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# Neuroprotection by bilobalide in ischemia: Improvement of mitochondrial function

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***Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.***

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Bilobalide, an active constituent of *Ginkgo biloba*, is known to have neuroprotective properties, but its mode of action remains unclear. In this study, bilobalide significantly reduced brain damage in mice (indicated by TTC staining) when given before transient middle cerebral artery occlusion (tMCAO). As measured by microdialysis in the ischemic striatum, local perfusion with bilobalide (10 μM) reduced ischemia-induced glutamate release by 70% while glucose levels were not affected. Mitochondria isolated from ischemic brain showed a decrease of respiration compared to non-ischemic controls. Treatment with bilobalide (10 mg/kg) before tMCAO improved respiratory capacity of complex I significantly when measured *ex vivo*. In addition, mitochondrial swelling induced *ex vivo* by calcium was used to estimate opening of the mitochondrial permeability transition pore. In this assay, the changes induced by tMCAO were completely reversed when mice had received pretreatment with bilobalide. We conclude that neuroprotection by bilobalide involves a mechanism in which the drug reverses ischemia-induced changes in mitochondria, leading to a reduction of glutamate release.

## 1. Introduction

Ischemic stroke is one of the leading causes of death and adult disability worldwide. However, in spite of intensive experimental and clinical research, only thrombolytic treatment has been found useful clinically while neuroprotective treatments aimed at preserving neurons and preventing neurodegeneration have not proven effective in humans (Carmichael 2005; Ginsberg 2008). Among the complex pathophysiology of brain ischemia, excitotoxicity induced by glutamate release, calcium influx, mitochondrial damage and oxidative stress have been identified as likely contributors to neuronal cell death (Smith 2004; Sims and Muyderman 2010). Several compounds that interfered with these processes were neuroprotective in animal models, but could not be given to patients in a preventive manner due to intolerable side effects. Chronic treatment of at-risk patients would be important, however, because after stroke, drugs do not reach the ischemic tissue any more due to severely limited blood flow in the core of the stroke (Lang et al. 2010).

*Ginkgo biloba* extracts such as the standardized extract EGb 761 are widely used in neurodegenerative disorders and are well tolerated at doses up to 240 mg/d. We and others have shown that EGb761 and bilobalide, a constituent representing 3% of EGb761, are neuroprotectants which scavenge oxygen radicals and reduce calcium-induced toxicity and deficits of the mitochondrial respiratory chain *in vitro* (Ahlemeyer and Krieglstein 2003; Eckert et al. 2005, 2012a). *In vivo*, in rodent stroke models, bilobalide reduced brain edema formation and infarct size and protected against glutamate-induced excitotoxic neuronal death (Chandrasekaran et al. 2001; Mdzinarishvili et al. 2007). The mechanism of action of bilobalide remained obscure although interactions with amino acid-mediated neurotransmission were reported (Kiewert et al. 2007, 2008). In the present study, we first confirmed neuroprotective activity of bilobalide in an experimental stroke model, specifically using transient occlusion of the middle cerebral artery (tMCAO). We then investigated bilobalide's effect on glutamate release, mitochondrial electron transport, and mitochondrial swelling due to opening of the mitochondrial permeability transition pore (mPT). We report evidence that bilobalide's neuroprotective actions involve reduction of excitotoxicity and protection of mitochondrial function.

## 2. Investigations and results

We first investigated neuroprotective effects of bilobalide in mice undergoing transient middle cerebral artery occlusion (MCAO) for 60 min. Brain slices were obtained 24 h after MCAO and stained with TTC (Fig. 1A). Striatal slices of stroked mice (Fig. 1A, middle panel) showed a clearly marked white necrotic area in the left hemisphere. Local perfusion of the striatum with bilobalide (10 μM in artificial cerebrospinal fluid, aCSF; see Experimental for composition) reduced the infarcted area and led to a pink-reddish staining which resembled control (sham-operated) animals (Fig. 1A, compare third to first panel). Calculations of the infarct area as shown in Fig. 1B indicate that the stroke area was reduced by 38% when bilobalide was infused ($p < 0.001$).

Figure 2 displays extracellular concentrations of glucose and glutamate, measured by microdialysis in the mouse striatum before, during and after 60 min of ischemia. Glucose levels

