levels of TNF-α, IL-1β, and IL-6 were measured in the hippocampus. A one-way ANOVA revealed significant between-group effects for the expression levels of proinflammatory cytokines ($F_{(5,30)} \geq 4.812$, $p < 0.002$). According to post-hoc analyses, the expression of TNF-α, IL-1β, and IL-6 was greater in the hippocampus of the BCCAo group than in the sham group (##$p < 0.01$, ###$p < 0.001$; Fig. 3A-B). Importantly, the BCCAo-related increases in hippocampal cytokine expression were attenuated by GBE treatment at doses of 20 and 40 mg/kg (**$p < 0.01$, ***$p < 0.001$; Fig. 3A-B). GBE also showed dose-related effects in mitigating increases in hippocampal proinflammatory cytokines after BCCAo.

Effects of GBE on the hippocampal TLR4 pathway

Thereafter, we investigated whether GBE affected TLR signaling, which is known to play an important role in glial-derived neuroinflammation (Yao et al., 2013). A one-way ANOVA revealed significant between-group effects for the expression of TLR4 ($F_{(5,30)} = 7.641$, $p < 0.001$), but not TLR2 ($F_{(5,30)} = 1.783$, $p = 0.147$), in the hippocampus. Treatment with GBE at a dose of 40 mg/kg suppressed the BCCAo-related increases in hippocampal TLR4 expression, in a dose-related manner (**$p < 0.01$; Fig. 4A, 4D). We further investigated the hippocampal expression of TLR4 downstream mediators, TRIF and MyD88 (Tanimura et al., 2008). One-way ANOVA showed significant effects between groups for the MyD88 levels ($F_{(5,30)} = 15.228$, $p < 0.001$), but not for the TRIF levels ($F_{(5,30)} = 1.723$, $p = 0.160$). GBE treatment attenuated the BCCAo-related increases in MyD88 expression at doses of 20 and 40 mg/kg in a dose-related manner (***$p < 0.001$; Fig. 4B, 4E). These results suggest that GBE alleviates the BCCAo-related changes in the TLR4-MyD88-dependent pathway in rats.

Effects of GBE on the hippocampal expression of RAGE and Ang-II

RAGE and Ang-II are known to elicit the production of proinflammatory cytokines in the CNS (Agarwal et al., 2013; Srikanth et al., 2011). Therefore, we investigated the effect of GBE on the expression of RAGE and Ang-II in the hippocampus. A one-way ANOVA revealed significant between-group effects for the expression of RAGE ($F_{(5,30)} = 3.605$, $p = 0.011$) and Ang-II ($F_{(5,30)} = 2.493$, $p = 0.047$). According to the post-hoc analyses, GBE treatment at a dose of 40 mg/kg inhibited the BCCAo-induced increases in RAGE and Ang-II expression (*$p < 0.05$, **$p < 0.01$; Fig. 4C and F); however, the reduction did not appear to be dose-related.

Effects of GBE on the hippocampal expression of MAPK pathway components

Moreover, we investigated the effect of GBE on MAPK signaling, which is known to regulate the cellular inflammatory response (Lin et al., 2012). We specifically examined the phosphorylation status of ERK, p38, and JNK in the hippocampus. A one-way ANOVA revealed significant between-group effects for the phosphorylation of ERK ($F_{(5,30)} = 6.386$, $p < 0.001$) and p38

