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Effect of a short- and long-term treatment with *Ginkgo biloba* extract on Amyloid Precursor Protein Levels in a transgenic mouse model relevant to Alzheimer's disease

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ABSTRACT

Several clinical trials have reported beneficial effects of the *Ginkgo biloba* extract EGb761 in the prevention and therapy of cognitive disorders including Alzheimer's disease (AD). The aim of the present long-term feeding trial was to study the impact of dietary EGb761 on Amyloid precursor protein (APP) metabolism in mice transgenic for human APP (Tg2576). Tg2576 mice were fed diets with and without EGb761 (300 mg/kg diet) for 1 and 16 months, respectively. Long-term treatment (16 months) with EGb761 significantly lowered human APP protein levels by ~50% as compared to controls in the cortex but not in the hippocampus. However, APP levels were not affected by EGb761 in young mice. Current data indicate that APP seems to be an important molecular target of EGb761 in relation to the duration of the *Ginkgo biloba* treatment and/or the age of the animals. Potential neuroprotective properties of EGb761 may be, at least partly, related to its APP lowering activity.

Introduction

The *Ginkgo biloba* extract EGb761 is commonly used in the prevention and therapy of vascular and cerebral disorders including Alzheimer's disease (AD) [1]. AD, a progressive neurodegenerative disorder, is characterized by extra cellular amyloid-β (Aβ)[1] plaques, intracellular neurofibrillary tangles and massive neuronal loss in the cortex and hippocampus [2–4]. EGb761 is a standardized extract of the leaves of the *Ginkgo biloba* tree and is characterized by its main fractions, the flavonols (mainly isorhamnetin, kaempferol and quercetin: 22–27%) and the terpenelactone (5–7%). These two fractions are thought to be, at least partly, responsible for potential neuroprotective properties of EGb761 [5–7]. Several human intervention trials reported beneficial effects of *Ginkgo biloba* in the prevention and therapy of neurodegenerative disorders [7–12]. These studies revealed a significant enhancement in cognitive functions [9–11], memory [12] and concentration in response to EGb761 treatment [7,8]. However, the placebo controlled double blind trials by Dodge et al. (2008) and van Dongen et al. (2003) did not show beneficial effects of EGb761 in age associated memory impairment in elderly people [13,14]. In spite of these contrary outcomes, meta-analyzes revealed modest beneficial effects of EGb761 on cognitive functions in patients with AD [5,15]. It has been shown in studies in cultured cells as well as in laboratory animals that EGb761 exhibits antioxidant [16–18] and anti-inflammatory activity [19], modulates neurotransmitters activity, and increases cerebral blood flow [6,20,21]. Furthermore, recent animal studies have shown that short-term treatment with EGb761 influences mRNA and protein expression of genes encoding for proteins involved in the pathogenesis of AD [22,23]. Additionally, in transgenic cell and animal models for AD it has been shown that Aβ aggregation [24,25] and Aβ induced oxidative stress was attenuated by EGb761 treatment [17,26]. Aβ is a cleavage product of the transmembrane amyloid precursor protein (APP). Two different proteases can cleave APP in different positions. While the cleavage by α-secretase occurs within the Aβ sequence and releases αAPP and a C83 fragment, the β-secretase generates βAPP and a C99 fragment containing the N-terminus of Aβ. Further cleavage of C83 and C99 by a γ-secretase leads to soluble, non toxic P3 and P6 fragments and Aβ and the P6 fragment,

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[1] *Abbreviations used: Aβ*, amyloid beta; *AD*, Alzheimer's Disease; *ADAM-10*, a disintegrin and metalloprotease-10; *APP*, amyloid Precursor protein; *APPbp1*, amyloid precursor protein binding protein 1; *BACE-1*, beta site amyloid precursor protein cleaving enzyme-1; *C*, control diet; *EGb761*, standardised *Ginkgo biloba* extract, *G*-EGb761 supplemented diet; *hPrP*, h amster prion protein; *huAPP*, human amyloid precursor protein; *msAPP*, mouse amyloid precursor protein; *NEP*, neprilysin; *o*, mice fed for 16 months; *y*, mice fed for 1 month.

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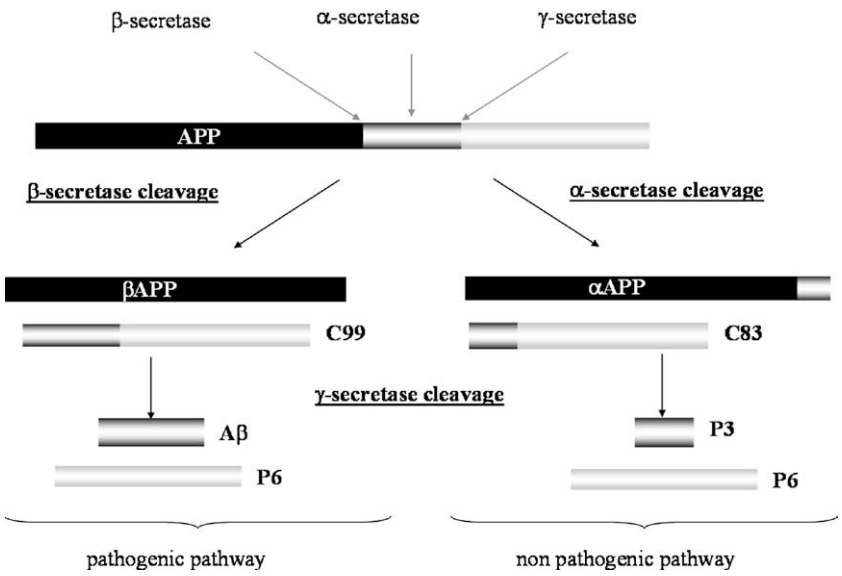