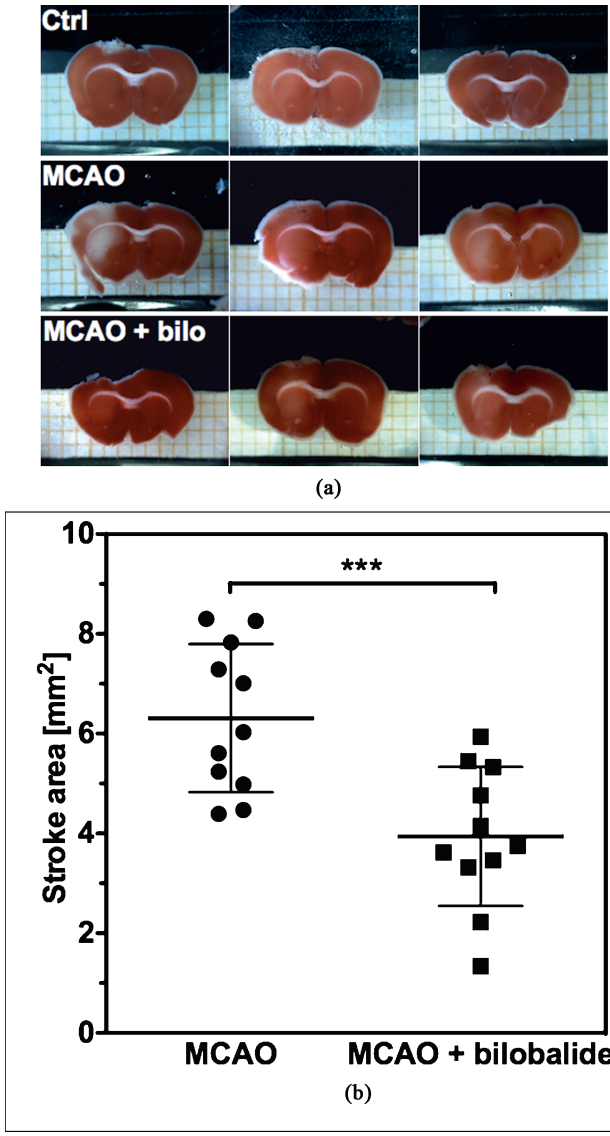


Fig. 1: (**A**) Stroke area 24 h after transient middle cerebral artery occlusion (tMCAO, 60 min). Brain slices were stained with 2,3,5-triphenyl-tetrazolium chloride (TTC). Healthy tissue is colored red, ischemic tissue remains white. Upper panel: control animals. Middle panel: mice with transient MCAO. Lower panel: mice with transient MCAO that were infused with bilobalide (10 μM in aCSF). (**B**) Stroke area in $mm^2$ was calculated from slices as shown in (A) using Image J software. Data is expressed as means ± S.D. of N = 11 experiments. ***, $p < 0.001$ (t-test, GraphPad Prism).

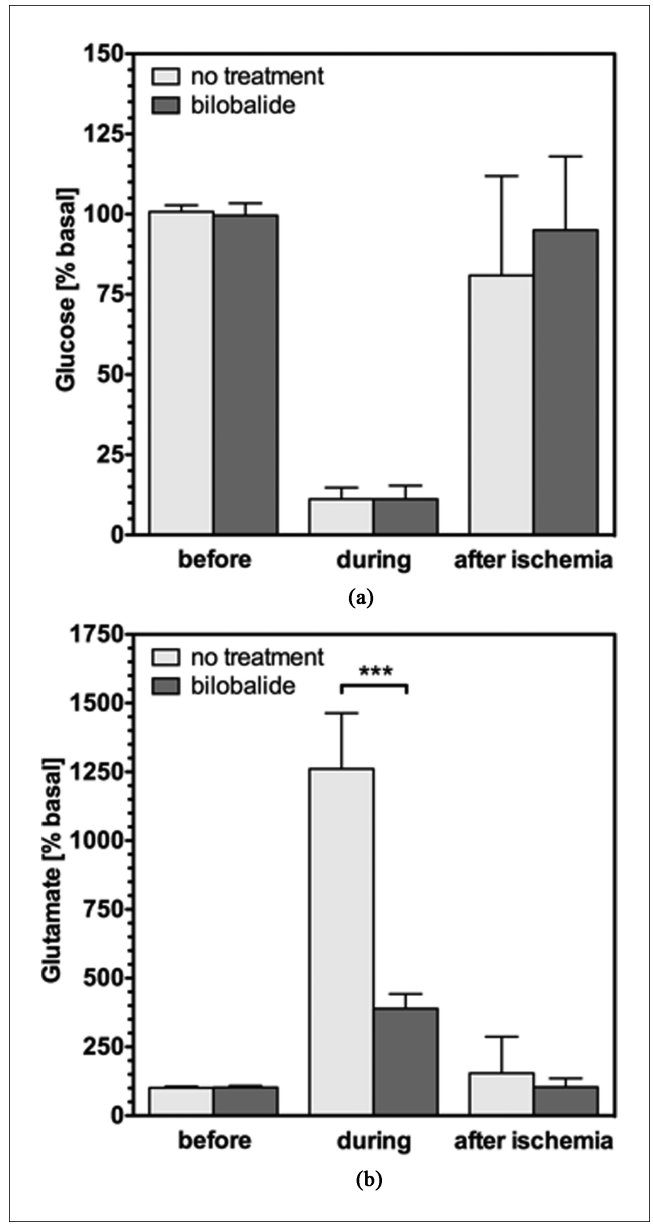


Fig. 2: Extracellular concentrations of (**A**) glucose and (**B**) glutamate in the mouse striatum before, during and after MCAO, as sampled by microdialysis. Values were calculated as percentages of the mean basal levels measured before ischemia. Control experiments are labeled "no treatment"; in experiments labeled "bilobalide", the compound was added to the microdialysis perfusion fluid (10 μM) for the duration of the experiment. Data is expressed as means ± S.D. of N = 8–12 experiments. ***, $p < 0.001$ (t-test, GraphPad Prism).

