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Ginkgo biloba extract (Egb 761) inhibits β-amyloid production by lowering free cholesterol levels

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Abstract

Ginkgo biloba extract (EGb 761) can improve cognitive function in patients with Alzheimer's disease, but the molecular mechanisms underlying this effect remain undefined. Because free cholesterol may be involved in the production of β-amyloid precursor protein and amyloid β-peptide, key events in the development of Alzheimer's disease, we examined EGb 761 in relation to cholesterol and amyloidogenesis. In aging rats, EGb 761 treatment lowered circulating free cholesterol and inhibited the production of brain β-amyloid precursor protein and amyloid β-peptide. Exposure of PC12 cells to EGb 761 decreased the processing of β-amyloid precursor protein and abolished cholesterol-induced overproduction of this protein. Exposure of human NT2 cells to EGb 761 decreased free cholesterol influx and increased free cholesterol efflux. Our findings indicate that free circulating and intracellular cholesterol levels affect the processing of β-amyloid precursor protein and amyloidogenesis. Our findings also provide the first demonstration that EGb 761 can influence these mechanisms.

Keywords: Neurodegeneration; Amyloid peptide; Cholesterol; Lipoproteins; *Ginkgo biloba*

1. Introduction

Ginkgo biloba leaves have been used for medical purposes for centuries in traditional Chinese medicine. However, currently, Ginkgo leaf extracts are more commonly associated with phytomedicine in Europe and America than with traditional Chinese medicine. The standardized Ginkgo leaf extract termed "EGb 761," which contains ~24% flavonol glycosides, ~6% terpene trilactones (ginkgolides, bilobalide), and ~7% proanthocyanidins and other constituents, is prescribed as therapy for a number of conditions. These include cerebrovascular insufficiency, peripheral vascular insufficiency, and for the cognitive impairment that is associated with aging and with neurodegenerative disorders such as Alzheimer's disease (AD) [1]. Substantial clinical and preclinical evidence indicates that EGb 761 opposes neural and vascular damage and has a multitude of beneficial effects on the central nervous system, actions that support its use in treating AD [2,3]. Most clinical studies involving AD patients have shown that EGb 761 treatment leads to modest improvement [4–7] or maintenance [7] of cognitive function, together with improvement of self-rated [8] or informant-rated [9] quality of life; however, the cellular and molecular mechanisms underlying this effect remain to be clarified.

In search of such mechanisms, we are examining the effects of EGb 761 in relation to amyloid β-peptide (Aβ), a group of hydrophobic peptides of 39-43 amino acid residues. These constitute a major component of senile plaques and vascular amyloid deposits of the brains of AD patients. The formation of these substances, via proteolytic cleavage of β-amyloid precursor protein (APP, existing as three major isoforms) is regarded as a crucial process in the pathogenesis of AD [10–13]. Because a growing body of epidemiological evidence indicates that high cholesterol levels might increase the risk for developing AD, and as cholesterol has been shown to modulate APP processing

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and affect APP mRNA expression [14–20], we have examined Aβ production in relation to cholesterol using both in vivo and in vitro models.

Our recent results have shown that EGb 761 inhibits the formation of Aβ-derived diffusible neurotoxic ligands and rescues PC12 neuronal cells from Aβ-induced cell death [21]. We also found that one physiological action of Aβ and APP involves their control of cholesterol transport and homeostasis [22]. In the present article, we report novel findings which have revealed that long-term treatment of aging Brown Norway (BN) rats with EGb 761 influences the production of brain APP and Aβ by lowering the levels of circulating free cholesterol. On the basis of these results and other findings described herein, we propose that one mechanism of action of EGb 761 that is relevant to its use in treating AD is its effect of decreasing the capacity of low-density lipoproteins (LDL) to carry free cholesterol to various tissues without affecting the capacity of high-density lipoproteins (HDL) to carry cholesterol back to the liver.

2. Methods and materials

2.1. Animals

Male Brown Norway rats, 18 months of age, were obtained from the National Institute on Aging. In this rat strain, as well as in other rat strains, age-related germ cell loss in the male begins focally at about 18 months of age and eventually leads to testicular atrophy [23,24]; therefore, rats of this age were considered appropriate for studying parameters that might be influenced by aging. Rats were housed at the Georgetown University Research Resources Facility under controlled light and temperature, with free access to rat chow and water. They were kept in groups of three animals each and allowed to acclimatize to their new conditions for 1 week before commencing experiments. Saline (0.9% NaCl in doubly distilled water) and EGb 761 (50 mg/kg) were administered orally every day for 28 weeks. The EGb 761 (lot K923) was kindly provided by the Institut Henri Beaufour (Les Ulis, France) and dissolved in doubly distilled water, and treatments were given at 10 AM. Subsequently, rats (10 control and 10 EGb 761-treated) were sacrificed 24 hours after the last treatment. Rat brains were excised and either fixed in 3.7% formaldehyde in PBS 1X solution, embedded in paraffin, and then 5-μm sections were cut for immunohistochemistry, or were stored at −70°C for further biochemical studies.

