

Published in final edited form as:

*Neurobiol Dis.* 2012 April ; 46(1): 180–189. doi:10.1016/j.nbd.2012.01.006.

## Preconditioning with Ginkgo biloba (EGb 761®) provides neuroprotection through HO1 and CRMP2

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### Abstract

*Ginkgo biloba*/EGb 761® (EGb 761) is a popular and standardized natural extract used worldwide for the treatment of many ailments. Although EGb 761 is purported to have a plethora of benefits, here, we were interested to study the neuroprotective properties of EGb 761 and its components and determine whether nuclear factor E2 (Nrf2)/heme oxygenase 1 (HO1) induction of the collapsin response mediator protein 2 (CRMP2) pathway contributes to neuroprotection. Mice were pretreated with EGb 761 or one of its constituents (bilobalide, ginkgolide A, ginkgolide B, and terpene free material [TFM]) for 7 days and then subjected to transient middle cerebral artery occlusion (tMCAO) and 48 h of reperfusion. All components except TFM significantly reduced infarct volumes and neurologic deficits. Next, we examined the antioxidant and neurotogenic properties of EGb 761 in primary neurons. Compared with vehicle-treated cells, pretreatment with EGb 761 significantly enhanced the survival of neurons exposed to tertiary butylhydroperoxide (*t*-BuOOH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and N-methyl-D-aspartate (NMDA). Bilobalide and ginkgolide A also protected cells against NMDA-induced excitotoxicity. Immunofluorescence and Western blot analysis showed that EGb 761 pretreatment significantly increased the protein expression levels of Nrf2, HO1, GAPDH,  $\beta$ -actin, CRMP2, and histone H3 during *t*-BuOOH-induced oxidative stress. These findings suggest that EGb 761 not only has antioxidant activity but also neurotogenic potential. Demonstrating such effects for possible drug discovery may prove beneficial in stroke and ischemic brain injury.

### Keywords

Collapsin response mediator protein 2; Ginkgo biloba; Heme oxygenase 1; Middle cerebral artery occlusion; Neuroprotection; Neurite growth

### Introduction

Used as an over-the-counter drug to enhance energy and memory, the standardized *Ginkgo biloba* leaf extract, or EGb 761® (EGb 761), is one of the most effective and commonly used nutraceuticals in the market. In Europe, EGb 761 is prescribed for multiple problems related to mental health and overall wellbeing. Several studies have shown EGb 761 to have neuroprotective properties, but the mechanism(s) underlying this effect has not been fully

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studied and requires further evaluation. Previously, our group has shown that heme oxygenase 1 (HO1) is essential for EGb 761 neuroprotection against ischemia in rodent models of transient middle cerebral artery (MCA) occlusion (*t*MCAO) (Saleem et al., 2008) and permanent distal MCA occlusion (*p*MCAO) (Shah et al., 2011). Furthermore, EGb 761 was shown to upregulate endothelial nitric oxide synthase and vascular endothelial growth factor in ipsilateral cortices of mice that have undergone experimental stroke, suggesting the possible contribution of these vasodilators (Shah, Nada et al. 2011).

Ischemic stroke is a leading cause of disability in the United States and worldwide (Elkins and Johnston, 2003). Insufficient oxygen and nutrients during cerebral ischemia triggers multiple biochemical cascades that result in axonal injury, breakdown of neuronal cytoskeleton, and neuronal degeneration and death (Won et al., 2002). Collapsin response mediator protein 2 (CRMP2) is crucial for axon outgrowth and determines the fate of axons and dendrites. It was originally identified as a signaling molecule required for growth cone collapse of dorsal root ganglion neurons in response to a repulsive guidance cue (Goshima et al., 1995). Overexpression of CRMP2 induces the formation of multiple axons, whereas knockdown of CRMP2 suppresses axon formation (Inagaki et al., 2001, Suzuki et al., 2003, Yoshimura et al., 2005), indicating that CRMP2 has a positive effect on axonal extension and plays a key role in dendrite specification and axon regeneration. CRMP2 colocalizes with F-actin in the growth cones of different types of neurons (Goshima et al., 1995, Minturn et al., 1995, Yuasa-Kawada et al., 2003) and also binds to actin, but its binding is not affected by phosphorylation (Arimura et al., 2005).

With the backdrop of failed neuroprotective agents in phase III clinical trials, there is an urgent prerequisite to develop neuroprotective agents that have few side effects and multiple mechanism(s) of action. In this study, we investigated the neuroprotective properties of EGb 761 and its components (bilobalide [BB], ginkgolide A [GA], ginkgolide B [GB], and terpene free material [TFM]) in a mouse *t*MCAO model with 48 h reperfusion. In addition, we explored the neuritogenic properties of EGb 761 in primary cultured neuronal cells by studying the roles of CRMP2, GAPDH, and  $\beta$ -actin and confirming Nrf2/HO1 involvement. We found that EGb 761 has the potential to reduce infarct volume in vivo, reduce neuronal apoptosis in vitro, and reverse excitotoxicity-induced cytoskeletal collapse.

## Materials and methods

### Animals

All animal protocols were approved by the University of Toledo Health Science Campus Institutional Animal Care and Utilization Committee. Guidelines of the National Institutes of Health were followed throughout the study. Male C57BL/6 mice (20-30 g) and timed 14-day pregnant female C57BL/6 mice were procured from Charles River Laboratories, Wilmington, MA.