dropped immediately upon MCAO to 10–15% of basal levels, whereas glutamate concentrations rose extensively to more than 1200% of baseline level. Both glucose and glutamate levels recovered rapidly when transient MCAO was terminated and reperfusion was allowed. Although reperfusion (as measured by laser Doppler) rarely reached pre-ischemia levels, glucose levels rose to >80% of pre-ischemia level, and glutamate levels almost completely reversed to basal values (<200% of basal). Importantly, in mice treated locally with bilobalide through the dialysis probe (10 μM in aCSF), glucose levels dropped identically as in untreated mice, indicating that ischemia was complete; however, the neurotoxic rise of glutamate was significantly attenuated and reached only 350% of baseline level ($p < 0.001$ vs. controls). After reperfusion, glutamate levels in bilobalide-treated mice returned to control levels (Fig. 2B).

Figure 3 illustrates the activity of mitochondrial respiration as measured in untreated (sham-operated) and bilobalide-treated mice. In Figs. 3 and 4, we used mitochondria from the same mouse brains, separated into the ischemic (left) and the non-ischemic ("healthy", right) hemisphere for direct comparison. As illustrated in Fig. 3A, transient ischemia caused a small reduction of mitochondrial respiration in the right (healthy) hemisphere but a much more distinctive reduction in the left (ischemic) hemisphere, compared to sham-operated controls. Interestingly, pretreatment with bilobalide (10 mg/kg i.p. 1 h before MCAO) improved oxygen flux in the ischemic hemisphere in a significant manner, compared to untreated mice. In absolute terms, in mitochondria that previously suffered from ischemia for 60 min, complex I activity was reduced to 16 % of controls (from 0.205 to 0.033 pmol/s*μg) whereas those from bilobalide-treated mice were only reduced by 46 % (to 0.094 pmol/s*μg). This difference was highly significant ($p < 0.01$). Interestingly, bilobalide's effect was restricted to complex I activity. As shown in Fig. 3B, oxygen flux measured in other respiratory states (complex II, IV, oxphos and ETS) was reduced after ischemia but was not affected by bilobalide (Fig. 3B).

