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BILOBALIDE, A COMPONENT OF THE *GINKGO BILOBA* EXTRACT (EGb 761), PROTECTS AGAINST NEURONAL DEATH IN GLOBAL BRAIN ISCHEMIA AND IN GLUTAMATE-INDUCED EXCITOTOXICITY

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Abstract - In this study, the effect of bilobalide, a purified terpene lactone component of the *Ginkgo biloba* extract (EGb 761), and EGb 761 against ischemic injury and against glutamate-induced excitotoxic neuronal death was compared. In the case of ischemic injury, neuronal loss and the levels of mitochondrial DNA (mtDNA)-encoded cytochrome oxidase (COX) subunit III mRNA in the hippocampal regions of gerbils was measured. A significant increase in neuronal death and a significant decrease in COX III mRNA were observed in the hippocampal CA1 neurons at 7-days of reperfusion after 5 min of transient global forebrain ischemia. Oral administration of EGb 761 at 25, 50 and 100 mg/kg/day and bilobalide at 3 and 6 mg/kg/day for 7 days before ischemia progressively protected hippocampal CA1 neurons against ischemia-induced neuronal death and reductions in COX III mRNA. In rat cerebellar neuronal cultures, addition of bilobalide or EGb 761 protected in a dose-dependent manner against glutamate-induced excitotoxic neuronal death [effective concentration (EC₅₀) = 5 µg/ml (12 µM) for bilobalide and 100 µg/ml for EGb 761]. These results suggest that both EGb 761 and bilobalide protect against ischemia-induced neuronal death *in vivo* and glutamate-induced neuronal death *in vitro* by synergistic mechanisms involving anti-excitotoxicity, inhibition of free radical generation, scavenging of reactive oxygen species, and regulation of mitochondrial gene expression.

Key words: Hippocampus, histology, *in situ* hybridization, mitochondrial mRNA, cytochrome oxidase

INTRODUCTION

A standardized extract of the leaves of *Ginkgo biloba*, e.g. EGb 761, has neuroprotective properties (13,31). For example, oral administration of EGb 761 has been shown to protect against neuronal death in the gerbil hippocampus after transient global ischemia (32,33), after focal ischemia induced by middle cerebral artery occlusion in rats (9,39), after ischemia-reperfusion injury in isolated hearts (26), and against ischemic injury in patients undergoing coronary bypass (27).

The extract (EGb 761) contains 24% flavonol glycosides, 6% terpene trilactones (ginkgolides A, B, C and bilobalide), about 7% proanthocyanidines, and other common compounds (13). It is generally hypothesized that such neuro- and cardio-protection is associated with the free-radical

scavenging and/or antioxidant properties of EGb 761, due to the flavonoid constituents of EGb 761 (8,12,13,14,31). However, to what extent the other components in the extract, particularly bilobalide - the terpene lactone component of the extract, could also contribute to protection against neuronal injury is unknown.

Recent results using brain slices and tissues suggest that bilobalide may be anti-excitotoxic and regulate mitochondrial metabolism including its gene expression. In rat hippocampal slices, hypoxia and *N*-methyl-D-aspartate (NMDA)-induced phospholipase A₂ activation and consequent phospholipid breakdown is inhibited in the presence of bilobalide (36). In rat cortical slices, addition of bilobalide reduced the hypoxia/hypoglycemia-stimulated release of the excitatory amino acid, glutamate (20,21). Oral administration of bilobalide protected rat brains against ischemia-induced alterations in activities of mitochondrial oxidative phosphorylation complexes (OXPHOS) I and III and thus delay the onset of ischemia-induced damage (3,18,19). In rat

Abbreviations: EGb 761: *Ginkgo biloba* extract; mtDNA: mitochondrial DNA; NMDA: *N*-methyl-D-aspartate; ROS: reactive oxygen species

pheochromocytoma PC12 cells differentiated with nerve growth factor, addition of bilobalide was shown to increase levels of mitochondrial DNA (mtDNA)-encoded cytochrome oxidase (COX) subunit III mRNA and NADH dehydrogenase subunit I mRNA (5,34), by a post transcriptional mechanism of mRNA stability (5,6). Taken together, these findings raised the possibility that bilobalide may be able to antagonize the response of the excitatory amino acid glutamate *in vitro* and protect vulnerable neurons against ischemic injury *in vivo*.