2.2. Cell culture

PC12 cells were cultured in 96-well plates in RPMI 1640 media supplemented with 5% equine serum and 10% fetal bovine serum, as we have previously described [21]. Human neuronal NT2 precursor cells (Ntera-2/D1) were obtained from Stratagene (Cedar Creek, TX) and cultured following the instructions of the supplier. Human LDL and HDL were purchased from Sigma (St. Louis, MO); [1,2-^{3}H(N)]-cholesterol (sp. act. 48.3 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA).

2.3. Immunohistochemistry

Sections were deparaffinized and were then immunostained using a mouse anti-APP monoclonal antibody that recognizes all three isoforms of APP (Clone 22C11; Chemicon International, Temecula, CA) at a concentration of 5 μg/mL and rabbit anti-$A\beta_{1-40}$ antiserum (Sigma) at 1:100. Immunoreactivity was detected using horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:500) (Transduction Laboratories, San Diego, CA). For negative controls, the primary antibody was replaced by 10% calf serum in PBS or non-immune IgG. Counterstaining was carried out with Mayer's hematoxylin (Sigma Diagnostics, St. Louis, MO).

2.4. Immunoblot (Western) analysis

Frozen brain specimens were homogenized and proteins were extracted with sample loading buffer for immunoblot analysis as described below. PC12 cells were cultured in six-well plates and treated for 72 hours with various concentrations of EGb 761 in the presence or absence of cholesterol. Cells were then washed with PBS and lysed with loading buffer. Proteins were resolved by 4–20% SDS-PAGE gel electrophoresis and electrotransferred onto nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were subjected to immunoblot analysis by incubation overnight with rabbit anti-APP (ZYMED Laboratories Inc., San Francisco, CA) at 0.5 μg/mL (primary antibody) and goat anti-rabbit IgG-horseradish peroxidase as the secondary antibody (1:7000) (ZYMED Laboratories Inc.). Protein bands were visualized using ECL reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Image-densitometric analyses of the immunoreactive protein bands were performed using OptiQuant-image analysis software (Packard BioScience, Meriden, CT). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal standard.

2.5. Cholesterol-protein binding blot assays

Cholesterol-protein binding assays were conducted as we have previously described [22]. In brief, various concentrations of isolated human lipoproteins (HDL and LDL), fetal bovine serum (FBS), or BN rat serum were incubated for 3 hours in medium containing 0.02 μCi ^{3}H-cholesterol in the presence or absence of increasing concentrations EGb 761 in a total volume of 20 μL at 37°C. After incubation, the samples were separated by 1.5% agarose (Type I-B, Sigma) gel electrophoresis and transferred to nitrocellulose membranes in 10 × SSC buffer. The membranes were exposed to a tritium-sensitive screen and analyzed by phospho-imaging using the Cyclone Storage phosphor system (Packard BioScience). Image-densitometric analysis of the radioactivity was performed as described above.