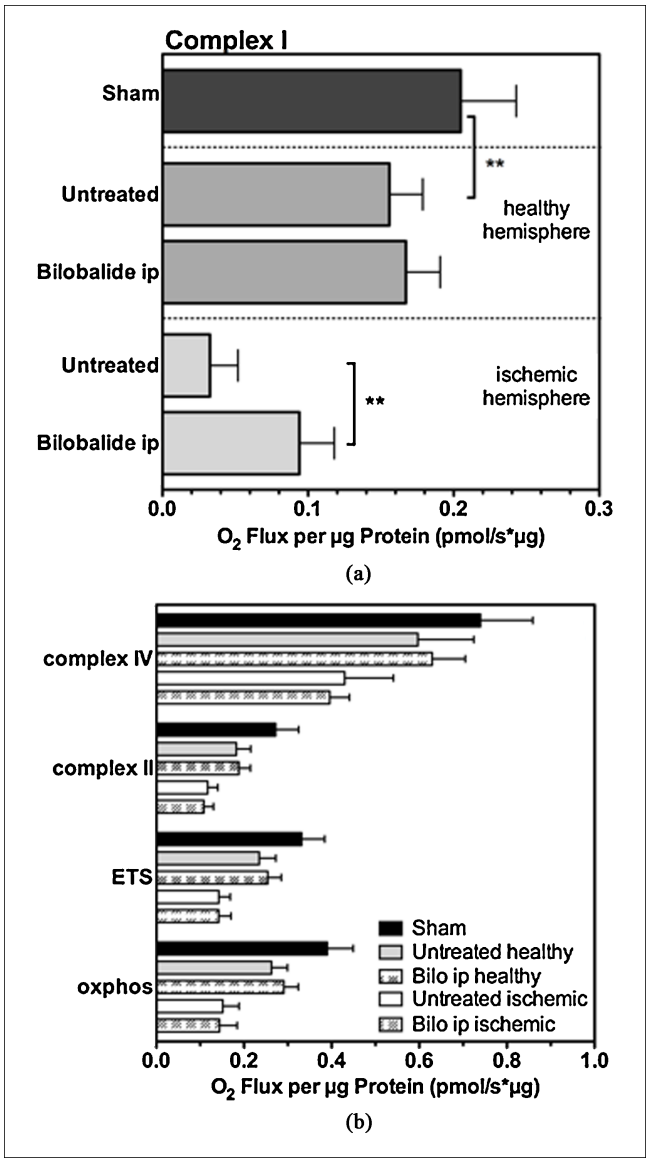


Fig. 3: Oxygen flux of mitochondrial respiration: (**A**) Flux in complex I of the electron transport chain (ETC). (**B**) Flux in complexes II and IV, electron transfer system (ETS) and oxidative phosphorylation state (oxphos). For the measurements, transient ischemia was induced by occlusion of the left MCA for 60 min; then, the mouse brain hemispheres were isolated separately. "Healthy" hemisphere denotes the right (unaffected) hemisphere, whereas "ischemic" hemisphere denotes the left hemisphere. "Sham"-treated mice did not sustain MCAO. "Untreated" mice underwent MCAO, but did not receive drugs. "Bilobalide ip" indicates that bilobalide (10 mg/kg i.p.) was administered systemically 60 min before MCAO (ex vivo-experiment). Data was obtained using a Clark-type electrode in a respirometer and were normalized to protein content. Data is expressed as means ± S.D. of N = 6-11 experiments. **, $p < 0.01$ (one-way ANOVA, Bonferroni post-test, GraphPad Prism).

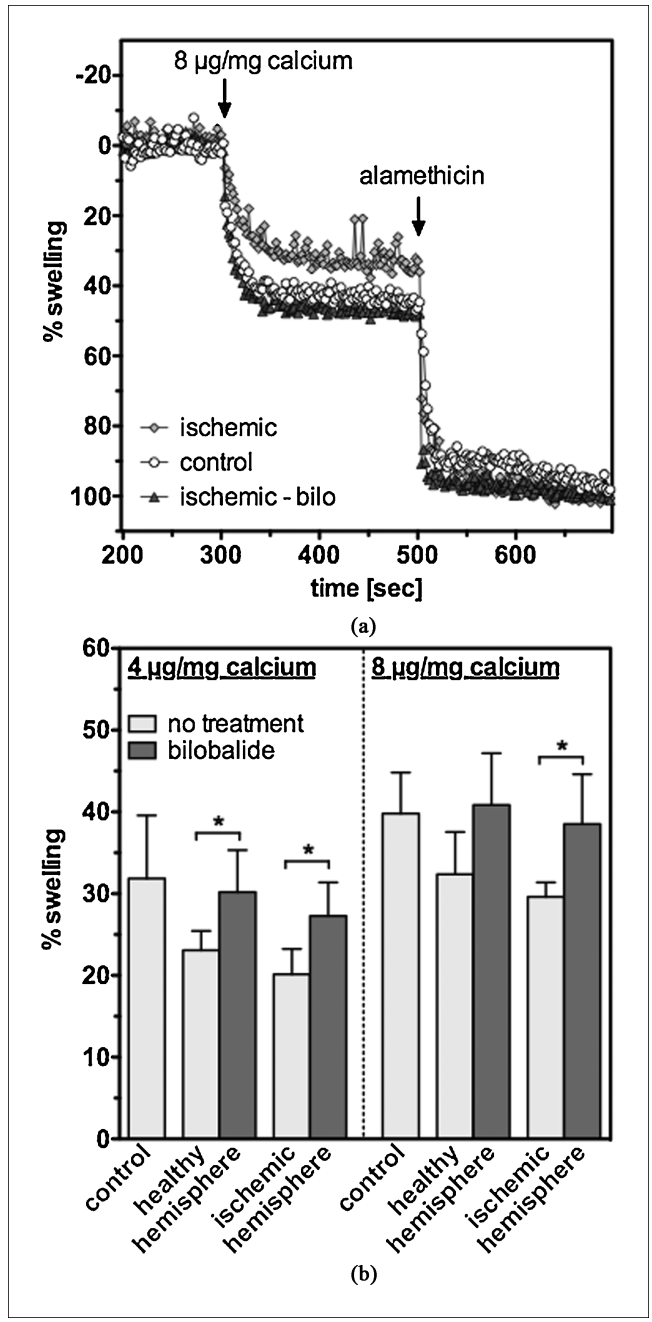


Fig. 4: Mitochondrial swelling caused by opening of the mitochondrial permeability pore (mPT). (**A**) Original data from photometric assay procedure reflecting mitochondrial swelling. The values were transformed into percentages, setting the values before calcium injection as 0% and after alamethicin (pore-opener) as 100%. (**B**) Summary of data obtained with two calcium concentrations (4 and 8 µg/mg protein) used to induce swelling. "Control" mice did not sustain MCAO. "Ischemic" mice sustained MCAO for 60 min. "Ischemic-bilo" mice underwent MCAO but had been pretreated with bilobalide (10 mg/kg i.p.) 1 h before MCAO. In (B), mitochondria were isolated separately for the right (healthy) and the left (ischemic) hemisphere, and mitochondrial swelling was induced by addition of calcium. Data is expressed as means ± S.D. of N = 4 experiments. *, $0.05 < p < 0.1$ (t-test, GraphPad Prism).

