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BILOBALIDE PREVENTS ISCHEMIA-INDUCED EDEMA FORMATION *IN VITRO* AND *IN VIVO*

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Abstract—EGb761, a standardized extract of *Ginkgo biloba*, has neuroprotective properties in animal models of ischemia, an activity that is partially attributed to its constituent, bilobalide. EGb761 has also been reported to inhibit edema formation induced by toxins such as triethyltin. The goal of this study was to test the activity of pure bilobalide to prevent edema formation in models of ischemia. Oxygen-glucose deprivation (OGD) in rat hippocampal slices served as a model of *in vitro*-ischemia. OGD caused cellular edema formation as indicated by an increase of slice water contents in 30 min. Bilobalide (1–10 μM) reduced slice water contents in ischemic slices in a concentration-dependent manner. As a model of *in vivo*-ischemia, we performed middle cerebral artery occlusion (MCAO) in mice. Permanent MCAO caused cell death and swelling of the ischemic hemisphere within 24 h. Pretreatment of the mice with bilobalide (10 mg/kg i.p.) reduced infarct area by 43% (as judged by 2,3,5-triphenyltetrazolium chloride (TTC) staining) and edema formation by 70% (as judged by hemispheric enlargement). In parallel experiments, pretreatment with bilobalide also reduced forebrain water contents in the ischemic hemisphere by 57%. As an alternative model of brain edema formation, we used water intoxication to increase brain water content; bilobalide, was, however, inactive in this model. We conclude that bilobalide strongly and specifically attenuates edema formation in models of brain ischemia *in vitro* and *in vivo*. Bilobalide may be therapeutically effective in brain edema which occurs secondarily to large hemispheric stroke and traumatic brain injury in humans.

Key words: brain water content, *Ginkgo biloba*, hippocampal slices, middle cerebral artery occlusion, oxygen-glucose deprivation, water intoxication.

Brain edema formation, as observed after large hemispheric stroke, is one of the most dangerous consequences of ischemic brain injury and a major determinant of survival after traumatic brain injury (Steiner et al., 2001; Unterberg et al., 2004). While water contents in healthy brains are homeostatically regulated by a variety of mechanisms (Kimelberg, 2004), breakdown of these mechanisms in ischemia, including severe dysregulations of ionic distributions, causes swelling of the brain and increased intracranial pressure (Steiner et al., 2001; Unterberg et al., 2004). Early brain swelling is known to be due to cytotoxic edema while vasogenic edema develops in a more delayed fashion (over hours). Among the brain regions, the hippocampus has been found to be highly susceptible to ischemia; pyramidal cells of the CA1 region are most sensitive (Schmidt-Kastner and Freund, 1991). In addition, astrocytes are well known to undergo swelling when excitotoxic concentrations of glutamate are present (Kimelberg, 2005; Seifert et al., 2006), and they may contribute strongly to ensuing changes of intracranial pressure.

Extracts of *Ginkgo biloba* such as EGb 761 are widely used for the treatment of progressive neurodegenerative disorders such as Alzheimer's disease (Oken et al., 1998; DeFeudis and Drieu, 2000). Experimental work during the last 20 years has also shown that ginkgo extracts and their constituents, such as ginkgolides and bilobalide, exert beneficial effects in animal models of acute neurodegeneration, e.g. in cerebral hypoxia and ischemia (Krieglstein et al., 1995; Pierre et al., 1999; Chandrasekaran et al., 2001). In an experimental model of hypoxia-induced phospholipid breakdown, we found that bilobalide, a sesquiterpene lactone which constitutes ca. 3% of EGb761, was the active constituent of the extract (Klein et al., 1997) acting in the submicromolar range (IC_{50}=0.38 μM). In further work, we described antagonistic effects of bilobalide on NMDA receptor-induced choline release from hippocampal slices (IC_{50}=2.3 μM) (Weichel et al., 1999). The neuroprotective properties of bilobalide have recently been reviewed (DeFeudis, 2002; Ahlemeyer and Krieglstein, 2003).