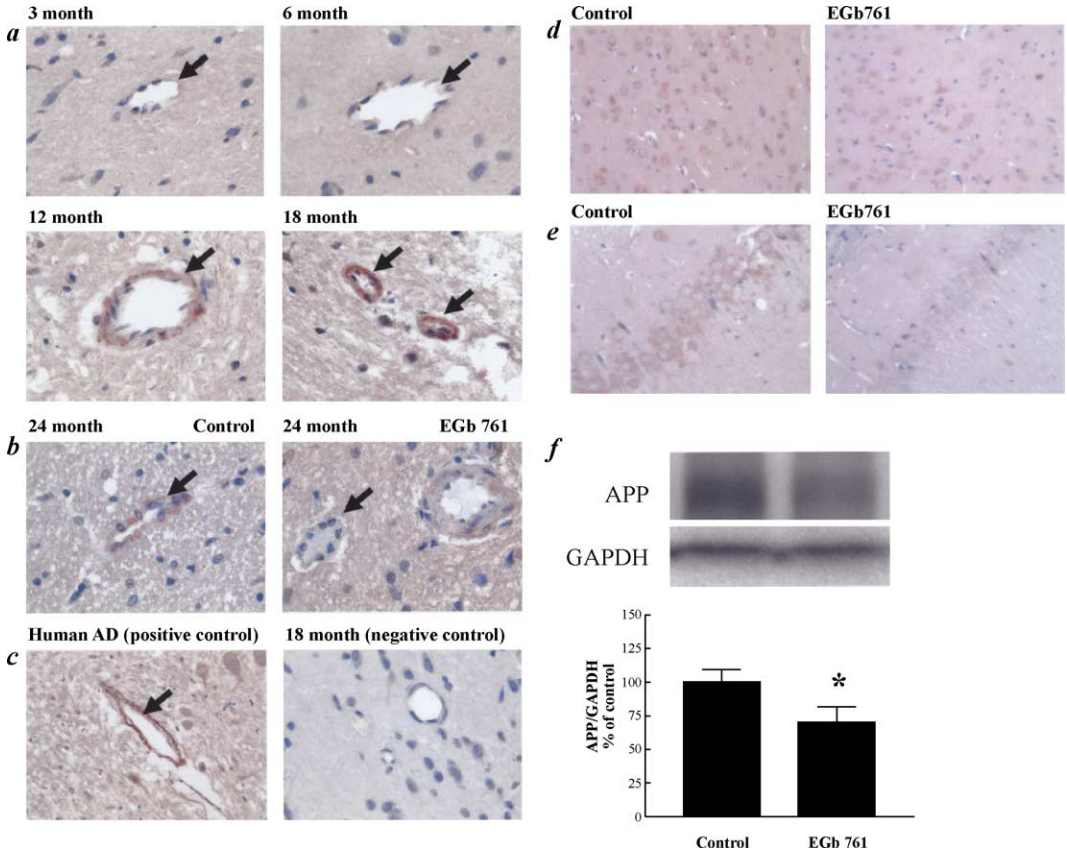

Fig. 1. Effect of *Ginkgo biloba* extract (EGb 761) on Aβ and β-amyloid precursor protein (APP) production in the Brown Norway (BN) rat brain. Immunohistochemical localization of Aβ accumulation in blood vessel walls of rat cerebral cortex: (*a*) as a function of age; (*b*) in 24-month-old rats after treatment with drug vehicle (*left*) or EGb 761 (*right*); (*c*) in the hippocampus of an AD patient (*left*) and in the cerebral cortex of an 18-month-old untreated (negative control, no primary antibody) rat (*right*). Results shown are representative of 4 independent experiments. Magnification, ×1000. Immunohistochemical staining showing APP in the cerebral cortex (*d*) and hippocampus (*e*) of control (vehicle-treated) and EGb 761-treated rats. Results shown are representative of 4 independent experiments. Magnification, ×400. (*f*) APP protein levels in control and EGb 761-treated rat brain extracts were determined by immunoblot analysis and quantified by image analysis. Upper section shows APP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels from a representative experiments. Lower section shows means ± SD of the densitometric analysis of the APP and GAPDH immunoreactive bands in brain extracts from three control and EGb 761-treated rats. $*P<0.05$.

2.6. ^{3}H-Cholesterol influx and efflux assays

To determine cholesterol influx, NT2 cells (2×10^{5}) in 24-well plates were incubated for 24 hours in media containing 0.5 mL 1% FBS or 50 μg/mL human LDL in the presence of 0.2 μCi ^{3}H-cholesterol and in the presence or absence of various concentrations of EGb 761. After incubation, the cells were washed with PBS and lysed in 1mL of 0.1 N NaOH. Radioactivity was measured by liquid scintillation spectrometry. Protein levels were quantified by

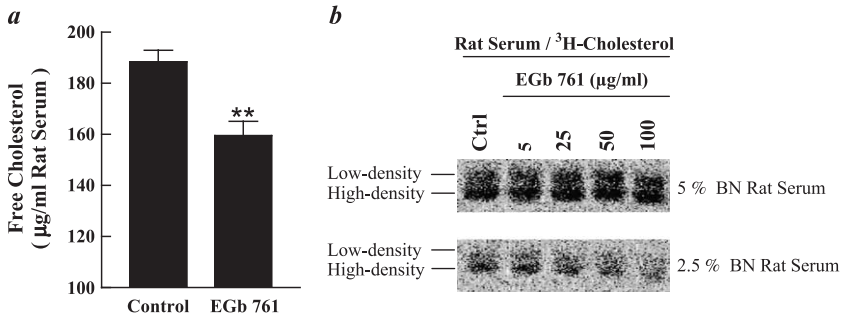

Fig. 2. Effect of EGb 761 on free cholesterol levels in BN rat serum. (*a*) Endogenous free cholesterol levels in the sera of control and EGb 761-treated BN rats. Means ± SEM for duplicate measurements from seven EGb 761-treated rats and seven age-matched controls. (*b*) Effect of EGb 761 on the binding of free cholesterol to proteins in BN rat serum, as examined by cholesterol-protein binding blot assay (CPBBA). Results shown are representative of four independent experiments. $**P<0.01$, $n=7$. Abbreviations as in Fig. 1.