As a further measure of mitochondrial damage, we determined calcium-induced swelling *ex vivo* in mitochondria isolated after 60 min of ischemia. The method is illustrated in Fig. 4A: in mitochondria incubated under control conditions, swelling is induced by exposure to high calcium levels. In this assay, the relative extent of swelling depends on the quality of the isolated mitochondria and is calculated relative to the extent of swelling observed after addition of alamethicin, a compound which opens the mitochondrial permeability pore (mPT). In our hands, higher calcium concentrations (8 vs. 4 µg calcium/mg protein) consistently induced more mitochondrial swelling (Fig. 4B). Ischemic mitochondria showed a decrease in swelling potential compared to non-ischemic control (Fig. 4), probably because limited swelling had already been induced by ischemia *in vivo* (the extent of swelling under basal conditions is not reflected in this assay). Application of bilobalide (10 mg/kg i.p.) one hour before transient MCAO significantly reduced this effect, and mitochondria from ischemic hemispheres reached swelling levels close to those of control brains (Fig. 4A and B). Notably, calcium-induced swelling was similar in "healthy" and "ischemic" mitochondria, a finding that indicates that all mitochondria,

irrespective of initial damage, are sensitive to calcium. In separate experiments, mitochondria incubated with cyclosporine A, which inhibits opening of the mPT pore, reduced calcium-induced swelling by 75% (4 μg/mg) or 65% (8 μg/mg; data not illustrated).

## 3. Discussion

Bilobalide is a promising compound for the treatment of cerebral ischemia. It is active in experimental stroke models (Ahlemeyer and Krieglstein 2003; Mdzinarishvili et al. 2007) and is well tolerated by humans, as illustrated by the wide use of bilobalide-containing *Ginkgo biloba* extracts. Therefore, it may be possible to use bilobalide and *Ginkgo* extracts for prevention of stroke-related consequences in patients with high risk for stroke. Moreover, brain permeability of the compound has been demonstrated (Lang et al. 2010), and brain levels were reached that correspond to active concentrations in *in vitro*-test systems. What is lacking in our understanding of the drug is the mechanism by which bilobalide affects nerve cell death. The present investigation shows that bilobalide may have neuroprotective properties through an influence on mitochondrial damage.

In the present study, we used a transient model of cerebral ischemia in which the middle cerebral artery was occluded for only 60 min before reperfusion was allowed. This model resembles a transient ischemic attack, or a stroke in which the clot was dissolved successfully. The damage seen with this type of stroke is less than with permanent MCAO (Mdzinarishvili et al. 2005), but it was chosen so that mitochondrial damage did not become too excessive to observe beneficial effects. We first used local infusions of bilobalide to demonstrate neuroprotection *in situ*, confirming that bilobalide has a local neuroprotective action in the brain (Fig. 1). We then sampled extracellular fluid by microdialysis in the striatum, in the core of ischemic damage, and found that bilobalide strongly reduced the release of glutamate, an excitatory amino acid, during ischemia (Fig. 2). Under ischemic conditions, glutamate is released in huge amounts because loss of ATP increases the intracellular sodium concentration and reverses the sodium-dependent glutamate transport (Nicholls and Attwell 1990; Danbolt 2001). High levels of glutamate are known to mediate neuronal cell death mainly through over-activation of NMDA receptors and resulting influx of calcium which overpowers the cell's ability to sequester it and leads to degeneration. Treatment with glutamate scavengers or blockers reduces neuronal cell death (Lipton 2007; Zlotnik et al. 2012). Bilobalide, however, was previously investigated for interactions with glutamate receptors, with largely negative results (Kiewert et al. 2007, 2008; Lang et al. 2011).

A major pathway of glutamate- and calcium-induced cell death is apoptosis, a cellular response to calcium overload that involves opening of the mitochondrial permeability pore (Halestrap 2009). In the following, we therefore focused on mitochondrial damage, using two *ex vivo*-assays: respirometry and mitochondrial swelling. Respirometry demonstrated (Fig. 3) that ischemia reduced oxygen flux in all complexes of the respiratory chain (see also Canevari et al. 1997 and Bouaziz et al. 2001). When we induced ischemia *in vivo* and isolated mitochondria separately for each hemisphere, mitochondrial respiration of complex I was most strongly suppressed in the ischemic hemisphere. Importantly, pretreatment with bilobalide significantly improved complex I function (NADH-quinone oxidoreductase; for review, see Mourier et al. 2011) leading to a three-fold higher activity *ex vivo* (Fig. 3A), while complexes II and IV were not affected (Fig. 3B). This finding confirms an earlier study in whole brain (Janssens et al. 2010) in which both complex I and III respiration were improved after treatment with bilobalide. While we can exclude a significant effect of bilobalide on complex II respiration, we cannot at present exclude an effect on the transfer of electrons from complex I to III, which is performed by coenzyme Q.

