Efficacy of Antioxidant Therapies in Transient Focal Ischemia in Mice

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Background and Purpose—Ginkgo biloba extract (EGb) and α-lipoic acid (LA) are commercially available “antioxidant supplements” with a variety of actions that may be beneficial during acute stroke. These actions include inhibiting platelet and leukocyte activation and adhesion, reducing free radical generation, and increasing cerebral blood flow. Both EGb and LA have been shown to be neuroprotective in cell culture and global central nervous system ischemia models. In this study we investigated the neuroprotective efficacy of EGb and LA in a clinically relevant, transient focal central nervous system ischemic model.

Methods—In the EGb study, 60 adult C57blk mice were randomized to treatment with EGb given orally (via gavage) for 7 days: low dose, 50 EGb mg/kg daily; high dose, 100 mg/kg daily; matched placebo. On day 7, reversible middle cerebral artery occlusion was produced by advancing a silicone-coated 8-0 filament into the internal carotid artery for 45 minutes followed by reperfusion. At 24 hours, the animals were evaluated on a 28-point clinical scale, and infarct volume was determined with the use of triphenyltetrazolium chloride. In the LA study, 24 C57blk mice were treated with 100 mg/kg SC of LA or placebo 1.5 hours before transient MCAO, as in the EGb study.

Results—In the EGb study, values for infarct volume at 24 hours were as follows (mean±SD): low dose (n=18), 13±5 mm³; high dose (n=22), 22±12 mm³; placebo (n=20), 20±10 mm³ (P=0.03 overall; P=0.02, low dose versus placebo). Infarct percentage of hemisphere values were as follows: low dose, 14±5%; high dose, 21±11%; placebo, 20±9% (P=0.03 overall; P=0.02, low dose versus placebo). Ten percent of the high-dose group showed significant intracerebral hemorrhage (ICH) within the infarct, while no ICH was seen in the other groups. Neurological function scores were as follows: low dose, 11.8±1.5; high dose, 11.4±1.7; placebo, 11.3±1.8 (P=NS). In the LA study, infarct volume was as follows: 100 mg/kg LA (n=12), 16.8±8.3 mm³; placebo (n=12), 27.2±14.6 mm³ (P<0.05). LA also produced a significant improvement in neurological function at 24 hours: LA, 9.5±1.2; placebo, 11.2±1.8 (P=0.02). There was no evidence of ICH in any of the animals.

Conclusions—Both oral EGb and LA therapies produced significant reductions in stroke infarct volume. However, for EGb this beneficial effect appears to be dose related, with higher doses potentially increasing the risk of ICH. (Stroke. 2001;32:1000-1004.)

Key Words: animal models ■ antioxidants ■ neuroprotection ■ oxygen radical ■ mice

Although restoring blood flow to the brain has been shown to be beneficial in both experimental and clinical stroke, there is increasing evidence that delayed reperfusion may actually potentiate central nervous system (CNS) ischemic injury.1-3 Further improvements in functional outcome after thrombolysis may be achieved by measures that counteract this reperfusion injury. Some of the mediators of reperfusion injury appear to be due to an inflammatory response involving free radical generation, activated leukocytes, and platelet-activating factor (PAF).1-3 Various commercially available “antioxidant supplements” appear to produce clinical benefit in several diseases. These supplements, including Ginkgo biloba extract (EGb) and α-lipoic acid (LA), have a multitude of biological effects that potentially may reduce reperfusion injury.4 EGb is extracted from the leaves and nuts of the Ginkgo biloba tree and has been used for centuries in Asia and Europe for a variety of disorders.4 It has recently been recognized in the United States after receiving Food and Drug Administration approval for clinical study in memory disorders.4,5 EGb extract contains flavonoids, which appear to possess strong free radical scavenging and anti-inflammatory properties,5-6 and terpenoids (ginkgolides A and B),7 which inhibit PAF and decrease free radical release.8 Treatment with EGb has shown beneficial effects in reducing reperfusion injury in CNS global ischemia and trauma experimental models.9

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LA is a thiol antioxidant (similar to glutathione) that is absorbed from the diet and crosses the blood-brain barrier. \(^\text{10}\) LA is taken up and reduced in cells to dihydrolipoate (DHLA), which is exported extracellularly. \(^\text{10}\) LA and DHLA have been shown to inhibit nuclear factor-κ (NF-κ) activation and decrease leukocyte adhesion receptor expression. \(^\text{11}\) LA has been shown to reduce infarct volume in permanent (clipped) focal middle cerebral artery occlusion (MCAO) models in both rats and mice. \(^\text{12,13}\) Since the inflammatory response appears to be more important in transient ischemia (reperfusion injury), LA may be more effective in a reversible MCAO model.

