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Ginkgolic acids induce neuronal death and activate protein phosphatase type-2C

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Abstract

The standardized extract from *Ginkgo biloba* (EGb 761) is used for the treatment of dementia. Because of allergenic and genotoxic effects, ginkgolic acids are restricted in EGb 761 to 5 ppm. The question arises whether ginkgolic acids also have neurotoxic effects. In the present study, ginkgolic acids caused death of cultured chick embryonic neurons in a concentration-dependent manner, in the presence and in the absence of serum. Ginkgolic acids-induced death showed features of apoptosis as we observed chromatin condensation, shrinkage of the nucleus and reduction of the damage by the protein synthesis inhibitor cycloheximide, demonstrating an active type of cell death. However, DNA fragmentation detected by the terminal-transferase-mediated ddUTP-digoxigenin nick-end labeling (TUNEL) assay and caspase-3 activation, which are also considered as hallmarks of apoptosis, were not seen after treatment with 150 μM ginkgolic acids in serum-free medium, a dose which increased the percentage of neurons with chromatin condensation and shrunken nuclei to 88% compared with 25% in serum-deprived, vehicle-treated controls. This suggests that ginkgolic acid-induced death showed signs of apoptosis as well as of necrosis. Ginkgolic acids specifically increased the activity of protein phosphatase type-2C, whereas other protein phosphatases such as protein phosphatases 1A, 2A and 2B, tyrosine phosphatase, and unspecific acid- and alkaline phosphatases were inhibited or remained unchanged, suggesting protein phosphatase 2C to play a role in the neurotoxic effect mediated by ginkgolic acids.

Keywords: *Ginkgo biloba* extract; EGb 761; 6-Alkyl salicylic acid; Fatty acid; Caspase-3; TUNEL

1. Introduction

EGb 761, a standardized extract from the leaves of *Ginkgo biloba*, has already been shown to exert neuroprotection in several in vitro and in vivo studies (reviewed by Ahlemeyer and Krieglstein, 1998). In addition, clinical studies showed the efficacy of an oral treatment with EGb 761 in human patients with dementia (Kanowski et al., 1996; Le Bars et al., 1997). EGb 761 is a complex mixture containing 24% flavonoid glycosides, 6% terpene lactones, some organic acids, and various other constituents (De Feudis, 1991). The neuroprotective effect of EGb 761 is suggested to be mediated by the terpene lactones, e.g. by ginkgolides and bilobalide (Bruno et al., 1993; Krieglstein et al., 1995; Klein et al., 1997; Rapin et al., 1998; Ahlemeyer et al., 1999). In contrast, ginkgolic acids and related alkylphenols have been reported to cause allergic skin inflammation (Hausen, 1998). Because of this and other undesired side effects (Siegers, 1999; Koch et al., 2000; Westendorf and Regan, 2000), the maximum level of ginkgolic acids in EGb 761 was restricted to an amount of 5 ppm. Since EGb 761 is used for human therapy of dementia, it is of interest to know whether these compounds also have neurotoxic effects, which may counteract neuroprotection mediated by the other constituents.

Protein phosphatase type-2C belongs to the class of protein serine/threonine-phosphatases. It is characterized by requiring Mg^{2+}-ions for activity (McGowan and Cohen, 1988). The cytosolic protein phosphatases 2Cα and 2Cβ isoenzmyes are ubiquitously expressed as monomeric proteins. Oxidizable unsaturated fatty acids, with a minimum chain length of 15 C atoms and a free negatively

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charged group, have been shown to activate protein phosphatase 2C and to induce neuronal apoptosis (Klumpp et al., 2001). As ginkgolic acids are structurally very similar, we determined whether these alkylphenols induce apoptosis in primary cultures of chick embryonic neurons and whether they specifically activate protein phosphatase 2C.

