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Inhibition of serum deprivation- and staurosporine-induced neuronal apoptosis by *Ginkgo biloba* extract and some of its constituents

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Abstract

Previous studies have already demonstrated that some constituents of an extract of *Ginkgo biloba* (EGb), such as ginkgolide B and bilobalide, protect cultured neurons from hypoxia- and glutamate-induced damage. This prompted us to investigate whether they were also able to inhibit neuronal apoptosis. We induced apoptosis in cultured chick embryonic neurons as well as in mixed cultures of neurons and astrocytes from neonatal rat hippocampus by serum deprivation and staurosporine. The increase in the percentage of apoptotic chick neurons from 12% in controls to 30% after 24 h of serum deprivation was reduced to control level by EGb (10 mg/l), ginkgolide B (10 μM), ginkgolide J (100 μM) and bilobalide (1 μM). After treatment with staurosporine (200 nM) for 24 h we observed 74% apoptotic chick neurons. This percentage of apoptotic neurons was reduced to 24%, 62% and 31% in the presence of EGb (100 mg/l), ginkgolide J (100 μM) and ginkgolide B (10 μM), respectively. Bilobalide (10 μM) decreased apoptotic damage induced by staurosporine treatment for 12 h nearly to the control level. In mixed neuronal/glial cultures, the extract of EGb (100 mg/l) and bilobalide (100 μM) rescued rat neurons from apoptosis caused by serum deprivation, whereas, bilobalide (100 μM) and ginkgolide B (100 μM) reduced staurosporine-induced apoptotic damage. Ginkgolide A revealed no anti-apoptotic effect in either serum-deprived or staurosporine-treated neurons. Our results suggest that EGb and some of its constituents possess anti-apoptotic capacity and that bilobalide is the most potent constituent.

Keywords: *Ginkgo biloba* extract; Neurons; Apoptosis; Bilobalide; Ginkgolide B; Ginkgolide J; Staurosporine; Serum withdrawal

1. Introduction

Apoptosis is being increasingly recognized as a prominent event in nervous system development (Oppenheim, 1991), but it is also associated with cerebral ischemia and several neurodegenerative diseases including Alzheimer's and Parkinson's disease (Thompson, 1995). Therefore, the inhibition of the apoptotic component of neuronal death is a new therapeutic strategy.

Extract of the leaves of *Ginkgo biloba* (EGb) was introduced in Europe in 1965 for the treatment of peripheral arterial occlusive disease and dementia in older patients (De Veudis, 1991; Kleijnen and Knipschild, 1992). EGb is a complex mixture containing 24% flavonoid glycosides, 6% terpene lactones, such as the ginkgolides A, B, C, J and bilobalide (also defined as the non-flavone fraction), some organic acids, and other various constituents (De Veudis, 1991). The early experiments of Oberpichler et al. (1988) showed that the non-flavone fraction, and not the flavonoid glycosides, protected brain tissue against hypoxic damage. This was further confirmed by studies of Krieglstein et al. (1995), who demonstrated that ginkgolide A, B and bilobalide reduced the infarct volume after focal ischemia in mice and rats and that ginkgolide B and bilobalide reduced the number of damaged neurons in culture after glutamate excitotoxicity and hypoxia. While these studies did not discriminate between necrotic and apoptotic cell death, Didier et al. (1996) showed an anti-apoptotic effect of EGb after olfactory nerve sectioning. Therefore, it was the aim of the present study to find out whether EGb and its constituents can protect neurons from apoptotic damage.

2. Material and methods

2.1. Chemicals

Minimal essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), Leibovitz's L15 medium,

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antibiotics, NUSERUM™ (25% fetal bovine serum, 75% supplements), fetal bovine serum, papain and supplement B27 were purchased from Life Technologies, Germany. Staurosporine, poly-L-lysine (MW 30 000–70 000), poly-L-lysine (MW 70 000–100 000), trypsin inhibitor, bovine serum albumin and dimethylsulfoxide (DMSO) were from Sigma–Aldrich, Germany. The Apop Tag Kit from Oncor Appligene, Gaithersburg, MD, USA, was used for terminal-deoxyribonucleotidyl transferase-mediated biotin–16-ddUTP nick-end labeling (TUNEL) staining. LI 1370 (a standardized EGb), the ginkgolides and bilobalide were gifts from Lichtwer Pharma (Berlin, Germany). EGb, ginkgolide A, B, and J and bilobalide were initially dissolved in pure DMSO and then diluted with culture medium to the final concentration, which was 0.1% in all experiments.

