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Review

Neuroprotective effects of *Ginkgo biloba* extract

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Abstract. *Ginkgo biloba* extract has been therapeutically used for several decades to increase peripheral and cerebral blood flow as well as for the treatment of dementia. The extract contains multiple compounds such as flavonoids and terpenoids that are thought to contribute to its neuroprotective and vasotropic effects. In this review, we summarize the experimental results on the mechanism of neuroprotection induced by standardized extract of *Ginkgo biloba* leaves (EGb 761) and its constituents. The effects described mostly in animals include those on cerebral blood flow, neurotransmitter systems, cellular redox state and nitric oxide level. Furthermore, we discuss the current status of clinical trials as well as undesired side effects of EGb 761.

Key words. Apoptosis; bilobalide; cerebral blood flow; EGb 761; flavone glycosides; ginkgolides; nitric oxide; reactive oxygen species.

Introduction

The dried leaves of *Ginkgo biloba* tree provide the crude drug from which the standardized *Ginkgo biloba* extract (EGb 761) is obtained. The defined, but complex product consists of two major groups of substances, the flavone glycosides (flavonoid fraction, 24%) and the terpene lactones (terpenoid fraction, 6%). The flavonoid fraction is primarily composed of quercetin, kaempferol and isorhamnetin glycosides [1–3], and the terpenoid fraction of ginkgolides, such as the ginkgolides A, B, C and J as well as bilobalide (fig. 1). In addition, the extract contains organic acids such as kynurenic acid (KYNA, fig. 1), 6-hydroxykynurenic acid (6-HKA, fig. 1), vanillic acid, shikimic acid and glucuric acid (fig. 1) as well as proanthocyanidines, glucose, rhamnose and other various constituents [1]. The amount of ginkgolic acids in EGb 761 is less than 0.0005%.

The neuroprotective effect of EGb 761 has been demonstrated in several in vitro and in vivo models [4]. In vitro, EGb 761 (10–100 μg/ml) protected cultured neurons against death induced by hypoxia [5], hydrogen peroxide [6-12], glutamate (fig. 2, [13, 14]), verapamil [14], amyloid β (Aβ, [15,16]), nitric oxide (NO, [17]), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, [18]) and cyanide [13]. In vivo, reduction of neuronal damage by EGb 761 [10–100 mg/kg, p.o. (per os), or i.p. (intraperitoneally)] has been observed after transient middle cerebral artery occlusion (MCAO) in rats [13,19] and gerbils [20–22], focal cerebral ischemia in mice and rats [13], hypoxia [23], heat stress [24], subchronic cold stress [25], amphetamine-induced behavioral sensitization [26] and in a transgenic mouse model of amyotrophic lateral sclerosis [27]. The ginkgolides (1–100 μM in vitro or 50–100 mg/kg in vivo), bilobalide (25–100 μM in vitro or 10 mg/kg in vivo) and in some cases also the flavonoid fraction (25–100 μg/ml in vitro or 40–100 mg/kg in vivo) have been shown to contribute to the neuroprotective effect of EGb 761.

Neurons can die either through an apoptotic or necrotic process. As apoptosis has been shown to play a dominant role in most of the neurodegenerative diseases as well as in stroke, the question arises whether EGb 761 can protect

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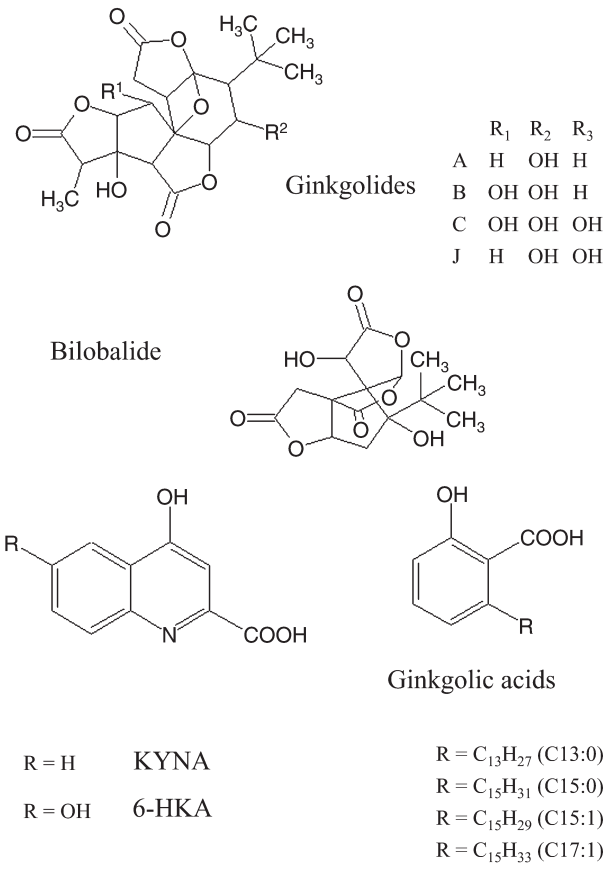

Figure 1. Chemical structure of some of the constituents of EGb 761 such as the ginkgolides A, B, C and J, bilobalide, ginkgolic acids, KYNA and 6-HKA.

neurons against apoptosis. The extract has been shown to reduce apoptosis induced by serum deprivation [28], staurosporine [28], hydroxyl radicals [6, 10, 12,29], olfactory nerve sectioning [30] and MCAO in rats [19]. The anti-apoptotic effect of EGb 761 has been characterized by detecting DNA fragmentation using the TUNEL (TdT-mediated nick end labeling) assay [19, 28] and DNA gel electrophoresis [6, 10, 12] as well as by measuring caspase-3 activity [19]. Among the constituents, bilobalide and ginkgolide B have been shown to protect cultured chick neurons against apoptosis caused by staurosporine and serum deprivation (fig. 3, [28]) and bilobalide inhibited DNA fragmentation induced by hydroxyl radicals in PC12 cells [11]. However, not the terpenoid, but the flavonoid fraction prevented hydrogen peroxide- and Aβ-induced increase in DNA fragmentation in hippocampal and cerebellar granule neurons, respectively [10, 15].