### Drug treatment

EGb 761, BB, GA, GB, and TFM were kindly provided by Dr. Willmar Schwabe Pharmaceuticals, Germany. Test drugs and vehicle (polyethylene glycol) were orally administered to mice daily for 7 days before ischemia at the following dosages: EGb 761, 100 mg/kg; BB, 6 mg/kg; GA, 6 mg/kg; GB, 6 mg/kg; and TFM, 10 mg/kg. Dosages were selected based on the amount of each constituent present in the EGb 761.

### Transient occlusion of the middle cerebral artery (*t*MCAO)

The middle cerebral artery occlusion procedure was carried out as published previously (Shah et al., 2006). Mice were anesthetized with halothane (Nicholas Piramal, India; 3%

initial, 1 to 1.5% maintenance) in O<sub>2</sub> and air (80%:20%). A 0.5-mm diameter microfiber was glued over the area of parietal cortex with cyanoacrylate glue (Super Glue Gel, Ross Products, Inc.) and connected to a laser-Doppler flowmeter (DRT4, Moor Instruments Ltd, Devon, England), which was used to confirm successful occlusion. Mice were turned to the supine position, and a midline incision was made in the neck to clear and expose the right common carotid artery (CCA), external carotid artery, and internal carotid artery; care was taken not to disturb the vagus nerve. A 7-0 Ethilon nylon filament (Ethicon, Inc., Somerville, NJ, USA), which had 5 mm of the tip coated with silicone (Cutter Sil Light and Universal Hardener, Heraeus Kulzer, GmbH, Hanau, Germany), was slipped into the internal carotid artery through the external carotid artery stump to block blood circulation to the MCA territory. The filament was carefully advanced up to 11 mm from the carotid artery bifurcation or until resistance was felt. Mice that did not attain at least an 80% decrease in cerebral blood flow were terminated from the study. Animals were kept in a humidity/temperature-controlled chamber at 32 °C to maintain their body temperature at 37 °C during the 90 min of MCA occlusion. For reperfusion, mice were briefly anesthetized, and the filament was withdrawn carefully without rupturing the arteries; open ends of arteries were cauterized to prevent bleeding. After the neck incision was sutured, mice were again placed in a humidity/temperature-controlled chamber for 2 h and then returned to their respective home cages.

### **Blood Gas Measurements**

Briefly, under an operating microscope mice (another cohort of animals) were placed in a porcine position and an incision was given on the limb and femoral artery was exposed. A PE-10 femoral artery catheter (Intramedic; BD Diagnostic Systems, Sparks, MD) attached to 1 ml syringe on one side was introduced in to it and fixed/secured with a silk suture. Blood was drawn intermittently at different intervals of time; 30 minutes before MCAO, 1 h after the initiation of MCAO, and 1 h after reperfusion. Blood samples were analyzed by blood gas analysis instrument (Rapiddlab 248; Chiron Diagnostic Corporation, Norwood, MA) for pH, PaO<sub>2</sub>, and PaCO<sub>2</sub> parameters.

### **Neurologic deficit score (NDS)**

Forty-eight hours after *t*MCAO, neurologic deficits were evaluated by an investigator blinded to treatment group using a previously modified 28-point scoring system (Saleem et al., 2009, Zeynalov et al., 2009). Motor deficits were evaluated by tests for body symmetry, gait, climbing, circling behavior, front limb symmetry, and compulsory circling; sensory deficits were evaluated by a whisker response test. Each test was graded from 0 (no deficit) to 4 (greatest deficit), establishing a maximum NDS of 28. After assessing weight loss, mice were sacrificed for measurement of infarct volume.

### **Infarct size and infarct volume analysis**

After 48 h of reperfusion, mice were anesthetized, and their brains dissected out. Coronal brain slices (2 mm) were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) and fixed in 10% buffered normal saline for 24 h. The slices were scanned individually by a video image analyzing system, and the infarct lesions were measured and analyzed by image analysis software SigmaScan pro 4 and 5 (Systat Inc., San Jose, CA, USA).

### **Neuronal Cell Culture**

On gestational day 17, pregnant mice were sacrificed by CO<sub>2</sub> overdose, and fetuses were collected in cold HBSS medium (Fisher Scientific, Hanover Park, IL, USA). The fetuses were decapitated and cortex was collected from each brain. The meninges were rapidly

removed, dissected into 5 ml of cold HBSS medium by trituration, and centrifuged at 18 °C for 3 min at 1000 × g. After the supernatant was removed, the cells were resuspended in 5 ml of 1X DMEM (Fisher) containing 0.25% trypsin (Fisher) and incubated at 37 °C under 5% CO<sub>2</sub> for 15 min. DNase solution (Roche Diagnostics, Indianapolis, IN, USA) was added to a final concentration of 0.02 mg/ml, and the suspension was incubated under the same conditions for another 5 min. After 5 ml of 1X DMEM containing 10% FBS was added to the trypsinized cells, the suspension was passed twice through a 70 μm nylon cell strainer (BD Falcon, Sparks, MD, USA) to remove the cell debris. Cells were spun down at 1000 × g for 3 min at 18 C, the supernatant was removed, and cells were resuspended in 5 ml of neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin (50 U/ml of medium), glutamine (2 mM), and B27 serum-free supplement (Invitrogen). This step was repeated twice, and then the cells were counted in trypan blue and plated in poly-L-lysine (50 μg/ml)-coated plates. Cultures were maintained at 37 C in 95% air and 5% CO<sub>2</sub>. All experiments were performed on day 14 of plating. Medium was changed every third day by replacing half of the old medium with fresh.

