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Effects of EGb 761® Ginkgo biloba Extract on Mitochondrial Function and Oxidative Stress

As major sources of reactive oxygen species (ROS), mitochondrial structures are exposed to high concentrations of ROS and may therefore be particularly susceptible to oxidative damage. Mitochondrial damage could play a pivotal role in the cell death decision. A decrease in mitochondrial energy charge and redox state, loss of transmembrane potential (depolarization), mitochondrial respiratory chain impairment, and release of substances such as calcium and cytochrome c all contribute to apoptosis. These mitochondrial abnormalities may constitute a part of the spectrum of chronic oxidative stress in Alzheimer's disease. Accumulation of amyloid beta (Aβ) in form of senile plaques is also thought to play a central role in the pathogenesis of Alzheimer's disease mediated by oxidative stress. In addition, increasing evidence shows that Aβ generates free radicals *in vitro*, which mediate the toxicity of this peptide.

In our study, PC12 cells were used to examine the protective features of EGb 761® (definition see editorial) on mitochondria stressed with hydrogen peroxide and antimycin, an inhibitor of complex III. In addition, we investigated the efficacy of EGb 761® in Aβ-induced MTT reduction in PC12 cells. Moreover, we examined the effects of EGb 761® on ROS levels and ROS-induced apoptosis in lymphocytes from aged mice after *in vivo* administration.
Here, we will report that EGb 761® was able to protect mitochondria from the attack of hydrogen peroxide, antimycin and Aβ. Furthermore, EGb 761® reduced ROS levels and ROS-induced apoptosis in lymphocytes from aged mice treated orally with EGb 761® for 2 weeks.
Our data further emphasize neuroprotective properties of EGb 761®, such as protection against Aβ-toxicity, and antiapoptotic properties, which are probably due to its preventive effects on mitochondria.

Pivotal Role of Mitochondria within the Cell

Mitochondria are essential for the maintenance of cell function and viability. They are often described as the 'power house of the cell'. The primary function of mitochondria is to produce ATP through the coupling of oxidative phosphorylation with respiration. The mitochondrial respiratory chain comprises five enzyme complexes: NADH-CoQ reductase (complex I), succinate CoQ reductase (complex II), ubiquinol-cytochrome c reductase (complex III), cytochrome c oxidase (complex IV), and F_1F_0-ATPase (complex V) (Fig. **1**). While the respiratory enzyme complexes transfer electrons to each other and ultimately to molecular oxygen, they translocate protons across the inner mitochondrial membrane. The proton gradient set up in this way provides the energy that drives the motor of the membrane-bound enzyme ATP synthase, which catalyses the conversion of ADP to ATP, completing the process of oxidative phosphorylation. The movement of protons has two major consequences: 1) it generates a pH gradient across the inner mitochondrial membrane with the pH higher in the matrix than in the cytosol (close to pH = 7); 2) it generates a voltage gradient (transmembrane potential, $\Delta\Psi_m$) across the inner mitochondrial membrane with the inside negative and the outside positive (estimated at –150 to –180 mV negative with respect to the cytosol). Changes in the latter can be readily determined with fluorescent dyes such as Rhodamine 123.

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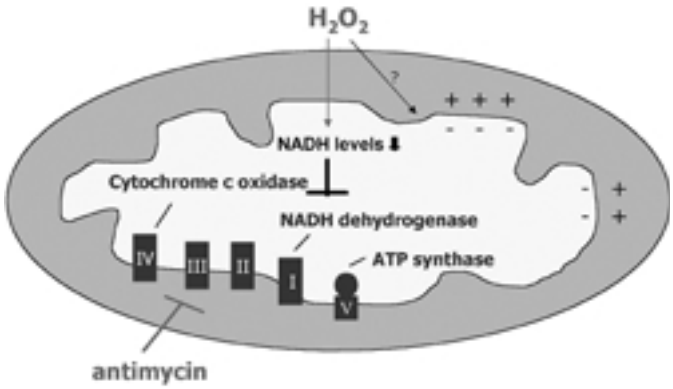

Fig. **1** **Mitochondrial targets with effects on membrane potential**. Antimycin is an inhibitor of complex III of the respiratory chain. Several early mitochondrial events develop after exposure to H_2O_2, including inhibition of α-ketoglutarate dehydrogenase and a decrease in steady state NADH levels that may consequently lead to an inhibition of complex I and a decrease in mitochondrial membrane potential ($\Delta\Psi_m$).

Mitochondria as Sources and Targets of ROS

The reactive oxygen species (ROS) category includes the superoxide anion radical as the primary product of one-electron dioxygen reduction, the extremely aggressive hydroxyl radical derived from subsequent reactions, singlet oxygen, and strong non-radical oxidants such as hydrogen peroxide. Furthermore, nitric oxide and its derivative peroxynitrite radical also belong to the ROS.