2.2. Cell cultures

Primary neuronal cultures were derived from 7-day-old chick embryo telencephalons as previously described by Pettmann et al. (1979). Briefly, the tissue was mechanically dissociated through nylon meshes of 48-μm mesh width and the resulting cell suspension was seeded at a density of 4×10^4 cells/cm^2 into poly-L-lysine (MW 70 000–100 000)-coated culture flasks. The cells were cultured in DMEM with 20% fetal bovine serum for 5 days before experiments. Mixed cultures of neurons and astrocytes were prepared by removing the hippocampi from neonatal Fischer 344 rats (P1–P2). Brain tissue was dissected and incubated for 20 min in Leibovitz's L15 medium supplemented with 1 mg/ml papain and 0.2 mg/ml bovine serum albumin. Thereafter, the cell suspension was layered onto growth medium containing 1% trypsin inhibitor plus 10% bovine serum albumin. After centrifugation at 600 rpm for 10 min, the pellet was resuspended and seeded into poly-L-lysine (MW 30 000–700 000)-coated Petri dishes. After 2 days, cultures were treated with cytosine-β-arabinofuranoside (1 μM) for 24 h to inhibit glial proliferation. The cells were cultured in MEM with 20% NUSERUM™ and supplement B27 for 12 days before experiments. The animal experiments were approved by the government ethics committee.

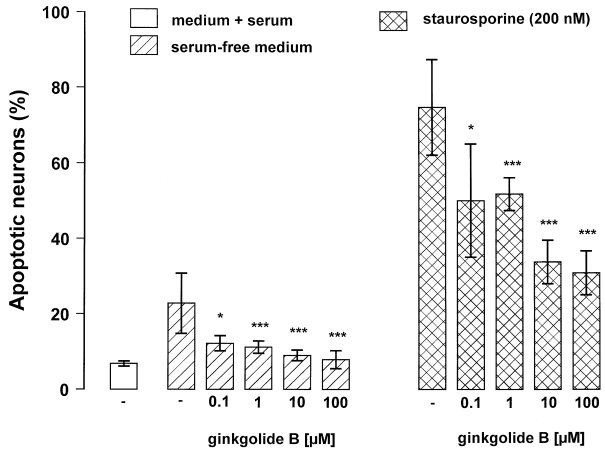

Fig. 2. Protective effect of ginkgolide B against apoptosis induced by serum deprivation and staurosporine. Chick neurons were incubated for 24 h in a serum-containing medium, serum-free medium or staurosporine (200 nM)-containing serum-free medium. The percentage of apoptotic neurons was evaluated by nuclear staining with Hoechst 33258. Values are means ± S.D. of six experiments. Different from the corresponding vehicle-treated cultures $^{*}P < 0.05$; $^{***}P < 0.001$ (ANOVA-1 with post-hoc Scheffé's test).

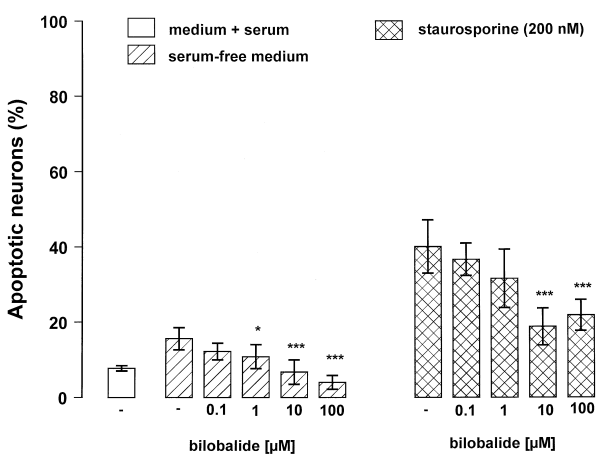

Fig. 1. Protective effect of *G. biloba* extract (EGb) against apoptosis induced by serum deprivation and staurosporine. Chick neurons were incubated for 24 h in serum-containing medium, serum-free medium or staurosporine (200 nM)-containing serum-free medium. The percentage of apoptotic neurons was evaluated by nuclear staining with Hoechst 33258. Values are means ± S.D. of six experiments. Different from the corresponding vehicle-treated cultures $^{*}P < 0.05$; $^{***}P < 0.001$ (ANOVA-1 with post-hoc Scheffé's test).