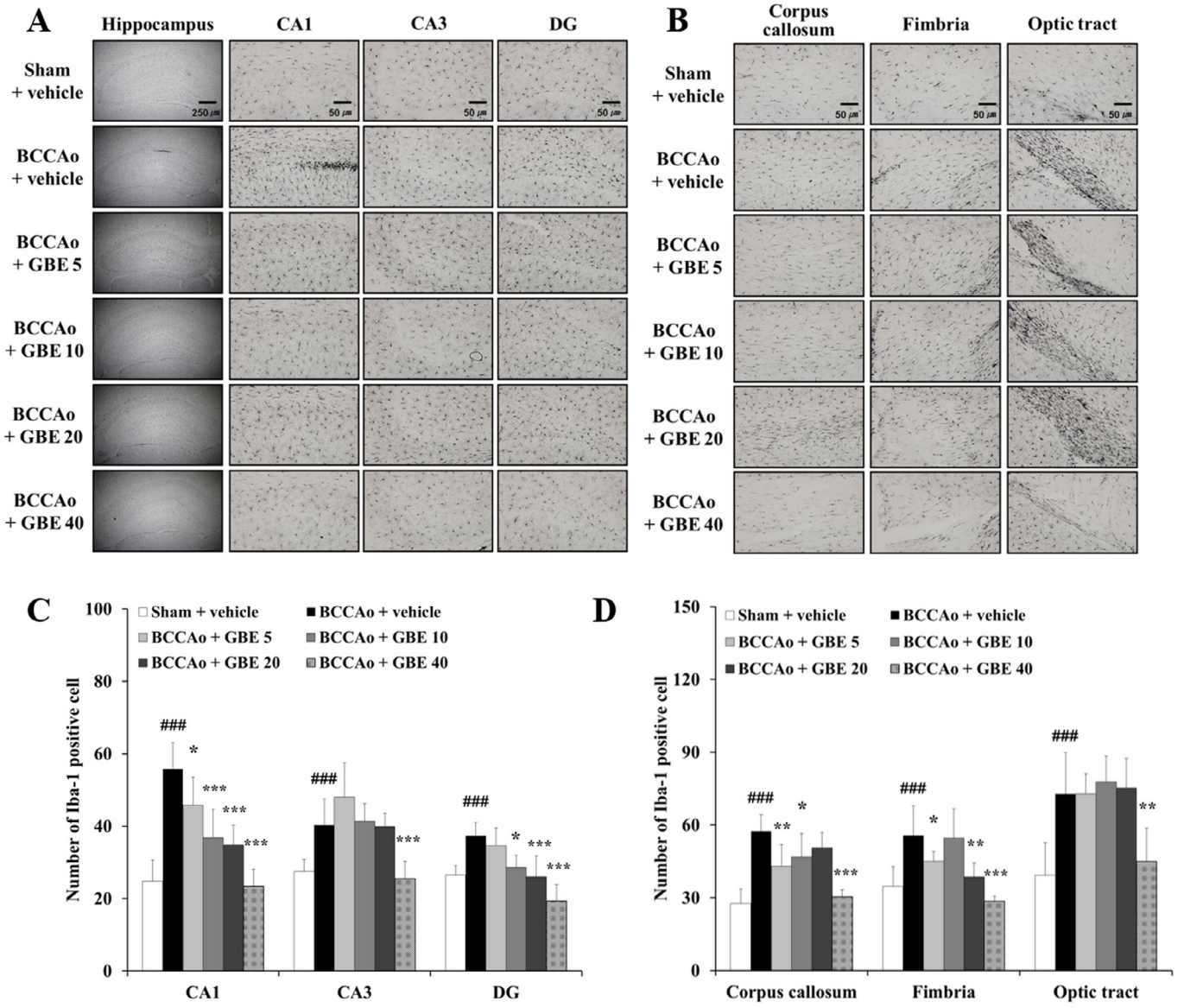

Fig. 1. GBE inhibits BCCAo-induced microglial proliferation in subregions of the hippocampus and white matter. (A and B) Representative Iba-1 staining in the hippocampal CA1, CA3, and dentate gyrus (DG) subregions (A) and in the corpus callosum, fimbria, and optic tract (B). (C and D) Measurement of the absolute numbers of Iba-1-positive microglia ($n=6$). ###$p < 0.001$ compared with sham plus vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with BCCAo plus vehicle. Original magnification was 40× for the entire hippocampus and 200× for other regions.

($F_{(5,30)} \geq 8.873$, $p < 0.001$), but not JNK ($F_{(5,30)} = 0.581$, $p = 0.714$). The total levels of ERK, p38, and JNK were unaffected by GBE treatment. As assessed by western blotting and further analyzed in post-hoc analyses, GBE treatment, particularly at a dose of 40 mg/kg, attenuated the BCCAo-induced changes in the phosphorylation status of ERK and p38 ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Fig. 5A-B), and showed a dose-related effect.

Effects of GBE on ChAT expression in the medial septum

Cholinergic dysfunction has been reported to develop in BCCAo rats (Choi et al., 2011), and has been found to be closely related to MAPK alterations in the brain (Williams et al., 2007). To examine the effects of GBE on cholinergic neurons, the number of ChAT-positive cells in the basal forebrain was estimated by using immunohistochemistry. Significant group effects were observed in the expression of ChAT ($F_{(5,30)} = 23.307$, $p < 0.001$). Post-hoc analyses revealed that the number of ChAT-positive cells in the basal forebrain was lower in the BCCAo group than in the sham group (###$p < 0.001$; Fig. 6A-B). However, the reduction in the number of ChAT-positive cells was significantly resolved by GBE treatment at doses of 20 and 40 mg/kg ($^{*}p < 0.05$, $^{***}p < 0.001$; Fig. 6A-B), and exhibited dose-related effects. These results indicate that GBE treatment may be neuroprotective for cholinergic neurons in BCCAo-induced CCH.

Discussion

CCH leads to working and reference memory impairments and white matter injury that resemble VaD (Jiwa et al., 2010). Although VaD is the second most common cause of dementia, no widely accepted treatment or cure is currently available (Guerra et al., 2015). Given that the number of VaD patients is increasing in various aging societies, several studies have focused on novel therapeutic and preventative approaches for VaD, as well as better diagnostic criteria (Kalaria, 2016). To our knowledge, this is the first report to

