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The ginkgo biloba extract (EGb 761) protects hippocampal neurons against cell death induced by β-amyloid

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Abstract

Substantial evidence suggests that the accumulation of β-amyloid (Aβ)-derived peptides, and to a lesser extent free radicals, may contribute to the aetiology and/or progression of Alzheimer's disease (AD). Ginkgo biloba extract (EGb 761) is a well-defined plant extract containing two major groups of constituents, i.e. flavonoids and terpenoids. It is viewed as a polyvalent agent with a possible therapeutic use in the treatment of neurodegenerative diseases of multifactorial origin, e.g. AD. We have investigated here the potential effectiveness of EGb 761 against toxicity induced by (Aβ)-derived peptides ($A\beta_{25–35}$, $A\beta_{1–40}$ and $A\beta_{1–42}$) on hippocampal primary cultured cells, this area being severely affected in AD. A co-treatment with EGb 761 concentration-dependently (10–100 μg/mL) protected hippocampal neurons against toxicity induced by Aβ fragments, with a maximal and complete protection at the highest concentration tested. Similar, albeit less potent protective effects were seen with the flavonoid fraction of the extract (CP 205), while the terpenes were ineffective. Most interestingly, EGb 761 (100 μg/mL) was even able to protect (up to 8 h) hippocampal cells from a pre-exposure to $A\beta_{25–35}$ and $A\beta_{1–40}$. EGb 761 was also able to both protect and rescue hippocampal cells from toxicity induced by H_2O_2 (50–150 μM), a major peroxide possibly involved in mediating Aβ toxicity. Moreover, EGb 761 (10–100 μg/mL), and to a lesser extent CP 205 (10–50 μg/mL), completely blocked Aβ-induced events, e.g. reactive oxygen species accumulation and apoptosis. These results suggest that the neuroprotective effects of EGb 761 are partly associated with its antioxidant properties and highlight its possible effectiveness in neurodegenerative diseases, e.g. AD via the inhibition of Aβ-induced toxicity and cell death.

Introduction

Substantial evidence indicates that β-amyloid (Aβ) peptides ($A\beta_{1–40}$ and $A\beta_{1–42}$) probably play a prominent role in the aetiology of Alzheimer's disease (AD, Roher *et al*., 1993; Harris *et al*., 1995). Aggregated Aβ protein fragments are one of the major constituents of senile plaques, a hallmark feature of the AD brain (Selkoe, 1991). Neurotoxic (Goodman *et al*., 1994; Barger *et al*., 1995; Bruce *et al*., 1996; Pike *et al*., 1997; Doré *et al*., 1997; Jordán *et al*., 1997) and apoptotic (Loo *et al*., 1993; Copani *et al*., 1995) effects of Aβ-related fragments ($A\beta_{25–35}$, $A\beta_{1–40}$ and $A\beta_{1–42}$) have been demonstrated in regions involved in the pathophysiology of AD, e.g. the hippocampus (Hyman *et al*., 1984). Although the precise mechanisms mediating the toxic properties of Aβ have yet to be fully established, it has been proposed that Aβ toxicity is associated with increases in reactive oxygen species (ROS, Goodman *et al*., 1994; Hensley *et al*., 1994; Behl *et al*., 1994; Barger *et al*., 1995; Schubert *et al*., 1995; Bruce *et al*., 1996; Pike *et al*., 1997) which are possibly involved in the progression of AD (Smith *et al*. 1991, 1996).

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Ginkgo biloba extract (EGb 761, Tanakan®, IPSEN Laboratories, Paris, France) is obtained from green leaves of the Ginkgo biloba tree according to a well-defined procedure (Drieu, 1986). The chemical constituents of EGb 761 include 24% flavonoids that are nearly exclusively flavonol-*O*-glycosides, 6% terpenoids (e.g. gingolides A, B, C, M, J and bilobalide), 5–10% organic acids, and >0.5% proanthocyanidins defined as flavonoid-based polymers (Drieu, 1986). EGb 761 displays, mainly via its flavonoid constituents, free radical scavenging and antioxidant actions (Marcocci *et al*., 1994a,b) that are probably associated with its protective actions in animal models of hypoxia (Oberpichler *et al*., 1988) and ischaemia (Droy-Lefaix *et al*., 1995; Szabo *et al*., 1997). Earlier studies have described its neuroprotective, nootropic and/or neurotrophic activities in the hippocampal formation (Rodriguez de Turco *et al*., 1993; Barkats *et al*., 1995; Kelche *et al*., 1996; Kristofikovà & Klaschka, 1997). EGb 761 has also been reported to reverse scopolamine-induced amnesia in rats (Chopin & Briley, 1992) and to facilitate learning in aged rodents (Winter, 1991; Stoll *et al*., 1996). EGb 761 is already recognized as a polyvalent therapeutic agent in the treatment of disturbances of multifactorial origin including cerebral insufficiency (Kleijnen & Knipschild, 1992) and mild cognitive impairments in elderly patients (Wesner *et al*., 1987; Rai *et al*., 1991). Interestingly, several clinical studies support the potential usefulness of EGb 761 in

AD and in vascular dementia (Hofferberth, 1994; Le Bars *et al*., 1997; Maurer *et al*., 1997; Oken *et al*., 1998).

Considering the purported antioxidant properties of EGb 761 (Marcocci *et al*., 1994a,b) and its possible therapeutic efficacy (Hofferberth, 1994; Le Bars *et al*., 1997; Maurer *et al*., 1997; Oken *et al*., 1998), we investigated the neuroprotective effects of EGb 761 against toxicity induced by different Aβ peptides ($A\beta_{25–35}$, $A\beta_{1–40}$ and $A\beta_{1–42}$) and H_2O_2 in rat hippocampal primary cell cultures.

Materials and methods

Materials

Ginkgo biloba extract (EGb 761), the flavonoid fraction (CP 205) and the terpenoid constituents bilobalide (CP 160) and ginkgolide B (BN 52021) were obtained from IPSEN Laboratories. The different fragments of Aβ peptides including $A\beta_{1–40}$ (lot number T-20513), $A\beta_{40–1}$ (lot number QL511) and $A\beta_{1–42}$ (lot number ZN327) were purchased from Bachem (Torrance, CA, USA). The fragment $A\beta_{25–35}$ was kindly provided by P. Gaudreau (CHUM, University of Montreal, Montreal, Canada). Materials used for cell cultures were obtained from Gibco BRL (Burlington, Ontario, Canada). Unless stated otherwise, all other chemicals were purchased from Sigma (St. Louis, MO, USA).