One aspect of ginkgo's potential therapeutic effects that has not been widely investigated is the anti-edema effect of the extracts. As early as 1986, ginkgo extract EGb761 was reported to inhibit toxic edema formation in white matter induced by triethyltin (TET). EGb761 both prevented TET-induced edema as well as accelerated recovery from prior exposure (Otani et al., 1986; Sancesario and Kreutzberg, 1986). Bilobalide was tentatively identified as the active constituent of the extract (Chatterjee et al., 1986; Sancesario and Kreutzberg, 1986). Later work demonstrated beneficial effects of EGb761 on cerebral edema formation induced by bromethalin, a toxin (Dorman et al., 1992), and by hyperthermia (Westman et al., 2000). Ginkgolide B was implicated in one study (Westman et al., 2000), but other active ingredients were not identified in these studies. As these previous studies suggested a potential anti-edema effect of bilobalide that may be therapeutically useful, we decided to probe its effects on the

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Abbreviations: ANOVA, analysis of variance; ECA, external carotid artery; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; OGD, oxygen-glucose deprivation; TET, triethyltin; TTC, 2,3,5-triphenyl-tetrazolium chloride.

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clinically important edema formation that is induced by ischemia. Our results establish bilobalide as a potential anti-edema drug and as a lead structure for further drug development.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley rats (250–350 g; Charles River, Wilmington, MA, USA) were kept under standardized 12-h light/dark, temperature (22 °C) and humidity (70%) conditions, with rat chow and water available *ad libitum*. All animal procedures were in accordance with NIH regulations and were registered with the Institutional Animal Care and Use Committee of TTUHSC. All efforts were made to minimize animal numbers and animal suffering.

Edema formation in hippocampal slices

Rats were briefly anesthetized in an isoflurane induction chamber and decapitated. Hippocampal slices (400 μm) were prepared as previously described (Weichel et al., 1999; Klein et al., 2003) and superfused (0.7 ml/min) at 35 °C with Tyrode solution of the following composition: NaCl, 137 mM; KCl, 2.7 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 1.2 mM; NaH_2PO_4, 0.2 mM; $NaHCO_3$, 11.9 mM; glucose, 5.6 mM. During the equilibration period, all superfusion solutions were continuously gassed with carbogen (95% O_2, 5% CO_2). For *in vitro*-ischemia (OGD, oxygen-glucose deprivation), the slices were superfused with solutions which did not contain glucose and which had been gassed with nitrogen instead of oxygen (95% N_2, 5% CO_2). Bilobalide was added in DMSO; control solutions contained the same amount of DMSO (0.1%) as bilobalide-containing solutions. Four lanes of slices were superfused in parallel for 30 min. At the end of the superfusion period, slices from each lane were collected, superficially dried, transferred to aluminum foil, and weighed ("wet weight"). They were then dried overnight at 105 °C in a desiccating oven and weighed again ("dry weight"). Slice water content was calculated according to [(wet weight−dry weight)/wet weight]×100.

Ischemic stroke by middle cerebral artery occlusion (MCAO)

In vivo-ischemia in the brain was induced as described in detail previously (Mdzinarishvili et al., 2005). Briefly, female CD-1 mice (26–32 g; Charles River) were anesthetized with 1.5% isoflurane in 30% O_2/70% N_2O. The skin was incised, and the left occipital and superior thyroid artery, branches of the external carotid artery (ECA), as well as the pterygopalatine artery were exposed, electrocoagulated, and cut. After occlusion of the common carotid artery by microclip, the left ECA was ligated, coagulated and cut distally to the cranial thyroid artery. A 21 mm monofilament nylon suture (5–0, Harvard Apparatus, Holliston, MA, USA; diameter of the heat-rounded tip: 0.2–0.3 mm) was inserted into the ECA and gently advanced through the internal carotid artery until its tip occluded the origin of the middle cerebral artery (MCA). Correct placement of the suture was monitored by a sudden drop of the local cortical blood flow in the left MCA territory to 10–15% of basal flow as monitored by laser-Doppler flowmetry. After successful occlusion, the monofilament was secured in place with ligature, and the skin incision was closed by surgical clips. Throughout surgery, temperature was maintained at 37 °C by a thermostatic blanket (rectal thermometer). Bilobalide (or vehicle, 10% DMSO in saline) was injected intraperitoneally 60 min before induction of ischemia at a dose of 10 mg/kg. MCAO was sustained for a period of 24 h, after which the animals were deeply anesthetized with isoflurane and killed by decapitation. The brains were quickly removed, sectioned coronally into 1 mm slices, and stained with 2,3,5-triphenyl-tetrazolium chloride (TTC). Images were acquired by digital camera, and areas of both hemispheres and the infarcted regions were quantified for each slice using Image J 1.30. Brain edema (brain swelling) were quantified by comparing the area of the ipsilateral (ischemic) hemisphere to the contralateral (non-ischemic) hemisphere ("hemispheric ratio"), as described previously (Kinouchi et al., 1991; Sydserff et al., 1996).