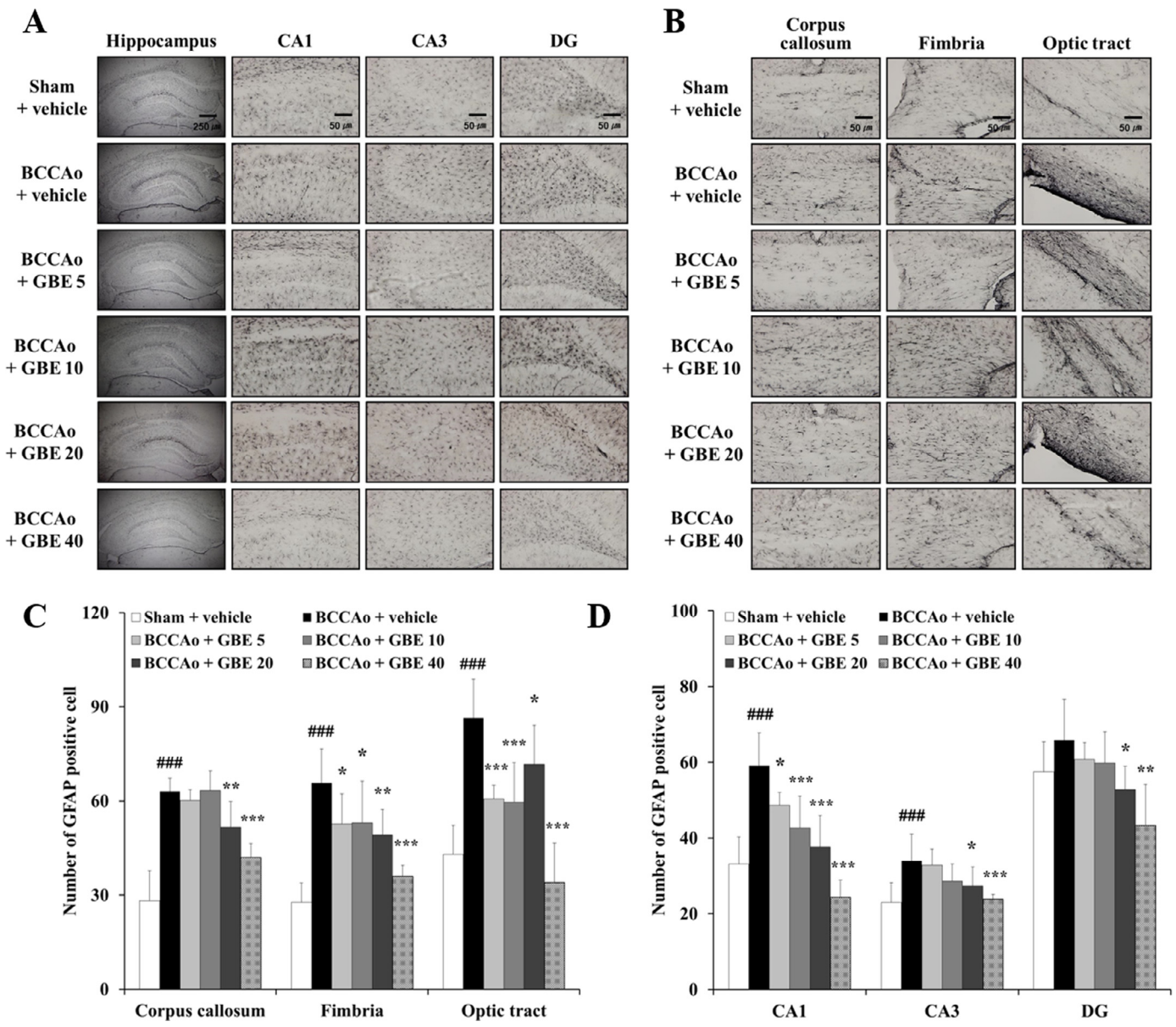

Fig. 2. GBE inhibits BCCAo-induced astrocyte proliferation in subregions of the hippocampus and white matter. (A and B) Representative GFAP staining in the hippocampal CA1, CA3, and dentate gyrus (DG) subregions (A) and in the corpus callosum, fimbria, and optic tract (B). (C and D) Measurement of the absolute numbers of GFAP-positive astrocytes ($n=6$). ###$p < 0.001$ compared with sham plus vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with BCCAo plus vehicle. Original magnification was 40× for the entire hippocampus and 200× for other regions.

show that GBE treatment ameliorates various pathological changes in the hippocampus and white matter in a rat model of BCCAo-induced CCH. We found that GBE resolved the CCH-related pathological changes, and accordingly may have therapeutic utility in the treatment of VaD.

GBE has traditionally been used for the treatment of age-related memory dysfunction. Bilobalide, ginkgolide A, ginkgolide B, and terpenes are thought to be the major active compounds in GBE that improve memory (Shah et al., 2011). Several studies on CCH models have demonstrated that GBE and its constituents are neuroprotective in nature. The white matter damage induced by BCCAo has been found to be attenuated by the administration of GBE (Kwak et al., 2012). Moreover, bilobalide has been reported to resolve the cognitive deficits in BCCAo through antioxidant and anti-apoptotic mechanisms (Li et al., 2013). In addition to oxidative stress and apoptosis, neuroinflammation and cholinergic dysfunction are the other common features of CCH. These features have been insufficiently studied in previous studies and were therefore the subject of our investigation.

A growing number of studies have reported that BCCAo elicits neuroinflammatory responses, including glial proliferation and the up-regulation of proinflammatory cytokines (Cai et al., 2010; Lee et al., 2013). Several studies have assessed the key roles of glia in neuroinflammation following central nervous system injury. Yasuda et al. demonstrated that the number of IL-1β-positive astrocytes is increased in the hippocampus 10 min after 4-vessel occlusion (Yasuda et al., 2011). Smith et al. reported that microglial responses following neuronal injury or ischemia led to the subsequent release of proinflammatory cytokines, including IL-1 and IL-6, and may have contributed to neurodegenerative pathology (Smith et al., 2012). Therefore, the inhibition of glial activation is a promising therapeutic strategy for CCH. In the present study, we found that GBE, particularly at higher doses, attenuated glial cell proliferation in the hippocampus and white matter, and re-