The overall objective of this study was to investigate the neuroprotective efficacy of EGb and LA in the treatment of CNS ischemic injury with an animal model that closely approximates clinical stroke with reperfusion.

**Materials and Methods**

**Experimental Design**

All animal procedures were approved by the Oregon Health Sciences University Institutional Review Board and were in accordance with guidelines for animal use published by the National Institutes of Health. In the EGb study, 60 C57Blk6 male mice were randomized to treatment with EGb761 (Dr Willmar Schwabe Pharmaceuticals) for 7 days. Oral EGb was gavaged at 100 mg/kg (high-dose group), 50 mg/kg (low-dose group), or matched placebo. A 20-gauge gavage needle was used, and weights were recorded daily for precise dosing. At 2 hours after treatment on day 7, the animal was subjected to CNS ischemia (see below). In the LA study, 24 C57Blk6 male mice were randomized to treatment with 100 mg/kg of LA (Sigma-Aldrich Chemical) or matched placebo given subcutaneously 1.5 hours before ischemia.

**CNS Ischemia Model**

Transient ischemia was produced by filament occlusion of the right MCA following a modification of the method originally reported by Koizumi et al. \(^\text{14}\) using nylon monofilaments (8-0) coated with silicone. The animals were anesthetized via inhalation mask with isoflurane, with a criterion cerebral blood flow between groups. Lesion volume at 24 hours was as follows (mean ± SD): low dose (n = 18), 13 ± 5 mm\(^3\); high dose (n = 22), 22 ± 12 mm\(^3\); placebo (n = 20), 20 ± 10 mm\(^3\) (P = 0.03 overall; df = 2; F = 3.89; P = 0.02, low dose versus placebo; df = 36; t test, 2.46). Lesion percentage of hemisphere values were as follows: low dose, 14 ± 5%; high dose, 21 ± 11%; placebo, 20 ± 9% (P = 0.03 overall; df = 2; F = 3.73; P = 0.02, low dose versus placebo; df = 36; t test, 2.52) (Table). Three of the animals in the high-dose group showed significant intracerebral hemorrhage (ICH) within the infarct, while no ICH was seen in the other groups (Figure). Neurological function scores were as follows: low

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**Results**

**Egb Study**

A total of 60 animals were evaluated. No behavioral side effects were seen with treatment before stroke. There were no differences in body weight or preischemic or postischemic cerebral blood flow between groups. Lesion volume at 24 hours was as follows (mean ± SD): low dose (n = 18), 13 ± 5 mm\(^3\); high dose (n = 22), 22 ± 12 mm\(^3\); placebo (n = 20), 20 ± 10 mm\(^3\) (P = 0.03 overall; df = 2; F = 3.89; P = 0.02, low dose versus placebo; df = 36; t test, 2.46). Lesion percentage of hemisphere values were as follows: low dose, 14 ± 5%; high dose, 21 ± 11%; placebo, 20 ± 9% (P = 0.03 overall; df = 2; F = 3.73; P = 0.02, low dose versus placebo; df = 36; t test, 2.52) (Table). Three of the animals in the high-dose group showed significant intracerebral hemorrhage (ICH) within the infarct, while no ICH was seen in the other groups (Figure). Neurological function scores were as follows: low
LA Study
A total of 24 animals were evaluated. No side effects were seen, and no differences in weight or cerebral blood flow were seen. Treatment with LA produced a significant reduction in lesion size at 24 hours: 100 mg/kg LA (n=12), 16.8±8.3 mm³; placebo (n=12), 27.2±14.6 (P<0.05; df=21; t test, 2.08). LA also produced a significant improvement in neurological function at 24 hours: LA, 9.5±1.2; placebo, 11.2±1.8 (P=0.02; df=21; t test, 2.49). There was no evidence of ICH in any of the animals.

Discussion
This study found that pretreatment with 2 different antioxidant supplements, EGb and LA, produced a significant reduction in lesion size at 24 hours: 100 mg/kg LA (n=12), 16.8±8.3 mm³; placebo (n=12), 27.2±14.6 (P<0.05; df=21; t test, 2.08). LA also produced a significant improvement in neurological function at 24 hours: LA, 9.5±1.2; placebo, 11.2±1.8 (P=0.02; df=21; t test, 2.49). There was no evidence of ICH in any of the animals.

In this study we found a 35% to 40% relative reduction in lesion size at 24 hours with EGb and LA. The magnitude of this reduction is similar to that seen in other studies involving anti-reperfusion injury agents in this model. We saw a treatment effect on functional outcome at 24 hours with LA but not EGb in this study. As is the case in clinical stroke, infarct size in the MCAO model does not always correlate with outcome. In prior studies we found a correlation of r=0.76 (R²=0.49) between the 28-point examination and infarct volume. It is unclear why a corresponding neurologically improved treatment was not seen with EGb treatment. This study also does not allow for a direct comparison of the relative efficacy or safety of EGb and LA because different treatment paradigms were used for the 2 agents.