2. Material and methods

2.1. Chemicals

Dulbecco's modified Eagle medium (DMEM), antibiotics and fetal bovine serum were purchased from Life Technologies, Germany. Cycloheximide, staurosporine, poly-L-lysine, Hoechst 33258, bovine serum albumin, casein, alkaline phosphatase (P7923), acid phosphatase (P0157), fluorimetric caspase-3 assay kit and dimethylsulfoxide (DMSO) were from Sigma-Aldrich, Germany. Protein phosphatases 2A (14–111) and 2B (14–104) were from Upstate Biotechnology, Germany, tyrosine phosphatase (539446) and protein phosphatase 1 (539555) from Calbiochem, Germany. Apotag Kit from Oncor, USA was used for TUNEL-staining. The bicincolinic acid protein assay kit was from Pierce, USA. Recombinant protein phosphatase 2C was obtained from cDNA clones encoding protein phosphatases 2Cα and 2Cβ from bovine retina, and expressed in *Escherichia coli* BL21(DE3)pLysS. Recombinant His-tag containing protein phosphatase 2C was purified from the soluble bacterial cell extract by chromatography on Ni-NTA agarose (Klumpp et al., 1998a). Ni-NTA agarose is a registered trademark of Qiagen, Germany. Ginkgolic acids were a gift from Dr. Willmar Schwabe & Co, Karlsruhe, Germany. About 97% of ginkgolic acids constitute four different salicylic acid derivatives with the following alkyl residues: C13:0 (11%), C15:0 (4%), C15:1 (43%) and C17:1 (39%). For calculating the concentrations, a mean molecular weight of 347 g/mol was used for this mixture of ginkgolic acids.

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-coated flasks. Cells were incubated in DMEM with 20% fetal bovine serum and antibiotics for 5 days and medium was exchanged every 2nd day. In these cultures, 98% of the cells were neurons as previously shown by an immunohistochemical demonstration of neurofilament NF160 (Ahlemeyer et al., 2000). This culture system was used because we wanted to study the direct effect of ginkgolic acids on neurons.

2.3. Drug treatment

Chick embryonic neurons were incubated for 24 h with ginkgolic acids in medium with and without serum. Cycloheximide was added concomitantly with ginkgolic acids. Ginkgolic acids were initially dissolved in pure DMSO and then diluted with culture medium to the final concentration of 0.016% DMSO in all experiments.

2.4. Cell viability

Cell viability was determined by trypan blue exclusion method. Cultures were incubated with 0.4% trypan blue in phosphate-buffered saline (PBS) for 5 min at 37 °C. Thereafter, the cultures were washed with PBS and the number of stained (non-viable) and unstained (viable) cells were counted in four areas of three different culture flasks. Cell viability was expressed as the percent ratio of unstained cells versus the total number of cells.

2.5. Nuclear staining with Hoechst 33258

Cell cultures were incubated for 10 min with 10 μg/ml of the DNA-fluorochrome Hoechst 33258 in methanol and then washed with methanol and PBS. Thereafter, nuclear morphology was observed under a fluorescence microscope. The number of neurons with a shrunken nucleus and condensed chromatin were counted in four areas of four different culture flasks and the results were expressed as the percent ratio of neurons with condensed chromatin and shrunken nuclei versus the total number of cells.

2.6. TUNEL-staining

To detect DNA fragmentation, the terminal-transferase-mediated ddUTP-digoxigenin nick-end labeling (TUNEL) reaction was applied to the neuronal cultures. Cell monolayers were fixed in methanol at -20 °C for 20 min and then incubated at 37 °C for 1 h with digoxigenin-labelled dd-UTP in the presence of terminal transferase. Thereafter, the reaction was stopped and anti-digoxigenin-antibodies coupled with peroxidase were added for further 30 min. Color reaction was performed by use of diaminobenzidine and H_2O_2. Cells were observed under a confocal laser scanning microscope (LSM 510, Zeiss, Germany).

2.7. Measurement of caspase-3 activity

To determine caspase-3 activity, a commercial assay kit was used. It was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amido-4-methylcoumarin (AMC) moiety. Briefly, cells were harvested and pelleted by centrifugation at $600 \times g$ for 5 min at 4 °C. The

Table 1
Ginkgolic acids decrease neuronal viability

Ginkgolic acids (μM)	Neuronal viability (%)	
	Medium with serum	Medium without serum
–	78.1 ± 4.5	61.8 ± 2.5
10	70.6 ± 6.2	64.7 ± 5.2
50	62.8 ± 4.0[a]	53.6 ± 7.6
100	54.0 ± 7.0[b]	49.6 ± 5.4[a]
250	34.5 ± 5.2[b]	18.8 ± 7.5[b]

Chick neurons were treated with vehicle or ginkgolic acids in the presence and absence of serum. Cell viability was determined by the trypan blue exclusion method. Values are given as means ± S.D.
[a] Different from corresponding vehicle-treated controls: $P < 0.05$.
[b] Different from corresponding vehicle-treated controls: $P < 0.001$.

supernatant was removed and the pellet was washed with PBS. Thereafter, the pellet was dissolved in lysis buffer containing 50 mM HEPES, pH 7.4, 5 mM 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) and 5 mM dithiothreitol and incubated for 20 min on ice, followed by centrifugation at 14,000 × *g* for 15 min at 4 °C. The supernatant was mixed with reaction buffer containing 10 mM Ac-DEVD-AMC, 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS and 5 mM dithiothreitol. Cultures treated for 8 h with 200 nM staurosporine in serum-free medium were used as positive controls. Fluorescence of AMC was measured at 360-nm excitation wavelength and 460-nm emission wavelength. Results were calculated using an AMC standard curve and are expressed as nmol AMC/mg protein/min. Protein was measured using the bicincolinic acid protein assay kit and bovine serum albumin as a standard.