### Cell viability assays

Neurons at a population of  $0.5 \times 10^6$  were plated in poly-L-lysine pre-coated 24-well dishes and pretreated with EGb 761 (0.1 mg/ml), BB (12 g/ml), GA (12 g/ml), GB (12 g/ml), or TFM (20 g/ml). After 6 h, one of the following stressors was added: tertiary butylhydroperoxide (*t*-BuOOH, 60 M), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 100 M), or N-Methyl-D-aspartate (NMDA, 100 M). After 18 h of exposure, cell viability assayed with the Promega cell proliferation assay kit (Promega, Madison, WI, USA). Optical density was measured at 570 nm. All experiments were repeated in triplicate with four separate batches of culture.

### TUNEL assay and immunocytochemistry

Neurons were seeded in 6-well dishes at a population of  $1.2 \times 10^6$  on sterilized coverslips coated with poly-L-lysine. On day 14, neurons were pretreated with EGb 761 (0.1 mg) and/or tin protoporphyrin-IX (SNPPIX, 10 μM) for 6 h. Then *t*-BuOOH (60 μM) was added for an additional 18 h. The TUNEL assay (Promega) was carried out according to the manufacturer's protocol. Briefly, coverslips were incubated with 1% albumin bovine fraction V (RPI, Mount Prospect, IL, USA) in 1 X PBS buffer at room temperature for 1 h. After being washed, the coverslips were incubated overnight at 4 °C with primary antibody, rabbit anti-HO1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by goat anti-rabbit secondary antibody (1:800; Jackson ImmunoResearch, West Grove, PA, USA). Coverslips were mounted with DAPI (Santa Cruz) and sealed. For CRMP2 and phalloidin staining, washed neurons were fixed with paraformaldehyde and treated with 0.3% triton X-100 for permeability. The fixed cells were blocked with 1% of BSA for 1 h at room temperature and then incubated overnight at 4 °C with rabbit anti-CRMP2 (1:8000; Millipore Corporation, Billerica, MA, USA). Slides were washed three times (10 min each) before incubation with secondary goat anti-rabbit IgG (1:800; Jackson ImmunoResearch). Slides were washed again and incubated with one unit of Phalloidin/slide (Invitrogen). Slides were washed again and mounted with DAPI and sealed. All experiments were repeated three times with three separate batches of cultures.

### Western blot analysis

Nuclear and cytosolic fractions were isolated from neuronal cells according to Shah et al. (Shah et al., 2011), and protein concentration was determined by Bio-Rad Bradford reagent (Bio-Rad, Hercules, CA, USA). Equivalent amounts of total cytoplasmic or nuclear protein (25 μg) were separated by SDS polyacrylamide gel electrophoresis on 10% gels. Proteins were transferred from the gel to PVDF membrane, blocked with 5% dry nonfat milk, and incubated with the following antibodies: rabbit anti-Nfr2 (1:1000; Santa Cruz), rabbit anti-

actin (1:2000; Sigma Aldrich), rabbit anti-HO2, (1:2000; Stressgen, MI, USA), rabbit anti-HO1 (1:1000; Stressgen), rabbit anti-CRMP2 (1:40,000; Millipore), rabbit anti-GAPDH (1:3000; Fisher) or rabbit anti histone H3 (1:2000; Fisher). After being washed with PBS-T buffer, membranes were incubated with secondary antibody (1:5000; Jackson ImmunoResearch). Images were analyzed by using Adobe Photoshop and ImageJ software provided by the NIH.

### Statistical analysis

Data were analyzed by Student's t-test, analysis of variance (ANOVA), or Newman-Keuls multiple range test. Comparisons of all physiological parameters and infarct volumes between treatment groups were made by one-way ANOVA with Newman Keuls post-hoc test. Neurologic deficits were analyzed by the non-parametric Kruskal-Wallis test. Data are presented as means  $\pm$  SEM. A value of  $p < 0.05$  was considered to be statistically significant.

## Results

### Pretreatment with EGb761 and its components improves neurologic deficit scores and reduces infarct size in mice

Mice randomized into different treatment groups were pretreated for 7 days with test drugs (EGb 761, BB, GA, GB, or TFM) and subjected to *t*MCAO. After 48 h of reperfusion, infarct volumes as analyzed by TTC staining were significantly lower in groups treated with EGb 761 ( $40.6 \pm 2.5\%$ ;  $p = 0.021$ ), BB ( $37.5 \pm 2.4\%$ ;  $p < 0.005$ ), GA ( $40.6 \pm 2.0\%$ ;  $p = 0.011$ ), and GB ( $39.5 \pm 2.1\%$ ;  $p = 0.007$ ) than in the group treated with vehicle ( $51.9 \pm 2.9$ ). Infarct volume in the TFM group was not significantly different from control (Fig. 1A). Similarly, the NDS was significantly reduced in the groups treated with EGb 761 ( $9.3 \pm 0.9$ ;  $p < 0.026$ ), BB ( $6.8 \pm 1.1$ ;  $p = 0.010$ ), or GB ( $7.3 \pm 1.7$ ;  $p = 0.030$ ) compared to the vehicle-treated group ( $13.5 \pm 1.1$ ). However, GA- and TFM-treated groups did not show a statistically significant difference (Fig. 1B). Furthermore, there were no differences in brain edema, weight loss, or physiological parameters (pH, PaCO<sub>2</sub>, PaO<sub>2</sub>) in any of the treatment groups (data not shown).