Hippocampal cell cultures were prepared from E19 foetuses obtained from Sprague–Dawley rats; the rats were killed by decapitation. Animal care was according to protocols and guidelines of the McGill University Animal Care Committee and the Canadian Council for Animal Care.

Mixed cell cultures

Mixed (glial/neuronal) hippocampal cells were plated at day 0 at a density of $\sim 5 \times 10^4$ viable cells per well in 96-well plates or 2.5×10^5 viable cells per well in 24-well plates. Cells were grown in *N*-2-[hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES)-buffered Dulbecco's modified Eagle's medium (DMEM) high glucose (pH 7.4) during 7 days, as previously described (Bastianetto *et al*., 1999). The experiments were performed using 7-day-old cultures, at which time the hippocampal neurons are fully differentiated (Mattson *et al*., 1991).

Enriched neuronal cultures

Enriched neuronal hippocampal cells were plated at a density of $\sim 5 \times 10^4$ viable cells per well in 96-well plates and grown in a neurobasal medium supplemented with L-glutamine (0.5 mM) and N2 1% (v/v), as previously described (Brewer *et al*., 1993). The initial medium was removed at day 3 and replaced with the same medium containing N2 supplement 1% (v/v). Cells were maintained in growth medium at 37 °C in a 95% air/5% CO_2 humidified atmosphere until the day of experiment (day 6).

Glial cell cultures

Dissociated hippocampal cells were plated on 25-cm^3 flasks (Corning, Cambridge, MA, USA) previously coated with poly-D-lysine (10 μg/mL) and were grown in a HEPES-buffered DMEM high glucose (pH 7.4) containing (in mM): sodium pyruvate, 1; KCl, 25; glucose, 28.5; and supplemented with 20% (v/v) foetal bovine serum (FBS, Immunocorp, Montréal, Québec, Canada). Glial cells were obtained by vigorously shaking cells every 3 days in a Hank's balanced salt solution. After being shaked, cells were grown in the same medium containing 20% FBS. At day 9, the cells were transferred in 96-well plates and kept in a 95% air/5% CO_2 humidified atmosphere (37 °C) until they reached confluence (12–15 days of plating).

Experimental treatments

Aβ-induced toxicity

On the day of experiment, the medium was removed and mixed cells were gently washed once with HEPES-buffered DMEM high-glucose medium. Cells were then incubated at 37 °C in the same medium without FBS and exposed to the freshly solubilized peptides $A\beta_{25–35}$, $A\beta_{1–40}$ or $A\beta_{1–42}$ for 24 h, in the presence or absence of EGb 761. Cells were also exposed to the reverse sequence peptide $A\beta_{40–1}$ to confirm the specificity of the toxic effects of the fragment $A\beta_{1–40}$. The effect of post-treatment with EGb 761 was studied by treating the cells from 4 to 8 h after being exposed to either $A\beta_{25–35}$ or $A\beta_{1–40}$. Mixed cell viability was quantified 24 h later using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (see below). The extent of cell injury caused by a 24-h exposure to $A\beta_{25–35}$ was evaluated using the fluorescence dye propidium iodide (PI, see below). Enriched neuronal and glial cell cultures were exposed to $A\beta_{25–35}$ for 24 and 48 h, respectively.

Hydrogen peroxide (H_2O_2)-induced toxicity

H_2O_2 is one of the major peroxides which reacts with iron or copper leading to the production of hydroxyl radicals ($OH^{\cdot}$) (Halliwell, 1992). Cells were incubated in HEPES-buffered DMEM high-glucose medium and co-treated with EGb 761 (10–100 μg/mL) and H_2O_2 (50–100 μM). After a 3-h incubation period, cell viability was determined using the MTT colorimetric assay (see below). The rescuing effect of EGb 761 was determined by treating hippocampal cells at different times following a 1-h exposure to H_2O_2 (50 μM). Cell viability was determined 24 h later using the MTT colorimetric assay (see below).

Assessment of cell viability and cell injury

MTT is an indicator of the mitochondrial activity of living cells and is widely used as an index of cell survival (Mattson *et al*., 1995). Aβ peptides induce an inhibition of MTT reduction (Behl *et al*., 1994; Doré *et al*., 1997). Experimentally, the culture medium was replaced with a DMEM high-glucose buffer containing freshly dissolved MTT (0.25 mg/mL). Following a 3-h incubation at 37 °C, living cells containing MTT formazan crystals were solubilized in a solution of anhydrous isopropanol–HCl 0.1 N. The optical density (OD) was determined at 570 nm using a micro-plate reader (Bio-Tek Instruments®, Ville St-Laurent, Québec, Canada).

The extent of neuronal injury was estimated by assessing the extent of neuronal uptake of the fluorescence dye PI, as described previously using hippocampal cell cultures (Alonso *et al*., 1994; Bruce *et al*., 1996). Briefly, the culture medium was replaced with a phenol red-free, HEPES-buffered Hanks-based medium, pH 7.4, containing PI (4.5 μg/mL). After a one-step wash, PI uptake into dead cells of each well was automatically quantified (excitation = 485 nm; emission wavelength at 640 nm) using a fluorescence plate reader (Bio-Tek Instruments®).

Measurement of intracellular reactive oxygen species

The accumulation of ROS was determined by analysing 2′,7′-dichlorofluorescein (DCF) fluorescence (Mattson *et al*., 1995). 2′,7′-dichlorofluorescein diacetate (DCF-DA, 25 μM; Molecular Probes, Eugene, OR, USA) was applied to the culture medium at the onset of either Aβ exposure, as described previously (Bruce *et al*., 1996). The freely cell-permeable DCF-DA is readily converted into 2′,7′-dichlorofluorescein which is able to interact with peroxides (primarily H_2O_2) to form the highly fluorescent DCF. DCF fluorescence was quantified (excitation = 485 nm, emission = 530 nm) using a fluores-

cence multiwell plate reader (Bio-Tek Instruments®), as described previously (Goodman *et al.*, 1994).

Measurement of mitochondrial reactive oxygen species

Dihydrorhodamine (DHR; Molecular Probes) has been reported to probe levels of mitochondrial ROS, in particular peroxynitrite (Kooy *et al.*, 1994), in hippocampal neurons (Mattson *et al.*, 1997). DHR localizes to mitochondria and yields the fluorescent dye rhodamine after being oxidized. Cells were incubated for 30 min in FBS-free DMEM containing 5 μM DHR and washed twice with phenol red-free Hank's balanced salt solution as previously described (Mattson *et al.*, 1997). DHR fluorescence was quantified within 30 min (excitation = 485 nm, emission = 510 nm) using a fluorescence multiwell plate reader (Bio-Tek Instruments®).