2.8. Measurement of phosphatase activities

Recombinant enzymes were used for the measurement of phosphatase activities because the amount of enzymes from neuronal cell extracts was limited and interference with endogenous lipids could be avoided. Protein phosphatases 2C and 2A activities were assayed at 30 °C for 10 min in 30 μl 20 mM Tris/HCl pH 7.5, 0.01% 2-mercaptoethanol, 0.7 mM Mg-acetate, 1.3 mg/ml bovine serum albumin, and 1 μM [^{32}P]casein (5×10^4 cpm) (McGowan and Cohen, 1988; Cohen, 1991). Reactions were terminated by the addition of 200 μl 20% trichloroacetic acid. After centrifugation at 10,000 × *g* for 5 min, 200 μl of the supernatant was analyzed for [^{32}P]phosphate content. Activity measurements were kept within the linear range of time and protein. The activities of the protein phosphatases 1 and 2B, tyrosine phosphatase, unspecific acid- and alkaline phosphatase were monitored at 37 °C by formation of *p*-nitrophenol from 0.5 mg/ml *p*-nitrophenyl phosphate (Garcia-Rozas et al., 1982). Determination of acid- and alkaline phosphatase activities were carried out in 1 ml 50 mM Na-acetate pH 5.5 and 50 mM Tris/HCl pH 7.5 with 1 mM Mg-acetate, respectively, and were stopped after sufficient colour development by the addition of 250 μl 13% K_2HPO_4. Activities of the protein phosphatases 1 and 2B and tyrosine phosphatase were determined in 100 μl 20 mM Tris/HCl pH 7.5, 1% glycerol, 0.1% 2-mercaptoethanol, and 5 mM $MnCl_2$ (PP1) or 1 mg/ml bovine serum albumin (tyrosine phosphatase), or 1 mM $MnCl_2$, 0.1 mM $CaCl_2$ and 10 μg/ml calmodulin (protein phosphatase 2B), and terminated by adding 25 μl 13% K_2HPO_4.

3. Results

In primary cultures of neurons from chick embryo telencephalons, the treatment with ginkgolic acids decreased neuronal viability as determined by the trypan blue exclusion method (Table 1) and increased the percentage of cells with condensed chromatin and shrunken nuclei in a dose-dependent manner in the absence as well as in the presence of serum (Fig. 1). Cell death induced by incubating chick neurons for 24 h with 250 μM ginkgolic acids in medium with serum showed signs of apoptosis as chromatin condensation and reduction of the nuclear size (Fig. 2). In addition, the protein synthesis inhibitor cycloheximide (0.1 μM) reduced the damage of the neurons (Fig. 3), suggesting an active type of cell death. However, DNA fragmentation as detected by the TUNEL assay (Fig. 2) and caspase-3 activation (Fig. 4) were reduced in cultures treated with 150 μM ginkgolic acids in serum-free medium compared with the respective serum-deprived, vehicle treated controls. In the corresponding sister cultures, we determined 87.8 ± 7.3% and 25.1 ± 7.1% neurons with condensed chromatin and shrunken nuclei in the presence

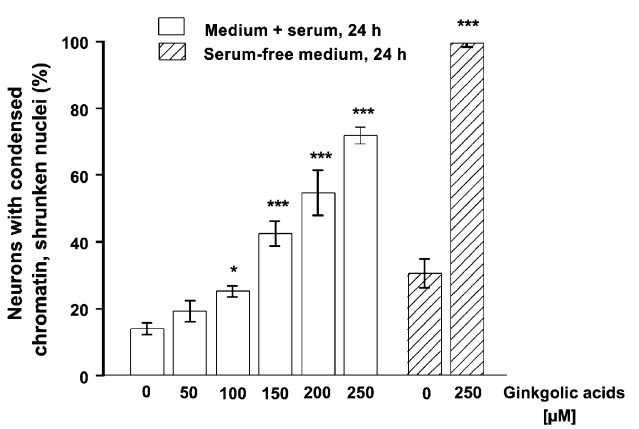

Fig. 1. Ginkgolic acids cause death of cultured chick neurons. Chick embryonic neurons were incubated for 24 h with vehicle or ginkgolic acids in the presence and absence of serum. The percentage of neurons with condensed chromatin and shrunken nuclei was determined by nuclear staining with Hoechst 33258. Values are given as means ± S.D. Different from corresponding vehicle-treated controls: $^{*}P < 0.05$; $^{***}P < 0.001$.