This study therefore investigated the protective effect of bilobalide and *Ginkgo biloba* extract: a/ against ischemia-induced neuronal death and ischemia-induced reductions in mitochondrial gene expression in gerbils, and b/ against glutamate-induced excitotoxic death in primary cultures of rat cerebellar granule neurons. Previous studies in ischemia-reperfusion showed that mitochondrial gene expression is a sensitive marker of mitochondrial dysfunction that occurs early in reperfusion (1). We used *in situ* hybridization of mtDNA-encoded COX III mRNA to monitor mitochondrial gene expression to test whether the protective mechanisms were activated. The results showed that oral administration of bilobalide and *Ginkgo biloba* extract protected brain against ischemia-induced delayed neuron death and reductions in mitochondrial gene expression in vulnerable neurons. Results obtained with primary cultures of rat cerebellar granule neurons suggested that both bilobalide and *Ginkgo biloba* extract protect against glutamate-induced neuronal death. These results suggest that the EGb 761 may mediate neuroprotection by multiple mechanisms involving anti-excitotoxicity, inhibition of free radical generation, scavenging of reactive oxygen species and regulation of mitochondrial gene expression.

MATERIALS AND METHODS

Animal experiments

Procedures involving animals and their care were conducted in conformity with institutional guidelines.

Male Mongolian gerbils (*Meriones unguiculatus*; Tumblebrook farm, West Brookfield, USA), aged 12 weeks and weighing 80-100 g were used. Animals were divided into seven groups and each group included six animals. The groups were sham operated controls, animals subjected to ischemia, animals pretreated orally with EGb 761 at 3 different doses, and animals pretreated with bilobalide at 2 different doses. Animals were subjected to 5 min global ischemia followed by reperfusion for 1 day or 7 days as described (7). One day or 7 days after ischemia, gerbils were anesthetized, decapitated and their brains were carefully removed and frozen in isopentane to -40°C for 1 min and stored at -70°C.

Drugs

Ginkgo biloba extract (EGb 761, from Beaufour Ipsen, Paris) was administered at 25, 50 and 100 mg/kg/day orally, and bilobalide was administered at 3 and 6 mg/kg/day orally for 7 days before ischemia. EGb 761 and bilobalide were solubilized in 2 drops of Tween 80 and water. The same pretreatment times and routes of administration of tween/water were used for sham-operated and ischemic control gerbils.

Histology and *in situ* hybridization

Brain sections (20 μ m) at dorsal hippocampal levels were cut on a cryotome at -20°C. Frozen adjacent brain sections from each animal were fixed, stained with cresyl violet (0.1%), and examined by light microscopy. *In situ* hybridization was done with α -³³P-dCTP labeled COX III probe (4).

The number of intact pyramidal cells with a distinct nucleus and nucleolus in a 1 mm length of the middle portion of the CA1 subfield was counted on the hippocampus (7). Values are expressed as the mean \pm SEM. Levels of COX III mRNA in neurons were determined by counting silver grains over neuronal cell bodies (7). Neuronal cell bodies were selected for counting from random fields of the middle portion of the CA1 subfield. Grain counts were determined over 10 randomly selected pyramidal neurons from each brain.

Data were analyzed by a one-way analysis of variance or repeated measures of analysis of variance. Tukey's post-comparisons procedure was then used to determine which pairs of means differed. Statistical significance was defined as value $p < 0.05$.

Primary rat cerebellar cultures

Cerebellar granule cell cultures were prepared from 7-day old Sprague-Dawley rats using a standard method (30). Neurons were plated at a density of 2×10^5 cells/cm² in 6-well tissue culture chambers coated with poly-L-lysine (MW 30 000 - 700 000) and cultured in Eagle's Basal Medium supplemented with Earle's salts, 10% inactivated fetal calf serum, 25 mM KCl and gentamycin (50 ng/ml). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% air. Twenty-four hr after plating, cytosine arabinoside (10 μ M) was added to the cultures to prevent growth of glial cells. To ensure sensitivity to glutamate, we routinely used 8 day-old cultures (8-DIV).