Assessment of Hoechst DNA staining of apoptotic nuclei

Nuclear staining was performed using the fluorescent nuclear dye Hoechst 33342, as described previously using hippocampal neurons (Jordán *et al.*, 1997). This method has been validated as an indicator of apoptotic cell death in combination with the cell-impermeant dye PI which is excluded (Belloc *et al.*, 1994). Following a 24-h exposure to $A\beta_{25-35}$, cells were washed in phenol red-free, HEPES-buffered Hank's (pH 7.4) and then incubated in the same medium for 15 min with Hoechst 33342 (final concentration 1 μg/mL). The microplate fluorescence reader (UV excitation and emission = 360 and 450 nm, respectively) was used to automatically quantify the number of apoptotic neurons by assessing the enhanced Hoechst 33342 staining.

Statistical analyses

Survival of vehicle-treated control groups not exposed to either Aβ-derived peptides, H_2O_2, or EGb 761 was defined as 100% and the number of surviving, dead and apoptotic cells in the treated groups was expressed as a per cent of control groups**.** One-way ANOVA followed by a Newman–Keuls multiple comparison test was used to compare control and treated groups with $P < 0.05$ being considered statistically significant. An unpaired *t*-test was used to compare vehicle-treated and EGb 761-treated control groups with $P < 0.05$ being considered statistically significant.

Results

Protective effects of EGb 761 against Aβ-induced toxicity

As estimated by the MTT assay, cell survival is significantly decreased after a 24-h exposure to either $A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{1-42}$ (Fig. 1a and c; $^*P < 0.001$). In contrast, the reverse sequence peptide $A\beta_{40-1}$ (5 μM) failed to affect cell survival compared with vehicle-treated control groups (94 ± 3 versus 100 ± 5; NS), revealing the specific nature of the observed effects. The toxic effects of the different β-amyloid peptides agree with previously reported data in primary hippocampal cells (Barger *et al.*, 1995; Bruce *et al.*, 1996; Doré *et al.*, 1997). A co-treatment of cells with EGb 761 (10–100 μg/mL) concentration-dependently protected cells against the toxicity induced by $A\beta_{25-35}$ (Fig. 1a). These effects were significant at the lowest concentration (10 μg/mL) tested here with a maximal and complete effect obtained at 100 μg/mL (Fig. 1a). Similar results were obtained using the PI toxicity assay as $A\beta_{25-35}$ produced increases in PI uptake which were dose-dependently attenuated by EGb 761 (10–100 μg/mL, Fig. 1b). EGb 761 also protected against cell death induced by either $A\beta_{1-40}$ or $A\beta_{1-42}$ (Fig. 1c). Figure 2 summarizes the morphological features of exposure to vehicle, $A\beta_{25-35}$ and combination of EGb 761 (100 μg/mL) and $A\beta_{25-35}$.

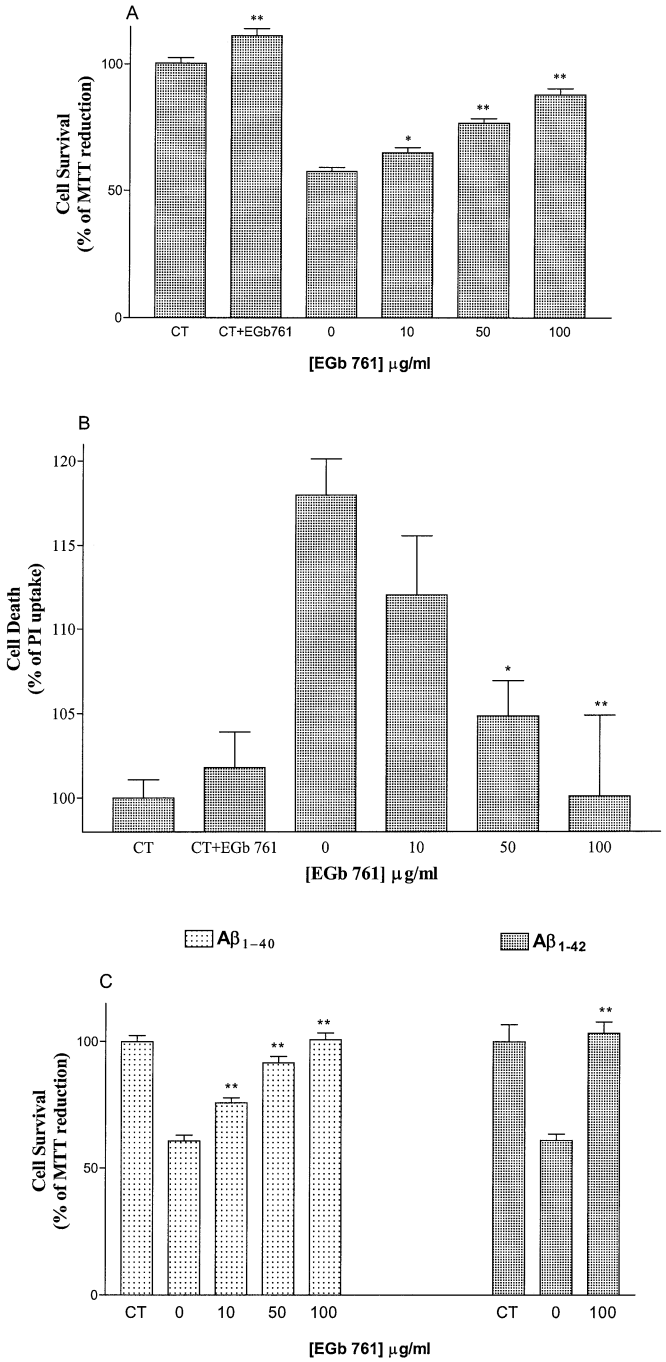

FIG. 1. Neuroprotective effect of EGb 761 against toxicity induced by a 24 h exposure to $A\beta_{25-35}$ (25 μM), as estimated by the MTT colorimetric (A) and PI fluorescence (B) assays, or to $A\beta_{1-40}$ (5 μM) and $A\beta_{1-42}$ (25 μM), as estimated by the MTT assay (C). Values represent mean ± SEM of at least three separate experiments, each performed in quadruplicate. $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle-treated groups.