Ginkgolic acids, which are removed from EGb 761 below a level of 0.0005%, have been recently described to cause death of cultured neurons in a concentration of 100 µM (fig. 4) and to increase the activity of protein phosphatase type 2C [31]. Ginkgolic acids-induced death showed features of apoptosis such as chromatin condensation, shrinkage of the nucleus and reduction of damage by protein synthesis inhibition, but also of necrosis, as cell death was not accompanied by an increase in DNA fragmentation and caspase-3 activity [31]. However, since the amount of ginkgolic acids in EGb 761 is less than 0.0005%, toxic effects in humans are not expected.

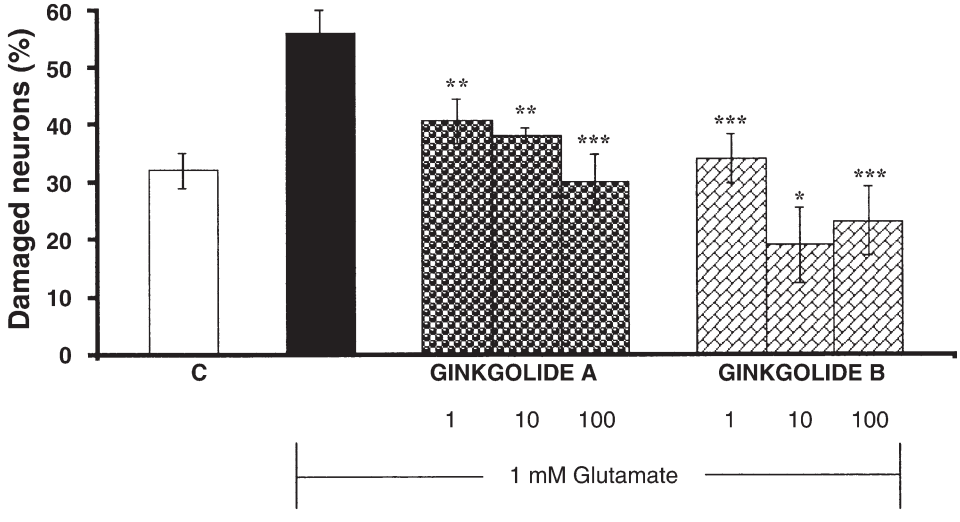

Figure 2. The ginkgolides A and B protected hippocampal neurons against damage caused by glutamate. Hippocampal cultures were exposed for 1 h to 1 mM glutamate or vehicle (C). The percentage of damaged neurons was determined 18 h later by the trypan blue exclusion method. Drugs were present in the culture medium from 30 min before up to 18 h after the exposure to glutamate. Means ± S.D. from six experiments are shown. Difference from the respective vehicle-treated control: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ [ANOVA (analysis of variance)-1 and posthoc Scheffé test] [13].

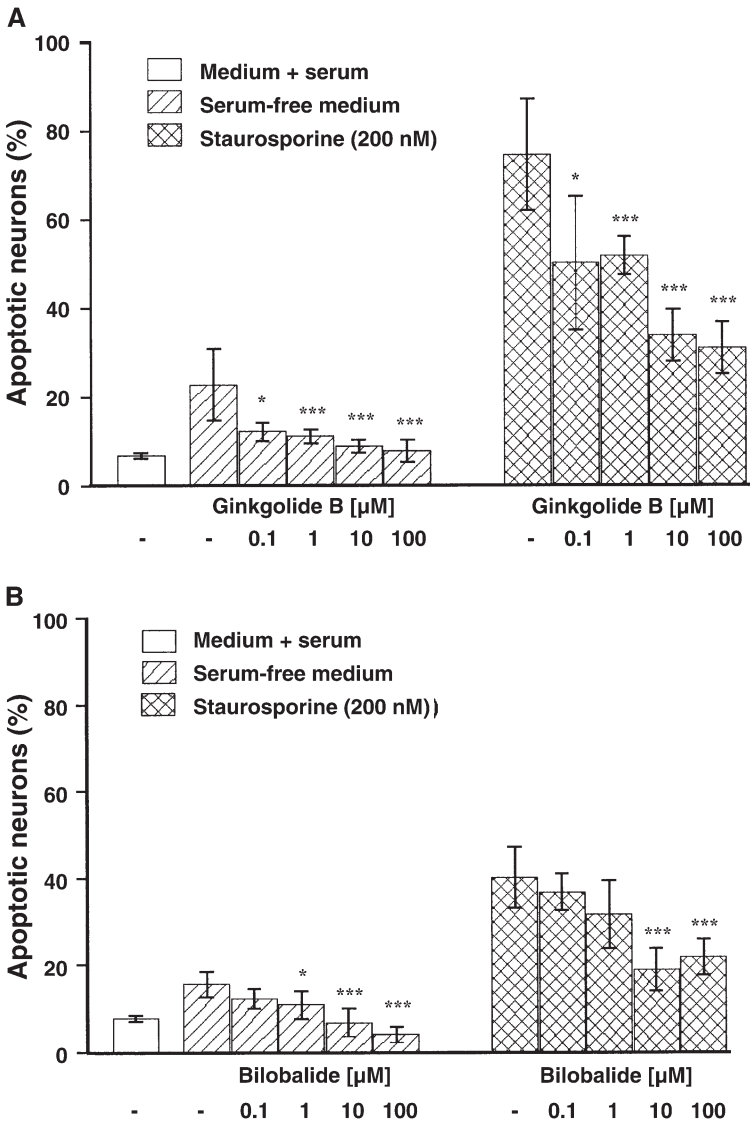

Figure 3. Ginkgolide B (*A*) and bilobalide (*B*) protected chick neurons against serum deprivation- and staurosporine-induced apoptosis. Neurons from chick embryo telencephalons were deprived of serum and were incubated with 200 nM staurosporine in serum-free medium for 24 (*A*) and 12 h (*B*) in the presence and absence of ginkgolide B (*A*) and bilobalide (*B*). Thereafter, the percentage of apoptotic neurons was determined by nuclear staining with Hoechst 33258. Means ± S.D. from eight experiments are shown. Difference from the respective vehicle-treated control: $*P < 0.05$; $***P < 0.001$ (ANOVA-1 and posthoc Scheffé test) [28].