### Neuroprotective effects of EGb 761 against oxidative stress and excitotoxicity in vitro

We used neuronal cell cultures to study the neuroprotective effects of EGb 761 and its components in vitro against oxidative stress induced by *t*-BuOOH and H<sub>2</sub>O<sub>2</sub>. Only EGb 761 (6 h pretreatment) significantly protected the neuronal cells from the effects of *t*-BuOOH and H<sub>2</sub>O<sub>2</sub> (Fig. 2A and B). BB, GA, GB, and TFM failed to provide any protection in terms of neuronal survival against these two stressors. Since, BB, GA, and GB pretreatment showed significant reduction of brain infarct volume against *t*MCAO, we were further interested to look into the other possible mechanisms of protection, such as against excitotoxicity. Therefore, we also investigated whether each agent could protect neurons against NMDA exposure. We found that 6 h of pretreatment with EGb 761, BB, GA, prevented the NMDA-induced loss of neuronal viability (Fig. 2C).

### Role of HO1 in neuroprotective mechanism of EGb 761

Next we investigated possible mechanism(s) by which EGb 761 might provide neuroprotection against the three different stressors. We pretreated neurons with EGb 761 in the presence and absence of *t*-BuOOH and/or SNPP1X (HO1 inhibitor). EGb 761 enhanced the induction of HO1 protein expression and provided significant protection to neurons from apoptosis induced by *t*-BuOOH-related oxidative stress (Fig. 3A and B). In the presence of the HO1 inhibitor, pretreatment with EGb 761 failed to protect neurons against the *t*-

BuOOH-induced neuronal death, suggesting that EGb 761 might protect neurons by inducing HO1 expression.

### Neurotogenic properties of EGb 761 and role of CRMP2

Next, we sought to understand how EGb 761 might promote neurite growth via mechanisms in addition to or downstream of HO1 induction. We tested the hypothesis that EGb 761 can protect neurons by inducing the expression of CRMP2 proteins in the axons and dendrites. Exposure of neurons to *t*-BuOOH for 6 h ruptured and collapsed F-actin filaments. In addition, the CRMP2 protein became dissociated from the axons and dendrites and was observed to be localized in the nucleus. Preincubation with EGb 761 for 6 h protected the neurons against *t*-BuOOH-induced oxidative stress by restoring F-actin filaments and CRMP2 expression in axons and dendrites, thereby inducing and enhancing neurite growth (Fig. 4). Furthermore, EGb 761 failed to restore F-actin filaments and CRMP2 expression in axons and dendrites in the presence of the HO1 inhibitor SNPP1X. When HO1 enzymatic activity was blocked by SnPP1X, EGb 761 no longer protected neuronal cells from apoptosis or neuronal degeneration. These results suggest a possible link between HO1 and CRMP2 signaling.

### Protein expression levels induced by EGb 761

Western blot analysis showed a significant reduction in protein expression of HO1, GAPDH,  $\beta$ -actin, nuclear Nrf2, and histone H3 in neuronal cells exposed to *t*-BuOOH. Little change was observed in the expression of HO2 and cytosolic Nrf2. Pretreatment for 6 h with EGb 761 before exposure to *t*-BuOOH restored the expression level of all proteins. Because F-actin collapsed and dissociated in neurons exposed to *t*-BuOOH,  $\beta$ -actin and histone expression levels also decreased in response to *t*-BuOOH. Consequently, neither could be used as a loading control (Fig. 5). Therefore, control band of the  $\beta$ -actin was used to normalize different protein bands and analysis made to draw corresponding graphs.

## Discussion

We hypothesized that EGb 761 and some of its components provide protection from ischemia-reperfusion injury through the Nrf2/HO1 and CRMP2 pathways. We observed significantly lower NDS in mice that were pretreated with EGb 761, BB, and GB and significantly smaller infarct volumes in those pretreated with EGb 761, BB, GA, and GB. Only EGb 761 ameliorated neuronal death against all three stressors: *t*-BuOOH, H<sub>2</sub>O<sub>2</sub>, and NMDA. EGb 761 pretreatment significantly decreased the number of TUNEL-positive cells, possibly by increasing HO1 expression, as the effect was lost in the presence of the HO1 inhibitor SNPP1X. Furthermore, EGb 761 reversed the stressor-induced decrease in CRMP2 protein level, but this effect also was abolished in the presence of SNPP1X. Finally, EGb 761 prevented the decreased expression of HO1, GAPDH,  $\beta$ -actin, nuclear Nrf2, and histone H3 observed in primary neurons exposed to *t*-BuOOH. These results suggest that EGb 761 provides neuroprotection through the Nrf2/HO1-induced CRMP2 pathway.