Although PI fluorescence in control groups treated with EGb 761 (100 μg/mL) was not modified (102 ± 2 versus 100 ± 1; NS), MTT values were found to be slightly, but significantly, increased in control groups treated for 24 h with EGb 761 (100 μg/mL) compared with vehicle-treated control groups (111 ± 3 versus 100 ± 2, $P < 0.05$). However, a 24-h exposure to EGb 761 (100 μg/mL) in the initial media did not increase MTT values (104 ± 2 versus 100 ± 2; NS). These data suggest that EGb 761 has little or no effect,

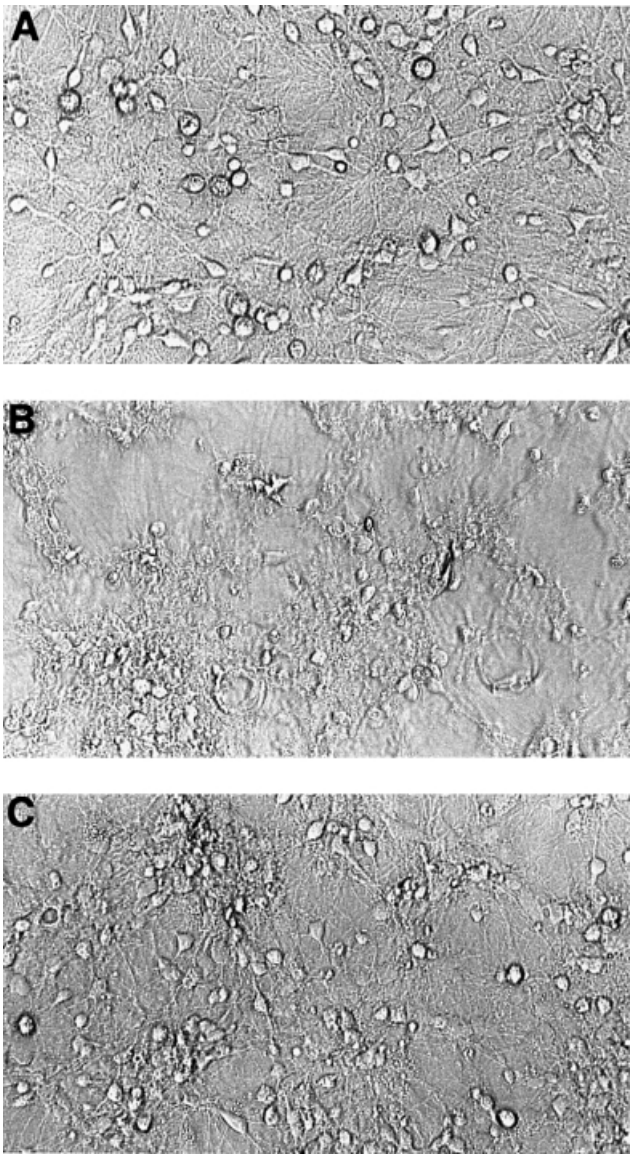

FIG. 2. Phase contrast photomicrographs showing mixed hippocampal cells exposed to vehicle (A), $A\beta_{25-35}$ (25 μM) (B), and to both EGb 761 (100 μg/mL) and $A\beta_{25-35}$ (25 μM) (C).

by itself, on cell division or neurite outgrowth, but that media changes and/or cell washes induce decreases in MTT values which are prevented in the presence of EGb 761.

We have investigated the possible differential effects of EGb 761 on pure neuronal and glial cell cultures that have been exposed to $A\beta_{25-35}$ (25 μM) for 24 and 48 h, respectively. The MTT assay revealed that the protective effects of EGb 761 tend to be more pronounced on glial than neuronal cells (Table 1).

We have also studied the effects of the major constituents of EGb 761 in regard to their respective amounts in the total extract. CP 205 (10–50 μg/mL), the flavonoid fraction of EGb 761, dose-dependently protected $A\beta_{25-35}$-induced toxicity, as measured 24 h later using MTT (Table 2), while the terpenoid constituents bilobalide (CP 160, 1–10 μg/mL) and ginkgolide B (BN 52021, 1–10 μg/mL) failed to display any protective effects (Table 2).

Interestingly, EGb 761 (100 μg/mL) is able to protect hippocampal cells from either $A\beta_{25-35}$ or $A\beta_{1-40}$-induced toxicity even if added up to 8 h post-Aβ treatments (Table 3). Similarly, EGb 761 (100 μg/mL) significantly attenuated PI uptake induced by $A\beta_{25-35}$ even if added up to 8 h $A\beta_{25-35}$ post-treatment (106 ± 6 versus 128 ± 2; $P < 0.01$), while PI fluorescence in control groups treated 8 h later with EGb 761 (100 μg/mL) was not modified (99 ± 2 versus 100 ± 2; NS).

TABLE 1. Effects of EGb 761 against toxicity induced by $A\beta_{25-35}$ in enriched neuronal and glial cell cultures from he rat, as estimated by the MTT assay

Treatment	MTT values (%)
Neuronal cells	
Control	100 ± 3
Control + EGb 761 (100 μg/mL)	105 ± 9
$A\beta_{25-35}$ (25 μM)	57 ± 5
$A\beta_{25-35}$ (25 μM) + EGb 761 (100 μg/mL)	84 ± 4*
Glial cells	
Control	100 ± 4
Control + EGb 761 (100 μg/mL)	107 ± 5
$A\beta_{25-35}$ (25 μM)	53 ± 4
$A\beta_{25-35}$ (25 μM) + EGb 761 (100 μg/mL)	111 ± 7*

Values represent mean ± SEM of at least three separate experiments, each performed in quadruplicate. *$P < 0.01$ compared with the group treated with $A\beta_{25-35}$ alone.

TABLE 2. Comparative effects of the flavonoid fraction (CP 205) and the terpenoid constituents (CP 160, BN 52021) of EGb 761 against toxicity induced by $A\beta_{25-35}$ in rat hippocampal cell cultures

Treatment	MTT values (%)
Control	100 ± 2
$A\beta_{25-35}$ (25 μM)	58 ± 2
$A\beta_{25-35}$ (25 μM) + CP 205 (10 μg/mL)	57 ± 3
$A\beta_{25-35}$ (25 μM) + CP 205 (25 μg/mL)	74 ± 3*
$A\beta_{25-35}$ (25 μM) + CP 205 (50 μg/mL)	75 ± 3*
$A\beta_{25-35}$ (25 μM) + CP 160 (1 μg/mL)	54 ± 2
$A\beta_{25-35}$ (25 μM) + CP 160 (5 μg/mL)	57 ± 2
$A\beta_{25-35}$ (25 μM) + CP 160 (10 μg/mL)	52 ± 2
$A\beta_{25-35}$ (25 μM) + BN 52021 (1 μg/mL)	55 ± 2
$A\beta_{25-35}$ (25 μM) + BN 52021 (5 μg/mL)	54 ± 2
$A\beta_{25-35}$ (25 μM) + BN 52021 (10 μg/mL)	50 ± 1

Values represent mean ± SEM of three separate experiments, each performed in quadruplicate. *$P < 0.01$ compared with groups treated with either $A\beta_{25-35}$ alone.