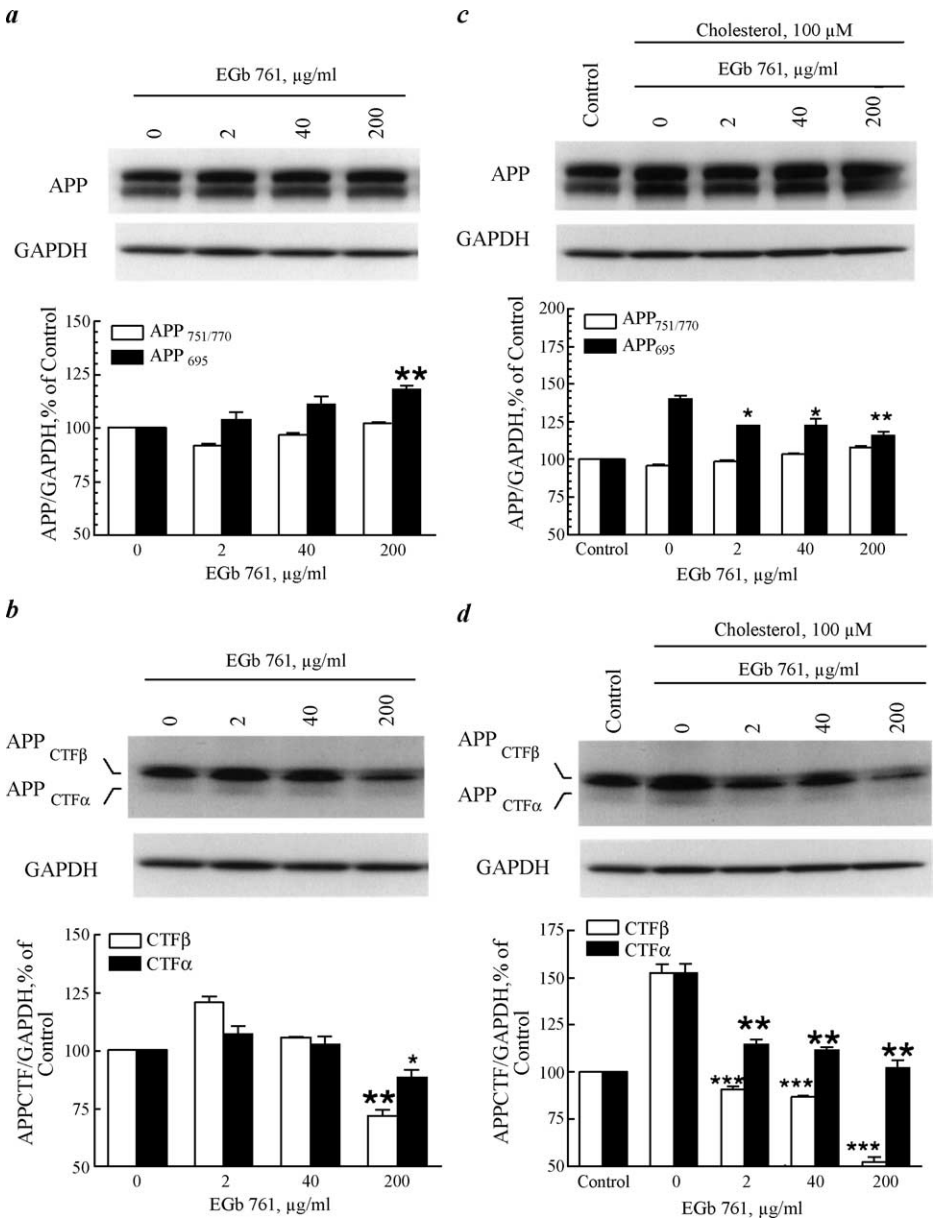

Fig. 3. Effect of EGb 761 on the production and processing of APP in PC12 cells. PC12 cells were treated with the indicated concentrations of EGb 761 in absence (*a, b*) or presence (*c, d*) of cholesterol. Immunoblot analyses (using specific antisera) and quantitative image analyses of $APP_{751/770}$ (*a, c, upper band*), APP_{695} (*a, c, lower band*), and APP C-terminal fragment (CTF)α/CTFβ (*b, d*) are shown. Significance, comparison with without EGb 761 treatment, is indicated as $*P<0.05$, $**P<0.01$, and $***P<0.001$. Results shown are representative of three independent experiments. Abbreviations as in Fig. 1.