EGb 761 is a multifunction antioxidant that has been shown to provide protection against cardiovascular and neurological disorders (Clark et al., 2001, Ahlemeyer and Kriegelstein, 2003, Chandrasekaran et al., 2003, Shah et al., 2011). Previous studies have shown EGb 761 to be protective in *t*MCAO and *p*MCAO models of ischemia and have implicated HO1 in the underlying mechanism. Whereas previous studies have investigated the effects of EGb 761 on stroke outcome at 24 h of reperfusion, here, we extended the survival time from 24 h to 48 h and tested not only EGb 761 but also its components. We found that 7-day pretreatment with EGb 761, BB, GA, and GB provides protection against transient ischemia. These results are in consonance with our previous study in a *p*MCAO model (Shah et al.,

2011). The fact that EGb 761 could protect even up to 48 h of *t*MCAO in present study, further consolidates its neuroprotective properties. Next, we sought to broaden our knowledge of the mechanism by which EGb 761 and its components provide neuroprotection. We found that only EGb 761 was able to rescue neurons *in vitro* against all three different types of stressors that we used—*t*-BuOOH, H<sub>2</sub>O<sub>2</sub>, and NMDA—highlighting its multipronged neuroprotective pathways. BB and GA also were able to rescue neurons against NMDA excitotoxicity, but no EGb 761 constituent was able to rescue neurons after exposure to *t*-BuOOH or H<sub>2</sub>O<sub>2</sub>. One of the possible reasons might be that BB acts through antioxidant and glutamate antagonism, while as GA acts through glutamate antagonism only. Another reason might be that in our studies, *t*-BuOOH and H<sub>2</sub>O<sub>2</sub> resulted in 80% of neuronal cell death as these two stressors work through multiple cell death signalling pathways and may be one of the caveats for not observing any protection with individual components. On the contrary, NMDA works mainly through excitatory mechanism initially and resulted in only 60% of neuronal cell death, subsequently making BB and GA potent enough to provide protection. Zhang et al. (Zhang et al., 2004) also observed that neurons in cell culture are injured worse by H<sub>2</sub>O<sub>2</sub> than by NMDA insult. BB not showing any protective effect in the present study may be attributed to higher neuronal death with H<sub>2</sub>O<sub>2</sub> insult. Further studies may be required to evaluate this claim. The fact that the various constituents were ineffective *in vitro* but effective *in vivo* may be attributable to the absence of cell type diversity in cell culture models.

Neurons are highly polarized, and the processes that extend from their cell bodies are specialized for receiving and transmitting information. These vital functions are based on their specialized architectures, which are composed of two structurally and functionally distinct parts, axons and dendrites (Craig and Banker, 1994). After cerebral ischemia, programmed neuronal cell death plays an important role in neuronal degeneration, but the underlying mechanisms are not fully understood. To promote functional recovery after ischemic injuries, it is vital to develop strategies that enhance both neuronal survival and regeneration. To validate the potential role of EGb 761 in inducing neurite growth, we used *t*-BuOOH (60 μM) to induce cell death and to study its effects on axons and dendrites. Immunofluorescence revealed that *t*-BuOOH dissociated F-actin, β-actin, and CRMP2 from axons and dendrites. Western blot analysis confirmed these results and also showed reduced protein expression levels of GAPDH, β-actin, and histone H3. Substantial evidence in the literature supports the theory that GAPDH and β-actin filaments are broken down and protein expression levels altered in neurodegenerative diseases (Zhu et al., 2005, Butterfield et al., 2010). Targeting this novel pathway could prove beneficial in neurodegenerative diseases where functional recovery is an important aspect of therapy. Hence, it is noteworthy that, in addition to upregulating HO1 and nuclear Nrf2, EGb 761 was able to restore F-actin, β-actin, GAPDH, histone H3, and CRMP2 in axons and dendrites.

CRMP2 is a developmentally regulated protein and highly expressed in the nervous system (Bretin et al., 2005). Overexpression of CRMP2 induces the formation of multiple axons and elongation of the primary axons in hippocampal neurons (Fukata et al., 2002a, Fukata et al., 2002b). Recent evidence suggests that CRMP2 is important in calcium regulation and excitotoxicity signaling (Bretin et al., 2006, Hou et al., 2009). CRMP2 is expressed as multiple isoforms (Bretin et al., 2005). The cytosolic active isoforms of CRMP2 have apparent masses between 63 and 66 kDa and the cleaved inactive CRMP2 forms have masses that range between 54 and 58 kDa. A number of studies have shown that CRMP2 becomes proteolytically cleaved during neuronal injury (Bretin et al., 2006, Brittain et al., 2011). In the present study, the cleaved 54 kDa CRMP2 was the only form observed to be expressed in apoptotic neurons, but multiple isoforms were detected after pretreatment of neurons with EGb 761, suggesting that CRMP2 is active in the protective process. However, in the presence of HO1 inhibitor, EGb 761 failed to protect neurons from apoptosis or

