Gingko biloba Extract (Egb 761) Prevents Ischemic Brain Injury by Activation of the Akt Signaling Pathway

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Abstract: Egb 761 is a standardized extract of Gingko biloba that exerts protective effects against ischemic brain injury. This study investigated whether Egb 761 modulates the neuroprotective effects through Akt and its downstream targets, Bad and FKHR. Adult male rats were treated with Egb 761 (100 mg/kg) or vehicle prior to middle cerebral artery occlusion (MCAO). Brains were collected 24 hours after MCAO and infarct volumes were analyzed. Egb 761 significantly reduced infarct volume. Potential activation was measured by phosphorylation of Akt at Ser473, Bad at Ser136, and FKHR at Ser256 using Western blot analysis. Egb 761 prevented the injury-induced decrease of pAkt and its downstream targets, pBad and pFKHR. Furthermore, Egb 761 prevented the injury-induced increase of cleaved caspase-3 levels. In conclusion, this study suggests that Egb 761 prevents cell death due to brain injury and that Egb 761 protection is affected by preventing the injury-induced decrease of Akt phosphorylation.

Keywords: Akt, Egb 761; Gingko biloba; Neuroprotection.

Introduction

The standardized extract Egb 761 is obtained from dried green leaves of Gingko biloba tree. This extract includes 24% flavonoids, 7% proanthocyanidins, and 6% terpenoids. The
flavonoid component is known to be a free radical scavenger and a potent antioxidant (Droy-Lefaix et al., 1995a; Oyama et al., 1996; Bastianetto et al., 2000; Calapai et al., 2000). EGb 761 prevents the neuronal cell death against oxidative stress (Ni et al., 1996). EGb 761 displays neuroprotective actions in animal models of hypoxia and ischemia (Oberpichler et al., 1988; Droy-Lefaix et al., 1995b; Ni et al., 1996; Pierre et al., 1999; Chung et al., 2006). EGb 761 increases the cerebral blood flow and reduces ischemic brain damage (Zhang et al., 2000). Furthermore, EGb 761 protects against endothelial dysfunction through the activation of Akt and subsequent eNOS phosphorylation (Koltermann et al., 2007).

The phosphatidylinositol-3 kinase (PI3-K)/Akt signal pathway is a key step for various biological effects mediating cell proliferation, growth, and survival (Datta et al., 1997; 1999). In the presence of survival factors, activated Akt phosphorylates its downstream targets, Bad and forkhead transcription factor (FKHR), and attenuates their pro-apoptotic actions. Phosphorylated Bad and phosphorylated FKHR interact with 14-3-3, which acts as an anti-apoptotic factor through interaction with pro-apoptotic molecules such as Bad and FKHR (Brunet et al., 1999; Datta et al., 1999; Masters et al., 2001; Nomura et al., 2002). However, apoptotic stimuli induce the de-phosphorylation of Bad and FKHR, and cause the release of these proteins from the interaction with pBad and 14-3-3 or pFKHR and 14-3-3. Consequently, de-phosphorylated Bad interacts with the anti-apoptotic protein Bcl-x(L), leading to release of Bax from the binding Bax and Bcl-x(L) (Yang et al., 1995; Zha et al., 1996). Bax promotes the release of cytochrome c from mitochondria into cytoplasm and the activation of the caspase cascade (Yang et al., 1995; Zha et al., 1996; Rena et al., 1999). Furthermore, the binding of 14-3-3 and phosphorylated FKHR anchors FKHR within cytoplasm and inhibits the translocation of FKHR into the nucleus. However, de-phosphorylated FKHR translocates into the nucleus, where it initiates a program of gene expression including Fas ligand expression. Fas ligand activates the cell surface of the Fas protein, which in turn activates a caspase cascade, thereby inducing cell death (Datta et al., 1999).

Although the protective effect of Gingko biloba extract has been well proven, the underlying molecular mechanisms and signaling pathways leading to Gingko's neuroprotective effect are unknown. Thus, this study examined the neuroprotective effect of EGb 761 against ischemic brain injury and investigated the neuroprotective role of EGb 761 through the activation of Akt and its downstream targets, Bad and FKHR.