the dye-binding assay of Bradford [25] using bovine serum albumin as standard. To determine cholesterol efflux, NT2 cells (2×10^5) were incubated in the presence of 0.2 μCi ^{3}H-cholesterol in 10% FBS media for 48 hours. After this incubation, the cells were washed with 10% FBS media twice and with FBS-free media twice and then incubated for 24 hours in 0.5 mL F12/Dulbecco's modified Eagle's medium (DMEM) containing 1% FBS or 50 μg/mL human HDL in the presence or absence of the indicated concentrations of EGb 761. Aliquots (100–200 μL) of media were used to determine radioactivity. Cells were washed with PBS and lysed in 1 mL of 0.1 N NaOH. Protein levels were quantified as described above.

2.7. Determinations of protein and serum free cholesterol levels

Microgram amounts of protein were quantified by the dye-binding assay of Bradford [25] using bovine serum albumin as standard [21]. The levels of free cholesterol in the sera of BN rats were determined using the cholesterol oxidase assay [26].

2.8. Statistical analyses

Statistical analyses were performed by one-way analysis of variance and unpaired Student *t* test using the INSTAT 3.00 package (GraphPad, San Diego, CA).

3. Results

3.1. EGb 761 treatment decreases APP and Aβ production in BN rat brain

As the generation of Aβ is crucial to the pathogenesis of AD, we evaluated its levels in the brains of aging BN rats. Our immunohistochemical results showed that the accumulation of Aβ in blood vessel walls of the cerebral cortex (cerebral Aβ angiopathy) increased as a function of age (Fig. 1a). To test for an effect of EGb 761 on this age-related process, we treated 18-month-old BN rats orally every day with either saline (0.9% NaCl solution) or EGb 761 (50 mg/kg) for 28 weeks. After this treatment, we found that the accumulation of Aβ in blood vessel walls of the cerebral cortex was decreased by EGb 761 treatment, as compared with controls (Fig. 1b). As noted in the Methods and materials section, for negative controls the primary antibody was replaced by 10% calf serum in PBS or non-immune IgG. Both controls gave identical results and the 10% calf serum in PBS data is shown. Sections of the hippocampus of an AD patient (positive control) and the cerebral cortex of an untreated 18-month-old BN rat (negative control) are also shown (Fig. 1c). We next examined APP expression in BN rat brain and found that EGb 761 decreased APP production in cerebral cortex (Fig. 1d) and hippocampus (Fig. 1e) after 28 weeks of treatment, as compared with controls. Further immunoblot analysis of brain extracts indicated that APP levels in BN rat brain were decreased by 32% after EGb 761 treatment compared with controls (Fig. 1f).

3.2. Egb 761 lowers circulating free cholesterol levels in BN rats

To examine the hypothesis that EGb 761 treatment might decrease brain levels of Aβ and APP by lowering free cholesterol levels, we measured the free cholesterol level of BN rat serum. Indeed, results obtained using the cholesterol oxidase assay indicated that circulating free cholesterol levels were lowered by 16%, from 188 ± 4.8 μg/mL serum to 159 ± 6.1 μg/mL serum, by EGb 761 treatment, as compared with controls (Fig. 2a). To explore further the mechanism underlying this effect of EGb 761, we tested its effect on the binding of free cholesterol to free cholesterol binding proteins using a cholesterol-protein binding blot assay (CPBBA) [35]. Our results indicated that EGb 761 decreased the binding of free cholesterol to the low-density more than to the high-density rat serum cholesterol binding protein (Fig. 2b).