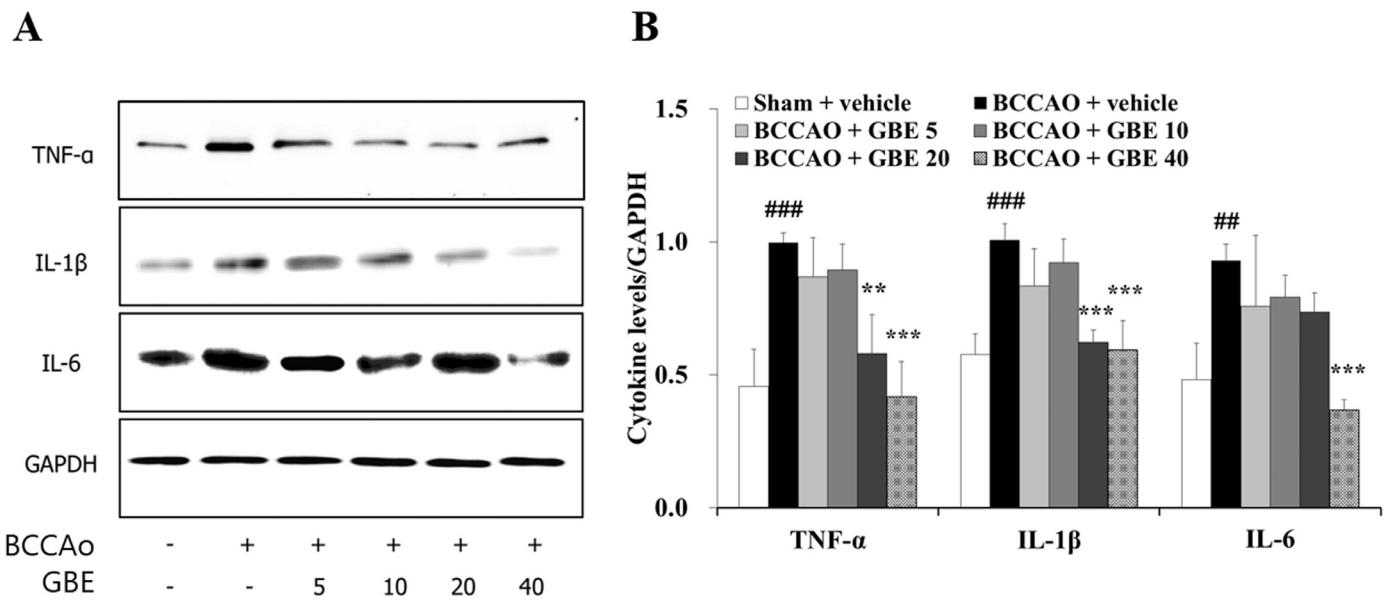

Fig. 3. GBE attenuates BCCAo-induced expression of proinflammatory cytokines in the hippocampus. (A) Representative western blots of proinflammatory cytokines in hippocampal tissues. GAPDH was used as the loading control. (B) Quantitative analysis of cytokine expression/GAPDH intensity ($n=6$). ##$p < 0.01$, ###$p < 0.001$ compared with sham plus vehicle; **$p < 0.01$, ***$p < 0.001$ compared with BCCAo plus vehicle.

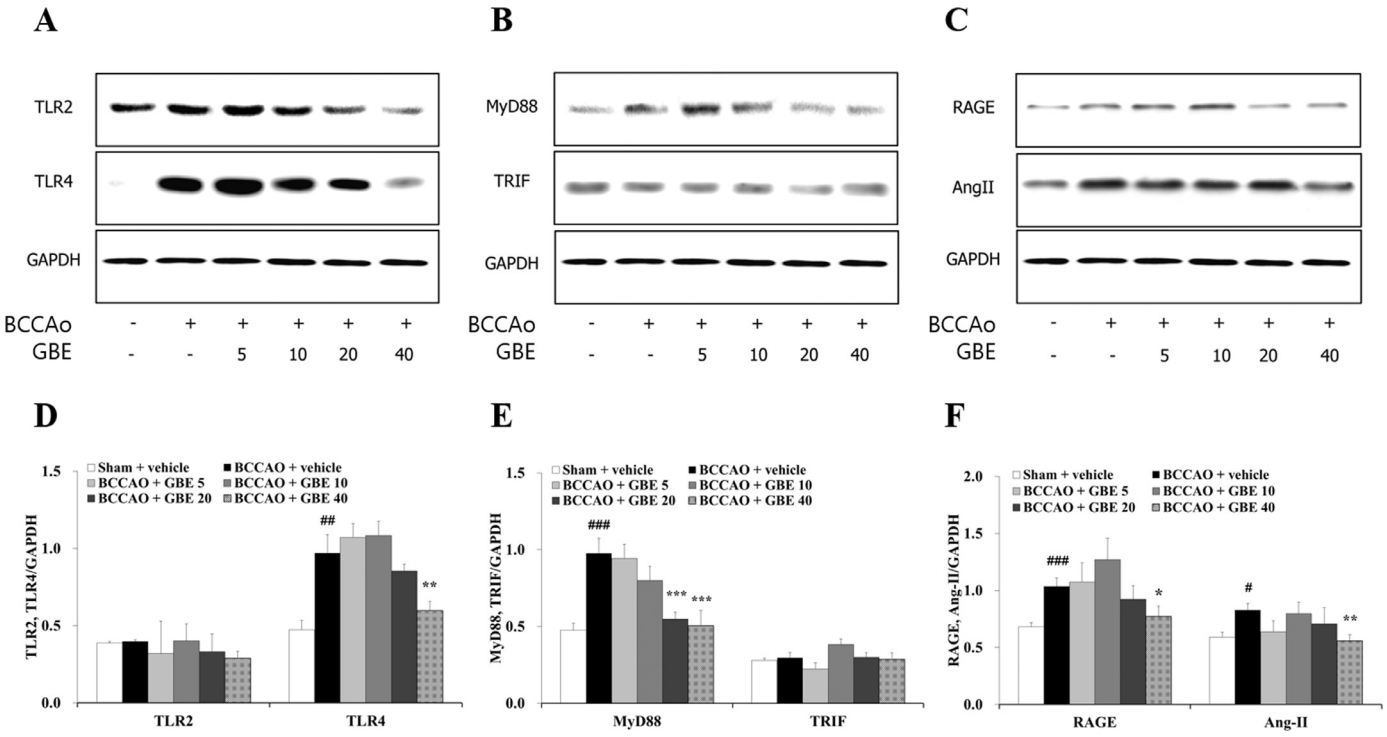

Fig. 4. GBE down-regulates the expression of TLR-related proteins, RAGE, and Ang-II following chronic BCCAo in the hippocampus. (A-C) Representative western blots of TLR2, TLR4, MyD88, TRIF, RAGE, and Ang-II in hippocampal tissues. GAPDH was used as the loading control. (D-F) Quantitative analysis of protein expression/GAPDH intensity ($n=6$). #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared with sham plus vehicle; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with BCCAo plus vehicle.

duced hippocampal proinflammatory cytokine production after BCCAo. Importantly, we decided to administer GBE orally to maximize the translational nature of our study, and initiated treatment on post-surgical day 21, based on the findings of a previous study wherein day 21 was estimated as the time of peak neuroinflammation after BCCAo (Bang et al., 2013). Our results support the hypothesis that GBE exerts an anti-inflammatory effect by inhibiting glial cell proliferation and activation.

