The *in vivo* neuromodulatory effects of the herbal medicine ginkgo biloba

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Extracts of *Ginkgo biloba* leaves are consumed as dietary supplements to counteract chronic, age-related neurological disorders. We have applied high-density oligonucleotide microarrays to define the transcriptional effects in the cortex and hippocampus of mice whose diets were supplemented with the herbal extract. Gene expression analysis focused on the mRNAs that showed a more than 3-fold change in their expression. In the cortex, mRNAs for neuronal tyrosine/threonine phosphatase 1, and microtubule-associated τ were significantly enhanced. Hyperphosphorylated τ is the major constituent of the neurofibrillary tangles in the brains of Alzheimer's disease patients. The expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-2, calcium and chloride channels, prolactin, and growth hormone (GH), all of which are associated with brain function, were also up-regulated. In the hippocampus, only transthyretin mRNA was upregulated. Transthyretin plays a role in hormone transport in the brain and possibly a neuroprotective role by amyloid-β sequestration. This study reveals that diets supplemented with Ginkgo biloba extract have notable neuromodulatory effects *in vivo* and illustrates the utility of genome-wide expression monitoring to investigate the biological actions of complex extracts.

Extracts prepared from medicinal plants and other natural sources contain a variety of molecules with potent biological activities. Unfortunately, it is often difficult to analyze the biological activities of these extracts because of their complex nature and the possible synergistic effects of their components. Genome-wide expression monitoring with high-density oligonucleotide arrays provides a simple way to examine the biochemical effects of herbal remedies and thereby gain insights into both their potential beneficial effects and negative side effects. Here, we apply this approach to the biological actions of *Ginkgo biloba*. Extracts of *Ginkgo biloba* leaves are being marketed as therapeutic dietary supplements to counteract a variety of disorders. Ginkgo leaf extract is commonly used to combat a variety of neurological disturbances such as Alzheimer's disease or various common geriatric complaints including vertigo, depression, short-term memory loss, hearing loss, lack of attention or vigilance (see the National Institutes of Health website on ginkgo biloba: http://ntp-server.niehs.nih.gov/htdocs/Chem_Background/ExecSumm/Ginkgo.html.) (1–3). Ginkgo biloba's antioxidant properties and/or free radical-scavenging properties are said to help prevent strokes and transient ischemic attacks (4). Although the putative therapeutic benefits of ginkgo biloba may reside in the synergistic effect of all of its components, isolated constituents have been found to be active in a variety of assays. For example, ginkgolide B has been shown to be a potent platelet activating factor antagonist, and the flavonoid fraction of ginkgo biloba, containing free radical scavengers, likely contributes to the antioxidant properties of the herb (4). To gain further insight into the biochemical effects of ginkgo biloba, we profiled the transcriptional effects of the herb in the cortex and hippocampus of mice using oligonucleotide microarrays.

Materials and Methods

Source of *Ginkgo biloba*. EGb761 is a standardized *Ginkgo biloba* leaf extract used extensively in clinical trials. The extract contains 24% flavone glycosides (primarily composed of quercetin, kaempferol, and isorhamnetin) and 6% terpene lactones (2.8–3.4% ginkgolides A, B, and C, and 2.6–3.2% bilobalide). Other constituents include proanthocyanadins, glucose, rhamnose, organic acids (hydroxykinurenic, kynurenic, protocatechic, vanillic, shikimic), D-glucaric acid, and ginkgolic acid (<5 ppm ginkgolic acids), and related alkylphenols. The EGb761 extract was obtained from Dr. Schwabe Pharmaceuticals (Karlsruhe, Germany).