Although the exact neuroprotective mechanism of EGb is not known, there is strong support for its role as a putative anti-reperfusion injury therapy. The flavonoid components have been shown to be responsible for directly scavenging free radicals, decreasing lipid peroxidation, and reducing free radical generation in experimental ischemia/reperfusion models. The terpenoid component (ginkgolides A and B) of EGb has been shown to improve blood flow and reduce thrombus formation by inhibiting PAF. The ginkgolides have also been shown to inhibit neutrophil activation, adhesion, and infiltration during reperfusion. EGb has also been shown to be beneficial in experimental global CNS ischemia models. Preischemic administration of ginkgolide B (50 mg/kg) was found to significantly reduce neuronal necrosis at 7 days in a transient forebrain ischemia model in rats. In the same model, Krieglstein et al found that EGb (100/kg IV) given 45 minutes after ischemia was able to increase cerebral blood flow and diminish delayed hypoperfusion. EGb also produced beneficial effects on functional outcome in a cerebral microemboli model in rats and in a global cerebral ischemia model in dogs. Clinically, EGb is readily available and widely used for the treatment of memory disorders. Numerous trials have found small but significant beneficial effects of EGb (120 to 240 mg/d) on memory and social function in early dementia. Potential bleeding complications of EGb are a concern since this population is also at increased risk for ICH because of coexisting hypertension and cerebral amyloid angiopathy. A recent case report described bilateral hematoma formation presumably due to EGb. Our study suggests that higher doses of EGb may increase the risk of hemorrhagic transformation after stroke. It is not known whether the risk of ICH would be further increased in patients on concurrent anti-
platelet, anticoagulant, or thrombolytic agents. Further dose finding, along with combination agent experimental stroke studies, may help to answer these questions.

The mechanism of action of LA appears to be due to its ability to substitute for glutathione. One of the major consequences of free radical injury is the depletion of the cellular antioxidant glutathione, leading to oxidation of protein thiols to disulfides and the loss of enzymes having thiol groups. Glutathione itself cannot be administered directly; however, LA is a thiol antioxidant that is absorbed from the diet and crosses the blood-brain barrier. Four antioxidant properties have been demonstrated: it has metal chelating capacity; it can scavenge reactive oxygen species; it can regenerate endogenous antioxidants, including vitamins E and C; and it can repair oxidative damage. LA and DHLA have been shown to inhibit NF-κ activation and decrease adhesion receptor expression. LA has been shown to be neuroprotective in several studies involving focal CNS ischemia. Wolz and Kriegstein studied LA in permanent focal MCAO models in both rats and mice when infarct volume was evaluated at 48 hours. In rats, 100 mg/kg SC of LA injected 2 hours before MCAO produced a significant reduction in infarct volume. In mice, a dose of 100 mg/kg SC at either 1 or 2 hours before ischemia produced a significant reduction in volume. Using the same permanent MCAO model,Prehn et al found that a dose of 50 mg/kg SC of DHLA given 30 minutes before ischemia produced significant infarct reduction in rats and mice. Panigrahi et al found that pretreatment with 25 mg of LA just before reversible global ischemia significantly improved mortality from 79% to 25%. Finally, Mitsui et al found that 100 mg/kg of LA given before ischemia produced significant protection in a peripheral nerve ischemia/reperfusion model. All of these successful studies, including ours, used pretreatment strategies. Although this might be clinically relevant if a patient was taking LA supplements before stroke, it does not address the potential of LA as an acute poststroke therapy. However, it appears that LA was well tolerated in all these trials, without any reports of ICH.

Conclusion

This study found that pretreatment with 2 commonly used antioxidant supplements, EGB and LA, reduced neurological injury in a transient focal CNS ischemic model. For EGB this protective effect appears to be dose sensitive, with higher dose of EGB potentially increasing the risk of ICH. The combined clinical use of EGB with antiplatelet, anticoagulant, and thrombolytic agents could potentially further increase the risk of ICH. Additional studies investigating the efficacy of delayed treatment and safety of these supplements in experimental stroke are needed before clinical studies are undertaken.

Acknowledgments

This study was supported by an American Heart Association Established Investigator Grant. EGB761 was kindly supplied by Dr Willmar Schwabe Pharmaceutical, Germany. The authors wish to thank Anne Tillinghast for assistance with this manuscript.

References


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Stroke. 2001;32:1000-1004
doi: 10.1161/01.STR.32.4.1000

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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