Mechanism of neuroprotection by EGb 761 and its constituents

Effects on cerebral blood flow and PAF antagonism

The ability of EGb 761 to increase blood flow in the whole body under normal conditions is well accepted. In the first characterization of EGb 761, an increase in blood flow through peripheral vessels of guinea pig leg was shown [32]. In the rat brain, EGb 761 increased the local cerebral blood flow (LCBF) by 50–100% in nearly all brain regions as evaluated by ^{14}[C]iodoantipyrine autoradiography [33]. The EGb 761-induced increase in LCBF has been related to the terpenoid fraction of EGb 761 (fig. 5).

After global ischemia, cerebral blood flow at first increases (postischemic hyperperfusion), but then it decreases for several hours to 40–60% of preischemic values (postischemic hypoperfusion). Administration of EGb 761 was able to reverse the hypoperfusion 15 and 45 min after 10 min of global cerebral ischemia in rats to nearly control levels, although the infarct volume was not reduced [13]. A less pronounced decrease in LCBF has been found after mild cerebral ischemia induced by microembolization in EGb 761, compared with vehicle-treated gerbils by Le Poncin-Laffite et al. [34]. The increase in LCBF under normal as well as ischemic conditions could be due to an increase in sympathetic activity.

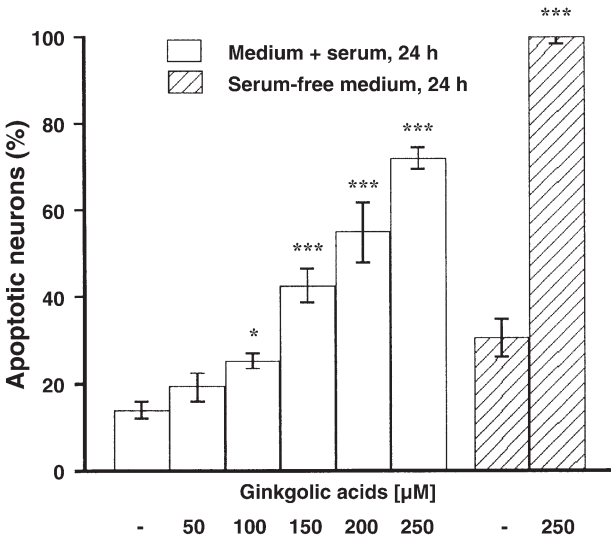

Figure 4. Ginkgolic acids cause death of cultured chick neurons. Chick embryonic neurons were incubated for 24 h with vehicle or ginkgolic acids in the presence and absence of serum. The percentage of neurons with condensed chromatin and shrunken nuclei was determined by nuclear staining with Hoechst 33258. Means ± S.D. from four experiments are shown. Difference from the corresponding vehicle-treated controls: $*P < 0.05$; $***P < 0.001$ [31].

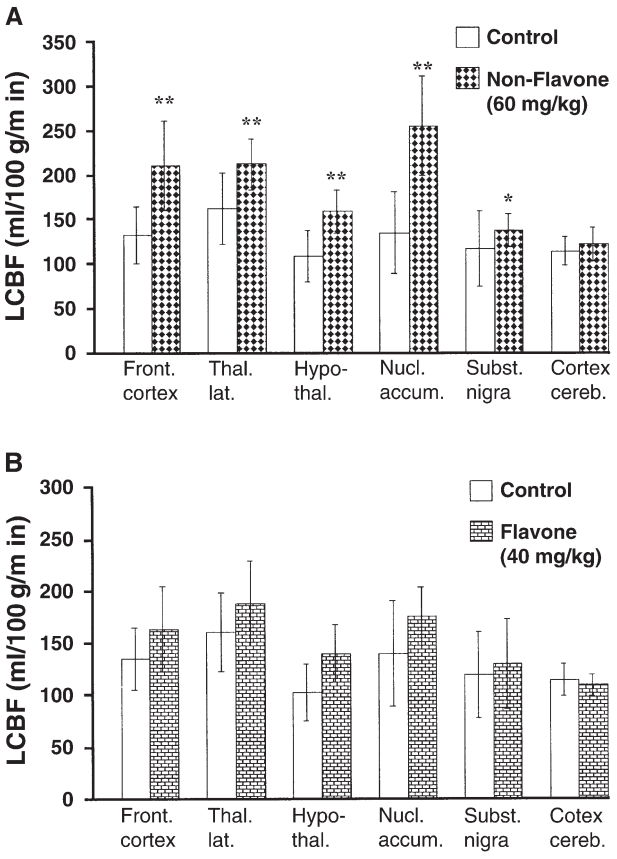

Figure 5. The non-flavone (*A*), but not the flavone (*B*) fraction of EGb 761 increased LCBF in defined rat brain regions. The flavone (40 mg/kg) and non-flavone (60 mg/kg) fraction of EGb 761 were administered (i.v.) for 20 min. Thereafter, LCBF was determined using the iodo-^{14}C-antipyrine technique. Means ± S.D. from six animals are shown. Differences between drug- and vehicle-treated animals: $*P < 0.05$; $**P < 0.01$ (ANOVA-1 and Scheffé test) [J. Krieglstein et al. unpublished results].

There is some experimental evidence that EGb 761 induces the release of catecholamines from their intracellular stores, thereby acting as an indirect sympathomimetic agent [35, 36]. Consistently, an increase in the level of biogenic monoamines has been measured in certain rat brain regions after treatment with EGb 761 [37]. In addition, EGb 761-induced increase in LCBF was discussed to be due to an increased synthesis of prostacyclin [38] and of nitric oxide (NO) as well as to the antioxidative capacity of EGb 761. Due to its antioxidative ability, EGb 761 prevented low-density lipoprotein (LDL) oxidation, thereby improving endothelial function and slowing down the process of atherosclerosis [39]; and it reduced formation of peroxynitrite from NO, thus leaving a sufficient amount of NO for vasodilatation. The level of NO increased by EGb 761 has been shown to be due to an activation of endothelial nitric oxide synthase (NOS) [40]. Consistently, dilatation of rat thoracic aorta by EGb 761 was inhibited by NOS inhibitors [41]. In addition, EGb 761 inhibited cyclic GMP (c-GMP) phosphodiesterase and thus prolonged the vasodilatatory effect of NO [42].