TABLE 3. Protective effects of EGb 761 (100 μg/mL) against toxicity induced by $A\beta_{25-35}$ or $A\beta_{1-40}$ in rat mixed hippocampal cell cultures

Treatment	MTT values (%)
Control	100 ± 2
Control + EGb 761 (4 h post-exposure)	105 ± 3
Control + EGb 761 (6 h post-exposure)	109 ± 3*
Control + EGb 761 (8 h post-exposure)	97 ± 2
$A\beta_{25-35}$ (25 μM)	64 ± 1
$A\beta_{25-35}$ (25 μM) + EGb 761 (4 h post-exposure)	98 ± 3**
$A\beta_{25-35}$ (25 μM) + EGb 761 (6 h post-exposure)	98 ± 6**
$A\beta_{25-35}$ (25 μM) + EGb 761 (8 h post-exposure)	97 ± 3**
$A\beta_{1-40}$ (5 μM)	61 ± 3
$A\beta_{1-40}$ (5 μM) + EGb 761 (4 h post-exposure)	110 ± 5†
$A\beta_{1-40}$ (5 μM) + EGb 761 (6 h post-exposure)	107 ± 4†
$A\beta_{1-40}$ (5 μM) + EGb 761 (8 h post-exposure)	97 ± 6†

Values represent mean ± SEM of at least three separate experiments, each performed in quadruplicate. *$P < 0.05$ control + EGb 761 (6 h post-exposure) versus vehicle-treated group, **$P < 0.05$ $A\beta_{25-35}$ + EGb 761 versus $A\beta_{25-35}$ alone, †$P < 0.05$ $A\beta_{1-40}$ + EGb 761 versus $A\beta_{1-40}$ alone.

Assessment of antioxidant activities of EGb 761

The antioxidant activities of EGb 761 were evaluated by assessing its ability to reduce levels of intracellular (DCF assay) and mitochondrial (DHR assay) ROS. As shown in Table 4, intracellular ROS (mainly peroxide) levels were found to be dose-dependently decreased when control groups were treated for 24 h with EGb 761 (10–100 μg/mL) compared with vehicle-treated control groups. Similarly, as measured by the DHR assay, EGb 761 (10–100 μg/mL) dose-dependently attenuated mitochondrial ROS (mainly peroxynitrite) levels, these effects being significant at the lowest concentration (Table 4).

TABLE 4. Comparison of the antioxidant activities of EGb 761 on intracellular (DCF assay) and mitochondrial ROS (DHR assay) levels in rat hippocampal cell cultures

Treatment	DCF fluorescence (%)	DHR123 fluorescence (%)
Control	100 ± 8	100 ± 2
Control + EGb 761 (10 μg/mL)	85 ± 6	88 ± 2*
Control + EGb 761 (50 μg/mL)	65 ± 10*	75 ± 2*
Control + EGb 761 (100 μg/mL)	49 ± 4*	64 ± 3*

Levels of DCF and DHR fluorescence were quantified 24 h and 30 min after exposure of cells to EGb (10–100 μg/mL), respectively. Values represent mean ± SEM of at least three separate experiments. $*P < 0.01$ compared with the group treated with control group.

Inhibitory effects of EGb 761 on ROS accumulation-induced Aβ peptides

Because hydrogen peroxide has been reported to mediate Aβ toxicity (Behl *et al.*, 1994), we studied the effects of EGb 761 on the intracellular peroxide as estimated by DCF assay. As previously reported in hippocampal cell cultures (Loo *et al.*, 1993; Barger *et al.*, 1995; Jordán *et al.*, 1997), a 24-h exposure to $A\beta_{25-35}$ resulted in significant increase ($*P < 0.001$) in DCF fluorescence (+51–67% above control values, Fig. 3a–d). These effects of $A\beta_{25-35}$ were strongly and dose-dependently inhibited by co-treatment with EGb 761 (10–100 μg/mL, Fig. 3a), as well as when EGb 761 (100 μg/mL) was added up to 8 h later (59 ± 7 versus 164 ± 11, $P < 0.01$). Similarly, EGb 761 was able to attenuate increase in levels of peroxide accumulation induced by $A\beta_{1-40}$ (62 ± 6 versus 123 ± 14, $P < 0.01$).

The flavonoid fraction CP 205 (10–50 μg/mL), but not the terpenoid constituents CP 160 (1–10 μg/mL) and BN 52021 (1–10 μg/mL), dose-dependently attenuated the vehicle-treated cells as well as $A\beta_{25-35}$-stimulated DCF fluorescence (Fig. 3b–d). Similar inhibitory effects were obtained with cells that were co-treated with CP 205 (25 μg/mL) and $A\beta_{1-40}$ (54 ± 3 versus 124 ± 14, $P < 0.01$).

Similarly, EGb (10–100 μg/mL) blocked the increase in DHR fluorescence induced by $A\beta_{25-35}$, these effects being significant at the lowest concentrations tested (Fig. 4).