prevent axon and dendrite collapse. The possible link between HO1 and CRMP2 may be the equimolar quantities of carbon monoxide (CO) formed during the catabolism of pro-oxidant heme (Kirkby and Adin, 2006). Like nitric oxide, CO is known to inhibit platelet aggregation and can produce relaxation in rabbit arteries *ex vivo* (Brune and Ullrich, 1987, Furchgott and Jothianandan, 1991). Several studies demonstrated that CO has the ability to bind to the iron atom of the heme moiety associated with soluble guanylyl cyclase, thereby activating the enzyme and increasing intracellular cyclic guanosine monophosphate (cGMP) production (Brune and Ullrich, 1987, Furchgott and Jothianandan, 1991, Christodoulides et al., 1995). Zhao et al. (Zhao et al., 2009) were able to demonstrate that cGMP can regulate axon branching by inhibiting glycogen synthase kinase 3  $\beta$  (GSK-3 $\beta$ ) via the activation of cGMP-dependent protein kinase 1. Another study by Yoshimura et al. (Yoshimura et al., 2005) showed that GSK-3 $\beta$  phosphorylates CRMP2, thus inactivating it and preventing the copolymerization of CRMP2 within tubulin and dissociation of CRMP2 from microtubulin. Another possibility was highlighted by a recent study by Tsai et al. (Tsai et al., 2010) and his group that showed EGb 761 reduced calpain activity, and small interfering RNA targeting HO1 abolished this reduction. The active calpain targets CRMP2 protein and cleaves it towards the C-terminal and causes its inactivation. Alternately, calpain is also activated by calcium influx when the cells become apoptotic during stress conditions (Hou et al., 2009). Furthermore, the effect of HO1 on soluble guanylyl cyclase (sGC) resulted in a several-hundred-fold stimulation of sGC. This activation results in a pronounced accumulation of cGMP, an important second messenger involved in a variety of physiological processes. cGMP regulates axonal guidance (Song and Poo, 1999) by activating cGMP dependent protein kinases and inhibiting the GSK-3 $\beta$  activity. In both ways, the inactive GSK-3 $\beta$  regulates actin filaments (Eickholt et al., 2002), while the active GSK-3 $\beta$  phosphorylates and inactivates CRMP-2. Since Nrf2 has already been shown to upregulate HO1 by binding to antioxidant response elements on its promoter region (Shah et al., 2010), the pathway through which Nrf2/HO1 mediates activation of CRMP2 and other proteins may be a novel target to maintain neuronal cytoskeleton under stress conditions. This pathway needs to be evaluated further and confirmed in future *in vivo* studies.

In conclusion, we showed that 7 days of pretreatment with EGb 761, BB, GA, and GB protected mice from transient ischemia after 48 h of reperfusion. *In vivo* results also corroborated well with the *in vitro* results, in which EGb 761 was found to provide neuroprotection against different stressors. Finally, we showed that EGb 761 pretreatment upregulates proteins (nuclear Nrf2, HO1, CRMP2, GAPDH,  $\beta$ -actin, and histone H3) that were downregulated by *t*-BuOOH-induced oxidative stress and demonstrated a link between HO1 and CRMP2 expression and the EGb 761 neuroprotection. These findings support our hypothesis that Nrf2/HO1-mediated upregulation of CRMP2 is vital for EGb 761 neuroprotection and provides a novel pathway for drug discovery in neurodegenerative diseases.

## Acknowledgments

This work was supported by a grant from the National Institutes of Health-National Center for Complimentary and Alternative Medicine (R00AT004197) to ZAS.

## Abbreviations

<b>BB</b>	bilobalide
<b>CRMP2</b>	collapsin response mediator protein 2
<b>EGb 761</b>	Ginkgo biloba/EGb 761®



<b>GA</b>	ginkgolide A
<b>GB</b>	ginkgolide B
<b>HO1</b>	heme oxygenase 1
<b>H<sub>2</sub>O<sub>2</sub></b>	hydrogen peroxide
<b>MCA</b>	middle cerebral artery
<b>NDS</b>	neurologic deficit score
<b>NMDA</b>	N-Methyl-D-aspartate
<b><i>t</i>-BuOOH</b>	tertiary butylhydroperoxide
<b>TFM</b>	terpene free material
<b>tMCAO</b>	transient middle cerebral artery occlusion
<b>TTC</b>	triphenyltetrazolium chloride

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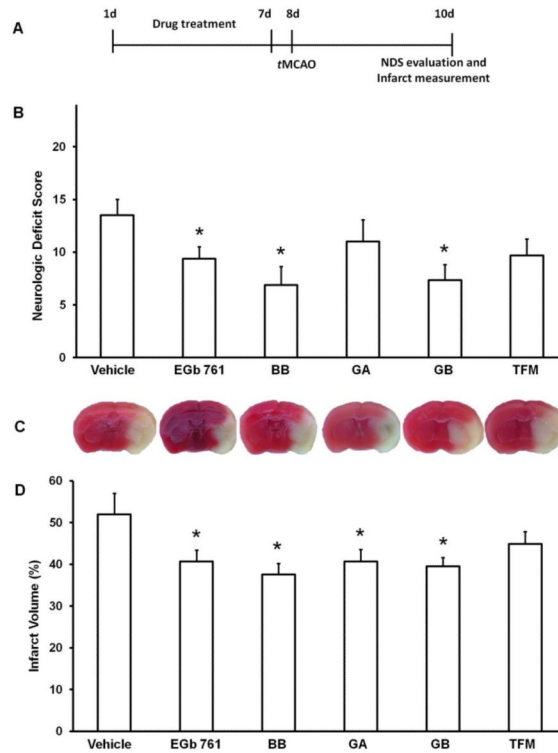
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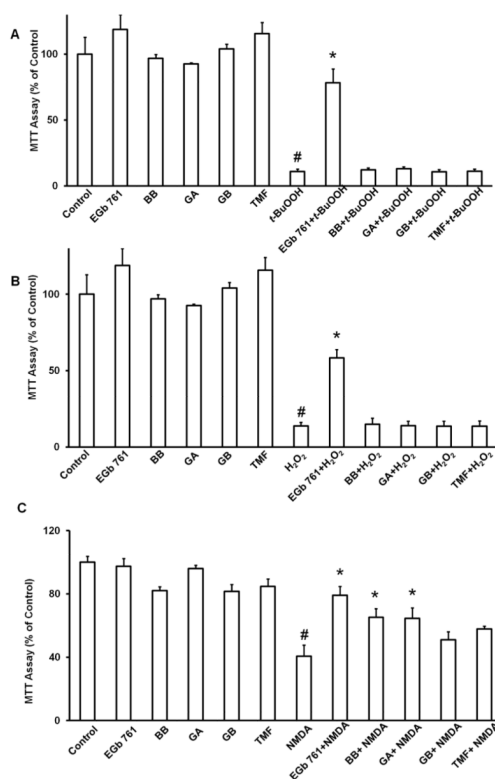
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### Highlights