Exposure of neurons to glutamate

On day 8 in culture, the cell culture medium was removed and stored. Neuronal cultures were washed once with prewarmed (37°C) Locke solution (134 mM NaCl, 5 mM KCl, 4 mM NaHCO₃, 5 mM HEPES, 2.3 mM CaCl₂ and 5 mM glucose) and incubated for 15 min. Immediately after, L-glutamate (50-250 μ M) and glycine (10 μ M) were added, and the cells were further incubated for 30 min at room temperature. After exposure to glutamate, the cells were washed and kept in the old culture medium without glutamate for up to 24 hr. Control cultures were treated with the vehicle for the same time period as that of glutamate treated cells. In case of experiments with bilobalide and EGb 761, they were added to Locke solution 30 min prior to exposure to glutamate. Both bilobalide and EGb 761 were dissolved in 60% alcohol and the final alcohol concentration was less than 0.01%. Stock solutions of MK-801 and 7-chlorokynurenic acid (7-CKYN) were prepared in water and added prior to the addition of glutamate.

Cell viability

Cell viability was determined using propidium iodide (PI). Non-viable granule cell neurons were quantified with cell-impermeant PI (10 μ g/ml). The number of cells that displayed PI fluorescence to the total number of cells in a field was determined.

Statistical analysis

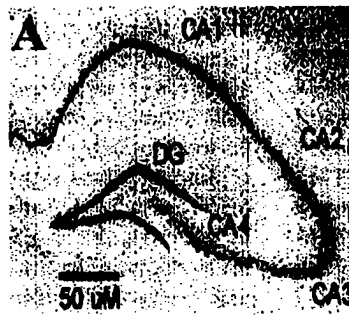
Results are expressed as the mean \pm SEM. Differences between controls and test samples were evaluated by ANOVA: Fischer's F-test was first used to compare between-groups and within-groups variance, if the former was significantly ($p < 0.05$) higher than the latter, individual groups were compared by Turkey's test for multiple comparisons.

RESULTS

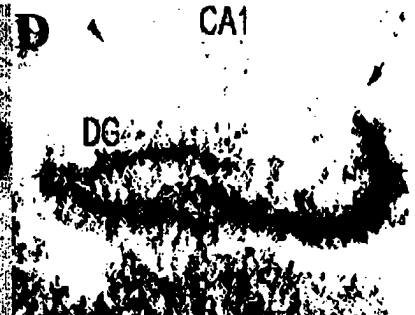
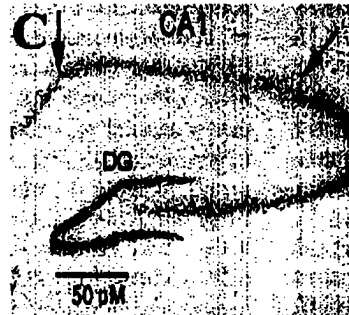
Changes in neuronal density and COX III mRNA and COX activity in hippocampal areas after ischemia and reperfusion

The normal distribution of neurons in dentate gyrus, hippocampal areas of CA4, CA3, CA2 and CA1 are shown in Fig. 1A. Photomicrographs that show delayed neuronal death

Non-ischemic



Ischemic



**Egb 761
(100 mg/kg/day)**



**Bilobalide
(6 mg/kg/day)**



Cresyl violet stain

COX III mRNA

Fig. 1 Neuroprotective effect of bilobalide and Egb 761 in gerbil brain ischemia. Histology and *in situ* hybridization for COX III mRNA, respectively, in hippocampal region of the gerbil brains in sham control (A-B), ischemic (C-D), pretreated orally with Egb 761 extract (100 mg/kg/day) (E-F) and pretreated orally with bilobalide (6 mg/kg/day) (G-H) is shown. Note a decrease in neuronal density and a complete loss of COX III mRNA in CA1 area in ischemic-animals (C-D) as compared to sham control (A-B). An increase in neuronal density and COX III mRNA *in situ* hybridization signals in CA1 area of animals pretreatment with Egb 761 (E-F) and bilobalide (G-H) was apparent. Or, Py, Rad and LMOL, oriens layer, pyramidal cell layer, stratum radiatum and lacunosum moleculare layer of the CA1 subfield, respectively. DG: dentate gyrus granule neurons; hippocampal areas are marked CA 4 to CA 1. Bar: 50 μm