To study the molecular mechanisms of the anti-inflammatory effect of GBE in CCH, we evaluated the expression of TLRs and TLR signaling-related proteins in the hippocampus. TLRs were found to be key target receptors for the innate immune response and proinflammatory cytokine production (Kawasaki and Kawai, 2014). In particular, TLR4 has been frequently cited in relation to neuroinflammation in the literature (Caso et al., 2007). In the present study, we found that GBE administration markedly down-regulated hippocampal TLR4 and MyD88 after BCCAo. These results suggest

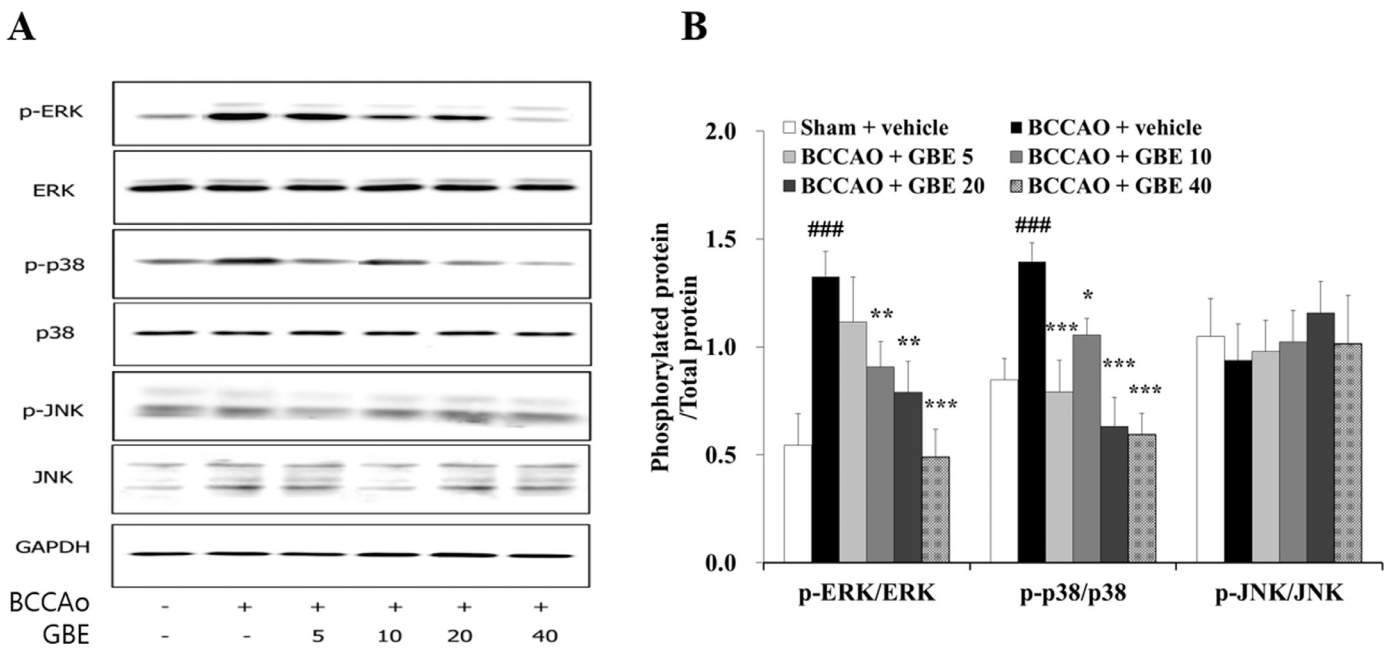

Fig. 5. GBE inhibits BCCAo-induced MAPK phosphorylation in the hippocampus. (A) Representative western blots of unphosphorylated and phosphorylated ERK, p38, and JNK in hippocampal tissues. (B) Quantitative analysis of phosphorylated MAPK expression/total MAPK intensity ($n=6$). ###$p < 0.001$ compared with sham plus vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with BCCAo plus vehicle.