Brain water contents were determined 24 h after MCAO by differential weighing. For this purpose, brain hemispheres were superficially dried, transferred to aluminum foil, weighed ("wet weight") and dried overnight at 105 °C in a desiccating oven. The dried slices were weighed again ("dry weight"), and total brain water was calculated according to [(wet weight−dry weight)/wet weight]×100.

Water intoxication

As an alternative *in vivo*-model of brain edema formation, we used water intoxication in mice (Manley et al., 2000; Amiry-Moghaddam et al., 2004). Briefly, male CD-1 mice (N=34) were given distilled water (20% of body weight) by rapid i.p. infusion. Desmopressin (DDVP) was added in a dose of 3 μg/kg to prevent renal elimination of excess fluid. Following water intoxication, the mice showed a decrease of spontaneous motility after 15 min which was accompanied by uncoordinated movements. After 30 min, the mice were rapidly anesthetized with isoflurane (4%) in an induction chamber, and decapitated. Brain water contents were determined by the differential weighing procedure described above. Mice received either bilobalide (10 mg/kg i.p.) or vehicle (saline containing 10% DMSO) 30 min before water infusion. Controls did not receive water but were killed 1 h after injection with saline (0.1 ml, with 10% DMSO) or bilobalide (10 mg/kg, i.p.).

Statistics

Data are shown as means±S.D. of N experiments. Statistical calculations were performed by GraphPad InStat 3.0 or by Prism 3.0, using analysis of variance (ANOVA) of paired or unpaired data as indicated in text and figure legends.

RESULTS

In vitro model of brain edema formation

Anti-edema effects of bilobalide were first tested in an *in vitro*-paradigm using rat hippocampal slices; edema formation in slices was quantified by measuring slice water contents using a differential weighing method as previously described (MacGregor et al., 2003). After 30 min of superfusion with control buffer (Tyrode solution), the water content of the slices was 80.7±1.0% (Fig. 1A). To mimic ischemic conditions, the slices were exposed to OGD, i.e. glucose was omitted from the superfusion buffer which was also gassed with nitrogen (MacGregor et al., 2003). Exposure of the slices to OGD increased water content by 3.05±0.43% (P<0.01). Bilobalide slightly reduced slice water content under control conditions and, importantly, almost completely prevented edema formation induced by OGD; in the presence of 10 μM bilobalide, the OGD-induced increase of water content was 0.55±0.22% when compared with incubations with bilobalide alone (Fig. 1A). This corresponds to an inhibition of edema formation of 82%. The effect of bilobalide was concentration-dependent; as shown in Fig. 1B, bilobalide also induced smaller