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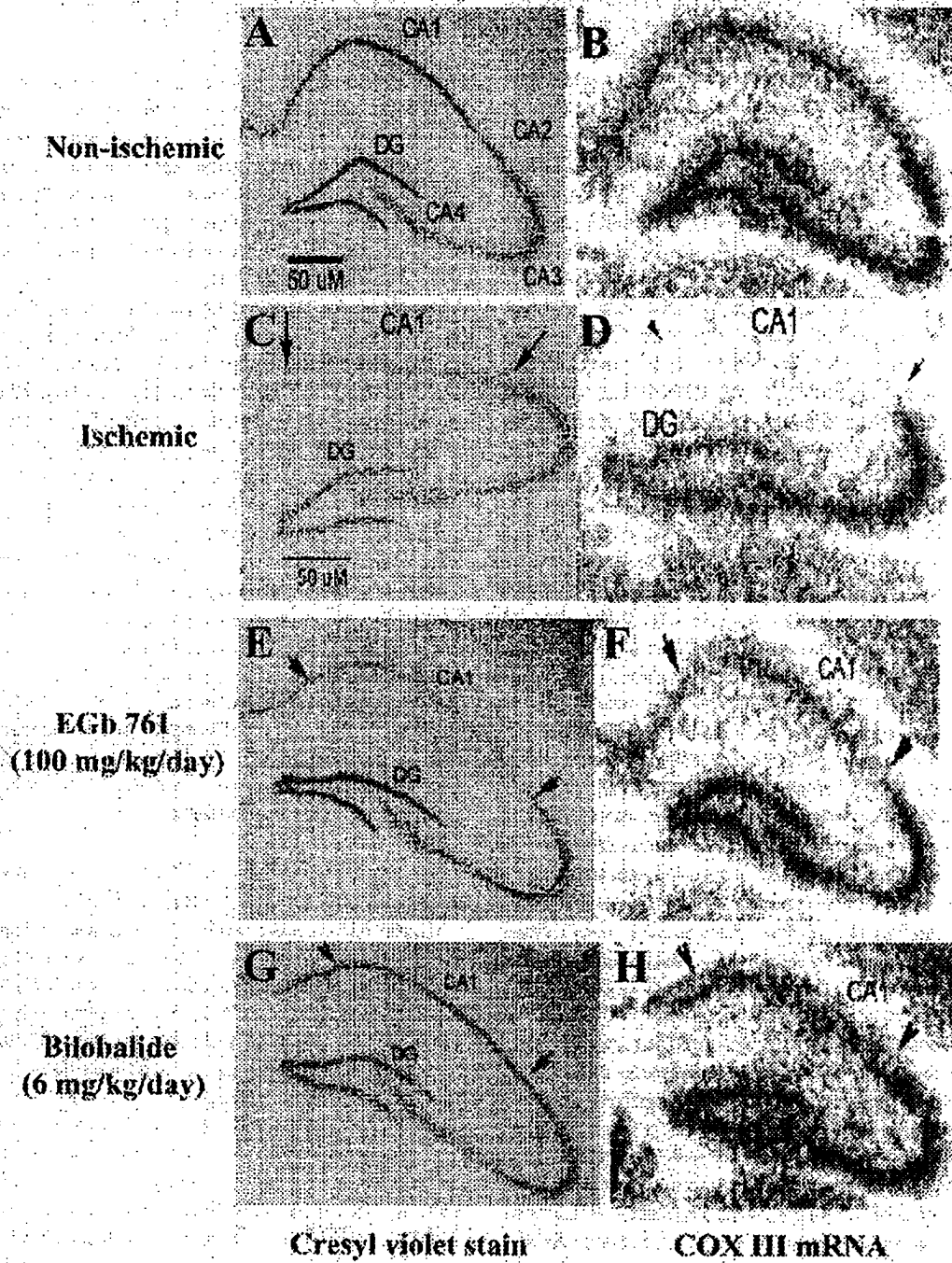


Fig. 1 Neuroprotective effect of bilobalide and EGb 761 in gerbil brain ischemia. Histology and *in situ* hybridization for COX III mRNA, respectively, in hippocampal region of the gerbil brain in sham control (A-B), ischemic (C-D), pretreated orally with EGb 761 extract (100 mg/kg/day) (E-F) and pretreated orally with bilobalide (6 mg/kg/day) (G-H) is shown. Note a decrease in neuronal density and a complete loss of COX III mRNA in CA1 area in ischemic animals (C-D) as compared to sham control (A-B). An increase in neuronal density and COX III mRNA *in situ* hybridization signals in CA1 area of animals pretreatment with EGb 761 (E-F) and bilobalide (G-H) was apparent. Cr, Py, Rad and LMOL, oriens layer, pyramidal cell layer, stratum radiatum and lacunosum moleculare layer of the CA1 subfield, respectively. DG, dentate gyrus granule neurons; hippocampal areas are marked CA 4 to CA 1. Bar: 50 μ m.