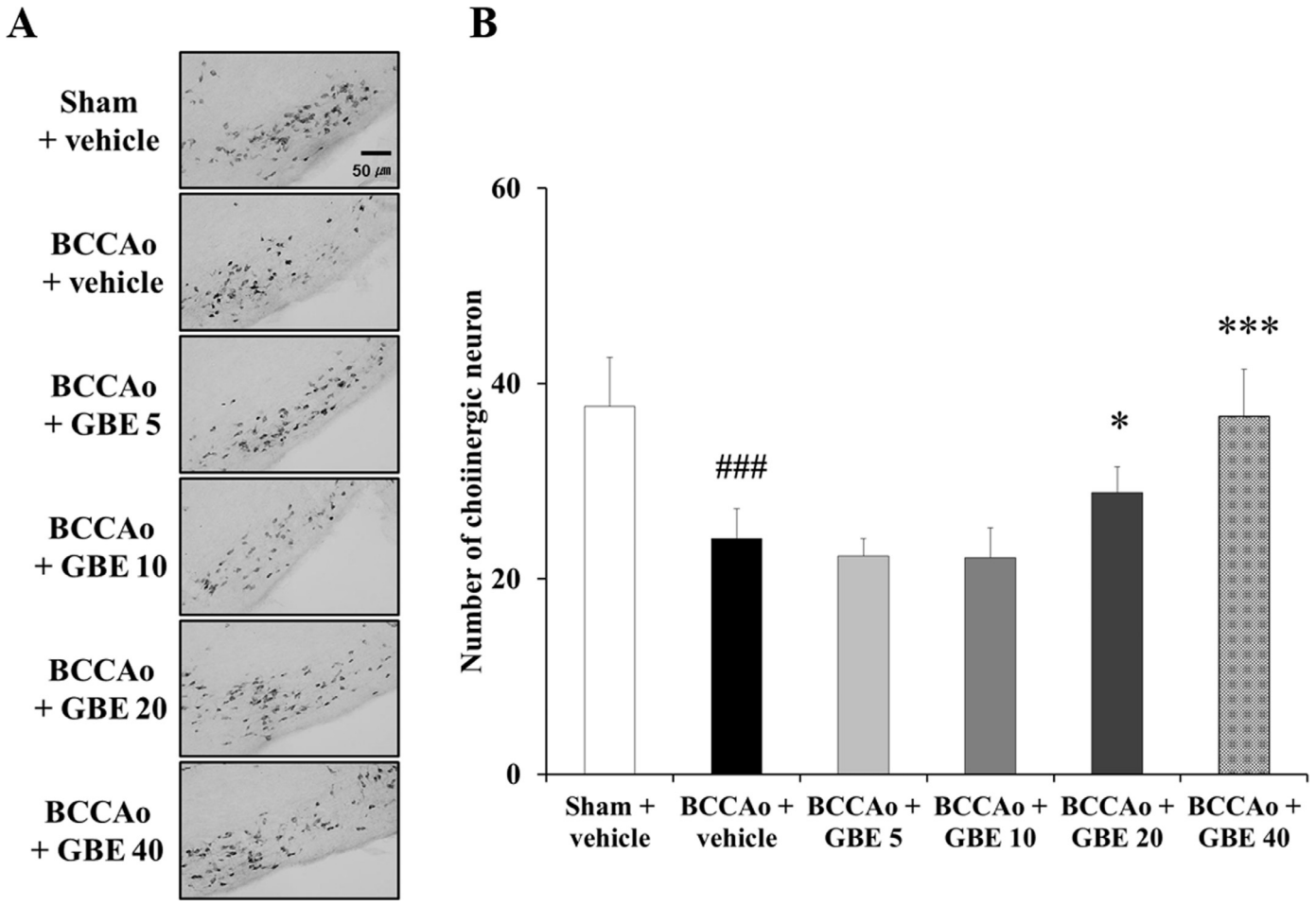

Fig. 6. GBE recovers ChAT-positive cholinergic neurons in the basal forebrain following chronic BCCAo. (A) Representative ChAT staining in the basal forebrain. (B) Measurement of the absolute numbers of ChAT-positive cholinergic neurons ($n=6$). ###$p < 0.001$ compared with sham plus vehicle; $^{*}p < 0.05$, $^{***}p < 0.001$ compared with BCCAo plus vehicle. Original magnification was 200×.

that GBE may have exerted anti-inflammatory effects via a mechanism involving the inhibition of MyD88-dependent TLR4 signaling.

Moreover, we investigated the expression of RAGE and Ang-II in the hippocampus following BCCAo and GBE treatment. RAGE has been reported to be responsible for the activation of inflammatory reactions and has a role in innate immunity (Yamamoto and Yamamoto, 2013). In contrast, Ang-II is a multifunctional peptide that has an important role in the production of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Jia et al., 2012). Our studies showed that treatment with GBE at a dose of 40 mg/kg significantly decreased hippocampal RAGE and Ang-II levels, thus indicating that GBE may regulate proinflammatory cytokines by inhibiting the expression of RAGE.

The activation of MAPKs, such as ERK, p38, and JNK, is correlated with the production of inflammatory mediators (Cho et al., 2015). Moreover, MAPK activation is considered to represent a primary consequence of MyD88-dependent TLR signaling (Kawai and Akira, 2010). Consistent with previous reports, hippocampal MAPKs such as ERK and p38 were found to be hyperphosphorylated in BCCAo rats (Lee et al., 2013). GBE treatment, particularly at a dose of 40 mg/kg, inhibited the BCCAo-induced phosphorylation of ERK and p38, without altering the total MAPK expression. Therefore, the inhibition of MAPK activation may be another mechanism by which GBE suppresses inflammation in CCH.

Finally, our study critically addressed the alterations in cholinergic function. Appropriate functionality of the cholinergic system is known to be essential for normal cognitive function. Approximately 40% of VaD patients showed significant reductions in acetylcholine (Ach) levels in the brain, and these alterations were thought to be responsible for memory dysfunction (Perry and Kalaria, 2002). BCCAo has been reported to decrease the expression of ChAT, an enzyme for Ach synthesis, in the basal forebrain of rats (Choi et al., 2011). Thus, the restoration of ChAT expression may be a useful therapeutic approach in VaD. Our data showed that GBE, particularly at a dose of 40 mg/kg, significantly increased ChAT expression in the basal forebrain of BCCAo rats. In addition, pretreatment with GBE has been shown to inhibit acetylcholinesterase activity, which could in turn increase the acetylcholine levels (Baradaran et al., 2012). These results readily explain the previous reports indicating improvements in cognitive function following GBE administration (Li et al., 2013). Nevertheless, additional studies are needed to directly observe the relationship between the effects reported herein and improved cognitive function, and furthermore to investigate the intracellular signaling pathways activated by GBE, such as cyclic adenosine monophosphate/protein kinase A-CREB.