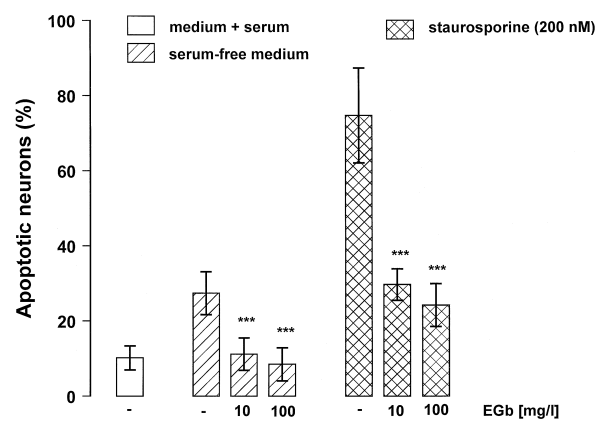

Fig. 3. Protective effect of bilobalide against apoptosis induced by serum deprivation and staurosporine. Chick neurons were incubated for 12 h in a serum-containing medium, serum-free medium or staurosporine (200 nM)-containing serum-free medium. The percentage of apoptotic neurons was evaluated by nuclear staining with Hoechst 33258. Values are means ± S.D. of six experiments. Different from the corresponding vehicle-treated cultures $^{***}P < 0.001$ (ANOVA-1 with post-hoc Scheffé's test).

