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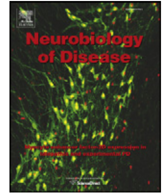

Preconditioning with *Ginkgo biloba* (EGb 761®) provides neuroprotection through HO1 and CRMP2

Shadia E. Nada, Zahoor A. Shah *

Department of Medicinal and Biological Chemistry, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, Toledo, OH, USA

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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 (*t*MCAO) and 48 h of reperfusion. All components except TFM significantly reduced infarct volumes and neurologic deficits. Next, we examined the antioxidant and neuritogenic 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_2O_2), 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, β-actin, CRMP2, and histone H3 during *t*-BuOOH-induced oxidative stress. These findings suggest that EGb 761 not only has antioxidant activity but also neuritogenic potential. Demonstrating such effects for possible drug discovery may prove beneficial in stroke and ischemic brain injury.

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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 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 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

Abbreviations: BB, bilobalide; CRMP2, collapsin response mediator protein 2; EGb 761, *Ginkgo biloba*/EGb 761®; GA, ginkgolide A; GB, ginkgolide B; HO1, heme oxygenase 1; H_2O_2, hydrogen peroxide; MCA, middle cerebral artery; NDS, neurologic deficit score; NMDA, N-Methyl-D-aspartate; *t*-BuOOH, tertiary butylhydroperoxide; TFM, terpene free material; *t*MCAO, transient middle cerebral artery occlusion; TTC, triphenyltetrazolium chloride.

* Corresponding author at: Department of Medicinal and Biological Chemistry, University of Toledo, 3000 Arlington Avenue, Toledo, OH, 43614, USA.
E-mail address: zahoor.shah@utoledo.edu (Z.A. Shah).

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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 β-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 (tMCAO)

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_2 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 min before MCAO, 1 h after the initiation of MCAO, and 1 h after reperfusion. Blood samples were analyzed by blood gas analysis instrument (Rapidlab 248; Chiron Diagnostic Corporation, Norwood, MA) for pH, PaO2, and PaCO2 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_2 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 $1000 \times g$. 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_2 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 $1000 \times g$ 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_2. 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 0.5×10^6 were plated in poly-L-lysine pre-coated 24-well dishes and pretreated with EGb 761 (0.1 mg/ml), BB (12 μg/ml), GA (12 μg/ml), GB (12 μg/ml), or TFM (20 μg/ml). After 6 h, one of the following stressors was added: tertiary butylhydroperoxide (*t*-BuOOH, 60 μM), hydrogen peroxide (H_2O_2, 100 μM), or N-Methyl-D-aspartate (NMDA, 100 μM). After 18 h of exposure, cell viability assayed with the Promega cell proliferation assay kit (Promega,

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