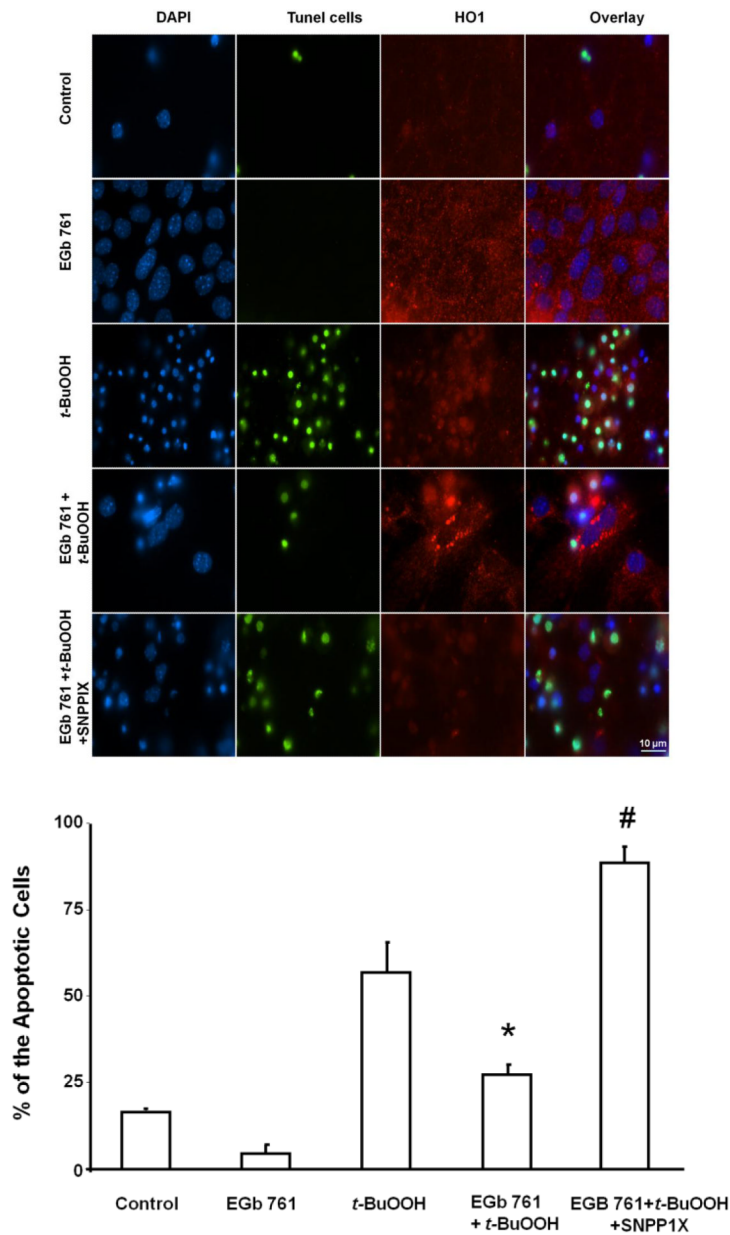
- >EGb 761 and its components are neuroprotective in a mouse transient ischemia model.
- >Putative mechanism of action is through the Nrf2/HO1-induced CRMP2 pathway.
- >EGb 761 can protect neurons *in vitro* from oxidative as well as excitatory toxicity.
- >EGb 761 induces neuritogenesis by upregulating CRMP2. >EGb 761 protected the filaments and restored filamental CRMP2 expression.



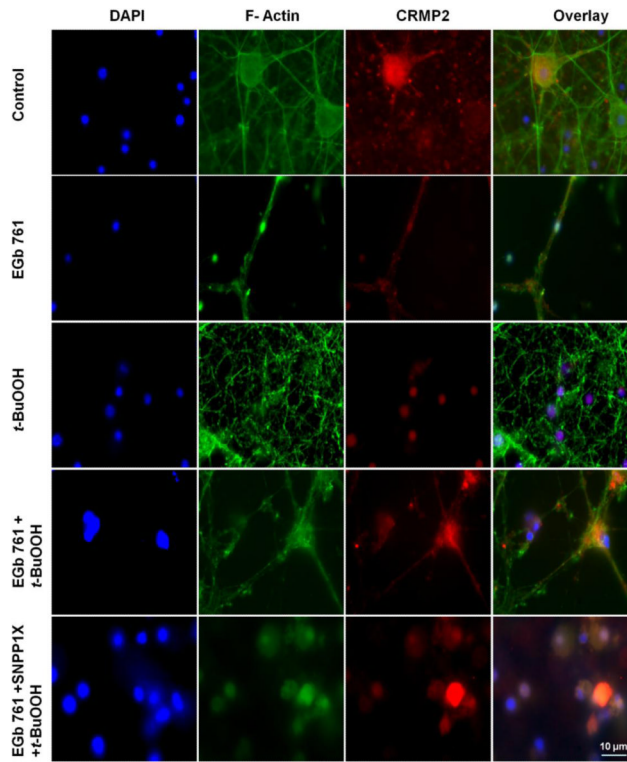
**Fig. 1.** Pretreatment with EGb 761 and its components reduces damage from stroke. (A) Schematic diagram of the experimental protocol. Mice were orally administered EGb 761 (100 mg/kg; n=9), BB (6 mg/kg; n=8), GA (6 mg/kg; n=6), GB (6 mg/kg; n=7), TFM (10 mg/kg; n=10), or vehicle control (n=4) once daily for 7 days; *t*MCAO was induced on day 8; mice were evaluated for neurologic deficit score (NDS) and sacrificed on day 10. (B) Compared with vehicle-treated controls, groups pretreated with EGb 761, BB, and GB had significantly lower NDS, (D) and those pretreated with EGb 761, BB, GA, and GB had significantly smaller infarct volumes. (C) Representative brain sections of mice showing infarct volumes of different treatment groups. Data are expressed as means  $\pm$  SEM;  $p$  0.05 vs. vehicle.