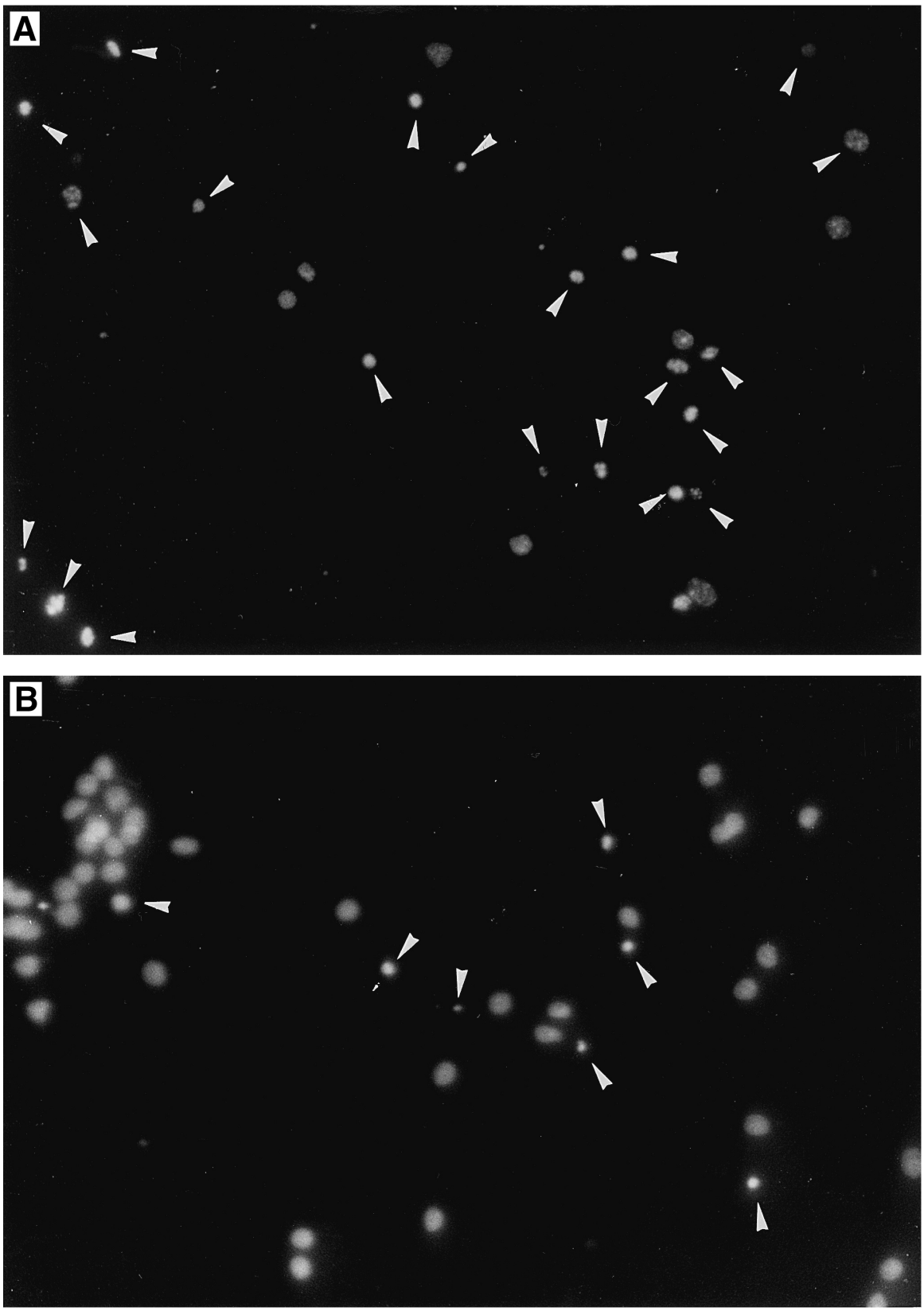

Fig. 4. Photomicrographs of chick embryonic neurons stained with the fluorescent nuclear dye Hoechst 33258 after 24 h of incubation with staurosporine (A) or staurosporine plus 10 μM ginkgolide B (B). White arrows indicate apoptotic cells.

Table 1
Neuroprotective effect of *G. biloba* extract (EGb) against apoptosis in chick embryonic neurons

Induction of apoptosis	EGb (mg/l)	Neuronal viability (%)
Medium + serum	–	76.0 ± 5.0
	0.1	78.2 ± 1.9
	1	81.6 ± 7.8
	10	88.2 ± 2.9[b]
	100	87.5 ± 6.1[a]
Serum-free medium	–	64.9 ± 9.0
	0.1	67.8 ± 5.4
	1	70.9 ± 4.9
	10	73.0 ± 2.6
	100	77.7 ± 4.1[a]
Staurosporine	–	16.9 ± 3.0
	0.1	22.7 ± 3.6
	1	20.7 ± 2.0
	10	28.9 ± 2.5[a]
	100	40.7 ± 1.9[c]

Cells were incubated for 24 h in serum-containing medium, serum-free medium or staurosporine (200 nM)-containing serum-free medium in the absence and presence of EGb.
Neuronal viability was determined by trypan blue exclusion.
Mean values ± S.D. of eight experiments are given.
Different from the corresponding vehicle-treated cultures: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ (ANOVA-1 with post-hoc Scheffé's test).

2.3. Drug treatment

Apoptosis was induced in chick embryonic neurons by serum deprivation and by treatment with 200 nM staurosporine in serum-free medium and in mixed neuronal/glial cultures from rat hippocampus by incubation in

Table 2
Neuroprotective effect of bilobalide and ginkgolide B against apoptosis in chick embryonic neurons

Induction of apoptosis	Drug concentration (μM)	Neuronal viability (%)	
		Bilobalide	Ginkgolide B
Medium + serum	–	88.6 ± 2.3	89.2 ± 2.7
	0.1	89.1 ± 2.4	87.7 ± 2.1
	1	91.4 ± 1.0	90.0 ± 1.7
	10	90.8 ± 1.9	91.4 ± 2.3
	100	93.4 ± 1.7	90.2 ± 2.9
Serum-free medium	–	70.3 ± 8.2	81.5 ± 5.6
	0.1	87.1 ± 2.9[c]	83.4 ± 5.7
	1	86.9 ± 4.2[c]	89.5 ± 3.2[a]
	10	89.7 ± 5.0[c]	91.4 ± 2.2[c]
	100	89.3 ± 2.9[c]	91.8 ± 2.3[c]
Staurosporine	–	31.4 ± 8.4	23.6 ± 2.0
	0.1	69.6 ± 5.7[c]	30.7 ± 6.2
	1	75.0 ± 4.1[c]	37.3 ± 8.9[b]
	10	83.1 ± 4.4[c]	47.4 ± 7.8[c]
	100	79.1 ± 6.9[c]	41.9 ± 8.0[b]