Mitochondria represent the major source of ROS. One consequence of oxidative phosphorylation is the generation of unpaired electrons. The interaction of these electrons with O_2 results in the generation of superoxide anions ($O_2^{\cdot}$), which are a highly reactive oxygen species. Superoxide dismutase (SOD) plays a protective role in all aerobic organisms by detoxifying the superoxide anion in a dismutase reaction producing hydrogen peroxide. Hydrogen peroxide, in turn, can be reduced to water (H_2O) by either glutathione peroxidase or catalase, or can itself produce an even more potent radical, the hydroxyl radical ($OH^{\cdot}$). Hydroxyl radicals are extremely reactive and can react with nearly all cellular macromolecules – including DNA, proteins, and membrane lipids. Being the major source of ROS, mitochondria are subjected to direct attack by large amounts of ROS in the cell, and might therefore be particularly susceptible to oxidative damage.

Mitochondria and Cell Death

Cell death constitutes one of the key events in cell biology. At least two models of cell death can be distinguished – apoptosis and necrosis. Apoptosis involves the regulated action of catabolic enzymes (proteases and nucleases). Characteristic features of apoptosis are changes in nuclear morphology and in chromatin biochemistry. When misregulated, apoptosis can contribute to various diseases that include cancer and neurodegenerative diseases [53]. In contrast to apoptosis, necrosis does not involve any regular DNA or protein degradation pattern, and is accompanied by swelling of the cytoplasm and mitochondrial matrix, which occurs shortly before the cell membrane ruptures.

Mitochondria have been found to play a central role in apoptosis, thereby exhibiting major changes in their structure and function [18,26]. A decrease in mitochondrial membrane potential is an early universal event of apoptosis [68]. In most apoptosis pathways, the release of mitochondrial cytochrome c and apoptosis-inducing factor are also key events in initiating the cascade of reactions leading to apoptotic cell death [48] (Fig. **2**). Cytochrome c release is clearly regulated by the pro- and anti-apoptotic proteins of the Bcl-family (bax, bak, bid as pro- and bcl-2 and bcl-xl as anti-apoptotic proteins) [54]. The mechanism by which cytochrome c activates the apoptotic cascade remains to be fully elucidated; however, it seems to activate caspase-9, which then cleaves downstream effectors and elicits the apoptotic response (Fig. **2**).

A mismatch between the production of prooxidants and antioxidants in cells might lead to oxidative stress. Mitochondria are very susceptible to oxidative damage. Mitochondrial membrane potential changes, mitochondrial respiratory chain impairment, and ATP depletion are characteristic consequences of oxidative stress. ATP deficiency further leads to a decrease in cell glutathione (GSH), which results in enhanced oxidative stress and triggers the vicious cycle of oxidative stress, mitochondrial dysfunction, and apoptosis.

Mitochondria, Oxidative Stress and Alzheimer's Disease

Mitochondrial abnormalities have been identified in a large proportion of neurodegenerative diseases [28,34,50,68]. Biochemical analysis of CNS tissue from patients with Alzheimer's disease (AD) has yielded evidence for abnormalities of components of the electron transport chain. In many AD patients, cytochrome c oxidase is impaired in the CNS – and even in other tissues, including platelets [7]. Several studies have suggested that β-amyloid (Aβ) may be directly toxic to isolated mitochondria [10] and that it also may cause a loss of cytochrome c oxidase activity in neurons in culture. Additionally, increasing evidence suggests a diminished activity of the α-ketoglutarate dehydrogenase complex (KGDHC) in brain tissue from AD patients [24,25].

The free radical hypothesis of aging according to *Harman* [27] posits that age-related accumulation of reactive oxygen species results in damage to major components of cells – nucleus, mito-

Fig. **2** **Mitochondrial role in the determination of life and death of the cell.** Mitochondria are very susceptible to oxidative damage. A decrease in mitochondrial membrane potential, a release of substances such as cytochrome c and apoptosis-inducing factor and an activation of the caspase cascade are characteristic features of apoptotic cell death. AIF: apoptosis-inducing factor; $O_2^{\cdot-}$: superoxide anion; $OH^{\cdot}$: hydroxyl radical

chondrial DNA, membranes and cytoplasmic proteins. Many authors have suggested that an imbalance between the generation of free radicals and antioxidants may be involved in the pathogenesis of most neurodegenerative diseases. The fact that age is the most important risk factor of sporadic Alzheimer's disease provides considerable support for the free radical hypothesis. Many considerations suggest that free radicals are involved in age-related pathologies including AD (for reviews, see [6,16,62]). Possibly, oxidative damage is the earliest event in AD [49].

During the last decade, considerable evidence has accumulated demonstrating oxidative stress products on certain cellular targets in AD:
- Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease [3].
- An Assessment of Oxidative Damage to Proteins, Lipids, and DNA in Brain from Patients with Alzheimer's Disease [42].
- High aggregate burden of somatic mtDNA point mutations in aging and Alzheimer's disease brain [39].
- Lipid peroxidation and advanced glycation end products in the brain in normal aging and in Alzheimer's disease [17,44].
- Increased activities of antioxidant enzymes in brains from Alzheimer's disease patients [58].

Effects of EGb 761® on Mitochondrial Function

Due to its free radical scavenging properties, EGb 761® Ginkgo biloba extract has been used for many years to treat age-related cognitive disorders. Several recent studies have clearly indicated the therapeutic potential of EGb 761® in AD [33,36–38].

Interestingly, increasing evidence provides support for protective effects of EGb 761® on the mitochondrial respiratory chain [32]. Therefore, it became increasingly important to find out the extent to which EGb 761® could protect mitochondrial function against deleterious insults.