in hippocampal CA1 neurons after ischemia/reperfusion are presented in Fig. 1C. In animals subjected to 5 min ischemia and 7-days reperfusion, there was a reduction in the number of CA1 neurons. In contrast, the dentate granule cells, CA4 and CA3 neurons displayed normal neuronal distributions (Fig. 1C). Neuronal death in hippocampal CA1 region of gerbils is delayed and occurs 3 to 4 days after an initial 5 min ischemic insult (22,33). Quantification of CA1 neuronal density (cells/mm) at 7-days reperfusion showed 80% decrease (225 ± 5.8 vs. 48.3 ± 11.4) in ischemic animals compared to controls.

In control brains sections, hybridization with mtDNA-encoded COX III probe revealed that grains for COX III mRNA were predominantly located in the neuronal cell bodies. Highest levels of COX III mRNA were found in neuronal cell bodies of dentate gyrus and hippocampal areas of CA4, CA3, CA2 and CA1 (Fig. 1B). In sections from animals subjected to 5 min ischemia and 7-days reperfusion, COX III mRNA in hippocampal CA1 cells was completely lost, while other hippocampal neurons such as CA4, CA3 and dentate granule cells showed almost no change in COX III mRNA levels (Fig. 1D). Quantification of the COX III mRNA (grain counts) over the cell body in CA1 neurons at 7-days of reperfusion showed >90% decrease (244 ± 7.8 vs. 22 ± 1.8) in ischemic animals compared to controls.

Effect of oral administration of Ginkgo biloba extract (EGb 761) on neuronal density, COX III mRNA and COX activity in hippocampal areas after ischemia and reperfusion

Ginkgo biloba extract (EGb 761) was administered at 25, 50 and 100 mg/kg/day orally for 7 days before ischemia. The animals were then subjected to 5 min ischemia and 7-days reperfusion. Adjacent brain sections at the hippocampal level were subjected to *in situ* hybridization and cresyl violet staining to determine the levels of COX III mRNA and distribution of neurons, respectively. The results obtained with the administration of the highest dose of EGb 761 are shown in Fig. 1. In animals that were pretreated with EGb 761 and then subjected to ischemia, increased levels of COX III mRNA and increased survival in CA1 neurons were observed compared to animals treated with the vehicle alone (compare Fig. 1C with 1E and Fig. 1D with 1F). Quantification of the neuronal density in the CA1 region indicated that survival of neurons increased from 48.3 ± 11.4 cells/mm (= 21 ± 5 % of control) in ischemic animals to 173.3 ± 25.5 cells/mm (= 76.9 ± 11.3 % of control) in animals that were pretreated with 100 mg/kg/day EGb 761. EGb 761 pretreatment resulted in a protection against loss of COX III mRNA levels within the CA1 neurons. The grain counts increased from 22 ± 3.8 (= 9.3 ± 1.3% of control) in ischemic animals to 88 ± 17 (= 77.2 ± 14.9% of control) in animals pretreated with EGb 761. Thus, in CA1 neurons of gerbils pretreated with EGb 761, there was

an increase in protection against neuronal death and ischemia-induced decrease in COX III mRNA levels.

Effect of oral administration of bilobalide on neuronal density COX III mRNA and COX activity in hippocampal areas after ischemia and reperfusion

Bilobalide was administered at 3 and 6 mg/kg/day orally for 7 days before ischemia. The animals were then subjected to 5 min ischemia and 7-days reperfusion. Brain sections at the hippocampal level were subjected to *in situ* hybridization and cresyl violet staining. The results obtained with administration of 6 mg/kg/day of bilobalide are shown in Fig. 1G and 1H. Increased levels of COX III mRNA, increased COX activity and increased survival of CA1 neurons were observed in animals that were pretreated with bilobalide and then subjected to ischemia compared to vehicle controls (compare Fig. 1C with 1G and Fig. 1D with 1H). In animals pretreated at 3 and 6 mg/kg/day with bilobalide, the level of COX III mRNA in CA1 neurons increased from 22 ± 3.8 (= 9.3 ± 1.3% control) in ischemic animals to 74 ± 7 (= 64.9 ± 6.2% of control) and 93.3 ± 17.3 (= 81.9 ± 15.2% of control), respectively. In animals pretreated at 3 and 6 mg/kg/day with bilobalide, the protection against loss of CA1 neurons increased from 48.3 ± 11.4 cells/mm (= 21 ± 5% control) in ischemic animals to 148 ± 5.8 cells/mm (= 65.7 ± 2.7% of control) and 186.7 ± 7.1 cells/mm (= 82.8 ± 3.1% of control), respectively. Thus, in gerbils pretreated with bilobalide, there was an increase in protection against neuronal death and ischemia-induced decrease in COX III mRNA in CA1 neurons.