**Fig. 2.** Effects of EGb 761 and its components on neuronal survival in vitro. Cells were grown in 24-well plates for 14 days and then pretreated with EGb 761 (100  $\mu$ g), BB (12  $\mu$ g), GA (12  $\mu$ g), GB (12  $\mu$ g), TFM (20  $\mu$ g), or vehicle control. Then they were exposed to (A) 60 M *t*-BuOOH, (B) 100 M H<sub>2</sub>O<sub>2</sub>, and (C) 100 M NMDA. Cell viability was assessed with the MTT assay and is shown as percent of control. Each experiment was conducted in triplicate and repeated four times with different primary culture batches. Data are expressed as mean  $\pm$  SEM; #*p* 0.05 vs. control, \**p* 0.05 vs. stressor.

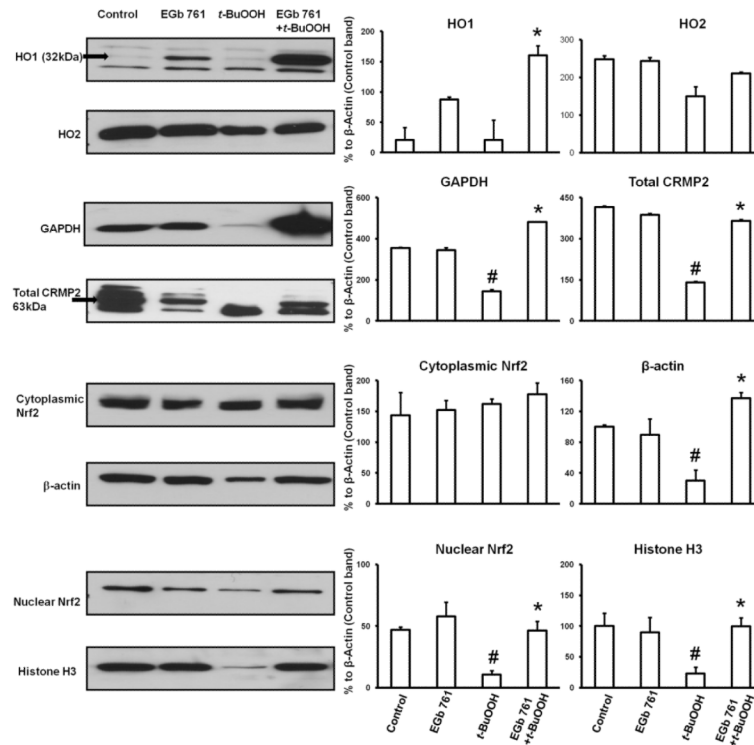


**Fig. 3.** EGb 761 protects neurons from apoptosis. Cultured cortical neurons from mice were pretreated for 6 h with vehicle or 100 μg/ml EGb 761 in the presence or absence of SNPP1X; then 60 μM *t*-BuOOH was added for an additional 18 h. (A) Immunocytochemical staining shows DAPI (blue), apoptotic cells (green), HO1 expression (red), and the overlay of all. Scale bar = 10 μm (B) The EGb 761-induced reduction in apoptosis was blocked by SNPP1X, indicating that the neuroprotection requires HO1. Each experiment was conducted in triplicate and repeated three times with different primary culture batches. Data are expressed as means ± SEM; *p* 0.05 vs. *t*-BuOOH; #*p* 0.05 vs. EGb 761+*t*-BuOOH.

**Fig. 4.**

EGb 761 induces neurite growth. Top panel: control neurons without any treatment. The nucleus is stained with DAPI (blue stain), F-actin (phalloidin green stain), and CRMP2 (red stain). The far right image shows the merged overlay of all the pictures. *t*-BuOOH treatment induced CRMP2 localization within the nucleus and the collapse of F-actin. Pretreatment with EGb 761 protected axons and dendrites from collapsing, as CRMP2 was detected in axons and dendrites. Each experiment was conducted in triplicate and repeated three times with different primary culture batches. Scale bar = 10 μm





**Fig. 5.** Effect of EGb 761 on protein expression. Western blot analysis was used to measure protein expression levels of HO1, HO2, GAPDH, β-actin, total CRMP2, Nrf2, and histone H3. Each experiment was conducted in triplicate and repeated three times with different primary cultures batches. Values shown are percent of β-actin control band except for nuclear Nrf2 protein which is expressed as mean ± SEM; # *p* 0.05 vs. Control; \**p* 0.05 vs. *t*-BuOOH.