Several select studies dealing with this topic are briefly outlined as follows:

In vitro studies:
- EGb 761® protects isolated liver mitochondria against anoxia/reoxygenation-induced injury [19]
- Bilobalide increased the levels of mtDNA-encoded COX subunit mRNA and protein levels in PC12 cells [12]
- Protection of Mitochondrial Respiration Activity by Bilobalide [32]
- EGb 761® and bilobalide inhibited hypoxia-induced decrease in ATP content in endothelial cells [31]

In vivo/Ex vivo studies:
- EGb 761® and bilobalide were shown to increase the respiratory control ratio of mitochondria isolated from orally treated rats [31]
- Bilobalide allows to maintain their respiratory activity under ischemic conditions by protecting complex I and III [29,30,32]
- EGb 761® administered orally for 7 days before ischemia protected neurons from a decrease in COX III mRNA [13]
- Treatment with EGb 761® prevented the age-related increase in oxidative damage [55]

EGb 761® Ginkgo biloba Extract Protects Mitochondria from Hydrogen Peroxide Attack

Mitochondrial membrane potential is an important marker for the function of mitochondria. A decrease in mitochondrial membrane potential has been related to cell death in different cell types [65].

In our study, PC12 cells were used to examine the protective features of EGb 761® on mitochondria challenged by hydrogen peroxide (H_2O_2). H_2O_2 is a membrane-permeable ROS widely used to induce oxidative stress. Several early mitochondrial events develop after exposure to H_2O_2, including decrease in $\Delta\Psi_m$, reduction of ATP levels and ATP/ADP ratio, inhibition of α-ketoglutarate dehydrogenase, and decrease of steady-state NADH levels (Fig. **1**) [15].

PC12 cells were treated in two different ways. First, efficacy of EGb 761® Ginkgo biloba extract in preventing H_2O_2-toxicity was tested. Here, PC12 cells were pretreated with 0.01 mg/ml or 0.1 mg/ml EGb 761® Ginkgo biloba extract. After pretreatment for one hour, H_2O_2 (0.5mM) was added and the mitochondrial transmembrane potential was determined after 6 h of H_2O_2-treatment. Second, the protective effect of EGb 761® on recovery after oxidative stress insult was tested. Thus, PC12 cells were also treated for 6 h with H_2O_2, but in this trial, we added EGb 761® 30 min after the onset of H_2O_2 exposure.

The membrane potential of the inner mitochondrial membrane was measured using Rhodamine 123 (R123) dye. The dye was added to the cell culture medium at a final concentration of 4 μM for 15 min. The cells were washed twice with HBSS, and fluorescence was determined with a fluorostar spectrometer at 507nm/529nm. Transmembrane distribution of the dye depends on the mitochondrial membrane potential ($\Delta\Psi_m$). Consequently, loading capability within the membrane decreases when the mitochondrial membrane potential declines after insult.

Our data show that EGb 761® exhibited protective efficacy against H_2O_2-induced mitochondrial dysfunction. Significant reduction of mitochondrial membrane potential changes was found at concentrations as low as 10 μg/ml EGb 761® compared to control cells, which were treated with H_2O_2 only (Fig. **3A**). Notably, EGb 761® also significantly reduced H_2O_2-induced decrease in $\Delta\Psi_m$ when added 30 min after the onset of H_2O_2 exposure (Fig. **3B**). During this time period, H_2O_2 can freely penetrate the cell membrane and easily damage intracellular membrane structures. On the basis of these results, it seems likely that the protective effect of EGb 761® is not only due to its radical-scavenging properties, since the reduction was still markedly present when cells were first exposed to oxidative stress. Thus, EGb 761® additionally affects the restoration of mitochondrial capacity after insult.