Finally, we were interested in the response of ischemic mitochondria towards calcium, because a significant protection of mitochondria may be reflected in a reduced propensity to swell and release apoptotic factors during calcium overload (Kristián and Siesjö 1998). This effect is mediated by opening of the mitochondrial permeability transition pore (mPT), which is located between the mitochondrial inner and outer membrane, formed by the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), cyclophilin-D and other molecules (see Eckmann et al. 2012). High calcium, but also oxidative stress and loss of ATP cause swelling of the mitochondria and opening of the pore allowing free diffusion of cytochrome c, a pro-apoptotic factor, through the mitochondrial membrane (Crompton 1999). In our hands, using two different calcium concentrations, all mitochondria, ischemic or not, responded to an enhanced calcium concentration by swelling (Fig. 4). Mitochondrial swelling was reduced when mitochondria were incubated with the inhibitor of pore formation, cyclosporin A, confirming the hypothesis. Some literature data indicate that ischemia causes a significant increase in mitochondrial swelling (Atif et al. 2009; Qi et al. 2010). In our experiments, however, mitochondrial swelling after ischemia was distinctively lower than control levels. These differing observations likely result from differences of assay conditions. Thus, previous studies used mitochondria which were isolated after ischemia and 22 h of reperfusion (Atif et al. 2009; Qi et al. 2010) so that there was a long time for recovery of mitochondrial functions during reperfusion. In our study, however, mitochondria were isolated immediately after ischemia, without reperfusion, so that ischemic mitochondria were already swollen when they were added to the spectrometer, and reacted less to the added calcium than "healthy" mitochondria. In agreement with our results, Sun et al. (2012) described an experimental ischemic group without reperfusion, showing nearly no mitochondrial swelling. Henceforth, the important finding in our study is that bilobalide reversed the effects of ischemia so that mitochondrial swelling in ischemic mitochondria, exposed to bilobalide, was identical to control mitochondria taken from sham-operated mice (Fig. 4).

Taken together, the present study considerably advances our knowledge about bilobalide's mechanism of action. Evidently, exposure to bilobalide affects glutamate release as well as mitochondrial function. We hypothesize a mechanism of action for bilobalide which is illustrated in Fig. 5. First, bilobalide stabilizes complex I activity under conditions of ischemia (Fig. 3). As complex I is most affected during ischemia, the three-fold increase of complex I function observed after bilobalide translates into a similarly increased oxygen flux *in vivo* when complex I substrates are in good supply (in contrast, succinate, the complex II substrate, may be low due to dysfunction of the Krebs cycle). Stabilization of complex I likely causes reduced loss of ATP, an effect that was previously shown with *Ginkgo* extract (Ahlemeyer and Krieglstein 2003), and a reduction of glutamate release (Fig. 2). Moreover, while glutamate is still increased three-fold under bilobalide, the response of the mitochondria (swelling and induction of apoptosis) is partially inhibited after bilobalide treatment due to stabilization of the mitochondrial permeability pore (Fig. 4). Thus, while the molecular target of bilobalide remains unknown, the newly found effects in ischemic brain and isolated mitochondria clearly point to an action on mitochondrial energy production, stabilizing both glutamate levels and mitochondrial swelling under ischemic stress. Reduction of apoptosis then leads to preservation of mitochondrial function which is directly demonstrated by TTC staining