Furthermore, EGb 761 has been shown to reduce ischemia-induced increase in blood viscosity and thrombocyte aggregation [43], and this effect has been related to its constituent ginkgolide B acting as a platelet activating factor (PAF) antagonist [44]. PAF plays an important role in the regulation of blood pressure, anaphylaxis, inflammation and in neuronal death after ischemic injury [45, 46]. Platelet aggregation induced by PAF, but not by oxidative stress and in response to collagen and thrombin was inhibited by EGb 761 as well as by ginkgolides A and B, whereas only EGb 761 reduced oxidative- as well as PAF-induced platelet aggregation [47]. This is reasonable, as ginkgolide B antagonizes PAF receptors [44], but its ability to scavenge reactive oxygen species (ROS) is still under discussion. The results further suggest that EGb 761 contains compounds in addition to ginkgolides A and B with an antioxidative capacity. In addition, EGb 761 may reduce thrombocyte aggregation by increased synthesis of prostacyclin [38], and it improved vessel function by inhibiting proliferation of vascular smooth cells which is responsible for the so-called restenosis after angioplasty [48]. When human umbilical endothelial cells are subjected to oxidative stress, polymorphonuclear neutrophils adhere to the endothelial plasma membrane accompanied by activation of tyrosine kinases and phosphorylation of focal adhesion kinase and the cytoskeletal proteins paxillin and p130cas [49]. EGb 761 inhibited adhesion of the neutrophils as well as activation

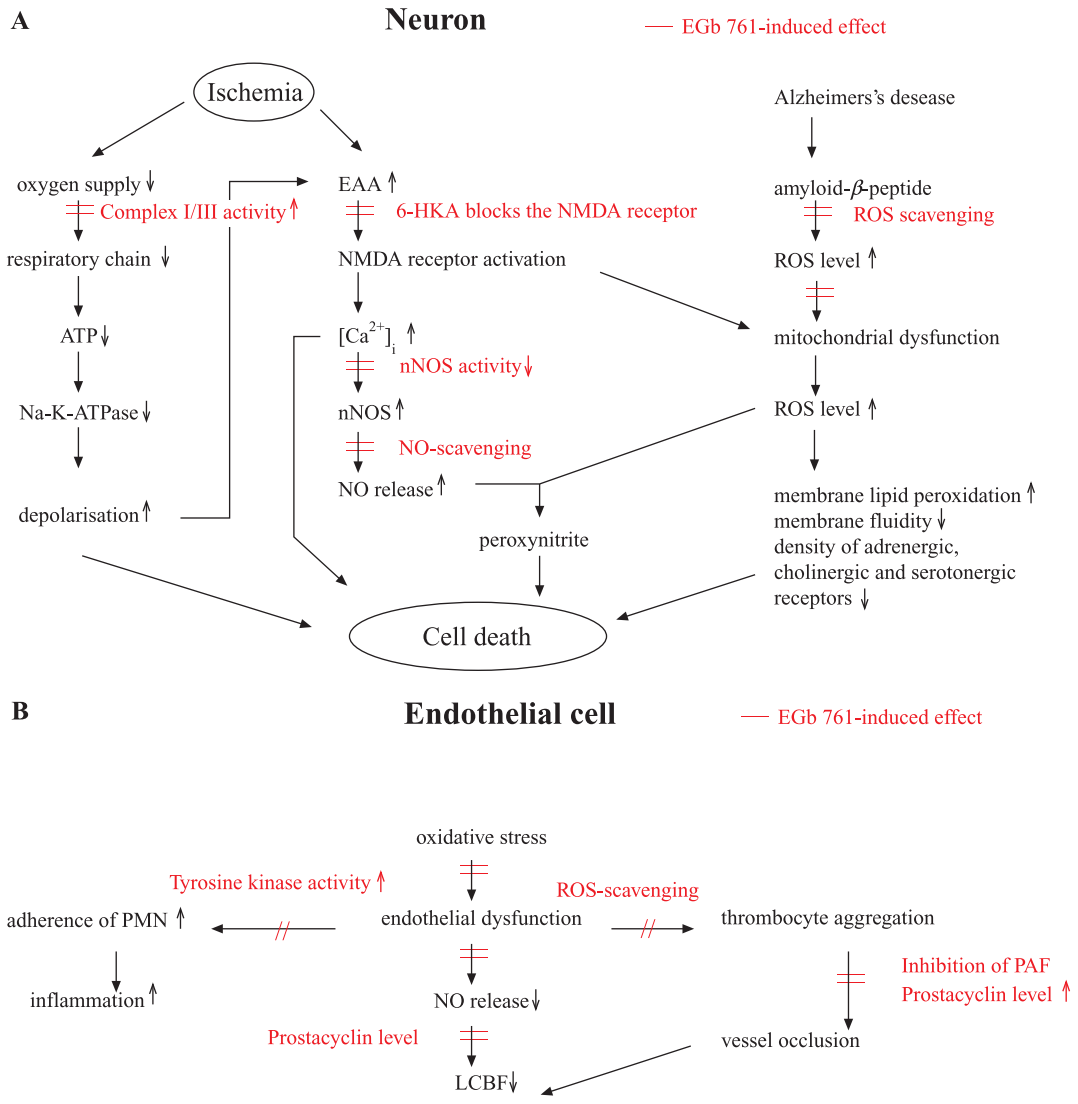

Figure 6. Scheme summarizing the supposed mechanism of neuroprotection by EGb 761. (*A*) Cerebral ischemia results in a loss of ATP due to the low oxygen supply followed by a decrease in the activity of the Na-K-ATPase, depolarization and a release of excitatory amino acids (EAA). The downstream mechanism of excitotoxicity includes an increase in the intracellular calcium concentration (Ca^{2+}_i), which in turn activates enzymes such as iNOS and disturbs mitochondrial function followed by an increase in the level of ROS and the formation of peroxynitrite. Similarly, Aß induces neuronal apoptosis in part by oxidative stress. (*B*) The severity of neuronal injury after ischemia also depends on the integrity of endothelial cells and of the vascular bed. Ischemia-induced endothelial dysfunction includes increased adherence of polymorphonuclear leukocytes (PMN), thrombocyte aggregation and reduced release of NO. The constituents of EGb 761 interfere with prodamaging pathways due to their ability to scavenge NO and ROS, preserve mitochondrial function, inhibit NMDA receptor activation, antagonize PAF, inhibit adhesion of inflammatory cells and increase the release of prostacyclin.