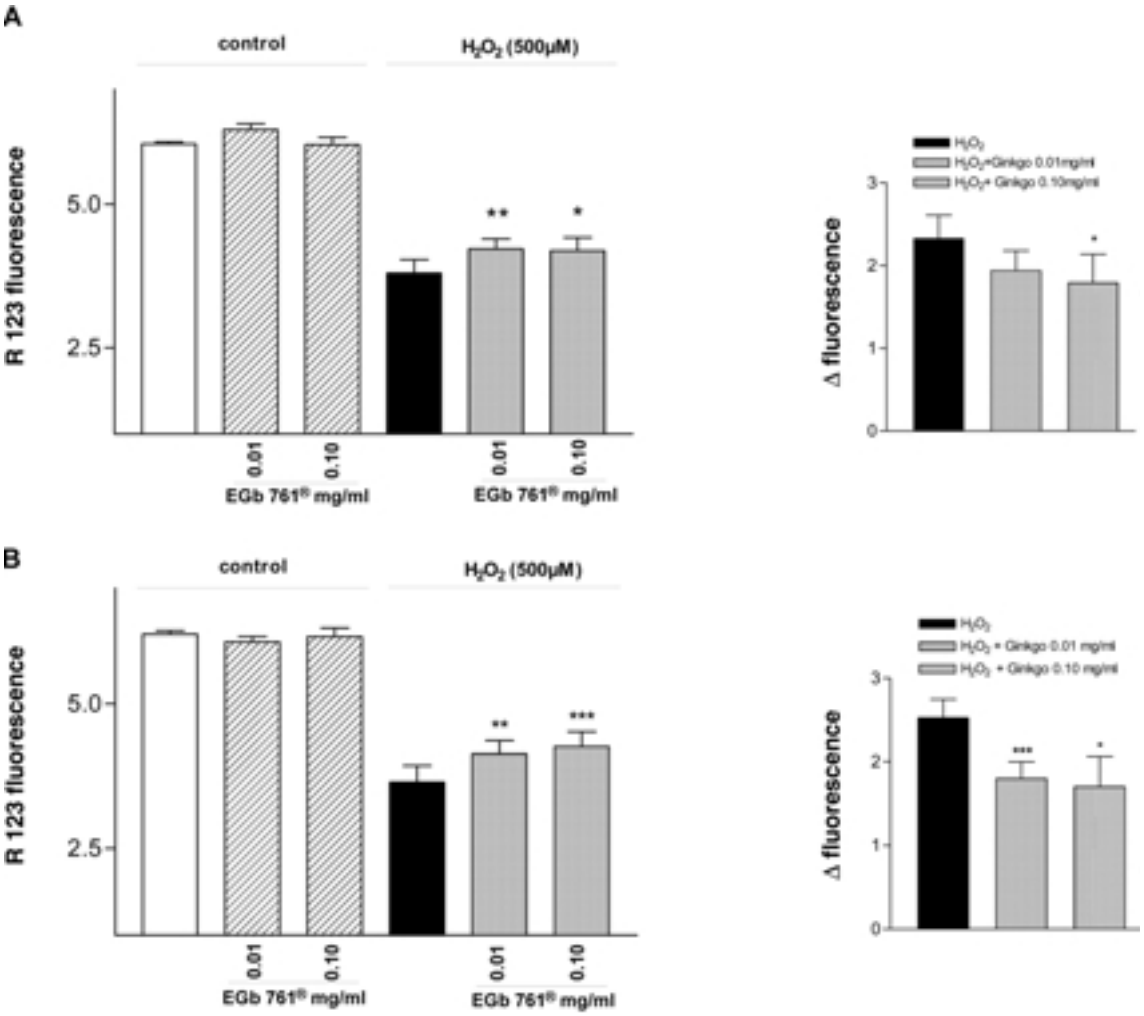

Fig. **3** **Protective effects of EGb 761® extract on H_2O_2-induced mitochondrial membrane potential changes**. **A**: PC12 cells were pretreated with 0.01 mg/ml and 0.1 mg/ml Ginkgo biloba extract EGb 761®. After 1 h pretreatment, H_2O_2 (0.5mM) was added and the mitochondrial transmembrane potential was determined after 6 h of H_2O_2-treatment. **B**: PC12 cells were also treated for 6 h with H_2O_2 (0.5mM), but in this trial, we added EGb 761® 30 min after the onset of H_2O_2 exposure. A and B: In both trials, EGb 761® significantly reduced the decrease in membrane potential evoked by treatment with H_2O_2. The left panels are showing the absolute values in the change of fluorescence intensities of Rhodamine 123 (R123) dye, and the right panels are presenting the difference in fluorescence levels (Δ fluorescence) between H_2O_2-treated cells in the presence or absence of EGb 761® and the corresponding controls. Data are expressed as means ± SEM (n = 6). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. H_2O_2, paired Student's *t*-test.

Pretreatment with EGb 761® Reduces Antimycin-Induced Mitochondrial Membrane Potential Changes

Defects in the electron transport chain within the mitochondria are major factors that contribute to the production of free radicals. Antimycin is a complex III inhibitor in the respiratory chain. An inhibition of complex III by antimycin results in a subsequent superoxide anion production.

In our study, we tested the effect of EGb 761® on antimycin-induced membrane potential changes in PC12 cells. The Tetramethylrhodamineethylester fluorescence dye was used to test acute and fast changes in $\Delta\Psi_m$, which exhibits a characteristic increase in fluorescence after challenging mitochondria with membrane potential decreasing drugs (Fig. **4**). PC12 cells were pretreated for 6 h with EGb 761®. Mitochondrial membrane potential was than recorded and antimycin (2μM) was added after 100 sec (Fig. **4**). Interestingly, EGb 761® was able to reduce the effects of antimycin on mitochondrial membrane potential probably by protecting complex III against the blocking effect of antimycin. A significant protection effect was observed at a concentration of 100 μg/ml EGb 761® (Fig. **4**). These results are also in accordance with the findings of *Janssens* et al., which also demonstrated bilobalide's protective effects on complex III under ischemia [29,30,32].