Animal Care and Diet Regimen. Twenty female, adult C57BL6 mice (Charles River Breeding Laboratories) were randomly allocated to the control (n = 10, initial average body weight 30.6 ± 1.1 g, mean ± SEM) or ginkgo (EGb761)-supplemented group (n = 10, 31.1 ± 1.0 g, mean ± SEM). The mice were housed separately in stainless steel cages for 4 weeks and maintained under standard conditions (22–24°C, 55% relative humidity, 14-h light/10-h dark cycle). Semisynthetic diets (disaccharides = 63.1%, protein = 17%, fat = 5.1%, fiber = 4%, metabolic energy = 15.1 MJ/kg) were purchased from Altromin (Lage, Germany). The control diet consisted of a low-flavonoid maintenance diet for adult mice (C1000 code #100E). The experimental diet was prepared by adding 300 mg/kg of the EGb761 extract. Animals were given free access to the pelleted diets; water and food not consumed by the mice each day were removed and replaced. Food consumption (of each animal) was monitored daily and body weights were recorded weekly.

After 4 weeks on the described diet (the 31st day of the experiment), blood from the animals was collected in heparinized syringes and pooled groupwise. The blood plasma was separated from lymphocytes and red blood cells by centrifugation (1,500 × g for 15 min) and stored at −80°C for further analysis. The hippocampi and cortex from each animal were rapidly dissected and immediately frozen on slabs of dry ice.

HPLC Analysis of Plasma Samples. Plasma samples were analyzed for quercetin, kaempferol, and isorhamnetin, the major flavonoid constituents found in standardized ginkgo extracts such as EGb761 (6, 7). The extraction procedure and HPLC conditions have been described above (5). Briefly, plasma samples were enzymatically treated with β-glucuronidase/sulfatase (Sigma), and the various flavonoids were detected with a fluores-

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EST, expressed sequence tag; RT-PCR, reverse transcription–PCR; GH, growth hormone.

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cence detector after postcolumn derivatization with $Al(NO_3)_3$. The HPLC system used was purchased from Jasco (Gross-Umstadt, Germany).

Total RNA Extraction, cRNA Preparation, and GeneChip Hybridization. The hippocampi from all 10 of the control or ginkgo biloba-treated mice were pooled and subsequently homogenized in Trizol reagent (GIBCO/BRL) according to the manufacturer's specifications. The cortices were treated identically. All total RNA samples were stored in 2-propanol at −80°C until further analysis. Total RNA (25 μg) was amplified and biotinylated with the protocol detailed by Affymetrix (Santa Clara, CA). MuU74 arrays (Affymetrix) representing ≈6,000 mouse full-length genes and 6,000 ESTs were used in each target hybridization. Two independent hybridizations were carried out on each sample, from which only those genes whose temporal and spatial expression level changed by at least 3-fold and with average difference values 200 in both data sets were selected.

Reverse Transcription (RT)-PCR Analysis of Cortex and Hippocampus Samples. RT-PCR was used to quantify transthyretin, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-2 channel, microtubule-associated τ, and tyrosine/threonine phosphatase 1 mRNA levels in the hippocampus or cortex of mice. After extraction, total RNA (10 μg) was transcribed into single-stranded cDNA by using SuperScript Choice (GIBCO/BRL). Duplex reactions with cDNA aliquots were conducted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous amplification standard. PCR conditions were optimized so that amplification of both GAPDH and the cDNA of interest was in the exponential phase. Each amplification cycle consisted of 1 min of denaturation at 95°C, 1 min of primer annealing at the appropriate temperature, followed by 1 min of extension at 72°C. The PCR buffer for tyrosine/threonine phosphatase 1 contained 5% (vol/vol) DMSO.