Protective effects of EGb 761 against toxicity induced by H_2O_2

A 3-h exposure to H_2O_2 results in a concentration-dependent decrease in cell survival as indicated by the MTT assay (Fig. 5). As expected

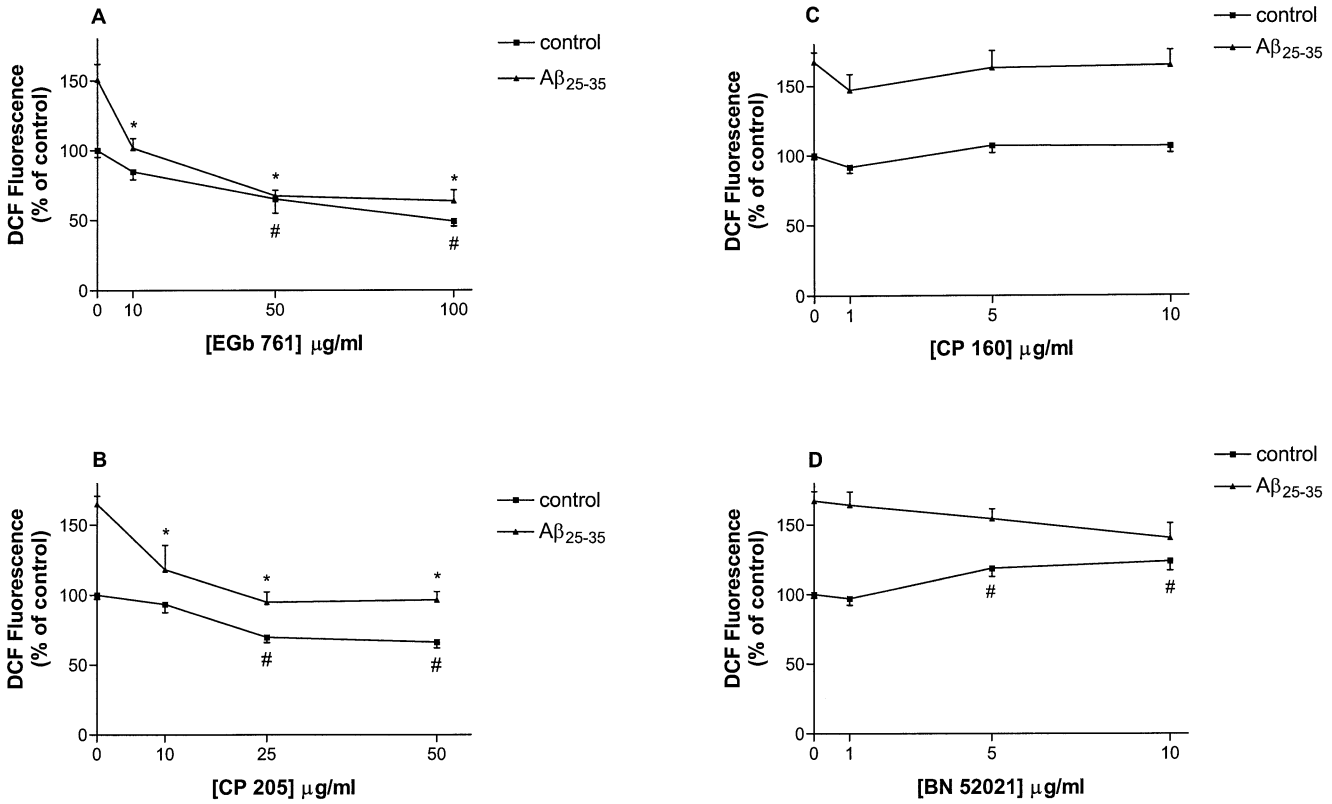

FIG. 3. Effects of EGb 761 (A), its flavonoid constituent CP 205 (B), and its terpenoid constituents CP 160 (C) and BN 52021 (D) on $A\beta_{25-35}$-induced oxidative stress in rat hippocampal mixed cell cultures. Drugs were applied at the onset of either $A\beta_{25-35}$ (25 μM) exposure or in vehicle-treated control group. Oxidative stress was determined 24 h after by quantification of DCF fluorescence as described in Materials and methods. Data represent mean ± SEM of at least three separate experiments. $\#P < 0.01$ compared with vehicle-treated groups. $*P < 0.01$ compared with groups treated with $A\beta_{25-35}$ alone.

on the basis of earlier data, the toxic effects of H_2O_2 were fully prevented by catalase (100 IU/mL), a well-known enzymatic antioxidant (data not shown). The toxicity induced by H_2O_2 (50–150 μM) was reduced, in a concentration-dependent manner, by a co-treatment with EGb 761 (25–100 μg/mL). The protective effect of EGb 761 was significant at 25 μg/mL and maximal at the highest concentration of EGb 761 tested here (100 μg/mL, Fig. 5). However, the flavonoid fraction CP 205 (25 μg/mL) failed to significantly protect against 50 μM H_2O_2-induced toxicity (66 ± 5 versus 62 ± 5, using MTT as index).

Effects of EGb 761 against $A\beta_{25-35}$-induced apoptosis

Staining with Hoechst 33343 revealed that a co-treatment with EGb 761 (10–100 μg/mL) inhibited apoptosis induced by $A\beta_{25-35}$ (Fig. 6). Interestingly, this inhibitory effect was still significant even if added up to 8 h later (Fig. 6).

Effects of various inhibitors against $A\beta_{25-35}$-induced cell death

As shown in Table 5, the selective inhibitors of lipoxygenase (nordihydroguaiaretic acid, 10 μM), protein kinase C (chelerythrine chloride, 1 μM) and phospholipase C (U-73122, 5 μM) as well as the L-type Ca^{2+}-channel blocker nitrendipine (50 μM) failed to protect against $A\beta_{25-35}$-induced toxicity.

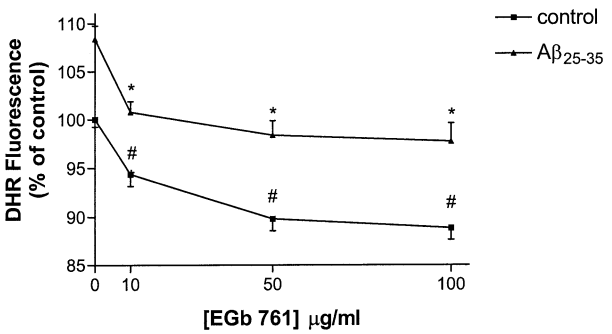

FIG. 4. Effects of EGb 761 on $A\beta_{25-35}$-induced mitochondrial peroxynitrite accumulation in rat hippocampal mixed cell cultures. Levels of DHR fluorescence were quantified 24 h later as described in Materials and methods. Data represent mean ± SEM of four separate experiments. #$P < 0.01$ compared with vehicle-treated groups. *$P < 0.01$ compared with groups treated with either $A\beta_{25-35}$ alone.

Discussion

The present study indicates that EGb 761 is able to completely protect, up to 8 h post-treatment, mixed hippocampal cells against Aβ peptides-induced toxicity. To our knowledge, this is the first demonstration of the protective activity of EGb 761 in cultured hippocampal cells. These data are of particular interest in the clinical context, given that the $A\beta_{1-40}$ and $A\beta_{1-42}$ fragments probably play a role in the neurodegenerative process occurring in AD (Roher *et al*., 1993; Harris *et al*., 1995) and the associated losses of hippocampal neurons (Hyman *et al*., 1984). It is our contention that EGb 761 can inhibit these events initiated by the production of Aβ peptides by blocking the production of free radicals and/or apoptotic events.