In Vivo Administration of EGb 761® Ginkgo biloba Extract Reduces ROS Levels and ROS-Induced Apoptosis in Lymphocytes from Aged Mice

Oxidative stress increases with age, and is a major factor in causing cellular damage and enhanced apoptosis in many tissues including brain and the immune system. Accordingly, we could show that peripheral lymphocytes in aged humans displayed a significantly higher content of apoptotic nuclei than young humans [57]. To exclude changes associated with AD, which may

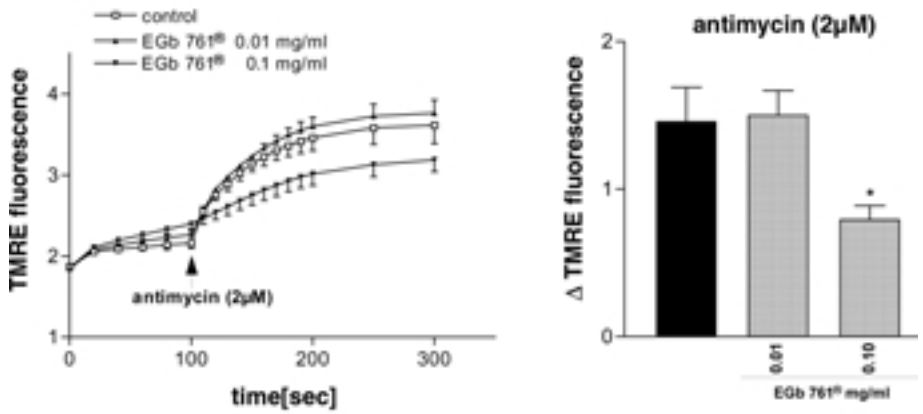

Fig. **4** **Pretreatment with EGb 761® reduced antimycin-induced changes in mitochondrial membrane potential evoked by inhibition of complex III.** PC12 cells were pretreated for 6 h with EGb 761®. Then, mitochondrial membrane potential was recorded with the dye Tetramethylrhodamineethylester (TMRE) and antimycin (2μM) was added after 100 sec (left panel). The right panel presents the difference in fluorescence levels (Δ TMRE fluorescence) between antimycin-treated cells in the presence or absence of EGb 761® after 300 sec and the corresponding basal fluorescence level before stimulation. Data are expressed as the means ± SEM (n = 4). *p < 0.05 vs. antimycin-control, Student's *t*-test.

already occur in presymptomatic human controls, we investigated T cells from young and old mice. T cells were isolated from the spleen of young mice (3 months) and old mice (24 months) and stimulated with an oxidative stress inducer [56]. 2-deoxy-D-ribose (d-Rib) depletes the intracellular pool of reduced glutathione, thereby promoting states of enhanced oxidative stress inside the cell [11,20,21,57]. T cells of the spleen from young and old mice were incubated for 24 h in the presence of 10mM d-Rib. Induction of oxidative stress revealed age-related changes in apoptosis. A significant increase towards higher apoptotic levels was found in cells from aged animals (Fig. **5A**) [56].

Since oxidative stress increases with age, old individuals may particularly benefit from treatment with EGb 761® [59,61]. Therefore, we treated aged female NMRI mice (24 months) with 100 mg/kg/day/p.o. of EGb 761® (dissolved in 0.2% agar) over a period of 14 days. The placebo-treated control group received vehicle only. Animals were sacrificed by cervical dislocation, and T lymphocytes from the spleen were isolated by negative depletion of B cells. After incubation of isolated lymphocytes from aged mice with d-Rib (10 mM), cells that have been chronically treated with EGb 761® for 2 weeks *in vivo* showed significantly lower levels of generated ROS compared to lymphocytes from placebo-treated control mice (Fig. **5C**). Ginkgo biloba therefore seems to be able to compensate for the enhancement of ROS during aging. In parallel, vulnerability to the induction of apoptosis was investigated in lymphocytes from the same animals. In agreement with our previous *in vitro* findings [56] that demonstrated the protective efficacy of EGb 761® against apoptosis, lymphocytes from aged mice treated with EGb 761® for two weeks revealed significantly lower levels of apoptotic cells after incubation with the ROS-generating agent d-Rib (10 mM, 24 hours) (Fig. **5B**). Notably, under chronic *in vivo* treatment, even low plasma concentrations of EGb 761® can provide sufficient protection against cell death.

Furthermore, our results are in accordance with findings indicating that Ginkgo biloba extract can reduce cell death in neurons after exposure to oxidative stress [51,47].

Thus, we can conclude that mice chronically treated with EGb 761® Ginkgo biloba extract appear to be protected against increased oxidative stress during aging. These findings may elucidate the therapeutic value of EGb 761® in restoring oxidant/antioxidant balance in relation to aging and age-related disorders.

Effects of β-Amyloid Peptide on Mitochondria

β-amyloid (Aβ), a 40–42 amino acid peptide that principally constitutes senile plaques, is thought to play a crucial role in the pathogenesis of AD. Supporting this hypothesis, Aβ has