Results and Discussion

High density oligonucleotide microarrays were used to analyze the changes in mRNA expression levels in mice whose diets were supplemented with ginkgo biloba. These microarrays represent all sequences (≈6,000) in the Mouse UniGene Database that have been functionally characterized, as well as ≈6,000 expressed sequence tag (EST) clusters. The effects of the herb on gene transcription were measured in the hippocampus and cerebral cortex of adult female mice (n = 10, per group) who were fed a diet either with or without ginkgo biloba (marketed for human consumption) for 4 weeks. Both feed intake and body weights of the mice were monitored; neither differed significantly between the control and experimental groups. The mean average of feed consumed by each animal per day was 3.8 ± 0.1 g, which corresponds to about 36 mg/kg of EGb761 (described in *Materials and Methods* below) per kg of body weight (for the experimental group). Final live weights for the control and experimental mice were 31.0 ± 1.1 g and 31.7 ± 0.8 g, respectively. The extract was thus well tolerated by the mice without any evidence of food aversion. After the 4-week diet regimen with ginkgo biloba, the hippocampi and cortices were removed and pooled with respect to both tissue type (hippocampus vs. cortex) and treatment (control vs. experimental). Quercetin, kaempferol, and isorhamnetin (major constituents of EGb761) concentrations in blood-plasma samples that were pooled group-wise were also measured as controls. Quercetin, kaempferol and isorhamnetin concentrations (12.0, 7.0, and 49.6 ng/ml) of the ginkgo-supplemented group were higher than those of the control group (4.8 and 3.2 ng/ml, respectively; isorhamnetin was not detectable in the control group), confirming absorption of at least some of the major components of the ginkgo leaf extract into the systemic circulation. Whereas quercetin and kaempferol most likely stem from the diet preparation, isorhamnetin may be partly derived from postabsorptive methylation of quercetin in the liver and intestinal mucosa (5). Total RNA was extracted from the hippocampi and cortices and processed to obtain biotinylated cRNA. The cRNA was subsequently fragmented and hybridized (two independent hybridizations) to high-density MuU74 Affymetrix GeneChip arrays.

Of the ≈12,000 combined genes and ESTs represented on the array, only 10 changed in expression level by 3-fold or more, and all were up-regulated. These findings are summarized in Table 1. In the hippocampus, only one gene was up-regulated greater than 3-fold; nine genes were up-regulated greater than 3-fold in

Table 1. The transcriptional effects of ginkgo biloba on the hippocampus and cortex (genes whose expression changed 3-fold or more)

Probe set	Gene/description	Fold change
Hippocampus		
Pre-albumin		
D00073	Transthyretin	16
Cortex		
Growth factors/Neuromodulators		
X02891	Growth hormone	11.1
X04418	Prolactin	11
Transcription factors		
Y07688	NfiX1 protein	7.3
U02098	Purinergic region binding protein α	5.1
Ion channels		
AF029347	Chloride channel protein 3	4.5
AF029347	Chloride channel protein 3	6.2
AF077739	Calcium channel	3.8
L32372	GluRB (AMPA-2)	3.9
Signal transduction		
X95518	Neuronal tyrosine/threonine phosphatase	7.3
Cytoskeletal		
M18775	Microtubule-associated τ	3.1
M18775	Microtubule-associated τ	4.4

the cortex. Most notably, transthyretin, AMPA-2 channel, neuronal tyrosine/threonine phosphatase 1, and microtubule-associated τ, all of which may have neuroprotective roles, were significantly up-regulated. Up-regulation of the expression of these genes was confirmed by RT-PCR. Ion channels, growth hormones, and transcription factors were also among those genes whose expression was enhanced by diet supplementation with ginkgo.

The hippocampus is part of the temporal lobe of the brain and is the center for learning and memory. Interestingly, the only gene on the array whose expression was up-regulated more than 3-fold in the hippocampus by dietary supplementation with ginkgo biloba (EGb761) encodes transthyretin. This protein is involved in the transport of thyroxine (a thyroid hormone) and retinol-binding protein in cerebrospinal fluid and serum (8) and was the most highly up-regulated protein in the profile, with a 16-fold enhancement. Thyroid hormones regulate neuronal proliferation and differentiation in discrete regions of the brain during development and are necessary for normal cytoskeletal assembly and stability as well as for neuronal proliferation and outgrowth (9). Transthyretin has also been shown *in vitro* to sequester amyloid-β (Aβ) protein and prevent Aβ aggregation from arising in amyloid formation (10). Evidence from *Caenorhabditis elegans* transgenic in human Aβ and transthyretin also suggests that the latter can inhibit Aβ amyloid formation in the kidney (11). In addition, transthyretin levels in cerebrospinal fluid have been found to be significantly decreased in Alzheimer's disease patients. Thus, one mechanism whereby ginkgo may exert neurological effects is by the modulation of transthyretin levels, and as a consequence, by either hormone transport or Aβ sequestration in the brain. It should be noted, however, that misfolding of transthyretin as a result of mutation can also result in fibrillogenesis (12).