We have investigated the mechanism(s) of action by which EGb 761 exerts its effects. To date, there is limited agreement in the literature concerning the cellular pathways involved in Aβ-induced cell death. One the most popular theories, however, suggests that Aβ-neurotoxicity results from induction of free radicals, presumably initiated either directly by Aβ peptides themselves (Hensley *et al*., 1994), or indirectly through increases in intracellular production of reactive oxygen species (Goodman *et al*., 1994; Behl *et al*., 1994; Schubert *et al*., 1995; Barger *et al*., 1995; Bruce *et al*., 1996; Pike *et al*., 1997). We observed here that a co- and, more interestingly, post-treatment with EGb 761 was able to completely inhibit $A\beta_{25-35}$-induced ROS accumulation. Indeed, we found that an exposure to H_2O_2, an hydroxyl radical donor (Halliwell, 1992) that has been reported to mediate Aβ protein toxicity (Behl *et al*., 1994), resulted in cell death which is inhibited by EGb 761. Moreover, as demonstrated by DHR fluorescence, EGb 761 was able to decrease mitochondrial ROS, in particular peroxynitrite, which have been reported to play a important role in the apoptotic and necrotic processes (Dawson & Dawson, 1996). These data are consistent with earlier data suggesting that the antioxidant activities and/or the peroxynitrite/hydroxyl radical scavenging properties of EGb 761 may be involved in its neuroprotective actions (Marcocci *et al*., 1994a,b; Oyama *et al*., 1994, 1996; Behar-Cohen *et al*., 1996). However, three lines of evidence suggest that other properties of EGb 761 are involved in its protective effect against Aβ toxicity. Firstly, less potent effects were seen with the flavonoid fraction of the extract, which is responsible for the antioxidant properties of EGb 761 (Marcocci *et al*., 1994b). Secondly, the intensity of the DCF and DHR fluorescence signals produced by both $A\beta_{25-35}$ or $A\beta_{1-40}$ are weak. Thirdly, the flavonol quercetin

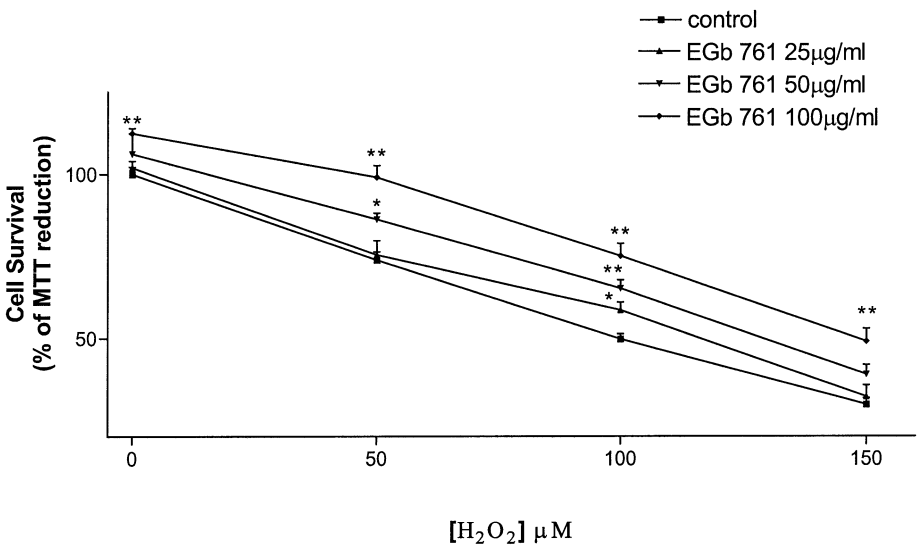

FIG. 5. Effects of a co-treatment with EGb 761 against toxicity induced by H_2O_2 (50–150 μM) in rat hippocampal mixed cell cultures. Concentration-dependent protective and rescuing effects of EGb 761 are observed against H_2O_2-induced toxicity. Cell survival was determined 3 h after exposure to drugs using the MTT assay. Values represent mean ± SEM of three separate experiments, each performed in quadruplicate. *$P < 0.05$, **$P < 0.01$ compared with groups treated with H_2O_2 alone.

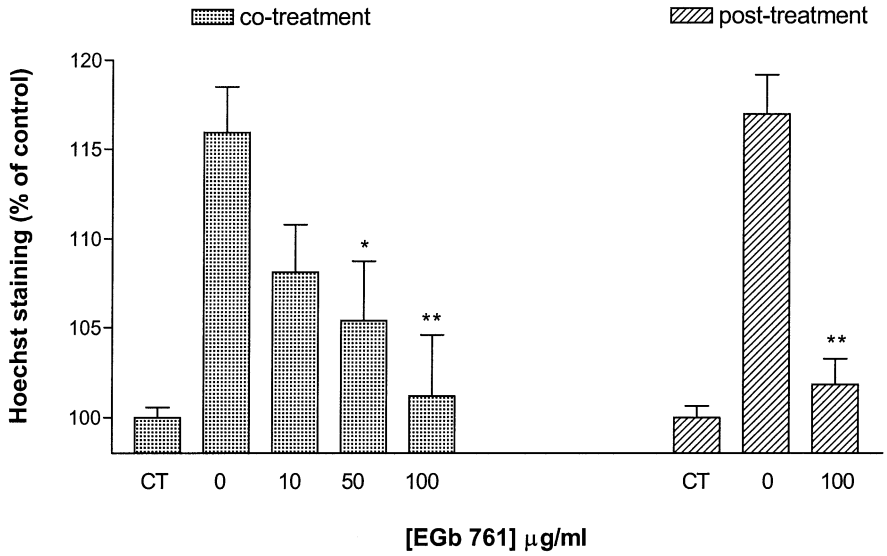

FIG. 6. Effects of EGb 761 on $A\beta_{25-35}$ (25 μM)-induced apoptosis in rat hippocampal mixed cell cultures. EGb 761 was applied concurrently with $A\beta_{25-35}$ or 8 h after a $A\beta_{25-35}$ exposure. The extent of apoptotic degeneration was quantified 24 h later using the fluorescent dye Hoechst 33343 as described in Materials and methods. Data represent mean ± SEM of at least three separate experiments, each performed in quadruplicate. $*P<0.05$, $**P<0.01$ compared with groups treated with $A\beta_{25-35}$ alone.