Cells were incubated for 12 h and 24 h in serum-containing medium, serum-free medium or staurosporine (200 nM)-containing serum-free medium in the absence and presence of bilobalide and ginkgolide B, respectively.
Neuronal viability was determined by trypan blue exclusion.
Mean values ± S.D. of eight experiments are given.
Different from the corresponding vehicle-treated cultures: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ (ANOVA-1 with post-hoc Scheffé's test).

Table 3
Neuroprotective effect of ginkgolide J against apoptosis in chick embryonic neurons

Induction of apoptosis	Ginkgolide J (μM)	Neuronal viability (%)	Apoptotic neurons (%)
Medium + serum	–	85.5 ± 6.9	9.7 ± 4.3
	1	78.2 ± 6.6	
	10	86.3 ± 3.4	
	100	91.2 ± 6.6	7.3 ± 1.7
Serum-free medium	–	72.5 ± 5.2	16.2 ± 5.0
	1	80.6 ± 4.8	11.5 ± 1.1
	10	85.7 ± 4.7[c]	11.1 ± 4.6
	100	87.9 ± 2.9[c]	7.9 ± 5.4[a]
Staurosporine	–	21.8 ± 4.0	74.7 ± 4.3
	100	57.4 ± 6.5[c]	61.9 ± 11.6[a]

Cells were incubated for 24 h in serum-containing medium, serum-free medium or staurosporine (200 nM)-containing serum-free medium in the absence and presence of ginkgolide J.
Neuronal viability and the percentage of apoptotic neurons were determined by trypan blue exclusion and by nuclear staining with Hoechst 33258, respectively.
Mean values ± S.D. of eight experiments are given.
Different from the corresponding vehicle-treated cultures: [a]$P < 0.05$, [c]$P < 0.001$ (ANOVA-1 with post-hoc Scheffé's test).

Table 4
Neuroprotective effect of EGb, ginkgolide B and bilobalide against apoptosis in rat hippocampal neurons

	TUNEL-positive rat neurons (%)
Controls 24 h	3.1 ± 3.5
Serum-free 24 h	14.9 ± 3.9[a]
Serum-free + 100 mg/l EGb 24 h	7.3 ± 4.2[a]
Serum-free + 100 μM ginkgolide B 24 h	9.8 ± 3.2
Serum-free + 100 μM ginkgolide J 24 h	9.7 ± 2.3
Staurosporine 24 h	64.3 ± 6.0[c]
Staurosporine + 100 μM ginkgolide J 24 h	51.4 ± 5.3[c]
Staurosporine + 100 μM ginkgolide B 24 h	42.5 ± 4.6[c]
Controls 24 h	13.1 ± 5.3
Staurosporine 24 h	64.3 ± 6.0
Staurosporine + 100 mg/l EGb 24	62.7 ± 11.5
Controls 12 h	15.6 ± 9.5
Serum-free 12 h	37.3 ± 7.4[c]
Serum-free + 100 μM bilobalide 12 h	20.7 ± 5.9[c]
Staurosporine 12 h	57.6 ± 3.4[c]
Staurosporine + 100 μM bilobalide 12 h	17.7 ± 7.8[c]

Apoptosis in mixed neuronal/glial cultures prepared from neonatal rat hippocampus was induced by serum deprivation and staurosporine (300 nM in serum-free medium).
Neuronal damage was evaluated by the TUNEL method.
Values are given as means ± S.D. of six experiments.
Significant differences with [a]$P < 0.05$, [c]$P < 0.001$ (Student's *t*-test).