The human cerebral cortex serves to control functions such as speech, memory, logical and emotional response, as well as consciousness, interpretation of sensation, and voluntary movement. Among the nine proteins with expression increased greater than 3-fold in the cortex of mice as a result of ginkgo biloba diet supplementation, neuronal tyrosine/threonine phosphatase 1 was up-regulated by 7-fold and microtubule-associated τ by 3- to 4-fold. Both proteins are associated with the formation/breakdown of intracellular neurofibrillary tangles, a hallmark lesion of Alzheimer's disease. Hyperphosphorylated τ has been found to be the major protein of these neurofibrillary tangles, possibly because of an imbalance of τ-kinase and phosphatase activities in the affected neurons (13). Hyperphosphorylated τ isolated from brains of those with Alzheimer's disease has been shown to be efficiently dephosphorylated *in vitro* by protein phosphatases 1, 2A, and 2B. Additionally, selective inhibition of protein phosphatase 2A by okadaic acid in metabolically competent rat brain slices has been shown to induce a hyperphosphorylation and accumulation of τ like that in Alzheimer's disease (14). Thus, upregulation of neuronal phosphatase 1 by ginkgo could play a neuroprotective role in the brain. The role of τ in neuronal axon elongation and in the regulation of microtubule assembly/organization remains controversial. A 3-fold overexpression of human τ in mice has been shown to cause widespread axonopathy with both neurofilament and microtubule accumulations (15). Surprisingly, however, cell death was not observed, and electron microscopic imaging did not reveal abnormal τ-positive filaments. Furthermore, the methods used to deplete cells of functional τ have given conflicting results. For example, antisense experiments and chromophore-assisted laser inactivation of τ have suggested requirement of τ in axonal outgrowth, whereas genetic knockout and immunodepletion studies have suggested that τ plays no role in this process (16).

The expression of mRNAs for ionotropic receptor AMPA-2 or GluRB ($\approx$4-fold), and two ion channels, ClCN3 or chloride channel protein 3 (4- to 6-fold), and calcium channel protein ($\approx$4-fold) were also up-regulated in the cortex. In the mammalian brain, rapid excitatory neurotransmission is mediated by ionotropic glutamate receptors comprised of the AMPA/kainate (non-NMDA) and *N*-methyl-D-aspartate (NMDA, or non-AMPA) receptor classes. These receptors play an integral role in synaptogenesis and the formation of neuronal circuitry and are involved in the processes of memory formation and learning (17). The AMPA receptors are encoded by four genes designated *gluRA* (*gluRI*), *gluRB* (*gluR2*), *gluRC* (*gluR3*), and *gluRD* (*gluR4*) and are composed of both Ca^{2+} permeable and impermeable forms. The GluRB or AMPA-2 subunit has been shown to render heterotrimeric AMPA receptor assemblies Ca^{2+}-impermeable. Interestingly, studies with animal models of transient forebrain ischemia and epilepsy have shown that GluRB expression is down-regulated in these neurons, which may give rise to the formation of Ca^{2+}-permeable AMPA receptors and enhanced toxicity of endogenous glutamate (18). Furthermore, mouse mutants with targeted AMPA receptor Ca^{2+} GluRB subunit alleles demonstrated increased AMPA receptor calcium permeabilities in pyramidal neurons and had mild to severe neurological dysfunctions, including epilepsy and deficits in dendritic architecture (19). Thus, upregulation of AMPA-2 receptor by ginkgo may represent another mechanism whereby the extract affects neurologic function (20).