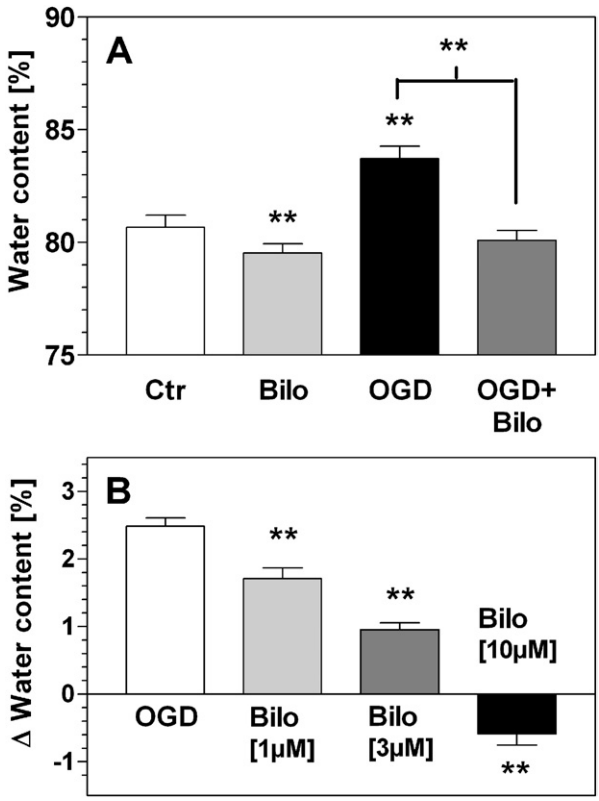

Fig. 1. Tissue water content in hippocampal slices. (A) Effects of bilobalide ("Bilo," 10 μM) on tissue edema induced by OGD. (B) Concentration–response relationship of bilobalide. Slices were exposed to OGD for 30 min. When bilobalide (1–10 μM) was present, it was added 5 min before OGD. Water contents were determined at the end of the superfusion procedure by differential weighing before and after drying the slices. Data in (A) are absolute values, data in (B) were expressed as differences from control incubations. Statistical significance was evaluated by paired ANOVA. ** $P<0.01$ vs. controls (Ctr) ($N=6$ for each series of experiments).

but significant decreases of edema formation at concentrations of 1 and 3 μM.

Brain edema formation induced by ischemic stroke *in vivo*

Neuroprotective and anti-edema effects of bilobalide were tested *in vivo* using MCAO in mice to induce focal cerebral ischemia. Fig. 2 shows a representative experiment in which infarct areas were stained 24 h after permanent occlusion with TTC (see Experimental Procedures). The areas of non-viable tissue, as indicated by pale color, were much smaller in the infarcted hemisphere when animals were pretreated with bilobalide (10 mg/kg i.p.) 1 h before MCAO (Fig. 2, right panel), compared with controls which were treated with vehicle (Fig. 2, left panel). Fig. 3A shows the averaged infarct areas as found in consecutive coronal slices. When infarct areas were calculated by averaging individual slices, the infarct area after stroke in control mice was 32.9±4.5% of the total brain area. Pretreatment with bilobalide was found to decrease infarct area to 17.0±2.8% which corresponds to a reduction of infarct volume by 43±9% ($P<0.01$) (all data mean±S.D., $N=6$).

In addition to infarct area, we also calculated the formation of edema *in vivo* by comparing the size of infarcted and contralateral hemisphere as described before ("hemispheric enlargement"; Elliott and Jaspar, 1949; Kinouchi et al., 1991; Sydserff et al., 1996). Twenty-four hours after MCAO, the ischemic side of the brain showed significant swelling compared with the control side (Fig. 3B; see also Fig. 2). The average increase of the ipsilateral (stroked) hemispheric area over the contralateral area was 19.9±6.5% (Fig. 3B). Slices from mice pretreated with bilobalide (10 mg/kg i.p.) had a much reduced swelling of brain tissue; the measured value (+6.0±2.2%) corresponds to a reduction of brain edema by 70±5% ($P<0.01$) when compared with untreated mice.

Brain water contents after stroke

As an alternative procedure to measure brain edema formation, we quantified brain water contents *in vivo* using a differential weighing procedure (Kinouchi et al., 1991; Gueniau and Oberlander, 1997). Water contents in the forebrain were compared in the stroked and non-stroked hemispheres of the same animals thereby increasing the statistical power of the method. As shown in Fig. 4, brain water contents in non-stroked hemispheres were 78.0±0.4% ($N=6$). MCAO induced an increase of water content to 81.5±0.8% in the ipsilateral hemisphere ($P<0.01$, ANOVA) indicating significant edema formation poststroke. Pretreatment with bilobalide (10 mg/kg i.p.) reduced edema formation in the stroked hemisphere but