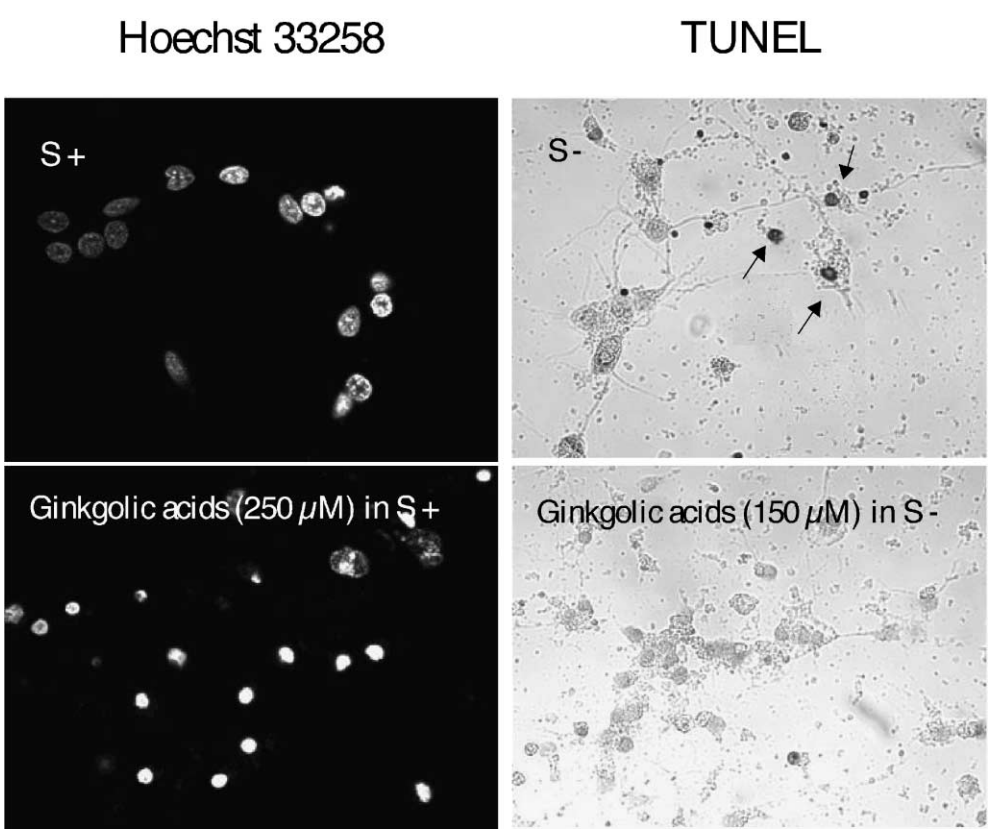

Fig. 2. Photomicrographs of neurons treated for 24 h with vehicle and ginkgolic acids in medium with serum (S +) and serum-free medium (S −). Note chromatin condensation and shrunken nuclei, but negative TUNEL staining in nearly all neurons treated with ginkgolic acids. Positive TUNEL staining in serum-deprived, vehicle-treated controls is indicated with black arrows.

and absence of 150 μM ginkgolic acids under serum-free conditions, respectively. Thus, ginkgolic acids caused a caspase-independent, active type of cell death accompanied by an apoptotic nuclear morphology without DNA fragmentation.

Ginkgolic acids are 6-alkyl salicylic acid derivatives and theoretically fulfill the structural requirements for activation of protein phosphatase 2C (Klumpp et al.,