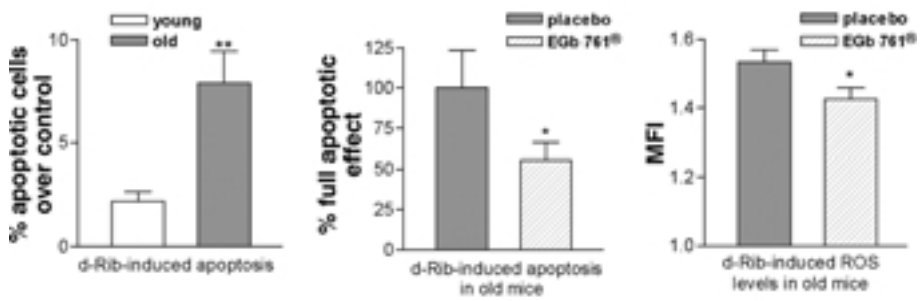

Fig. **5** **Treatment with EGb 761® for 2 weeks (100 mg/kg/day) significantly decreased oxidative stress levels and ROS-induced apoptosis in lymphocytes from aged mice. A:** Apoptosis in T cells from young (3 months, n = 9) and old (24 months, n = 13) mice. T cells from the spleens were incubated for 24 h at 37 °C in the presence of d-Rib (10 mM). Data are expressed as the means ± SEM; **p < 0.01, Student's *t*-test. **B:** Effect of oral EGb 761® treatment on ROS-induced apoptosis (d-Rib, 10 mM) in T cells from old mice. Mean levels of the d-Rib-induced apoptosis of the placebo group were set 100% (100% = full apoptotic effect). Apoptosis was reduced by EGb 761® by about 45% compared to the cells from placebo-treated old mice. Data are expressed as the means ± SEM (n = 13 per group). *p < 0.05 vs. placebo, Student's *t*-test. C: Effect of oral EGb 761® treatment on d-Rib-evoked (d-Rib, 10 mM) ROS levels in T cells from old mice. Treatment of old mice with EGb 761® orally for 2 weeks significantly decreased ROS levels – corresponding to lower MFI values – in lymphocytes from aged mice. Data are expressed as the means ± SEM (n = 11 per group). *p < 0.05 vs. placebo, Student's *t*-test.

been demonstrated to be directly toxic to cultured neurons; aggregation of Aβ into fibrils is apparently required for its cytotoxic effect [52]. Moreover, several findings have indicated that neuronal cell death associated with Aβ peptide is apoptotic in nature [23, 35, 41]. One possible mechanism for initiating apoptosis could be the generation of free radicals by the peptide [9] leading to lipid peroxidation and oxidative stress. Furthermore, oxidative stress induces intracellular accumulation of Aβ [43]. Nevertheless, the biochemical mechanisms underlying Aβ toxicity remain largely unknown. One consistent observation on Aβ cytotoxicity is the rapid inhibition of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to formazan [1]. Therefore, understanding how Aβ inhibits cellular MTT reduction could provide an insight into the mechanisms of Aβ neurotoxicity. MTT is a tetrazolium salt that forms purple-colored, water-insoluble formazan upon reduction. Because only living cells can reduce MTT, MTT reduction has been developed into one of the most widely used methods for measuring viability. Besides the assumption that MTT is reduced by active mitochondria in living cells, new results suggest that Aβ inhibits cellular MTT reduction by dramatically enhancing MTT formazan exocytosis, indicating an altered cellular signal transduction pathway [40]. Furthermore, both oxidative stress-dependent and independent effects of Aβ seem to be detected using MTT reduction [1].

Protective Effects of EGb 761® Ginkgo biloba Extract on Toxicity Induced by Oxidative Stress and Aβ

The ability of oxidative stress and/or Aβ to induce cell death and the protective effects of Ginkgo biloba extract on preventing this induction have been investigated during the last years in several studies, such as:

- EGb 761® protects and rescues hippocampal cells against nitric oxide-induced toxicity and Aβ-induced cell death [4, 5]
- Protective effect of bilobalide against NO-induced toxicity in PC12 cells [63]
- Preventive effect of EGb 761® or bilobalide on apoptosis in rat cerebellar neuronal cells induced by hydroxyl radical and H_2O_2 [14, 47, 69, 70]
- Ginkgo biloba extract protects neurons against oxidative stress induced by H_2O_2 [51]
- Inhibition of serum deprivation-induced neuronal apoptosis by EGb 761® [2]
- Protective effects of bilobalide on ROS- and on Aβ 25 – 35-induced apoptosis in PC12 cells [72, 73]
- EGb 761® rescues Aβ-induced cell death in PC12 cells [71]

Pretreatment with EGb 761® Prevented Aβ-Induced MTT Reduction in PC12 Cells

In our study, PC12 cells were pretreated with EGb 761® at different concentrations (0.01 – 0.5 mg/ml) for 24 h and were then exposed to Aβ 25 – 35 (1μM) for further 24 h incubation at 37 °C. EGb 761® prevented Aβ-induced toxicity (Fig. **6**) in a concentration-dependent manner. Significant effects of EGb 761® in preventing Aβ toxicity were seen at a concentration of 0.25 and 0.5 mg/ml (Fig. **6**). Our results agree with data from *Yao* et al. demonstrating protective effects of EGb 761® (0.2 mg/ml) on Aβ 1 – 42-induced inhibition of MTT reduction at 0.5μM and higher concentrations [66]. In addition, we monitored the kinetics of Aβ (1μM)-induced MTT reduction inhibition in the presence or absence of EGb 761® (0.25 mg/ml) in PC12 cells (Fig. **7**); MTT absorption values were recorded over a time period of 4 h after the addition of MTT reagent, demonstrating a clear increase in MTT absorption after 4 hours. In parallel, the production time course for formazan by reduction of MTT over a time period of 4 h was recorded by photomicrography. Importantly, both assays clearly show that the pretreatment with EGb 761® prevented Aβ-induced inhibition of MTT reduction and, notably, that the kinetic was similar to that of untreated control cells (Fig. **7**). Aβ-treated cells already exhibited formazan crystals 30 min after the addition of MTT reagent, whereas untreated control cells and cells treated with Aβ in the presence of EGb 761® started unequivocally later with the exocytosis of formazan (Fig. **7**, right panel). Thus, our results confirm the results of *Liu* et al., suggesting a scenario in which Aβ causes a dramatically enhancing MTT formazan exocytosis that may indicate an alteration of intracellular vesicle transport [40]. On this basis, we devised a model for the mechanisms of Aβ-induced inhibition of MTT reduction and, in addition, potential cellular targets for the protective effects of EGb 761® on Aβ-evoked MTT toxicity (Fig. **8**). First, EGb 761® can prevent direct inhibiting effects of Aβ on the reduction of MTT to formazan by redox enzymes. Second, EGb 761® may inhibit the Aβ-evoked acceleration of exocytosis of formazan to the outer side of the cell membrane. The latter effect is probably related to the strong membrane-destabilizing properties of Aβ fragments [22, 45, 46].