3.3. EGb 761 inhibits the overproduction of APP induced by free cholesterol in PC12 cells

To test further the hypothesis that EGb 761 treatment decreases the production of Aβ and APP in BN rat brain by

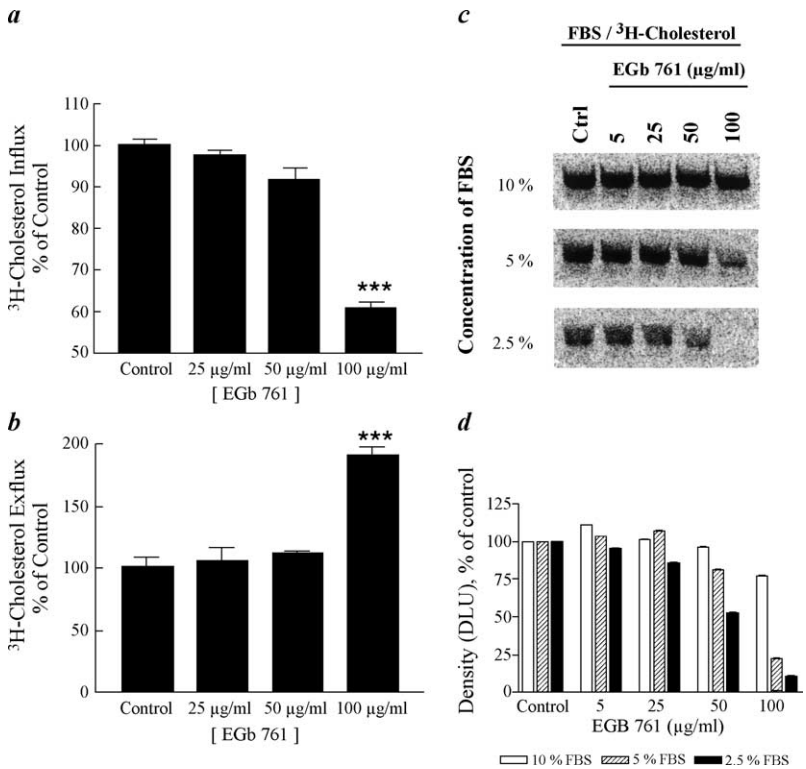

Fig. 4. Effect of EGb 761 on fetal bovine serum (FBS) lipoprotein-mediated cholesterol fluxes in NT2 cells. (*a*) ^{3}H-cholesterol influx into NT2 cells; (*b*) ^{3}H-cholesterol efflux from NT2 cells; means ± SD from four independent experiments ($n = 12$); (*c*) effect of EGb 761 on the binding of free cholesterol to lipoprotein in FBS, as examined by CPBBA; results shown are representative of four independent experiments. Significance (compared with control) is indicated. ***$P < 0.001$. Abbreviations as in Figs. 1 and 2.

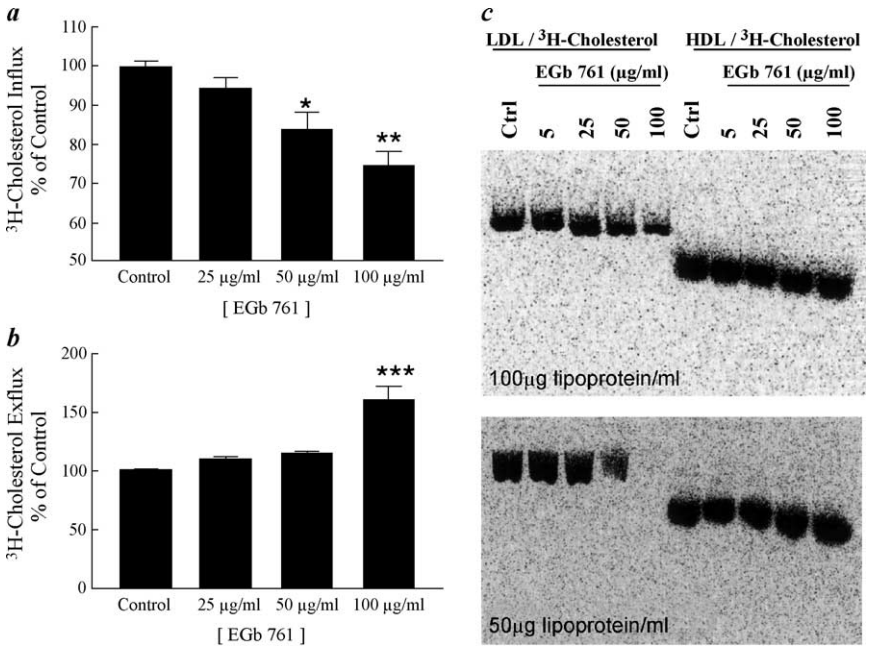

Fig. 5. Effect of EGb 761 on human low-density lipoprotein (LDL)– and high-density lipoprotein (HDL)–mediated cholesterol fluxes in NT2 cells. (*a*) ^{3}H-cholesterol influx into NT2 cells; (*b*) ^{3}H-cholesterol efflux from NT2 cells; means ± SD from four independent experiments (n = 12); (*c*) effect of EGb 761 on the binding of free cholesterol to human LDL and HDL, as examined by CPBBA; results shown are representative of four independent experiments. Significance (compared with control) is indicated. *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviations as in Figs. 1 and 2.