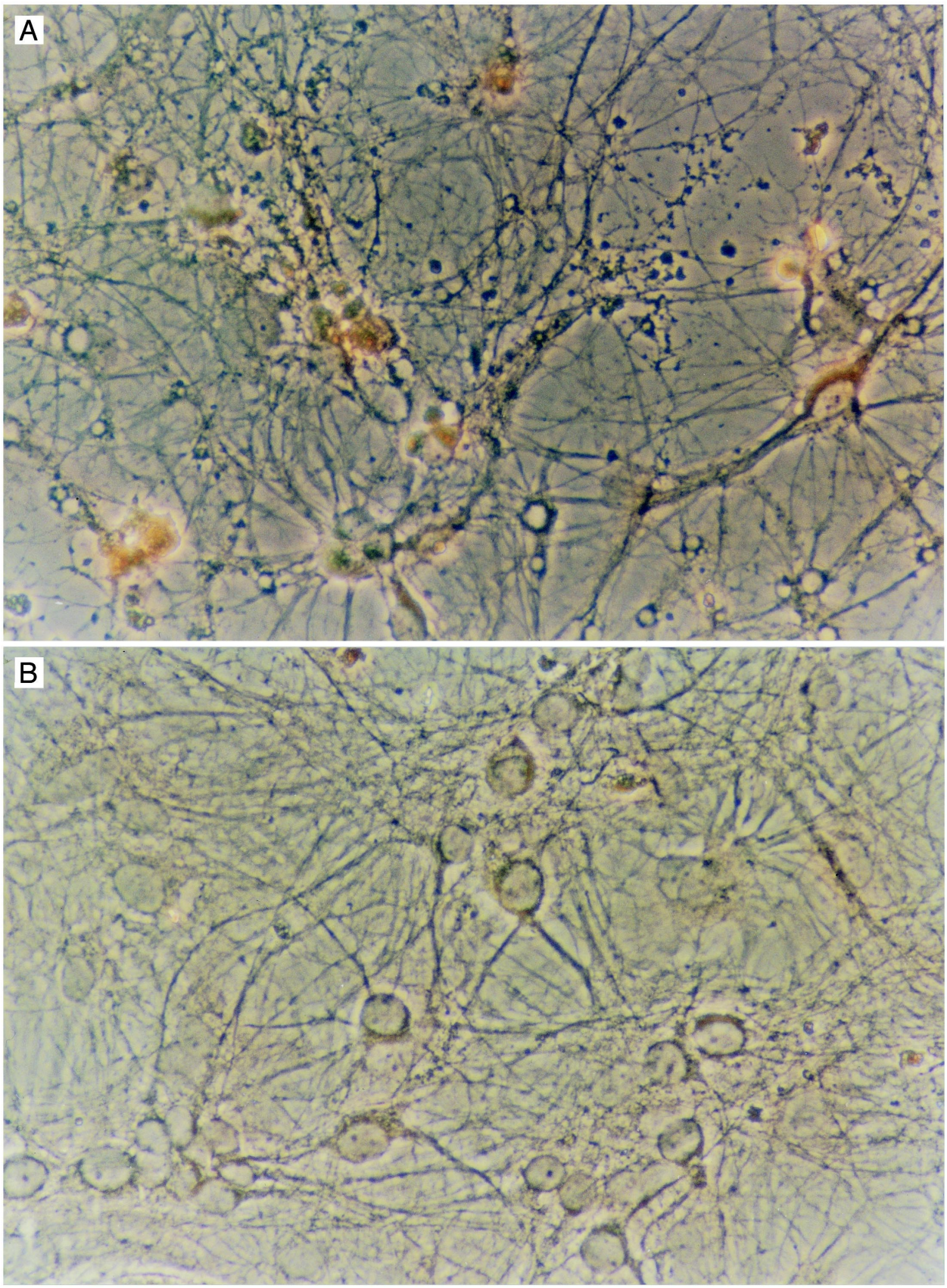

Fig. 5. Photomicrographs of mixed cultures of neurons and astrocytes after 12 h of incubation with staurosporine (A) or staurosporine plus 100 μM bilobalide (B). Brown color indicates TUNEL-positive neurons.

serum-free medium without supplement B27 and by treatment with 300 nM staurosporine in serum-free medium without supplement B27. Drugs were added simultaneously with the induction of apoptosis. The experiments were performed for 24 h, except for the study with bilobalide. Bilobalide had to be re-added every 3 h because of its short half-life time. Because bilobalide was maximally soluble at 0.25 M, the experimental time was limited to 12 h—otherwise the DMSO concentrations would have exceeded 0.1%.