Materials and Methods

Experimental Animals and Drug Treatment

Sprague-Dawley rats (male, 210–230 g, n = 60) purchased from Samtako Co. (Animal Breeding Center, Osan, Korea), were randomly divided into 3 groups, sham-operated group, vehicle-treated group, and EGb 761-treated group (n = 20 per group). A single dose of EGb 761 (100 mg/kg, Yuyu, Seoul, Korea) or vehicle alone was given via an intraperitoneal injection 1 hour before the onset of middle cerebral artery occlusion (MCAO) (Lee et al., 2002). Pretreatment with EGb 761 resulted in optimal neuroprotection in rats subjected to MCAO (Lee et al., 2002). Animals were allowed to have free access to food and water. All
procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health.

**Middle Cerebral Artery Occlusion**

Focal cerebral ischemia was induced by intra-arterial suture occlusion of the right MCAO (Longa et al., 1989). Before surgery, animals were anesthetized with sodium pentobarbital (100 mg/kg). The right common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A 4/0 monofilament nylon suture with its tip slightly rounded by heat was inserted from the external into the internal carotid artery until the tip occluded the origin of the middle cerebral artery. Animals were allowed to awaken from the anesthesia and temporarily transferred to a cage with heating lamp. At 24 hours after the onset of permanent occlusion, animals were decapitated and brains were rapidly removed.

**Quantification of Ischemic Damage**

The brains were cut into coronal slices of 2 mm in thickness. These slices were incubated for 20 min in a 2% triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) and fixed in 10% formalin. The stained slices were photographed by a Nikon CoolPIX990 digital camera (Nikon, Tokyo, Japan) and measured for the ischemic lesion by Image-ProPlus 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The ischemic lesion percentage of each slice was calculated by the ratio of the infarction area to the whole slice area.

**Western Blot Analysis**

The brains were rapidly removed and dissected into right cerebral cortex. Tissue samples were snap frozen and lysed in buffer [1% Triton X-100, 1 mM EDTA in 1 × PBS (pH 7.4)] containing 10 μM leupeptin and 200 μM phenylmethylsulfonyl fluoride. The lysates were sonicated and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were collected and the protein concentration of each lysate was determined by using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. Total protein (30 μg) was applied to each lane on 10% SDS–polyacrylamide gels. After electrophoresis and immunoblotting, the poly-vinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with the following primary antibodies: anti-Akt, anti-phospho-Akt(Ser473), anti-Bad, anti-phospho-Bad(Ser136), anti-FKHR, anti-phospho-FKHR(Ser256), anti-cleaved-caspase-3, and anti-α tubulin (diluted 1: 1,000, Cell Signaling Technology, Beverly, MA, USA). The membrane was incubated with secondary antibody (1: 5,000, Pierce) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used for detection according to the manufacturer’s protocol. The intensity analysis of Western blot was carried out by using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, USA) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, USA).
Statistical Analysis

All data are expressed as mean ± S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Student’s t-test. The difference for comparison was considered significant when *p < 0.05.

Results

Neuroprotective Effect of EGb 761

Our results confirmed that EGb 761 had a neuroprotective effect against ischemic brain injury. TTC staining showed that EGb 761 administration significantly reduces infarct volume, compared to the vehicle-treated animals (Fig. 1A). The ischemic lesion area was 37.65 ± 3.25% and 12.25 ± 1.75% in vehicle- and EGb 761-treated animals, respectively (Fig. 1B).

Phosphorylation of Akt, Bad, FKHR by EGb 761

To elucidate the neuroprotective mechanism of EGb 761 in ischemic brain injury, we investigated the phosphorylation of Akt at Ser473, Bad at Ser136, and FKHR at Ser256 in the cerebral cortex by Western blot analysis. Ischemic brain injury induced a decrease of pAkt levels, and EGb 761 administration prevented injury-induced down-regulation of pAkt.
levels of pAkt were $0.67 \pm 0.02$ and $1.03 \pm 0.04$ in cerebral cortex of vehicle- and EGB 761-treated animals, respectively (Fig. 2). Also, EGB 761 administration prevented injury-induced down-regulation of pBad and pFKHR. The level of pBad was $0.48 \pm 0.08$ in cerebral cortex of vehicle-treated animals, whereas it was $1.09 \pm 0.06$ in EGB 761-treated animals. The levels of pFKHR were $0.36 \pm 0.04$ and $0.78 \pm 0.02$ in cerebral cortex of vehicle- and EGB 761-treated animals, respectively (Fig. 3). The expression levels of Akt, Bad, and FKHR were consistently maintained in both vehicle- and EGB 761-treated animals.
Figure 4. Western blot analysis of cleaved caspase-3 in cerebral cortex from sham-operated, EGb 761- and vehicle-treated rats prior to MCAO. Each lane represents an individual experimental animal. Densitometric analysis is represented as an arbitrary unit (A.U.), normalized by α-tubulin. Data (n = 10) are represented as mean ± S.E.M. *p < 0.05.