of tyrosine kinases, thereby interrupting infiltration of inflammatory cells into the tissue [49].

Effects on neurotransmitter systems

Effects on the adrenergic system

In the study of Brunello et al. [50], EGb 761 enhanced the release of noradrenaline in rat brain, and after treatment for at least 4 weeks, it reduced the density of cerebral β-adrenoceptors as an adaptive mechanism. However, other studies showed a decrease in the noradrenaline level [51] and no change in the β-adrenoreceptor density in the rat hippocampus by EGb 761 [52]. With respect to hippocampal α_2-adrenoceptors, EGb 761 prevented age-related decrease in receptor density [53]. Therefore, the effect of EGb 761 on brain adrenergic system is less clear and needs further investigations.

Effects on the dopaminergic system

There are some indications that EGb 761 influences the dopaminergic system. In a mouse model of MPTP-in-

duced toxicity, the extract inhibited the degeneration of dopaminergic neurons in the striatum [18, 54, 55], suggesting that inhibition of reuptake of dopamine and 1-methyl-4-phenyl-1,2,3,β-tetrahydropyridine (MPTP) and of monoamine oxidase activity were involved in EGb 761-induced neuroprotection. EGb 761 did not change the density of dopamine (D_2) receptors [52].

Effects on the cholinergic system

It is well known that the density of acetylcholine receptors in rat hippocampus decreases with age. This decline could be blocked by EGb 761 and was found only in older animals with decreased acetylcholine receptor densities, but not in younger rats [52]. In hippocampal slices, hypoxia and NMDA-induced release of choline and activation of phospholipase A2 have been shown to be prevented by bilobalide [5, 56].

Effects on the serotoninergic system

Older rats show a decrease in the serotonin (5-HT) level as well as a reduced number of 5-HT_{1A} receptors in the brain [54]. With respect to 5-HT receptors, chronic treatment with EGb 761 reversed the age-related decrease in 5-HT_{1A} receptor density [57], which was suggested to be due to the ability of EGb 761 to inhibit lipid peroxidation and membrane destruction as well as to modulate receptor synthesis [58, 59]. Huguet et al. [57] assumed that the effect of EGb 761, which also blocked the age-related decrease in acetylcholine- and α_2-adrenoceptors, was a general effect, independent of the receptor type. With respect to the 5-HT level, Petkov et al. [50] found an increase in nearly all brain structures of old rats treated with EGb 761, except for the pons. Similarly, EGb 761 enhanced 5-HT uptake in synaptosomes from mouse cortex, and this effect was related to the flavonoid fraction, especially to quercetin, which is the major aglycone of the ginkgo flavone glycosides [60]. In addition, there is experimental evidence that EGb 761 enhances stimulation of 5-HT_{1A} receptors by preventing their desensitization after subchronic cold stress [25]. In rats, EGb 761 induced a stimulus control similar to that of 5-HT_{1A} receptor agonists, and indeed, changes in behavior induced by EGb 761 were antagonized by the selective 5-HT_{1A} receptor antagonist WAY 100635 [61]. In monoaminoxidase A-deficient mice, EGb 761 reduced 5-HT-mediated aggression [62], suggesting that EGb 761 may interact with signaling pathways controlling serotonergic activity and particularly those related to stressful situations [63].

Effect on the glutamatergic and GABAergic system

In the mouse hippocampus, bilobalide increased the level of γ-aminobutyric acid (GABA) as well as the protein level and activity of glutamic acid decarboxylase, the rate-limiting enzyme of GABA synthesis [64]. Bilobalide did not change glutamate levels in the hippocampus [64], and thus it increased the ratio of inhibitory to excitatory neurotransmitters. Other constituents of EGb 761 modulating the glutamatergic system are KYNA and 6-HKA [65]. KYNA belongs to the group of low-affinity metabotropic glutamate receptor (mGluR) antagonists and interferes with the glycine B site of the NMDA receptor [66, 67]. The 6-hydroxy derivative of KYNA inhibited NMDA and α-amino-3-hydroxy-5-methylisoxazol-4-propionic acid (AMPA) receptors (GluR1-4), but with different selectivity. While potency at NMDA receptors was roughly halved, the affinity at AMPA receptors increased eight-fold [68]. As antagonists of the low-affinity mGluR, GluR and AMPA receptors inhibit excitotoxicity and thus have potential clinical application as anticonvulsants, the question arises whether KYNA and 6-HKA contribute to the neuroprotective effect of EGb 761. On the one hand, KYNA was found to accumulate in the brain if given systemically [66], but on the other, passive diffusion of KYNA and 6-HKA through the blood-brain barrier appears unlikely because of their low lipophilicity [68]. It is not yet clear whether both constituents are of relevance for neuroprotection induced by EGb 761.