TABLE 5. Lack of effects of NDGA, nitrendipine, chelerythrine chloride and U-73122 against toxicity induced by $A\beta_{25-35}$ in rat hippocampal cell cultures

Treatment	MTT values (%)
Control	100 ± 1
$A\beta_{25-35}$ (25 μM)	67 ± 2
$A\beta_{25-35}$ (25 μM) + NDGA (10 μM)	65 ± 4
$A\beta_{25-35}$ (25 μM) + Nitrendipine (50 μM)	64 ± 2
$A\beta_{25-35}$ (25 μM) + Chelerythrine chloride (1 μM)	66 ± 2
$A\beta_{25-35}$ (25 μM) + U-73122 (5 μM)	74 ± 4

Values represent mean ± SEM of three separate experiments, each performed in quadruplicate.

potently inhibited ROS accumulation induced by $A\beta_{25-35}$ but failed to significantly protect hippocampal cells from $A\beta_{25-35}$-induced toxicity (data not shown). Taken together, these results suggest that, in addition to inhibiting ROS, the protective effect of EGb 761 results from at least two distinct mechanisms (e.g. apoptosis) acting in concert.

Our data and those of others (Didier *et al.*, 1996; Ni *et al.*, 1996) suggest that EGb 761 is able to attenuate $A\beta_{25-35}$-induced apoptosis in hippocampal neuronal cultures, a process that may be relevant to neurodegenerative events occurring in AD (Hyman *et al.*, 1984; Johnson, 1994). Interestingly, this effect was still evident even if EGb 761 was applied 8 h post-$A\beta_{25-35}$ treatment. $A\beta_{25-35}$ and H_2O_2 share the ability to induce apoptotic cell death in neuronal cultures, although the existence of a direct causal link has yet to be fully established (Loo *et al.*, 1993; Copani *et al.*, 1995; Whittemore *et al.*, 1995). We can speculate that the protective effect of EGb 761 on $A\beta_{25-35}$-induced apoptosis and on H_2O_2-induced toxicity could be, at least in part, causally related. This hypothesis is supported by the fact that EGb 761 can prevent hydroxyl radicals-induced apoptosis in cultured neurons (Ni *et al.*, 1996).

It has also been suggested that the neurotoxic action of Aβ peptides is associated to an elevation of $[Ca^{2+}]_i$ through their ability to stimulate free radical production (Goodman *et al.*, 1994; Barger *et al.*, 1995; Zhou *et al.*, 1996) in addition to modulate effectors involved in the phosphatidylinositol hydrolysis cascade including phospholipase C and arachidonic acid (Cowburn *et al.*, 1996). Activation of these effectors initiates a cascade of intracellular events that modulate the activity of protein kinases (Cowburn *et al.*, 1996). Therefore, we have investigated if the neuroprotective action of EGb 761 involves its ability to inhibit the elevation of $[Ca^{2+}]_i$ (Oyama *et al.*, 1993) and intracellular enzymes implicated in the elevation of $[Ca^{2+}]_i$, e.g. protein kinase C (Rogue & Malviya, 1996) and phospholipase C (Rodriguez de Turco *et al.*, 1993). We observed that neither nitrendipine, a L-type calcium channel blocker, chelerythrine chloride, a protein kinase C inhibitor, nor U-73122, a phospholipase C inhibitor, were able to protect against $A\beta_{25-35}$-induced toxicity. A lack of effect was also observed with nordihydroguaiaretic acid (NDGA), an antioxidant and selective lipoxygenase inhibitor with reported neuroprotective effects against Aβ toxicity (Goodman *et al.*, 1994). Taken together, these data suggest that the modulation of these intracellular signalling molecules are not directly involved in the neuroprotective actions of EGb 761 against Aβ toxicity.

EGb 761 is a standardized natural extract containing 24% flavonoids and 6% terpenoids including ginkgolides and bilobalides (Drieu, 1986). We have observed that the protective effects of EGb 761 partly involved its flavonoid fraction which is mainly responsible for the antioxidant and free radical scavenging properties of EGb 761 (Marcocci *et al.*, 1994b). These data are in agreement with the protective role of the flavonoid constituents of EGb 761 in models of oxidative neuronal injury (Oyama *et al.*, 1994; Ni *et al.*, 1996) and support the role of free radicals in mediating $A\beta_{25-35}$-induced toxicity. Besides the free radical theory, inflammatory events have been reported to occur in AD and others in neurodegenerative diseases (McGeer & McGeer, 1995). EGb 761 displays anti-inflammatory effects and protective actions against brain damage probably through its terpenoids ginkgolides (Panetta *et al.*, 1987; Oberpichler *et al.*, 1990). Indeed, the ginkgolide B (BN 52021) acts as an antagonist of platelet-activating factor (PAF, Panetta *et al.*, 1987), a factor that induces pro-inflammatory effects (McGeer & McGeer, 1995) and shares with Aβ peptides the ability to damage vascular tissues (McGeer & McGeer, 1995; Thomas *et al.* 1996). However, we have found that neither the ginkgolide B (BN 52021) nor bilobalide (CP 160) displayed any protective effects suggesting that the PAF antagonistic properties of EGb 761 are not involved in the neurodegenerative processes induced by Aβ peptides.

In summary, we observed that EGb 761 is able to completely protect rat hippocampal primary cells against Aβ peptides and H_2O_2-induced toxicity and apoptosis. These effects are not fully attributable to the antioxidant properties of EGb 761 and may depend upon the polyvalent action of its constituents which include flavonoids. The

protective/rescuing properties of EGb 761 support the therapeutic usefulness of EGb 761 in treating aged-related central nervous system disorders, e.g. AD (Hofferberth, 1994; Le Bars *et al*., 1997; Maurer *et al*., 1997; Oken *et al*., 1998), and are particularly exciting considering the limited clinical arsenal available to persons suffering from these diseases.

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Abbreviations

Aβ, β-amyloid; AD, Alzheimer's disease; DCF, 2′,7′-dichlorofluorescein; DHR, dihydrorhodamine; DMEM, Dulbecco's modified Eagles medium; EGb 761, Gingko biloba extract; FBS, foetal bovine serum; HEPES, *N*-2-[hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NDGA, nordihydroguaiaretic acid; PAF, platelet-activating factor; PI, propidium iodide; ROS, reactive oxygen species.

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