**Inhibition of Cleaved Caspase-3 by EGb 761**

Figure 4 demonstrates that the levels of cleaved caspase-3 were increased in the cerebral cortex of vehicle-treated animals. However, in the presence of EGb 761, cleaved caspase-3 levels were decreased in the cerebral cortex. Cleaved caspase-3 levels were 1.08 ± 0.04 and 0.51 ± 0.03 in cerebral cortex of vehicle- and EGb 761-treated animals, respectively.

**Discussion**

The *Gingko biloba* extract, EGb 761, exerts a neuroprotective effect against permanent and transient focal cerebral ischemia (Pierre et al., 1999; Lee et al., 2002; Chung et al., 2006). EGb 761 also protects hippocampal neurons against cell death induced by beta-amyloid toxicity (Bastianetto et al., 2000). Furthermore, beneficial effects of EGb 761 on various neurodegenerative disorder and peripheral arterial occlusive disease have been reported in clinical trials (DeFeudis et al., 2000; Ramassamy et al., 2007). EGb 761 has a potent free radical scavenging activity and antioxidant effect (Droy-Lefaix et al., 1995b; Oyama et al., 1996; Bastianetto et al., 2000; Calapai et al., 2000). Furthermore, excessive free radical production caused by ischemic injury induces protein oxidation and DNA damage (Slemmer et al., 2008). In addition to the antioxidant function of EGb 761, we propose that EGb 761 elicits neuroprotective effect by activation of a survival pathway. A previous study suggests the fact that EGb 761 mediates the Akt signaling pathway (Koltermann et al., 2007). That study confirms that EGb 761 administration significantly decreases the cerebral infarct volume following focal cerebral ischemia by using TTC stain. Furthermore, EGb 761...
treatment mediates this neuroprotective effect by enhancing anti-apoptotic signals through Akt and its downstream targets, Bad, and FKHR.

The PI3-K/Akt signal pathway mediates the suppression of cell death through several downstream targets, including Bad, forkhead transcription factors, and caspase-9 (Datta et al., 1997; Brunet et al., 1999). The phosphorylation of Akt, Bad, and FKHR is a critical step for the enhancement of cell survival. Egb 761 treatment acutely enhanced the phosphorylation of Akt (Koltermann et al., 2007). However, there is little information on other downstream targets of Akt such as Bad and FKHR. This study focused on the neuroprotective effect of Egb 761 on the activation of Akt and its down-stream targets, Bad and FKHR, in ischemic brain injury. Our results show that the total levels of Akt, Bad, and FKHR were not changed in vehicle- and Egb 761-treated animals. However, the levels of pAkt, pBad, and pFKHR were decreased in the brain injury and that Egb 761 administration inhibits down-regulation of these proteins. Apoptotic stimuli can induce de-phosphorylation of Bad and release Bad from its interaction with pBad and 14-3-3, resulting in the activation of Bax and caspase cascade (Brunet et al., 1999; Datta et al., 1999; Rena et al., 1999). Furthermore, apoptotic conditions induce de-phosphorylation of FKHR and initiate Fas ligand gene activation and caspase cascade activation (Brunet et al., 1999). Thus, the maintenance of phosphorylated Bad and phosphorylated FKHR is an essential step to block apoptosis. In regions of ischemic injury, pBad and pFKHR levels are decreased, Egb 761 administration prevents the injury-induced decreased levels of pBad and pFKHR. Moreover, our data demonstrate that Egb 761 treatment prevents the injury-induced increase of cleaved caspase-3. Thus, we hypothesized that the maintenance of pBad and pFKHR by Egb 761 treatment during ischemic brain injury blocks the activation of the caspase cascade and contributes to the neuroprotective activity of Egb 761.

In conclusion, this study suggests that Egb 761 plays a potent neuroprotective role by preventing the injury-induced decrease of pAkt and its down-stream targets, Bad and FKHR. Thus, Egb 761 prevents the injury-induced inactivation of the Akt signaling pathway, thereby preventing neuronal cell death.

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References