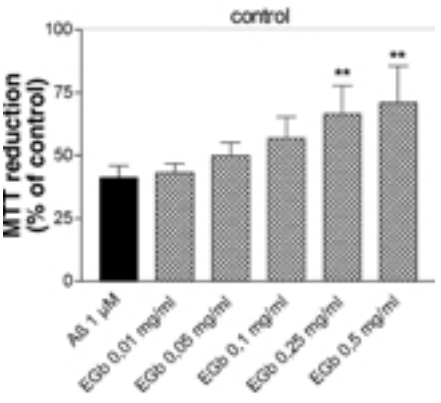

Fig. **6 Pretreatment with EGb 761® prevented Aβ-induced inhibition of MTT reduction in PC12 cells.** PC12 cells were pretreated with EGb 761® at different concentrations (0.01 – 0.5 mg/ml) for 24 h and were then exposed to Aβ 25 – 35 (1μM) for further 24h. EGb 761® prevented in a concentration-dependent manner Aβ-induced toxicity. EGb 761® at a concentration of 0.25 and 0.5 mg/ml significantly inhibited Aβ-induced reduction of MTT. Data are expressed as the means ± SD (n = 4 per treatment). $^{**}p < 0.01$ vs. Aβ, ANOVA followed by Tukey's *post hoc* test.

Conclusions

There is convincing evidence to support the hypothesis that oxidative stress plays a crucial role in aging and in the pathogenesis of Alzheimer's disease. Many risk factors in AD, such as age and β-amyloid load, have been shown to precipitate oxidative stress pathways. Preclinical evidence suggests a valuable role of antioxidant treatment to protect against free radical-induced cell death. Available clinical trial data are promising, and support continued investigation.

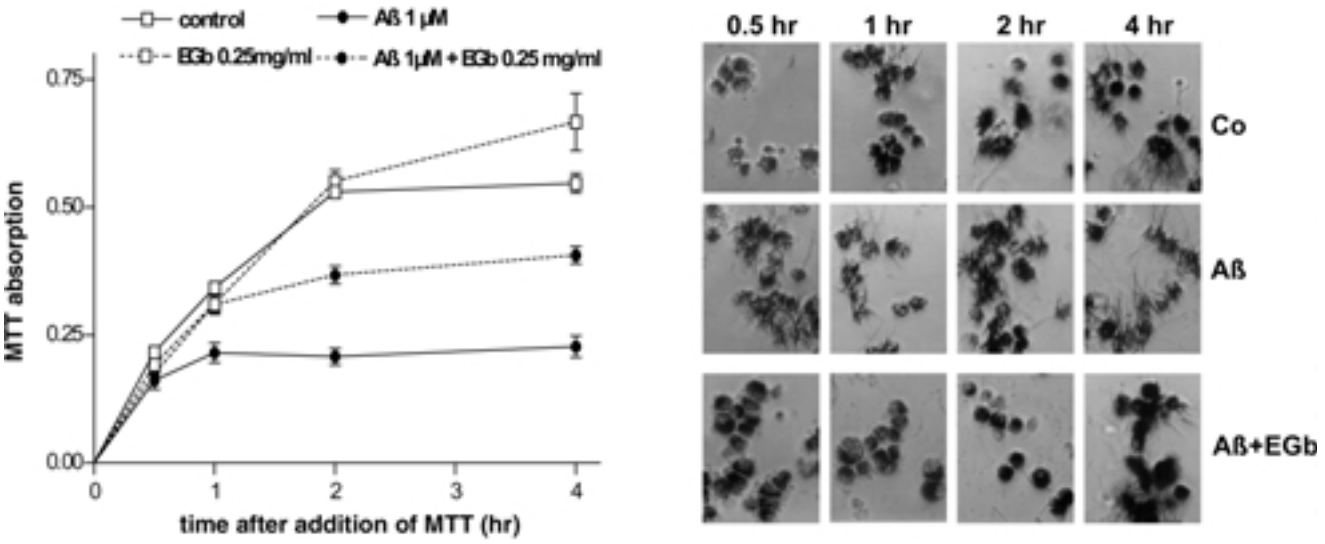

Fig. **7** Kinetic of Aβ-induced inhibition of MTT reduction to formazan and influence of EGb 761® treatment. Kinetic of the Aβ (1µM)-induced inhibition of the reduction of MTT in the presence or absence of EGb 761® (0.25 mg/ml) in PC12 cells. Left panel: MTT absorption values were recorded over a time period of 4 h after the addition of MTT reagent. Data are expressed as the means ± SD (n = 2 per treatment, each performed in triplicates). Right panel: In parallel, time course of production of formazan by reduction of MTT over a time period of 4 h was recorded by photomicrographs. Pretreatment with EGb 761® prevented Aβ-induced inhibition of MTT reduction and the kinetic was similar to that of untreated control cells.