Our research revealed that GBE affected neuroinflammation and the cholinergic system following CCH, but the exact mechanism underlying this pathological improvement remains unclear. However, a recent study reported that the anti-inflammatory function of GBE might involve the enhancement of autophagy. In particular, treatment with GBE significantly induced autophagy in an amyloid precursor protein-transgenic mouse model of Alzheimer's disease, and reduced the expression of NLRP3, which is a key component of the inflammasome (Liu et al., 2015). Since NLRP3 affects the activation of proinflammatory cytokines (Schroder and Tschopp, 2010), GBE treatment may inhibit inflammatory responses.

Conclusion

Overall, our data provide evidence indicating that GBE improves BCCAo-induced VaD-like pathology by alleviating glial proliferation, neuroinflammation, and cholinergic deficits. Given that CCH may damage the hippocampus and white matter in VaD, GBE may be a promising therapeutic treatment for patients with VaD.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Agarwal, D., Dange, R.B., Raizada, M.K., Francis, J., 2013. Angiotensin II causes imbalance between pro-and anti-inflammatory cytokines by modulating GSK-3β in neuronal culture. Br. J. Pharmacol. 169, 860–874.
Bang, J., Jeon, W.K., Lee, I.S., Han, J.S., Kim, B.Y., 2013. Biphasic functional regulation in hippocampus of rat with chronic cerebral hypoperfusion induced by permanent occlusion of bilateral common carotid artery. PLoS One 8, e70093.
Baradaran, A., Rabiei, Z., Rafieian, M., Shirzad, H., 2012. A review study on medicinal plants affecting amnesia through cholinergic system. J. HerbMed. Pharmacol. 1, 3–9.
Cai, Z.Y., Yan, Y., Chen, R., 2010. Minocycline reduces astrocytic reactivation and neuroinflammation in the hippocampus of a vascular cognitive impairment rat model. Neurosci. Bull. 26, 28–36.
Caso, J.R., Pradillo, J.M., Hurtado, O., Lorenzo, P., Moro, M.A., Lizasoain, I., 2007. Toll–like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation 115, 1599–1608.
Cho, K.O., La, H.O., Cho, Y.J., Sung, K.W., Kim, S.Y., 2006. Minocycline attenuates white matter damage in a rat model of chronic cerebral hypoperfusion. J. Neurosci. Res. 83, 285–291.
Cho, Y.-C., Ju, A., Kim, B.R., Cho, S., 2015. Anti-inflammatory effects of Crataeva nurvala Buch. Ham. are mediated via inactivation of ERK but not NF-κB. J. Ethnopharmacol. 162, 140–147.
Choi, B.R., Kwon, K.J., Park, S.H., Jeon, W.K., Han, S.H., Kim, H.Y., Han, J.S., 2011. Alternations of septal-hippocampal system in the adult Wistar rat with spatial memory impairments induced by chronic cerebral hypoperfusion. Exp. Neurobiol. 20, 92–99.
Farkas, E., Luiten, P.G., Bari, F., 2007. Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. Brain Res. Rev. 54, 162–180.
Guerra, A., Petrichella, S., Vollero, L., Ponzo, D., Pasqualetti, P., Määttä, S., Mervaala, E., Bressi, F., Iannello, G., Rossini, P., 2015. 56. Neurophysiological features of motor cortex excitability and plasticity in subcortical ischemic vascular dementia: a TMS mapping study. Clin. Neurophysiol. 126, e13.
Jia, L., Li, Y., Xiao, C., Du, J., 2012. Angiotensin II induces inflammation leading to cardiac remodeling. Front. Biosci. 17, 221–231.
Jiwa, N.S., Garrard, P., Hainsworth, A.H., 2010. Experimental models of vascular dementia and vascular cognitive impairment: a systematic review. J. Neurochem. 115, 814–828.
Kalaria, R.N., 2016. Neuropathological diagnosis of vascular cognitive impairment and vascular dementia with implications for Alzheimer's disease. Acta Neuropathol 131, 659–685.
Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11, 373–384.
Kawasaki, T., Kawai, T., 2014. Toll-like receptor signaling pathways. Front. Immunol 5.
Kehr, J., Yoshitake, S., Ijiri, S., Koch, E., Nöldner, M., Yoshitake, T., 2012. Ginkgo biloba leaf extract (EGb 761®) and its specific acylated flavonol constituents increase dopamine and acetylcholine levels in the rat medial prefrontal cortex: possible implications for the cognitive enhancing properties of EGb 761®. Int. Psychogeriatr 24, S25–S34.
Kim, M.-S., Jeon, W.K., Lee, K.W., Park, Y.H., Han, J.-S., 2015. Ameliorating effects of ethanol extract of Fructus mume on scopolamine-induced memory impairment in mice. Evid. Based Compl. Altern. Med. 2015 Article ID: 102734.
Kwak, P., Lim, S.C., Han, S.-R., Shon, Y.-M., Kim, Y.-I., 2012. Supra-additive neuroprotection by renexin, a mixed compound of ginkgo biloba extract and cilostazol, against apoptotic white matter changes in rat after chronic cerebral hypoperfusion. J. Clin. Neurol. 8, 284–292.
Kwon, K.J., Kim, M.K., Lee, E.J., Kim, J.N., Choi, B.R., Kim, S.Y., Cho, K.S., Han, J.S., Kim, H.Y., Shin, C.Y., Han, S.H., 2014. Effects of donepezil, an acetylcholinesterase inhibitor, on neurogenesis in a rat model of vascular dementia. J. Neurol. Sci. 347, 66–77.
Lana, D., Melani, A., Pugliese, A.M., Cipriani, S., Nosi, D., Pedata, F., Giovannini, M.G., 2014. The neuron-astrocyte-microglia triad in a rat model of chronic cerebral hypoperfusion: protective effect of dipyridamole. Front. Aging. Neurosci. 6, 1–17.
Lee, K.M., Bang, J.H., Han, J.S., Kim, B.Y., Lee, I.S., Kang, H.W., Jeon, W.K., 2013. Cardiotonic pill attenuates white matter and hippocampal damage via inhibiting microglial activation and downregulating ERK and p38 MAPK signaling in chronic cerebral hypoperfused rat. BMC Complement. Altern. Med. 13, 334.
Li, W.Z., Wu, W.Y., Huang, H., Wu, Y.Y., Yin, Y.Y., 2013. Protective effect of bilobalide on learning and memory impairment in rats with vascular dementia. Mol. Med. Rep. 8, 935–941.