means of an action related to its effect of decreasing serum free cholesterol, we examined the effect of the extract on neuronal APP and Aβ synthesis. PC12 cells were treated with EGb 761 for 72 hours; proteins were separated by electrophoresis; and $APP_{751/770}$, APP_{695}, and APP C-terminal fragment (CTF) α/CTF β levels were determined by immunoblot analyses using specific antisera and quantified by image analyses. For these in vitro studies, we used an EGb 761 preparation that is devoid of proanthocyanidins (IPS200) that may interact with proteins in a nonspecific manner. EGb 761 did not significantly affect $APP_{751/770}$ protein levels (Fig. 3a). However, APP_{695} protein levels were increased in the presence of increasing concentrations of EGb 761; that is, by 9% at 2 μg/mL to 20% at 200 μg/mL IPS200 (Fig. 3a). To evaluate the influence of EGb 761 on APP processing, we determined the levels of C-terminal fragments (CTFα and CTFβ) of APP, 12-kDa peptides produced by α-secretase or β-secretase cleavage of APP, in the same blots using specific antisera to CTFα/CTFβ. CTFβ levels were decreased by 30% in response to a 72-hour treatment with 200 μg/mL EGb 761, compared with control (Fig. 3b). In contrast, CTFα levels were less changed by the EGb 761 treatment.

Considering previous results that have indicated that cholesterol modulates APP and Aβ production and APP processing [14–20], we treated PC12 cells for 72 hours with or without increasing concentrations of EGb 761 in the presence of 100 μmol/L cholesterol. EGb 761 and cholesterol did not significantly influence $APP_{751/770}$ protein level (Fig. 3c). However, the APP_{695} protein level was increased by 39% in the presence of 100 μmol/L cholesterol, as compared with control, and EGb 761 inhibited this cholesterol-induced overproduction of APP_{695} in a concentration-dependent manner (Fig. 3c). The effect of EGb 761 on APP processing in the presence of 100 μmo/L cholesterol was then investigated. We found that the levels of CTFα and CTFβ of APP were increased by about 50% in the presence of 100 μmol/L cholesterol, and that EGb 761 treatment (2–200 μmol/L) abolished this effect of cholesterol on APP processing (Fig. 3d). At a concentration of 200 μg/mL, EGb 761 also blocked the basal production of CTF (Fig. 3d).

3.4. EGb 761 treatment affects free cholesterol influx and efflux in NT2 cells by inhibiting its binding to LDL

EGb 761 treatment decreased free cholesterol influx into human NT2 cells in a concentration-dependent manner, this decrease being about 40% at the 100 μg/mL concentration of the extract, as compared with control (Fig. 4a). In contrast, EGb 761 increased cholesterol efflux in a concentration-dependent manner, this increase being about 85% at the 100 μg/mL concentration of the extract (Fig. 4b). Using CPBBA, we found that EGb 761 influenced these cholesterol fluxes by acting directly on the lipoprotein-cholesterol interaction that occurred in the serum (FBS) that was used in these cell culture experiments. EGb 761 inhibited free cholesterol binding to lipoproteins in a concentration-dependent manner (Fig. 4c).

HDL and LDL are the main carriers of free cholesterol that mediate its transport between peripheral tissues and the

liver [4–7]. To clarify further the mechanism of action of EGb 761 on circulating free cholesterol and free cholesterol transport, we substituted human LDL and HDL for FBS in the culture medium and examined the effect of EGb 761 on free cholesterol influx and efflux in NT2 neuronal cells using CPBBA. The data showed that EGb 761 decreased free cholesterol influx (Fig. 5a) and increased efflux (Fig. 5b). CPBBA results showed that EGb 761 inhibited free cholesterol binding to LDL but did not affect free cholesterol binding to HDL, although it did increase HDL density or increase HDL negative charge (Fig. 5c).

4. Discussion

High cholesterol levels affect the generation of APP and APP processing, effects that might be linked to AD [14–20]. We have recently demonstrated that one of the physiological functions of APP and Aβ is to control free cholesterol transport. Specifically, in the presence of high cholesterol levels, APP synthesis (expression) is increased and the processing of APP for the formation of Aβ and Aβ peptide fragments is enhanced, resulting in increased Aβ production [22]. In addition, we reported that Aβ can influence the capacity of lipoprotein and apolipoprotein to carry free cholesterol [22]. Other studies have revealed that hypercholesterolemia is associated with both cardiovascular disease and AD [27], and that treatment with EGb 761 can benefit patients with cerebrovascular insufficiency and AD [2,3]. It is these findings that led us to examine EGb 761 in relation to brain Aβ, APP and circulating free cholesterol levels in search of a mechanism that might underlie its beneficial effects in AD.