Antioxidant activity

Oxidative stress describes a condition in which cellular antioxidant defenses are insufficient to keep the levels of ROS below a toxic threshold (fig. 6). Oxidative stress represents a key factor in various acute and chronic neurodegenerative diseases as well as in stroke, and thus antioxidants are therapeutically used for such illnesses [69]. The antioxidative property of EGb 761 has already been recognized by Doly et al. [70]. The authors measured a reduced membrane lipid peroxidation in the isolated rat retina in the presence of EGb 761. Similarly, Pincemail et al. [71] showed that the production of free radicals could be blocked by adding EGb 761 to the reaction mixture. EGb 761 protected cultured neurons against oxidative stress caused by hydrogen peroxide and iron sulfate [6, 7, 9, 10, 12] and apolipoprotein E [72] as well as neural tissue against cerebral ischemia [22]. Whether the flavonoid or the terpenoid fraction of EGb 761 is responsible for the antioxidative property of EGb 761 is still a matter of discussion. In acellular systems, the flavone glycosides scavenged superoxide anions, hydroxyl radicals and peroxyl radicals [73–77], and prevented lipid peroxidation [78]. It is thought that the polyphenol structure of flavonoids allows scavenging of free radicals and the chelation of transition metals including iron [78]. Quercetin [79] and myricetin especially profoundly reduced oxidation of *tert*-butylhydroperoxide. However, there is limited knowledge about the intestinal uptake of flavone glycosides; the extent of degradation to various

phenolic acids, some of which still possess radical scavenging ability [80]; and on the permeability of flavonoids through the blood-brain barrier. Therefore, it remains open whether the flavone glycosides of EGb 761 or their metabolites reach concentrations in the brain high enough for sufficient radical scavenging. Contrasting data exist showing the terpene lactones to react with ROS in acellular systems. Bilobalide and the ginkgolides B, C and J have been shown to react [81] as well as not to react with superoxide anion [82]. With respect to ginkgolide A, both studies consistently showed a lack of superoxide scavenging activity of this constituent. In cellular systems, the antioxidative ability of EGb 761 has been attributed to the flavonoid fraction [10, 83] as well as to bilobalide [11, 22, 84, 85], which might be due to differences in the models as well as the type of oxidative stress used in these studies.

In addition, there is evidence that the constituents of EGb 761 stabilize the cellular redox state rather than have a direct radical scavenging effect. EGb 761 has been shown to increase the protein level and activity of antioxidant enzymes such as superoxide dismutase and catalase in rat hippocampus [86] and rat ileum [87] as well as of glutathione (GSH) reductase in mouse liver [88]. Similarly, the activity of γ-glutamylcysteinyl synthetase, the rate-limiting enzyme of GSH synthesis, was enhanced by EGb 761 [89].

Furthermore, EGb 761 particularly inhibited oxidative stress in the mitochondria. This is of importance, as mitochondrial DNA is a major target of free-radical attack. Oxidative damage of mitochondria is a primary event in the aging process. Wu et al. [90] evaluated the effects of EGb 761 and its flavonoid constituent tamarixetin on aging of *Caenorhabditis* elegans. EGb 761 and tamarixetin increased life span by 5 and 25%, respectively, and EGb 761 enhanced the resistance of the worm to acute oxidative and thermal stress. In isolated mitochondria, the effect of EGb 761 and its constituents on the respiratory chain (activities of complexes I–IV), on the activity of cytochrome c oxidase, NADH dehydrogenase and ADP phosphorylation versus oxygen consumption after anoxia/reoxygenation and during aging have been studied. Anoxia/reoxygenation decreased ADP phosphorylation versus oxygen consumption in isolated mitochondria from rat brain [91] and rat liver [92], and this was preserved by EGb 761. Bilobalide inhibited ischemia-induced decrease in the activities of complexes I and IV, allowing mitochondria to maintain their respiratory activity [84]. In addition, the GSH/GSSG (oxidized form of GSH) ratio in rat mitochondria decreased during aging, and this decrease was reversed by EGb 761 [93]. Recently, EGb 761 and bilobalide have been shown to increase the messenger RNA (mRNA) and protein level of subunit 1 of mitochondrial NADH dehydrogenase, thereby decreasing stage 4 respiration, whose increase is thought to be responsible for oxidative damage in mitochondria [85].

Effects at the cellular NO level

NO is a messenger molecule synthesized from L-arginine by NOS. The three isoforms of NOS, neuronal NOS (nNOS, NOS-I), endothelial NOS (eNOS, NOS-III) and inducible NOS (iNOS, NOS-II), are expressed in different tissues and cells. NO plays a vital role in diverse biological responses, such as regulation of vascular tone, neurotransmission, and antiviral and immune defense. In the central nervous system, NO is synthesized by neuronal NOS and functions as a synaptic signaling molecule [94]. In some cells, NO donors [95, 96] or NO produced by constitutive nNOS [97] limited apoptosis induced by trophic factor deprivation in primary neurons and PC12 cells. These reports concluded that NO-induced neuroprotection was due to activation of soluble guanylate cyclase. In addition, NO prevented tumor necrosis factor-α and actinomycin D-induced apoptosis in MCF-7 cells [98] by suppressing increases in caspase-3-like activity [99,100]. NO S-nitrosylates the active site cysteine present in all caspases [101]. When produced in excess, NO can lead to neuronal cell death [102–104], and cytotoxicity is associated with activation of poly (ADP-ribose) polymerase [103] and the formation of peroxynitrite by reaction with superoxide [105]. Increased nNOS expression has been associated with a variety of neurologic disorders, including stroke, Parkinson´s and Alzheimer's disease [106]. EGb 761 was already described in 1994 as an NO scavenger [76]. In addition, neurotoxicity induced by the NO-generating substance sodium nitroprusside was reduced by EGb 761 [17]. Beside its NO-scavenging ability, EGb 761 has been shown to reduce NO release after ischemia in the brain [21] and heart [107], after heat stress [24] and from lipopolysaccharide-stimulated macrophages [108] by reducing iNOS mRNA and protein expression [91, 108, 109]. It is still open which constituent of EGb 761 is responsible for reduction of the NO level. On the one hand, bilobalide reduced neurotoxicity induced by the NO donor morpholinosydnonimine [9], and the ginkgolides A and B inhibited NO production of lipopolysaccharide-stimulated microglia [91]. On the other hand, EGb 761 and the flavonoid fraction, but not bilobalide and ginkgolide B, inhibited sodium nitroprusside-induced death in rat primary hippocampal cultures [17], and the authors suggested that a radical scavenging effect of the flavone glycosides abolished the formation of peroxynitrite as a reaction product of NO and superoxide anion.