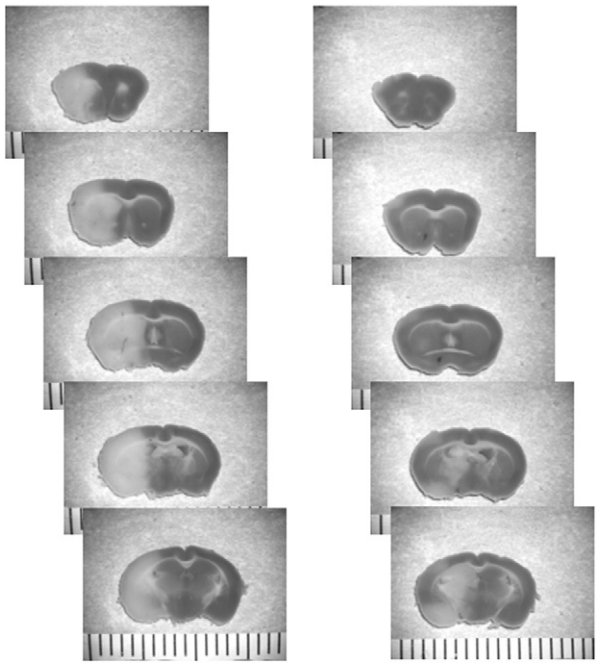

Fig. 2. Effects of bilobalide on cell death induced by MCAO in the mouse. Left panel: Mouse treated with vehicle (0.3 ml saline i.p. containing 10% DMSO) 1 h prior to MCAO. Right panel: Mouse treated with bilobalide (10 mg/kg i.p.) 1 h prior to MCAO. The animals were killed 24 h past stroke induction, five slices of 1 mm thickness were cut coronally and stained by TTC. Areas with insufficient mitochondrial activity to reduce TTC are indicated by pale white color.

did not affect water contents in the contralateral hemisphere (Fig. 4). The increase of water content after ischemia was reduced from 3.54±0.32% (control) to 1.53±0.60% by pretreatment with bilobalide which corresponds to an average decrease of 57% ($P<0.01$, *t*-test).

Brain edema induced by water intoxication

To test the specificity of bilobalide's anti-edema effects, we used water intoxication as an alternative *in vivo*-model of brain edema formation (Manley et al., 2000; Amiry-Moghaddam et al., 2004). In this series of experiments, control mice had brain water contents of 77.7±0.5% ($N=10$) (Fig. 5). Water infusion caused signs of toxicity (hunched posture, reduced spontaneous motility) after about 15 min and, after 30 min, an increase of brain water contents to 79.2±0.5% ($P<0.01$, ANOVA). Pretreatment of the mice with bilobalide (10 mg/kg i.p.) 30 min before water injection slightly reduced water contents in both control and water-intoxicated mice (Fig. 5). The increase of water content following infusion of distilled water, was, however, not significantly affected by pretreatment with bilobalide. The average increase of brain water in water-intoxicated control mice was 1.46±0.48%; in bilobalide-treated mice, this value was slightly reduced 1.30±0.27% ($P>0.3$, *t*-test).

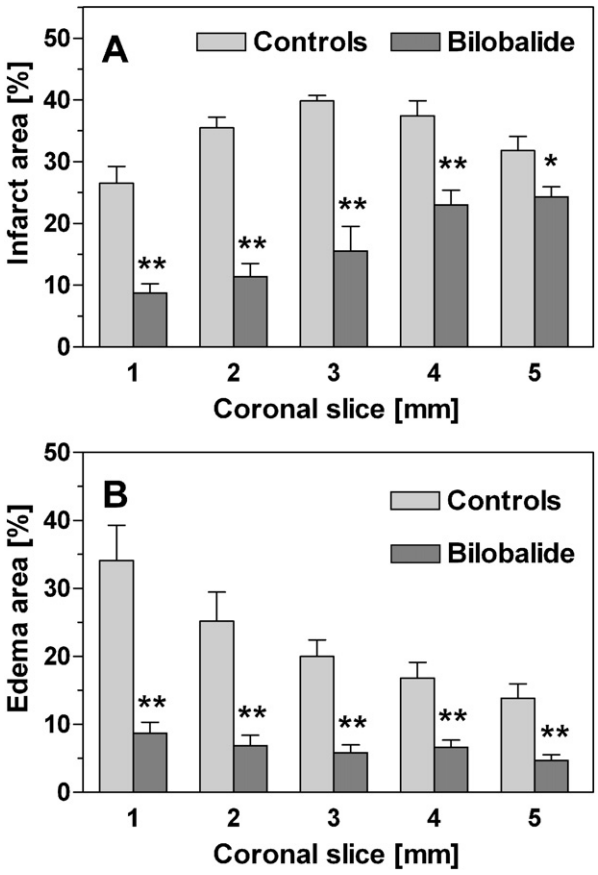

Fig. 3. Effects of bilobalide on (A) infarct area and (B) edema formation induced by MCAO in the mouse. Infarct areas and edema ratios were measured 24 h after MCAO in consecutive brain slices taken (cf. Fig. 2) from mice pretreated with vehicle (controls) or bilobalide (10 mg/kg i.p.). Infarct areas in (A) were calculated as percentage of infarct areas over total brain area in each slice and averaged over six animals. Edema formation was calculated as hemispheric enlargement and is given as percentages (relative increase of the brain area in the infarcted hemisphere vs. the contralateral hemisphere). Results are mean±S.E.M., $N=6$.