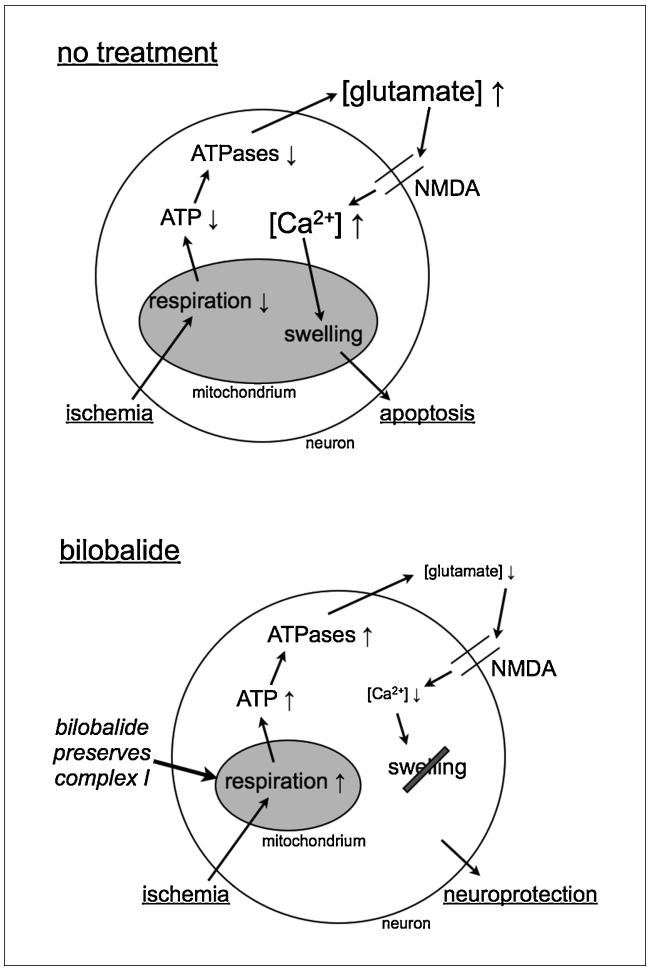


Fig. 5: Simplified pathophysiology of brain ischemia and neuroprotective mechanism of bilobalide. After MCAO (upper panel, "no treatment"), ischemia reduces mitochondrial respiration caused by a lack of oxygen and glucose in the affected tissue. This causes reduced ATP levels, membrane depolarization and reversal of glutamate pumps leading to the release of massive amounts of glutamate. Excitotoxic glutamate concentrations in the extracellular space cause calcium influx through NMDA receptors, causing cellular calcium overload, opening of the mitochondrial permeability transition pore, mitochondrial swelling and finally apoptosis. In the presence of bilobalide, complex I respiration is preserved, and much less glutamate is released thus attenuating excitotoxicity, calcium influx and mitochondrial swelling.

(Fig. 1). In summary, then, bilobalide protects complex I function during ischemia, reduces loss of ATP, reduces release of glutamate and NMDA receptor activation, and reduces calcium-induced swelling of mitochondria (Fig. 5). This mechanism of action, which is now supported by a range of findings from experimental stroke research, is in line with our understanding of ischemic pathways of neurodegeneration and should facilitate entry of the drug into clinical studies.

## 4. Experimental

### *4.1. Animals and drugs*

Female CD-1 mice (28–32 g, Charles River) were kept under standardized conditions: 12h-light/dark circle, 22 °C temperature, 70% humidity, food and water available *ad libitum*. All animal procedures were carried out to minimize animal suffering in accordance with German and European law. The study was registered with the local authorities (Regierungspräsidium Darmstadt). Bilobalide was obtained in pure form (>99% purity) from Dr. Willmar Schwabe Co. For the experiments, mice were randomly distributed in two experimental groups: Either vehicle or bilobalide (10 mg/kg in Ringer-Lactate solution) were administered 60 min before onset of ischemia by i.p. injection. For microdialysis experiments, bilobalide was added to the perfusion fluid in a concentration of 10 μM.

### *4.2. Middle cerebral artery occlusion (MCAO)*

Transient *in vivo*-ischemia in mouse brain was induced by occlusion of the middle cerebral artery (MCAO) as previously described (Mdzinarishvili et al. 2005). Briefly, mice were anesthetized with isoflurane (2% in synthetic air) and kept at 37 °C using a thermostatic blanket coupled to a rectal thermometer (Harvard/Hugo Sachs, March-Hugstetten, Germany). Through a cervical incision, the left bifurcation of the common carotid artery (CCA) was dissected and all three branches (CCA, external (ECA) and internal (ICA) carotid artery) were ligated. A 20 mm monofilament (Doccol, Redlands, California; size 6-0) was inserted into the ECA and gently advanced through the ICA into the brain until its tip occluded the origin of the middle cerebral artery (MCA). Local cerebral blood flow was measured by laser Doppler flowmetry (Moor Instruments, Devon, UK; AP –0.5, L + 3.5 from bregma) and dropped to 10-15% of basal flow during occlusion. After 60 min of occlusion, the filament was removed to allow reperfusion to at least 50% of basal flow. The skin incision was closed with surgical clips and mice were allowed to recover in their home cages.

### *4.3. Microdialysis*

For microdialysis experiments, a self-made dialysis probe was implanted into mouse striatum one day prior to MCAO. Mice were anesthetized with isoflurane (2% in synthetic air) and placed in a stereotaxic frame. The probe (exchange length: 2,5 mm) was implanted using the following coordinates from bregma: AP +0.5 mm; L + 2.2 mm; DV -3.8 mm (Franklin and Paxinos 1997) and fixed at this position with Multilink Automix (Ivoclar Vivadent AG, Schaan, Liechtenstein). Mice were allowed to recover over night in their home cages. Perfusion of the microdialysis probe was started 1 h before MCAO, was sustained while the MCAO surgery was performed and was continued for 2 h after MCAO. The perfusion fluid was artificial cerebrospinal fluid (aCSF: 147 mM NaCl; 4 mM KCl; 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$), the perfusion rate was 2 μl/min, and samples were collected in 10 min intervals. 24 h after MCAO, mice were deeply anesthetized with isoflurane and euthanized by decapitation. Brains were quickly removed, sectioned coronally into 1 mm slices and stained with 2,3,5-triphenyl-tetrazolium chloride (TTC). Images were acquired by a Dino-Lite camera and areas of both hemispheres and the infarct regions were quantified for each slice using Image J 1.30.