In the vascular system, NO, derived from eNOS at low levels, acts as a vasodilatator. eNOS-catalyzed synthesis of NO is impaired in atherosclerotic lesions and in cells

treated with atherogenic factors, such as oxidized lipoproteins. iNOS is primarily expressed in macrophages and upon induction; a high level of NO can be sustained for a prolonged period of time. Excessive production of NO injured endothelial cells. EGb 761 as well as ginkgolide B and bilobalide reduced levels of NO metabolites released from macrophages, whereas they did not affect eNOS-mediated NO production [110]. However, Li et al. [40] measured an increase in eNOS activity in cultured endothelial cells by EGb 761. Similarly, in the rat, the precontracted aortic ring was dilated upon administration of EGb 761 and quercetin. This vasodilatation was inhibited by NOS inhibitors, suggesting that the increase in cerebral blood flow by EGb 761 is induced by an increase in eNOS activity. Thus, EGb 761 seems to increase e-NOS-mediated NO production, resulting in vasodilatation, whereas it inhibits iNOS-mediated production of an excessive amount of NO in macrophages (which might be accompanied by increased ROS production and the formation of peroxynitrite) (fig. 6).

Other mechanisms

Other mechanisms which may be involved in EGb 761-induced neuroprotection are modulation of ion homeostasis, glucocorticoid level, Aβ aggregation and synthesis of growth factors.

Neuronal death during and after ischemic injury is accompanied by disturbances in ion homeostasis. For example, due to the lack of energy, the activity of Na-K-ATPase decreases, followed by depolarization and an influx of sodium ions into the cell. EGb 761 prevented the ischemia-induced decrease in Na-K-ATPase activity as well as brain edema and lipid peroxidation [111, 112]. Li et al. [40] found activation of transient outward potassium channels by EGb 761 using excised inside-out patches. Interestingly, increased extracellular potassium concentration promotes dephosphorylation of Na-K-ATPase, thereby stimulating its transport activity. In addition, EGb 761 increased the mRNA level of GluR2 and voltage-dependent chloride (Cl3CNG) and calcium (CaCNG2) channels [113]. As stimulation of GluR receptors renders them permeable for Ca^{2+}, and chloride and calcium channels are important to maintain ion homeostasis, these upregulations may represent another mechanism by which EGb 761 improves neurological function.

Although glucocorticoids are essential for adaptation to acute physical stress, they have a broad pathogenic potential at increased levels, including neurotoxicity [114]. Glucocorticoid-induced neurotoxicity plays a critical role in neural development, aging as well as in neurological diseases related to hippocampal damage [115]. In rats, EGb 761 and its constituent ginkgolide B specifically reduced the ligand binding as well as the mRNA and protein level of the adrenal mitochondrial peripheral benzodiazepine receptor. As activation of this receptor is the rate-determining step in steroid biosynthesis, EGb 761 reduced glucocorticoid synthesis [116, 117]. EGb 761-induced decrease in glucocorticoid level prevented glucocorticoid receptor desensitization and the development of amphetamine-induced and glucocorticoid-mediated stereotyped behavioral changes in rats [26].

Using mRNA microarrays, Watanabe et al. [113] measured EGb 761-induced changes in mRNA levels in the hippocampus and cortex of normal mice. EGb 761 was administered orally for 4 weeks at a dose of 300 mg/kg (which is three-fold higher than the dose used in most of the other studies). Only differences in the mRNA level of EGb 761- and vehicle-treated animals higher than factor 3 were considered relevant. EGb 761 increased the mRNA level of microtubuli-associated tau protein as well as of neural protein phosphatase type 1, which is known to dephosphorylate hyperphosphorylated tau protein. When hyperphosphorylated, tau dissociates from the microtubuli and aggregates intracellularly due to its low solubility, which is suggested to be a key event in the pathogenesis of Alzheimer's disease. Thus, a decrease in the mRNA level of hyperphosphorylated and microtubuli-associated (nonaggregating) tau protein by EGb 761 may contribute to its beneficial effect on the neurological outcome of patients with dementia of the Alzheimer type. In addition, EGb 761 increased the mRNA level of transthyretin in mouse hippocampus [113]. Transthyretin has been shown to be involved in the transport of thyroxin and retinol-binding protein in cerebrospinal fluid and serum [118], but also to sequester Aβ protein in vitro thus preventing Aβ aggregation from arising in amyloid formation [119].

Furthermore, mRNA expression of growth hormone (GH) and prolactin in mouse cortex were upregulated by EGb 761 [112]. GH is an anabolic hormone that stimulates most target cells to grow in size and to proliferate, and GH receptors are present in the brain [120]. GH has beneficial effects on certain functions such as memory, mental alertness, motivation and working capacity in adults [121]. Similarly to GH, prolactin induced the proliferation and differentiation of brain cells, especially of embryonic astrocytes [122]. In the study of Zheng et al. [123], bilobalide increased the mRNA and protein expression of glial-derived neurotrophic factor and vascular endothelial growth factor in cultured rat cortical astrocytes. Glial-derived neurotrophic factor has the ability to promote survival of dopaminergic neurons in the substantia nigra, and the vascular endothelial growth factor participates in the defense system of the central nervous system.

The relevance of EGb 761-induced changes in the mRNA level of the above-mentioned ion channels, enzymes, re-

ceptors, microtubule proteins and growth factors for its neuroprotective ability is yet not clarified.

Beneficial effects of EGb 761 on neurological outcome

Several studies tried to find out whether the neuroprotective effect of EGb 761 against various types of injury also results in an improved neurological outcome. In most cases, changes in behavior, learning and memory ability under normal conditions as well as after injury were measured by passive avoidance tests. In streptozotocin-treated rats, impaired behavior was significantly slowed down by EGb 761 [124]. In addition, significantly improved memory after bilateral occlusion of the carotid arteries as well as after scopolamine-induced amnesia was observed in rats treated with EGb 761 [125]. In the study of Stoll et al. [126], EGb 761-treated old mice showed better passive avoidance learning than vehicle-treated animals. In asymptomatic human volunteers, EGb 761 improved memory, particularly working memory [127].