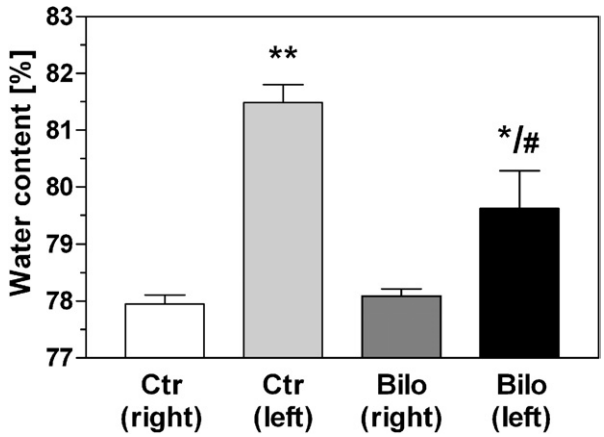

Fig. 4. Effects of bilobalide on brain water contents. Brain edema formation was induced by MCAO in the mouse. Twenty-four hours after MCAO, hemispheric brain water contents were measured in the forebrains of mice pretreated with vehicle ("Ctr") or bilobalide ("Bilo"; 10 mg/kg i.p.), by the differential weighing procedure. MCAO was induced in the left hemispheres; the right hemispheres represent healthy tissue. Results are mean±S.E.M., $N=6$.

DISCUSSION

The goal of the present project was the characterization of bilobalide as a potential agent to inhibit the formation of brain edema. We first used an *in vitro*-model of edema formation, i.e. exposure of hippocampal slices to OGD which leads to uptake of water as an indicator of cellular

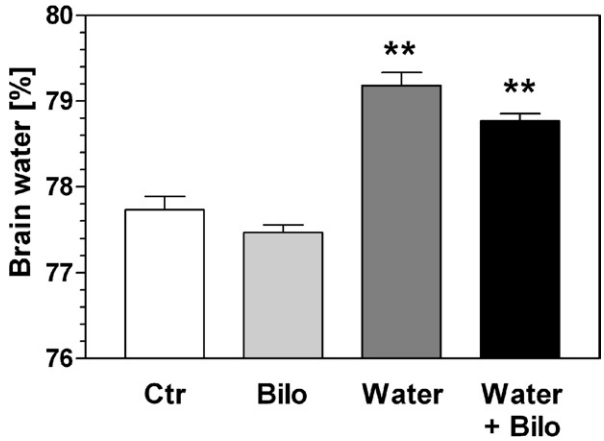

Fig. 5. Water intoxication in mice: effect of bilobalide. Water intoxication was induced by rapid i.p. infusion of distilled water (20% of body weight) plus desmopressin (3 μg/kg). Mice were killed after 30 min, and brain water was determined by differential weighing of total forebrain tissue before and after drying of the brains. Bilobalide (10 mg/kg i.p.) or vehicle (Ctr) was administered 30 min before water intoxication. Statistical significance was evaluated by ANOVA for independent data from $N=34$ experiments ($F_{3,33}=29.6$; $P<0.01$). ** $P<0.01$ vs. Ctr.

edema formation. According to previous studies, preparation and superfusion of hippocampal slices per se already cause a swelling of slices over 30–60 min which is accompanied by sodium and calcium uptake (Siklos et al., 1997). Bilobalide slightly reduced water contents under basal conditions (Fig. 1A), probably by interfering with ionic movements, although its mechanism of action is unknown (see below). During ischemia (OGD), an increase of sodium and calcium uptake was observed in earlier studies to which voltage-operated cation channels as well as glutamate receptors of the AMPA and NMDA subtypes contributed (LoPachin et al., 2001; MacGregor et al., 2003). In our hands, bilobalide (1–10 μM), a constituent of *Ginkgo biloba*, was very effective in preventing OGD-induced edema formation by more than 80%; thus, its efficacy was similar to antioxidants, sodium channel blockers and glutamate receptor antagonists tested in previous studies (LoPachin et al., 2001; MacGregor et al., 2003). Bilobalide acted in the low micromolar range, with a half-maximum inhibition of edema formation occurring at approximately 3 μM (Fig. 1B).