Fig. 1. Scheme of APP processing.

respectively (Fig. 1). Released Aβ can undergo proteolytic degradation by several proteases including nepriylsin (NEP) [4,27]. However, little is known how EGb761 affects the processing of APP, a crucial step in the pathogenesis of AD, generating neurotoxic Aβ. Thus, the aim of the present study was to investigate the short (1 month) and long-term (16 months) effect of dietary EGb761 supplementation on APP metabolism in a transgenic mouse model relevant for Alzheimer disease.

Materials and methods

Tris (hydroxymethyl) aminomethane (TRIS), sodium chloride, sodium dodecyl sulfate (SDS) and Tween 20 were obtained from Carl Roth (Karlsruhe, Germany). Sodium deoxycholate and nonylphenyl-polyethylene glycol (NP-40) were obtained from Sigma Aldrich (Steinheim, Germany).

The standardized *Ginkgo biloba* extract (EGb761) was a kind gift from Dr. Schwabe pharmaceuticals (Karlsruhe, Germany). EGb761 was manufactured according to the German Federal Health Authority (BGA/BfArM Kommission E, 1994) using acetone/water as extraction solvent and subsequent purification steps. The composition of the used extract is described in Augustin et al. 2008 [28].

Animals

Transgenic Tg2576 mice expressing the Swedish mutation of human APP_{695} (APPK670N/M671L) at high levels under the control of the hamster prion protein promoter were used in this study. Transgenic mice were bred by mating Tg2576 males with C57B6/SJL F1 females. Transgenity of the animals was tested for at 3 weeks of age by PCR using tail biopsy samples as described in Hsiao et al. 1996 [29,30].

At the age of 4 months, female Tg2576 were randomly divided into four groups ($n=10$ for the short-term treatment of 4 weeks and $n=6$ for the long-term treatment of 16 months; body weight 21.5 ± 1.2 g; mean ± SEM).

Prior to the intervention period, mice were adapted to a low-flavonoid control diet (C1000, Altromin; Lage, Germany) for 1 week. During intervention (4 weeks and 16 months, respectively), mice were fed either the control diet (C) or EGb761 supplemented (300 mg/kg diet) diet (G). Mice were housed under standard conditions (21–23 °C, 50–60% humidity, 14-h light/10-h dark cycle) and body weights were recorded weekly. Animal care and experimental procedures were conducted according to the German Guidelines and Regulations on Animal Care (Deutsches Tierschutzgesetz, 2006) and were approved by the University of Kiel Committee on Animal Care. At the age of 5 and 20 months, respectively, mice were decapitated and brain tissues (cortex and hippocampus) rapidly dissected. Blood was taken from all animals and samples from each dietary group were pooled.

Determination of flavonoid content in plasma

Plasma was obtained after centrifugation (2000*g* for 10 min, 4 °C) and stored at −80 °C until analysis. Flavonols were analyzed in plasma aliquots by HPLC with postcolumn derivatization as described previously [22]. All plasma samples were treated with β-glucuronidase/sulfatase (Carl Roth, Karlsruhe, Germany) prior to extraction of flavonols.

Gene expression analysis

Half of each cortex and hippocampus (short-term experiment: $n=10$ and long-term experiment: $n=6$) were immediately suspended in RNA*later*™ RNA stabilisation reagent (Qiagen, Hilden, Germany) and incubated over night. Total RNA was extracted according to the RNeasy™ Lipid Tissue Protocol (Qiagen). RNA integrity, concentration and purity were checked as previously described in Augustin et al. (2008) [28]. RNA aliquots were stored at −80 °C until analysis.

Mouse APP (msAPP), ADAM10 a protease with α-secretase activity, NEP, APP binding protein (APPbp1) and human APP (huAPP) primer pairs were designed to the corresponding mRNA as shown in Table 1 using Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm). The GenBank Accession Nos. are: P05067 for the human APP, P12023 for mouse APP, O35598 for ADAM10, Q8VBW6 for APPbp1 and Q61391 for NEP. The mRNA and primer sequences are given in Table 1. Primer pairs were obtained from MWG (Ebersberg, Germany). QuantiTectP™ Primer Assay (Qiagen) was used for 18S rRNA amplification, with a product of 149 bp. For one-step quantitative reverse transcriptase polymerase chain reaction (one-step qRT-PCR) two aliquots of RNA were amplified using QuantiTect™SYBR™Green RT-PCR Kit (Qiagen). External relative standard curves of total RNA were determined with each run. Data was normalized by dividing the expression level of target genes by the level of 18S rRNA. Each PCR reaction (final volume 20.0 μL)