Lin, M.C., Hui, C.F., Chen, J.Y., Wu, J.L., 2012. The antimicrobial peptide, shrimp anti-lipopolysaccharide factor (SALF), inhibits proinflammatory cytokine expressions through the MAPK and NF-kappaB pathways in Trichomonas vaginalis adherent to HeLa cells. Peptides 38, 197–207.

Liu, X., Hao, W., Qin, Y., Decker, Y., Wang, X., Burkart, M., Schötz, K., Menger, M.D., Fassbender, K., Liu, Y., 2015. Long-term treatment with Ginkgo biloba extract EGb 761 improves symptoms and pathology in a transgenic mouse model of Alzheimer's disease. Brain Behav. Immun. 46, 121–131.

Nooshinfar, E., Lashgari, R., Haghparast, A., Sajjadi, S., 2008. NMDA receptors are involved in Ginkgo extract-induced facilitation on memory retention of passive avoidance learning in rats. Neurosci. Lett. 432, 206–211.

Paganelli, R.A., Benetoli, A., Milani, H., 2006. Sustained neuroprotection and facilitation of behavioral recovery by the Ginkgo biloba extract, EGb 761, after transient forebrain ischemia in rats. Behav. Brain Res. 174, 70–77.

Perry, E., Kalaria, R., 2002. Neurotransmitter control of the cerebral vasculature and abnormalities in vascular dementia. In: Vascular Cognitive Impairment. Martin Duniz Ltd, London, UK, pp. 167–185.

Rocher, M.-N., Carré, D., Spinnewyn, B., Schulz, J., Delaflotte, S., Pignol, B., Chabrier, P.-E., Auguet, M., 2011. Long-term treatment with standardized Ginkgo biloba Extract (EGb 761) attenuates cognitive deficits and hippocampal neuron loss in a gerbil model of vascular dementia. Fitoterapia 82, 1075–1080.

Schroder, K., Tschopp, J., 2010. The inflammasomes. Cell 140, 821–832.

Shah, Z.A., Nada, S.E., Doré, S., 2011. Heme oxygenase 1, beneficial role in permanent ischemic stroke and in Gingko biloba (EGb 761) neuroprotection. Neuroscience 180, 248–255.

Smith, J.A., Das, A., Ray, S.K., Banik, N.L., 2012. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. Brain Res. Bull. 87, 10–20.

Srikanth, V., Maczurek, A., Phan, T., Steele, M., Westcott, B., Juskiw, D., Münch, G., 2011. Advanced glycation endproducts and their receptor RAGE in Alzheimer's disease. Neurobiol. Aging 32, 763–777.

Tanimura, N., Saitoh, S., Matsumoto, F., Akashi-Takamura, S., Miyake, K., 2008. Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. Biochem. Biophys. Res. Commun. 368, 94–99.

Tulsulkar, J., Shah, Z.A., 2013. Ginkgo biloba prevents transient global ischemia-induced delayed hippocampal neuronal death through antioxidant and anti-inflammatory mechanism. Neurochem. Int. 62, 189–197.

Ueno, Y., Koike, M., Shimada, Y., Shimura, H., Hira, K., Tanaka, R., Uchiyama, Y., Hattori, N., Urabe, T., 2015. L-carnitine enhances axonal plasticity and improves white-matter lesions after chronic hypoperfusion in rat brain. J. Cerebr. Blood. F. Met. 35, 382–391.

Williams, B., Granholm, A.C., Sambamurti, K., 2007. Age-dependent loss of NGF signaling in the rat basal forebrain is due to disrupted MAPK activation. Neurosci. Lett. 413, 110–114.

Xin, Y., Nan, C., Chun-Hua, M., Jing, T., Jian-An, B., Zong-Qi, C., Zu-Tao, C., Li-Yan, M., 2015. Ginkgo biloba extracts attenuate lipopolysaccharide-induced inflammatory responses in acute lung injury by inhibiting the COX-2 and NF-κB pathways. Chin. J. Nat. Med. 13, 52–58.

Yamamoto, Y., Yamamoto, H., 2013. RAGE-mediated inflammation, type 2 diabetes, and diabetic vascular complications. Front. Endocrinol. 4, 21–23.

Yao, L., Kan, E.M., Lu, J., Hao, A., Dheen, S.T., Kaur, C., Ling, E.-A., 2013. Toll-like receptor 4 mediates microglial activation and production of inflammatory mediators in neonatal rat brain following hypoxia: role of TLR4 in hypoxic microglia. J. Neuroinflamm. 10, 23.

Yasuda, Y., Shimoda, T., Uno, K., Tateishi, N., Furuya, S., Tsuchihashi, Y., Kawai, Y., Naruse, S., Fujita, S., 2011. Temporal and sequential changes of glial cells and cytokine expression during neuronal degeneration after transient global ischemia in rats. J. Neuroinflamm. 8, 2094–2098.

Zhang, Z., Peng, D., Zhu, H., Wang, X., 2012. Experimental evidence of Ginkgo biloba extract EGB as a neuroprotective agent in ischemia stroke rats. Brain Res. Bull. 87, 193–198.