Effects of bilobalide and EGb 761 on glutamate-induced acute cell death in cerebellar granule neurons

Cerebellar granule neurons were maintained in culture for 8 days. At 8 DIV, the cell culture was exposed to glutamate (100 μM) for 30 min in the presence of 10 μM glycine. After exposure to glutamate, cells were washed and kept in the old culture medium without glutamate for up to 24 hr. Control cultures were treated with the vehicle for the same time period as that of glutamate treated cells. Neuronal viability was determined by propidium iodide (PI) staining. Exposure of cerebellar granule neurons to 100 μM glutamate for 30 min decreased the neuronal viability ($60 \pm 7\%$ PI-positive neurons). The glutamate-induced neurotoxicity was blocked by the selective NMDA-receptor antagonist MK-801 (10 μM) and by the glycine antagonist 7-chlorokynurenic acid (10 μM).

Addition of bilobalide or EGb 761 to the Locke solution for 30 min prior to the addition of glutamate (100 μM) was able to reduce glutamate-induced neurotoxicity in a dose-dependent manner. The inhibitory concentrations (IC₅₀) were determined to be 5 μg/ml for bilobalide (n= 4; Fig. 2A) and 100 μg/ml for EGb 761 extract (n= 4; Fig. 2B).

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Fig. neu with (B) 10 μ IC₅₀

DISCUSSION

The protective effect of the *Ginkgo biloba* extract against cerebral ischemia and hypoxia in rats has been previously demonstrated (32). Spinnewyn *et al.* (33) showed that repeated oral administration of EGb 761 at 15, 30 and 60 mg/kg/day for 14 days protected hippocampal CA1 neurons from transient ischemia-induced delayed neuronal death. Recent studies have shown a protective effect of *Ginkgo biloba* extract against neuronal death in rats after focal ischemia induced by middle cerebral artery occlusion (9,39). The present study expands upon these results by demonstrating that bilobalide, a specific component of *Ginkgo biloba* extract, exhibits protection against delayed ischemic neuronal death similar to that observed with administration of EGb 761.

In animals subjected to 5 min ischemia and 7-days reperfusion, there was a significant reduction in mtDNA-encoded COX subunit mRNA levels and COX activity in the vulnerable neurons of the hippocampus (1). In a recent study, using *in situ* hybridization results with COX III probe, we showed that a significant decrease in COX III mRNA levels occurs in ischemic animals compared to non-ischemic controls at 1-day of reperfusion (7). There was no significant neuronal death at 1-day of reperfusion, suggesting that the reduction in COX III mRNA level precede neuronal death. Oral administration of EGb 761 and bilobalide prior to ischemia protected against ischemia-induced reduction in COX III mRNA at 1-day and at 7-days of reperfusion (7). The preservation of mtDNA-encoded COX III mRNA level in CA1 neurons at 1-day of reperfusion in animals that were pretreated with bilobalide or EGb 761 suggest that the extract contains substances that act at early stages of reperfusion to reduce ischemic brain damage (7).

The mechanism of neuroprotection against ischemic injury by EGb 761 is not completely understood. The extract contains 24% flavonol glycosides and 3% bilobalide (13). The flavonol glycosides has the ability to scavenge free-radicals and the extract is thus able to prevent the release of free radicals and protect neurons against injury (11,13,25,26,29,31,37). The cellular damage that occurs during cerebral ischemia and reperfusion is at least partly due to oxidative stress (15,16,17,24,35). Antioxidants are shown to be neuroprotective in various models of ischemia (10). Therefore, the protection observed with the *Ginkgo biloba* extract could potentially be entirely attributed to the antioxidant capacity of the extract.