Current status on clinical trials and undesired side effects of EGb 761

EGb 761 is mainly recommended for the treatment of peripheral arterial diseases, and for vascular and neurodegenerative dementia. In recent years, therapeutic use of EGb 761 has extended to diseases such as tinnitus of circulatory origin [128], equilibrium disorders, severe acute mountain sickness and intermittent claudication [129].

More than 40 clinical trials have been published on treatment of cerebral insufficiency with EGb 761. Among these studies, 8 were judged to be adequate in terms of their design and 7 showed a positive effect of EGb 761 [128, 130]. However, the number of studies to be considered becomes much smaller if one applies updated assessment criteria such as operationalized diagnosis of dementia, inclusion of mild and moderate, but not severe cases, and demonstration of statistically significant effects at three efficacy levels (psychopathological, psychometric and behavioral). The most relevant studies on the efficacy of EGb 761 in outpatients with Alzheimer's disease and with multiinfarct dementia were published by Kanowski et al. [131] and Le Bars et al. [132]. In the study of Kanowski et al. [131], 156 patients (222 patients at entry) finished the prospective, randomized, double-blind, placebo-controlled, multicenter study lasting for 24 weeks. The patients received a daily oral dose of 240 mg of EGb 761 or placebo. Clinical global impressions for psychological assessment were chosen for evaluation, and clinical efficacy was assessed by means of responder analysis. Proof of efficacy was carried out using a validated measurement instrument. There was a significant difference in the number of responders at the end of the treatment (28% for EGb 761 compared with 10% for placebo), suggesting that EGb 761 is of clinical efficacy in the treatment of outpatients with dementia. A subgroup analysis according to the type of dementia revealed that both patients with Alzheimer's disease and those with multiinfarct dementia responded to treatment with EGb 761; the response in the Alzheimer subgroup, however, was, consistently larger. In the second placebo-controlled, double-blind, randomized trial, 137 patients (327 patients at entry) were treated for 52 weeks with 120 mg of EGb 761 [132]. The patients treated with EGb 761 showed significant improvement in learning, memory, visual and spatial orientation, and social behavior. Wettstein [133] compared the efficacy of cholinesterase inhibitors and EGb 761 in Alzheimer's disease of published placebo-controlled studies and found no significant difference in delay of symptom progression in patients treated with donezepil, rivastigmine, metrifonate or EGb 761, suggesting that all drugs are equally effective in the treatment of mild to moderate Alzheimer's dementia. Another retrospective analysis explored whether the therapeutic effect of EGb 761 in Alzheimer's disease depends on baseline severity [134]. Treatment effect favorable for EGb 761 could be observed with respect to cognitive performance ($P = 0.02$) and social functioning ($P = 0.0001$), regardless of the stage of dementia. However, improvement was observed only in the group of patients with very mild cognitive impairment, while in more severe dementia, the mean effect of EGb 761 should be considered in terms of stabilization or slowing down of worsening. Recently, Solomon et al. [135] evaluated whether extract of *Ginkgo biloba* improves memory in healthy elderly volunteers as measured by objective neuropsychological tests and subjective ratings. The authors found no significant differences for any outcome measure. Since EGb 761 and its constituents have been shown in many different experiments to act on neurons under pathobiochemical conditions, it may be assumed that EGb 761 is effective in patients with cerebral disorders, but not in healthy volunteers.

No serious adverse effects have been noted in any clinical trial. In rare cases, mild gastrointestinal complaints, nausea, dizziness, headache, dry mouth, sleep disturbances, transient cyanosis of nails and lips, and allergic skin reactions have been reported. EGb 761 seemed to be well tolerated. Therefore, the data obtained so far show a positive therapeutic risk-benefit relationship.

Ginkgo biloba-containing drugs are one of the top sellers for herbal remedies in many European countries as well as in United States. In Germany, EGb 761 was standardized to the pharmacopeial monograph for *Ginkgo biloba* referring to the German Commission E monograph. According to this monograph, EGb 761 contains 22–27% flavone glycosides, 2.8–3.4% ginkgolides and 2.6–3.2%

bilobalide, whereas the content of ginkgolic acids must be lower than 0.0005% because of their allergenic and neurotoxic potential [31, 136–139]. However, to ensure the same efficacy of different extracts of *Ginkgo biloba*, these brands should not only contain the same amount (pharmaceutical equivalence), but also the same rate and extent of dissolution and absorption (bioequivalence) of active ingredients. Until now, less attention has been paid to the bioavailability of EGb 761, and even less is known about the pharmacokinetic profile of the constituents of EGb 761 in humans [140]. Recently, the dissolution rates of the constituents of extracts of *Ginkgo biloba* on the US market and of standardized EGb 761 were measured at gastric pH [141]. The dissolution rates of some of these extracts were not in accordance with standardized EGb 761 [141]. Consistent with the finding of nonequivalence between different extracts of *Ginkgo biloba*, Guidetti et al. [12] determined different IC_{50} values (78 and 186 µg/ml, the concentration where damage was inhibited by 50%) for the neuroprotective effect against hydrogen peroxide-induced injury using two different commercially available extracts of *Ginkgo biloba*. Bioequivalence is a prerequisite not only to guarantee the efficacy, but also for the safety of a herbal remedy. The doses of herbal compounds such as extract of *Ginkgo biloba* should be carefully monitored to prevent rare, but potential disastrous results. There are eight single case reports of subarachnoid hemorrhage and bleeding which are suggested to be caused by EGb 761 and to be related to its ability to antagonize PAF [142–149].

Conclusion

From various in vivo and in vitro experiments, it is concluded that constituents of *Ginkgo biloba* have neuroprotective properties. PAF antagonism, free-radical and NO scavenging, interaction with neurotransmitters and even induction of growth factors are possible mechanisms of action. Clinical studies suggest a clear positive therapeutic risk-benefit relationship using the EGb 761 in the treatment of mild to moderate dementia.

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