Our in vivo results, obtained with aging rats, show for the first time that a complex plant extract, EGb 761, can decrease the production of APP and Aβ in the brain by an action that is associated with a lowering of the level of circulating free cholesterol. The age-related accumulation of fibrillar Aβ deposits that occurs in cerebral blood vessels, and that is associated with an accumulation of Aβ in the basement membrane and a degeneration of adjacent smooth muscle cells of blood vessel walls [13,28–30], was also decreased by treatment with the extract. Collectively, these in vivo results support an action of EGb 761 of inhibiting the cerebral amyloidogenesis and amyloid angiopathy that characterize all forms of AD.

Our in vitro results with PC12 cells are the first to provide evidence that a complex plant extract, EGb 761, can prevent the over-production of APP and Aβ induced by free cholesterol. The findings that EGb 761 increased APP_{695} protein levels and inhibited cholesterol-induced over-production of APP_{695} while not influencing $APP_{751/770}$ protein levels in PC12 cells indicate that a certain constituent(s) of the extract have some selectivity for neurons, since APP_{695} is expressed predominantly in neuronal cells of the central nervous system, whereas the $APP_{751/770}$ isoforms are found in both neuronal and non-neuronal cells [31–33]. The results of our experiments on APP processing by PC12 cells are also of interest. It is known that CTFβ, an Aβ-bearing membrane-associated C-terminal derivative formed via the action of β-secretase, is subsequently cleaved by γ-secretase to release amyloidogenic Aβ species ($Aβ_{1-40}$, or less commonly the highly fibrillogenic species, $Aβ_{1-42}$ or $Aβ_{1-43}$), and that the formation of CTFα via α-secretase–catalyzed cleavage of APP within its Aβ domain (at Lys^{16} and Leu^{17}) leads to the formation of $Aβ_{17-40}$, thereby precluding formation of the full-length Aβ peptide and signifying operation of the non-amyloidogenic pathway [8,9,31–33]. Thus, the actions of EGb 761 of decreasing CTF levels and abolishing cholesterol-induced increases in CTF levels indicate that the extract has some potential for counteracting APP processing, as well as the deleterious effects of cholesterol on APP processing.

Our in vitro experiments with NT2 cells provide the first results that indicate that a complex plant extract, EGb 761, can decrease the capacity of LDL to carry free cholesterol. This finding is of considerable interest in light of recent studies that have shown that increases in cellular cholesterol concentration can increase the synthesis of Aβ, and that reducing the cellular cholesterol level of both neurons and peripheral cells (e.g., with inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase or methyl-β-cyclodextrin) can enhance the non-amyloidogenic α-secretase pathway and decrease the production of Aβ [14,18,34]. Because, as we have previously shown, free cholesterol can bind to the α-secretase cleavage site of Aβ [22], and because cholesterol is also involved in the conversion of nontoxic soluble Aβ to its toxic aggregated form in AD brain [35], we suggest further that certain EGb 761 constituents might compete with free cholesterol for interaction with Aβ, and thereby decrease its aggregation and toxicity.

One issue that requires consideration is the relationship between the concentrations of EGB 761 used in these in vivo studies versus in vitro studies, as well as the relevance of these concentrations to humans. As noted earlier EGb 761 is a complex mixture of hundreds of chemical constituents [2]. Because its pharmacological active constituents terpene trilactone ginkgolides represent approximately 2–3% [2,36], these compounds were recently used to monitor its bioavailability in rats [36]. In this study, Biber [36] demonstrated that after oral administration of 100 mg/kg EGb 761 in rats the concentration of ginkgolides in the blood reached 2–3 μg/mL [36], a concentration suggesting the presence of 100 μg/mL EGb 761 in blood. Interestingly, similar concentrations of ginkgolides were found in the blood of human subjects treated with 240 mg/day of EGb 761 [37], a dose used to stabilize the disease progression in patients with Alzheimer's disease [4,6,9,38].

Although much further study is required concerning the exact identities of the chemical constituents of EGb 761 that are responsible for the findings described here, our results do provide some insight concerning the basic mechanism(s)

underlying the therapeutic effect of the extract in AD patients. The results provided here may also be applied more generally in explaining the therapeutic effects of EGb 761 on vascular dementia, cerebrovascular insufficiency, and other cardiovascular disorders.

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