2.4. Cell viability

The viability of the neurons was determined by the trypan blue exclusion method. The percentage of viable cells was calculated by counting the stained (non-viable) and the unstained (viable) cells.

2.5. TUNEL staining

Rat hippocampal neurons with damaged DNA were detected by TUNEL staining. Nick-end labeling of biotin–ddUTP by using terminal transferase was performed in methanol-fixed cell monolayers for 1 h at 37°C. After the enzyme reaction was blocked, digoxigenin antibodies coupled to peroxidase were added and labeling was visualized by using hydrogen peroxide and diaminobenzidine. TUNEL-positive cells were brown (Fig. 5). In the negative control, lacking the terminal transferase, we found no labeled neurons (data not shown). The results are expressed as the percentage of TUNEL-positive neurons.

2.6. Nuclear staining with Hoechst 33258

Chick embryonic neurons were incubated for 15 min with 10 μg/ml Hoechst 33258 in methanol. Thereafter, the cells were washed with methanol and phosphate-buffered saline and were observed under a fluorescence microscope. Cells with a small nucleus, a high fluorescence intensity (due to chromatin condensation) or nuclear fragmentation were considered as apoptotic neurons.

3. Results

In primary cultures of chick embryonic neurons, serum deprivation or treatment with staurosporine (200 nM) reduced cellular viability and increased the percentage of apoptotic cells compared to that of control cultures. Staurosporine treatment was always performed in serum-free medium (Wiesner and Dawson, 1996; Prehn et al., 1997), because serum protected the neurons against staurosporine-induced apoptosis. For example, we determined that $36 \pm 6\%$ and $76 \pm 5\%$ of the neurons ($n = 8$) were apoptotic when the cells were incubated for 24 h with 200 nM staurosporine in the presence and absence of serum, respectively. For comparison, we found in the same series of experiments after 24 h of incubation in serum-containing and serum-free medium $17 \pm 6\%$ and $33 \pm 4\%$ apoptotic neurons ($n = 8$), respectively. Therefore, we used serum deprivation and treatment with staurosporine in serum-free medium as two ways to induce apoptosis to a lesser and greater extent. EGb, ginkgolide B, ginkgolide J and bilobalide were able to reduce apoptotic damage under both damaging conditions (Figs. 1–4, Tables 1–3). Ginkgolide A failed to protect chick embryonic neurons after both serum deprivation and staurosporine treatment (data not shown).

In mixed cultures of neurons and astrocytes from neonatal rat hippocampus we also found an increase in the percentage of TUNEL-positive neurons after incubation of the cells in serum-free medium without supplement B27 as well as after staurosporine treatment compared to the percentage in control cultures. EGb reduced the percentage of TUNEL-positive neurons after incubation in serum-free medium without supplement B27 (Table 4), but not after staurosporine treatment. Ginkgolide B reduced staurosporine-induced, but not serum deprivation-induced apoptotic damage (Table 4). Bilobalide protected rat hippocampal neurons after incubation in serum-free medium without supplement B27 as well as after staurosporine treatment (Fig. 5, Table 4). Ginkgolide A (data not shown) and ginkgolide J had no effect (Table 4).

4. Discussion

Previous work already demonstrated the neuroprotective potency of EGb and its constituents ginkgolide A, ginkgolide B, and bilobalide in different in vivo and in vitro models (Ahlemeyer and Krieglstein, 1998). Because these studies did not discriminate between necrotic and apoptotic cell death, we attempted to find out whether EGb and the constituents ginkgolides A, B, J and bilobalide were able to inhibit neuronal apoptosis. We found that EGb, ginkgolides B and J and bilobalide had anti-apoptotic effects. Their ability to inhibit apoptosis seemed to depend on the species and on the age of the animal from which the neurons were derived and on the model used for the induction of apoptosis. For example, ginkgolide J reduced apoptotic damage of chick embryonic neurons, but not of neurons derived from neonatal rat hippocampus. In addition, in mixed neuronal/glial cultures from neonatal rat hippocampus ginkgolide B had protective effects after staurosporine treatment, but not after serum deprivation. Furthermore, we found differences in the anti-apoptotic potency of EGb and its constituents.