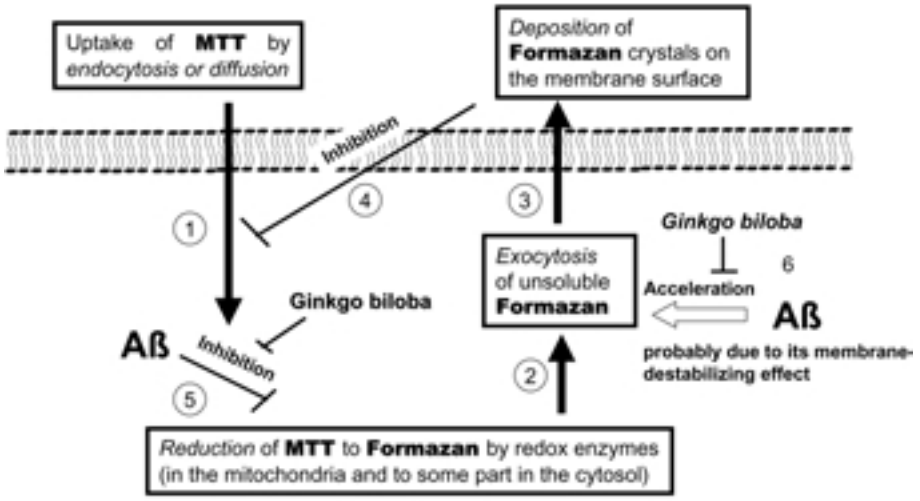

Fig. **8** Model of Aβ-induced inhibition of MTT reduction and potential cellular targets for the protective effects of EGb 761®.

Flavonoid and/or bilobalide constituents of EGb 761® and/or their metabolites appear to be involved in mediating such free radical scavenging and antioxidant actions. In addition, EGb 761® extract exhibits neuroprotective effects, such as protection against Aβ-toxicity, and anti-apoptotic properties – probably due to its protective effects on mitochondria. While the effects of flavonoid constituents of EGb 761® have been shown to be mainly due to antioxidant properties, EGb 761® seems to have a complex mode of action, which is – at least in part – independent from antioxidant effects. Furthermore, these effects seem to play a significant role in relation to the beneficial therapeutic effects of the EGb 761® extract, observed in the clinical setting.

Due to the simplicity of treatment, ease of access, and low costs, antidementive drugs such as Ginkgo biloba seem to be a highly attractive treatment possibility in delaying or slow down the aging process and disease manifestation.

Synopsis

Mitochondria as the major target of EGb 761®

The data summarized in the present communication strongly indicate that most of the neuoprotective and/or the antipoptotic of EGb 761® can be explained by its free radical scavenging and mitochondria potecting properties. While for the first effect the flavonoids seem to be most important, bilobalide seems to be the major constituent for the latter effect (Table **1**).

Table **1** The three components of mitochondrial protection by EGb 761®

1.	Direct free radical scavenging properties (mainly flavonoids)
2.	Mitochondrial stabilization (mainly bilobalide)
3.	Modulation of chloride conductance (bilobalide and ginkgolides)

Furthermore, when viewing the in vivo pharmacology of EGb 761®, it is remarkable that many of its effects especially regarding to affect and cognition are usually much more pronounced under conditions of impaired brain function (Ihl, this issue; Müller and Chatterjee, this issue). It is quite interesting that most if not all of those conditions of impaired brain function (aging, hypoxia, glucose deficits, radiation), which are specifically sensitive to (subchronic) EGb 761® treatment, are associated with mitochondrial dysfunction or even damage. Moreover, according to the modified β-amyloid cascade hypothesis, synaptic failure due to mitochondrial dysfunction seems to be the initial very early event in AD pathology [8, 34, 61]. Thus, perfectly in line with the key role of mitochondria for impaired brain function in aging and even AD are the numerous findings indicating stabilization of impaired mitochondrial functions by EGb 761®. Again, a major player in this aspect is bilobalide [30, 31, 32, 67].

One other mechanism might indirectly contribute to the mitochondria-protecting effects of EGb 761® (Table **1**). The function of bilobalide and ginkgolides has recently been associated with proteins related to receptor gated cloride channels (Chatterjee et al., this issue). By modulation of chloride condutance, stabilizing effects on mitochondrial function have been described [59, 60]. Thus, this mechanism will additionally contribute to the beneficial effects of EGb 761® on mitochondria, but will also directly affect neuronal function (see Müller and Chatterjee, this issue).

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