Similarly, ion homeostasis is important to the maintainance of normal brain function, whereas the failure of ion homeostasis may result in neurodegeneration and abnormal neuronal processes. Diet supplementation with ginkgo biloba resulted in up-regulation of both a calcium channel (CACNG2) and a chloride channel (ClCN3). ClCN3 or chloride channel protein 3 is a voltage-gated chloride channel that is expressed primarily in tissues derived from neuroectoderm; its expression in the brain is particularly evident in the hippocampus, olfactory cortex, and olfactory bulb (21). CACNG2 encodes for a voltage-dependent calcium channel. Although the exact biological function of this gene has not been elucidated, genetic mutations in voltage-dependent calcium channels have been associated with spinocerebellar ataxia in humans and with ataxia, progressive cerebellar degeneration, and epilepsy in mice (22).

Interestingly, ginkgo biloba also up-regulated expression of two growth factors, growth hormone (GH; 11-fold) and prolactin (11-fold) in the cortex. GH is an anabolic hormone that stimulates most target cells to grow in size and divide. Although its major effects are directed to the growth of skeletal muscles and long bones of the body (23), GH receptors are also present in the brain. Observations indicate that the hormone may cross the blood–brain barrier through receptor-mediated mechanisms (24). Within the past decade, studies have revealed that GH may exert significant effects on the central nervous system. Cognitive impairments are well known hallmark features of GH deficiency, and clinical studies have reported psychological improvements (in mood and well being) and beneficial effects on certain functions including memory, mental alertness, motivation, and working capacity in adults receiving GH replacement therapy. Moreover, GH therapy in children deficient in this protein have been reported to produce marked improvement in their behavior (24).

Prolactin is a peptide hormone structurally similar to GH. While prolactin plays important roles in reproduction, it is now well established that the functions of prolactin are complex and may include over 300 distinct biological activities (25). Among these activities may be neurological functions, although it is not clearly understood whether the central effects of prolactin are as a neurotransmitter and neuromodulator, or as a central cytokine in the regulation of vascular growth and/or glial functions.

Nonetheless, prolactin has been shown to induce proliferation and differentiation in embryonic astrocytes (26). Moreover, targeted disruption of prolactin in mice resulted in diminished activity of tuberoinfundibular dopaminergic neurons in these mice as demonstrated by the greatly reduced levels of dopamine and tyrosine hydroxylase (27).

Finally, ginkgo biloba also increased expression of two transcription factors, the purinergic region binding protein α, (Pur-α; 5-fold), and nuclear I/X or NfiX protein (7-fold). Although the functions of these proteins have not been extensively investigated, it is known that Pur-α binds both single-stranded DNA and RNA and has been shown to regulate transcription by RNA polymerases of the Pol II class and some Pol III enzymes. The protein has been shown to attenuate transcription of a variety of mRNAs expressed in neural cells. For example, BC1 (whose RNA is distributed in neuronal dendrites as RNA–protein complexes) has been demonstrated to bind Pur-α, linking the BC1-RNP complex to microtubules (28). Fe65, an adaptor protein that interacts with Alzheimer β-amyloid precursor protein, is also transcriptionally regulated by Pur-α (29). NfiX seems to encode a dimeric DNA-binding protein that is involved both in the initiation of adenovirus DNA replication and in the stimulation of transcriptional activation (30).

Although clinical studies have demonstrated that EGb761, a standardized extract of ginkgo biloba, has some effect in the amelioration of mild to moderate dementia in patients with Alzheimer's disease and with other neurological disorders, the mechanism(s) underlying its neuroprotective benefits have remained unclear. We have shown here that a variety of neuronal genes are induced in the hippocampus and cortex by dietary supplementation with ginkgo biloba. However, it is not clear what effects these changes in mRNA expression have on overall brain function. Further studies will be required to fully investigate both possible beneficial and harmful effects of ginkgo biloba. Nonetheless, this experimental approach can provide a rational framework for a more detailed exploration of the biochemical activities of ginkgo biloba extract as well as other complex extracts and may facilitate the isolation of their bioactive components.

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