Glucose and glutamate concentrations in the microdialysis samples were determined by a CMA-600 microanalyzer (CMA Microdialysis, Stockholm, Sweden). Lower limits of detection for glucose and glutamate were 20 and 1 μM, respectively.

### *4.4. Isolation of mitochondria*

For the isolation of mitochondria, mice were decapitated after 60 min of ischemia (induced by MCAO) and brains were removed quickly. The cerebellum was discarded, and the two hemispheres were separated by a section along the longitudinal fissure. Mitochondria from the right (healthy) and the left (ischemic) hemisphere were isolated separately. Sham-operated mice were anesthetized but did not undergo MCAO surgery. The hemispheres were homogenized with a Potter S Homogenizer (Sartorius BBI System, Göttingen, Germany) in 2 ml Mitochondrial Respiration Medium ("MiR05") enriched with protease inhibitor (Lassnig et al. 2011). Mitochondria were isolated from the homogenates by multiple centrifugation steps (Lassnig et al. 2011).

### *4.5. Mitochondrial assays: respirometry*

80 μl of the mitochondrial suspension (see 4.4.) and 2 ml MiR05 medium were injected into a chamber of the Oroboros® Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). The two chambers of the oxygraph allowed simultaneous measurement of oxygen flux in two hemispheres. Activity of mitochondrial respiration was determined by injecting several substrates and inhibitors of the respiratory chain using a complex pipetting scheme (elaborated by E. Gnaiger, see also Hand et al. 2010 and Eckert et al. 2012b). First, complex I respiration was determined by adding pyruvate (5 mM) and malate (2 mM) as substrates for complex I and ADP (2 mM) as substrate for complex V. The oxphos state represented full oxidative phosphorylation after adding succinate (10 mM), a substrate for complex II. Addition of cytochrome c (10 μM) served as quality control, because oxygen flux increases only when mitochondria are damaged during isolation. Leak state was reached with addition of oligomycin (2 μg/ml), inhibiting complex V (not relevant in this study). The electron transfer system (ETS) was reflected after injection of the uncoupler carbonylcyanide-p-trifluoromethoxyphenylhydrazon (FCCP, 1.5 μM). Complex II respiration was determined indirectly by adding rotenone (0.5 μM), a specific complex I inhibitor. Antimycin-A inhibited physiological respiration by blocking complex III, and the remaining residual oxygen consumption (ROX) was subtracted from all measured data. Maximum complex IV respiration was determined by addition of tetramethyl-phenylendiamine (TMPD) as electron

donor. For re-oxygenation, chambers were opened for approximately 10 min two times during the procedure.

### *4.6. Mitochondrial assays: permeability transition pore*

Mitochondrial swelling due to opening of the mitochondrial permeability transition pore (mPT) was also measured *ex vivo* in isolated mitochondria with or without MCAO pretreatment. The separated hemispheres were pottered in 15 ml isolation buffer (IB) + 12% Percoll by a Dounce homogenizer (Sims 1990). Mitochondria were separated in a 24%/40% Percoll gradient and centrifuged at 30,700 x g for 7 min. The extracted mitochondrial fraction was diluted with 5 ml IB and centrifuged (16,700 x g, 12 min) to remove Percoll. The pellet was resuspended in IB and adjusted to protein content (2.75 mg/ml). Absorption of the mitochondrial solution was observed over 750 s with an Aminco Spectrometer Bowman Series 2 at 540 nm (SLM Spectronic Instruments; Rochester, USA). The stirred glass cuvette contained 1.1 ml of measuring buffer (Hansson et al. 2004). 27.5 µg mitochondria were incubated with 2.2 µl glutamate (5 mM) and 2.2 µl malate (5 mM) for 3 min. After the addition of 0.5 µl oligomycin (1 µg/ml) the measurement was started. After 60 s either cyclosporine A (2 µl; 1 µM in ethanol) or the same amount of ethanol was injected. At 120 s, 4.4 µl ADP (20 µM) were added. Swelling was induced by addition of 10 µl calcium (either 4 or 8 µmol/mg protein) at 300 s. Injection of alamethicin (5.5 µl; 7.5 µg/ml) at 500 s resulted in maximal mitochondrial swelling, providing the 100% swelling value. The percentages of swelling in Fig. 4 were calculated by using the absorbance before calcium injection as 0% and the absorbance after alamethicin injection (pore opener) as 100% value. The quality of mitochondria was verified by measuring cytochrome c loss in the Oroboros® Oxygraph-2k as described above.

### *4.7. Statistical analysis*

Data are given as means ± S.D. of N experiments. Data in Fig. 3 was compared by one-way ANOVA with Bonferroni post-test. Data in Figs. 1, 2 and 4 were compared by two-tailed t-test (software: GraphPad Prism 5.0).

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