Though the flavonoids and ginkgolides may be partly responsible for the neuroprotection afforded by EGb 761, the significance of the present investigation is the ability of the other terpene lactone of the extract to exert protection by an apparently different mechanism. In cell-free experiments, addition of bilobalide exhibits little if any ability to scavenge superoxide and hydroxyl radicals (26). Addition of EGb 761 and ginkgolide B but not of bilobalide protected primary cultures of hippocampal neurons against oxidative stress-induced neural death (28). The total flavonoid component of EGb 761 and a mixture of flavonoids and terpenes protected cerebellar granule cells from oxidative damage and apoptosis induced by hydroxyl radicals, whereas total terpenes of EGb 761 did not protect against apoptosis (38). The nitric oxide (NO)-induced increase in free radical accumulation and in cell death in rat primary mixed hippocampal cell cultures were blocked by either EGb 761 (10-100 µg/ml) or its flavonoid fraction CP 205 (25 µg/ml) but not by the terpenoid constituents of EGb 761 (2). Thus, the mechanism of protection by bilobalide may not be related to a direct antioxidant activity.

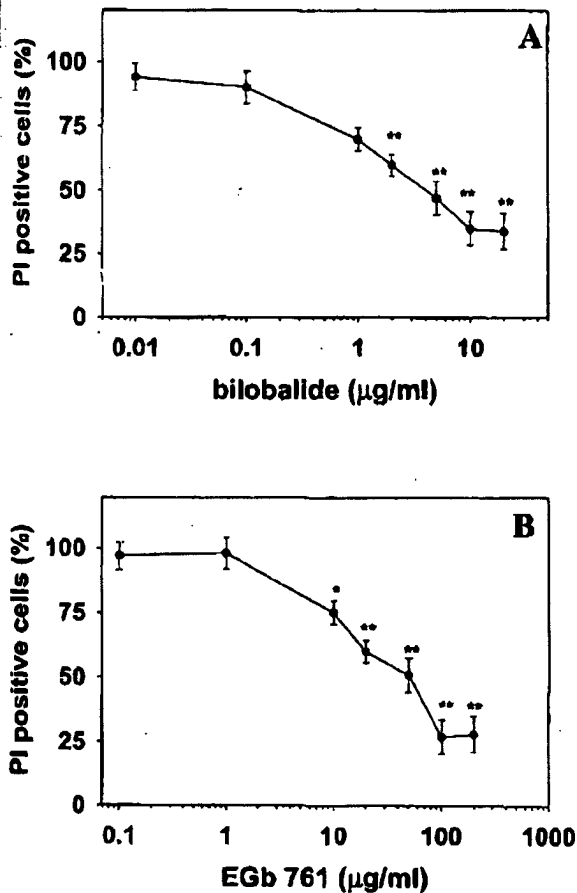


Fig. 2 Dose-dependent inhibition of glutamate-induced neurotoxicity by bilobalide and EGb 761. Cultures were treated with the indicated concentrations of bilobalide (A) or EGb 761 (B) for 30 min, then exposed for 30 min to 100 µM glutamate + 10 µM glycine. Neuronal viability was measured 24 hr later. The IC₅₀ for bilobalide was 12 µM and EGb 761 was 100 µg/ml.

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Our results demonstrating the ability of bilobalide to reduce glutamate-induced excitotoxic neuronal death suggests that this agent may also act at the level of NMDA receptor release or activation. Recently it has been shown that NMDA-induced phospholipase A2 activation and phospholipid breakdown in rat hippocampal slice cultures is inhibited by the addition of bilobalide and that increasing glycine concentration antagonizes the effect of bilobalide (36). Addition of bilobalide was shown to reduce the release of the excitatory amino acid, glutamate in rat cortical slices (20,21). These results suggest that the neuroprotective properties of bilobalide may, in part, be mediated through its ability to decrease in the excitotoxic effects of this neurotransmitter.

In conclusion, our results suggest that both EGb 761 and bilobalide are protective against ischemia-induced neuronal death *in vivo* and glutamate-induced neuronal death *in vitro* by synergistic mechanisms involving anti-excitotoxicity, inhibition of free radical generation, scavenging of reactive oxygen species, and preservation of mitochondrial gene expression against ischemia-induced decreases. This effect might explain the apparent therapeutic potential of *Ginkgo biloba* extract in cerebrovascular and neurodegenerative disorders (13,23).

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