To test *in vivo*-activity of bilobalide, we employed MCAO as an experimental model to induce focal brain ischemia. When used in the mouse, MCAO causes the formation of a large infarct area in cortex and striatum, with some damage also observed in the hippocampus (Mdzinarishvili et al., 2005). A variety of pharmacological agents is known to suppress neuronal cell death after experimental ischemia and/or traumatic brain injury (Lees, 2000; Royo et al., 2003) but anti-edema effects of compounds have been less frequently reported. In the present experiments, we confirm an earlier report (Krieglstein et al., 1995) that bilobalide, at a dose of 10 mg/kg, reduces the infarct area after MCAO as judged by TTC staining of viable cells (Fig. 2). More importantly, we report that edema formation in the brain is strongly inhibited by the compound. When judged from measurements of hemispheric enlargement, brain edema was inhibited by 70% (Fig. 3B). When brain water content was used as parameter of edema formation, pretreatment of bilobalide decreased edema formation by 57% (Fig. 4). The similarity of these results attests to the validity of the two methods to measure edema formation.

Water intoxication causes hyponatremia and hyposmolarity in plasma and is followed by the development of brain edema (Olson et al., 1990; Gullans and Verbalis, 1993). Brain water uptake following water intoxication has recently been shown to depend on aquaporin-4, and swelling of astrocytic endfeet is the most likely cellular consequence of water intoxication (Manley et al., 2000). In the present study, we used water intoxication to test the selectivity of bilobalide's anti-edema action. Bilobalide, while slightly reducing brain water contents in both control and water-treated mice, was not significantly active in this model. Thus, bilobalide does not seem to block water transport under hyposmolar conditions, and its effects were selective for edema formation induced by ischemia.

An important question that was not addressed in the present study is the molecular mechanism of bilobalide's anti-edema action. Previous work has shown that inhibitors of sodium and calcium influx, as well as antioxidants, protect against edema formation, at least in *in vitro*-models (LoPachin et al., 2001; MacGregor et al., 2003). Bilobalide can protect mitochondrial energetics and Na,K-ATPase activity under conditions of ischemia (Pierre et al., 1999; Chandrasekaran et al., 2001), and we and others have provided evidence that bilobalide interferes with chloride fluxes through ligand-operated receptor channels (Chatterjee et al., 2003; Klein et al., 2003). These effects may contribute to an improved maintenance of ionic balances in ischemic areas. As water enters the cells together with ions, improved maintenance of ionic gradients of sodium and chloride would explain bilobalide's beneficial effects. Astrocytes may be potential targets of bilobalide because they contribute strongly to brain swelling (see introduction), and bilobalide was recently shown to increase the expression of glial growth factors in astroglial cultures (Zheng et al., 2000). However, more work is needed to identify the molecular target(s) of this important natural compound.

Summarizing, the present results identify bilobalide as a rather potent neuroprotective agent which reduces brain edema formation under ischemic conditions by more than 50%. This effect is of sufficient magnitude to be of therapeutic relevance. Bilobalide's potency in the low micromolar range would make the compound a potential drug in its own right, or it may serve as a lead compound for the development of more potent derivatives. Bilobalide probably reaches therapeutic levels in the brain, as shown in this and previous studies, but as yet, there are no pharmacokinetic studies with pure bilobalide that would allow the definition of an active brain level of the drug. Rats dosed orally with 30–100 mg/kg ginkgo extract EGb761 (which contains 3% bilobalide) had plasma levels of bilobalide of 0.5–1.3 μM (Biber, 2003). With linear pharmacokinetics, 10 mg/kg of bilobalide (as used in the present study) would therefore be expected to produce plasma levels of ca. 5 μM, a concentration that was active in the *in vitro*-experiments (Fig. 1). It remains an open question whether ginkgo extracts would yield anti-edema effects in humans, but the current study would seem to encourage investigations into this important issue of human health.

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