Our results are consistent with the anti-apoptotic effect of EGb described by Didier et al. (1996). In their study, sectioning of the olfactory nerve induced apoptosis in adult rats (evaluated by measuring the thickness of neurites and by DNA fragmentation) and the apoptotic rate was reduced

in EGb (100 mg/kg)-pretreated animals. The authors suggested that the antioxidant property of EGb was responsible for the inhibition of apoptosis. However, our study evaluated for the first time the anti-apoptotic effect of different constituents of EGb.

Although the mechanisms sustaining the neuroprotective effect by EGb are not fully clarified, effects on cerebral blood flow, on cerebral glucose and lipid metabolism and on neurotransmitter systems have been discussed (Ahlemeyer and Krieglstein, 1998). In our study, we induced apoptosis by serum deprivation or addition of staurosporine. Both treatments lead to an increase in the production of reactive oxygen species (Barroso et al., 1997; Prehn et al., 1997), an effect which can be blocked by antioxidants (Prehn et al., 1997). Reactive oxygen species play a dominant role in neuronal apoptosis (Coyle and Puttfarcken, 1993; Olanow, 1993), and it has been shown that the overexpression of endogenous antioxidant systems, such as superoxide dismutase (Greenlund et al., 1995) or the protooncogene bcl-2 (Hockenberry et al., 1993; Kane et al., 1993), prevents apoptotic cell death. Therefore, the anti-apoptotic action of EGb may be mediated by its radical scavenging capacity (Droy-Lefaix, 1997). The antioxidant capacity of EGb has been related to different constituents. For example, the flavonoid glycosides have been shown to scavenge hydroxyl radicals (Husain et al., 1987), superoxide anions (Robak and Gryglewski, 1988) and lipid peroxides (Deby et al., 1993), whereas, the terpene lactones preferentially scavenge superoxide anions (Marcocci et al., 1994). However, it has been recently published that ginkgolide B, ginkgolide J and bilobalide, but not ginkgolide A, react with the superoxide anion (Scholtyssek et al., 1997). This is in parallel with our finding of an anti-apoptotic effect in chick neurons of the ginkgolides B, J and bilobalide, but not ginkgolide A.

Processes downstream of the antioxidant effect of EGb may be also involved in the inhibition of apoptosis. Suppression of the production of reactive oxygen species reduces lipid peroxidation and membrane viscosity and enhances membrane fluidity (Heron et al., 1980). During the executive phase of apoptosis the distribution of membrane lipids changes and especially that of phosphatidylserine, which moves from the inner membrane leaflet to the outer cell surface. This process serves as a recognition signal for phagocytes (Reutlingsperger and van Heerde, 1997) and can be blocked by EGb. In addition, mitochondrial dysfunction due to oxidative stress—a key event during neuronal apoptosis—has also been reported to be suppressed by EGb (Sastre et al., 1998).

Ginkgolide B may act not only as a radical scavenger, but also as a PAF antagonist (Nunez et al., 1986; Touvay et al., 1986; Panetta et al., 1987), thereby suppressing the PAF-induced generation of reactive oxygen species (Bazan et al., 1991). Furthermore, EGb increases the availability of acetylcholine in brain tissue (Kristofikova and Klaschka, 1997). Acetylcholine plays a crucial role in memory and learning processes, and a loss of basal forebrain cholinergic neurons is directly related to apoptosis-associated Alzheimer's disease (Bartus et al., 1982).

According to studies on the bioavailability of the constituents of EGb in humans (Fourtillan et al., 1995), a single oral dose of 120 mg EGb leads to a blood concentration of 0.56 μM ginkgolide A, 0.44 μM ginkgolide B and 1.61 μM bilobalide. To our knowledge, there is no information about the concentrations of the constituents of EGb in brain tissue after oral administration of EGb to humans. Because the blood levels of the constituents of EGb are in the same range as those that have anti-apoptotic effects and because lipophilic drugs accumulate in brain tissue after passing through the blood–brain barrier, our data might have a relevance for human therapy.

In summary, our data showed that EGb and some of its constituents protected cultured neurons from apoptotic death induced by serum deprivation and treatment with staurosporine and that bilobalide was the most effective constituent. Further studies are needed to clarify the mechanism of action of each of these constituents.

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