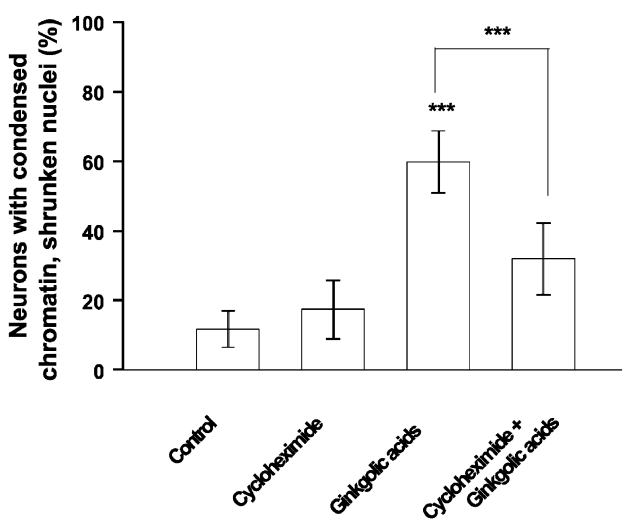

Fig. 3. Cycloheximide reduces ginkgolic acid-induced neuronal death. Chick embryonic neurons were incubated for 24 h with vehicle (controls), 250 μM ginkgolic acids or 0.1 μM cycloheximide in medium with serum. The percentage of neurons with condensed and shrunken nuclei was determined by nuclear staining with Hoechst 33258. Values are given as means ± S.D. Different from vehicle-treated controls: $^{***}P < 0.001$; significantly different: $^{***}P < 0.001$ (ANOVA-1 and post hoc Scheffé-test).

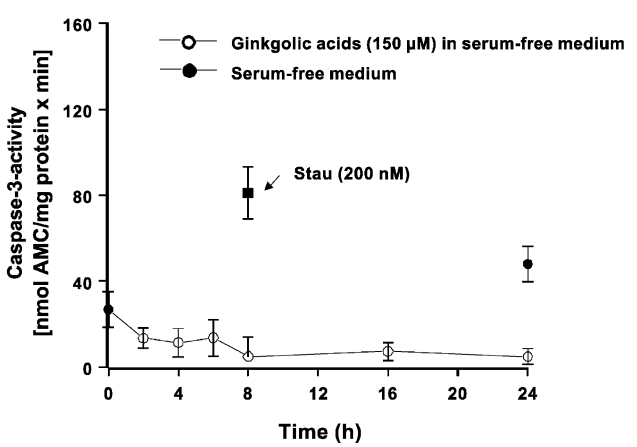

Fig. 4. Ginkgolic acids-induced neuronal death is independent of caspase-3 activation. Chick embryonic neurons were incubated with vehicle or 150 μM ginkgolic acids in serum-free medium for the indicated time period. Cultures treated for 8 h with 200 nM staurosporine in serum-free medium were used as positive control. Caspase-3 activity was determined by a commercial assay kit. Caspase-3 activity is expressed as nmol AMC/min/mg protein. Values are given as means ± S.D.

1998b). Protein phosphatase 2Cα was strongly activated by ginkgolic acids with a maximal rate of stimulation (14-fold) at 600 μM ginkgolic acids (Fig. 5). Sensitivity of protein phosphatase 2Cβ to ginkgolic acids was 40% less compared with that of protein phosphatase 2Cα (data not shown). Activation of protein phosphatase 2C by ginkgolic acids was biphasic and decreased beyond a concentration of 600 μM (Fig. 5). An artefact of ginkgolic acids acting on the substrate [^{32}P]casein could be excluded, because in contrast to protein phosphatase 2C, dephosphorylation of [^{32}P]casein by protein phosphatase 2A was inhibited by ginkgolic acids. Salicylic acid, the ginkgolides A and B as well as bilobalide did not activate protein phosphatase 2C (data not shown). We also determined the effect of ginkgolic acids on other dephosphorylating enzymes, such as the unspecific acid- and alkaline phosphatases as well as the protein phosphatases 1, 2A and 2B and tyrosine phosphatase. Alkaline phosphatase activity was not affected by ginkgolic acids (Fig. 5). The activities of protein phosphatases 1A, 2A and 2B, tyrosine phosphatase and acid phosphatase were inhibited (Fig. 5). Of note, the catalytic part of cytosolic tyrosine phosphatase was highly sensitive towards ginkgolic acids (IC_{50} = 3 μM, Fig. 5).

4. Discussion

Ginkgolic acids represent a major group of constituents in crude extracts of *Ginkgo* leaves. These compounds have been shown to possess allergenic as well as cytotoxic and genotoxic properties (Hausen, 1998; Siegers, 1999; Koch et al., 2000; Westendorf and Regan, 2000). Due to these adverse effects, guidelines of several regulatory authorities require the removal of these substances from therapeutically used *Ginkgo* extracts below a limit concentration of maximally 5 ppm. The *Ginkgo* extract EGb 761 meets these requirements. In previous experiments with rats, we demonstrated that EGb 761 increased cerebral circulation and improved energy metabolism under hypoxic conditions (Krieglstein et al., 1986). The protective effect of the extract was found to be caused mainly by a fraction which contained ginkgolides and bilobalide (Oberpichler et al.,

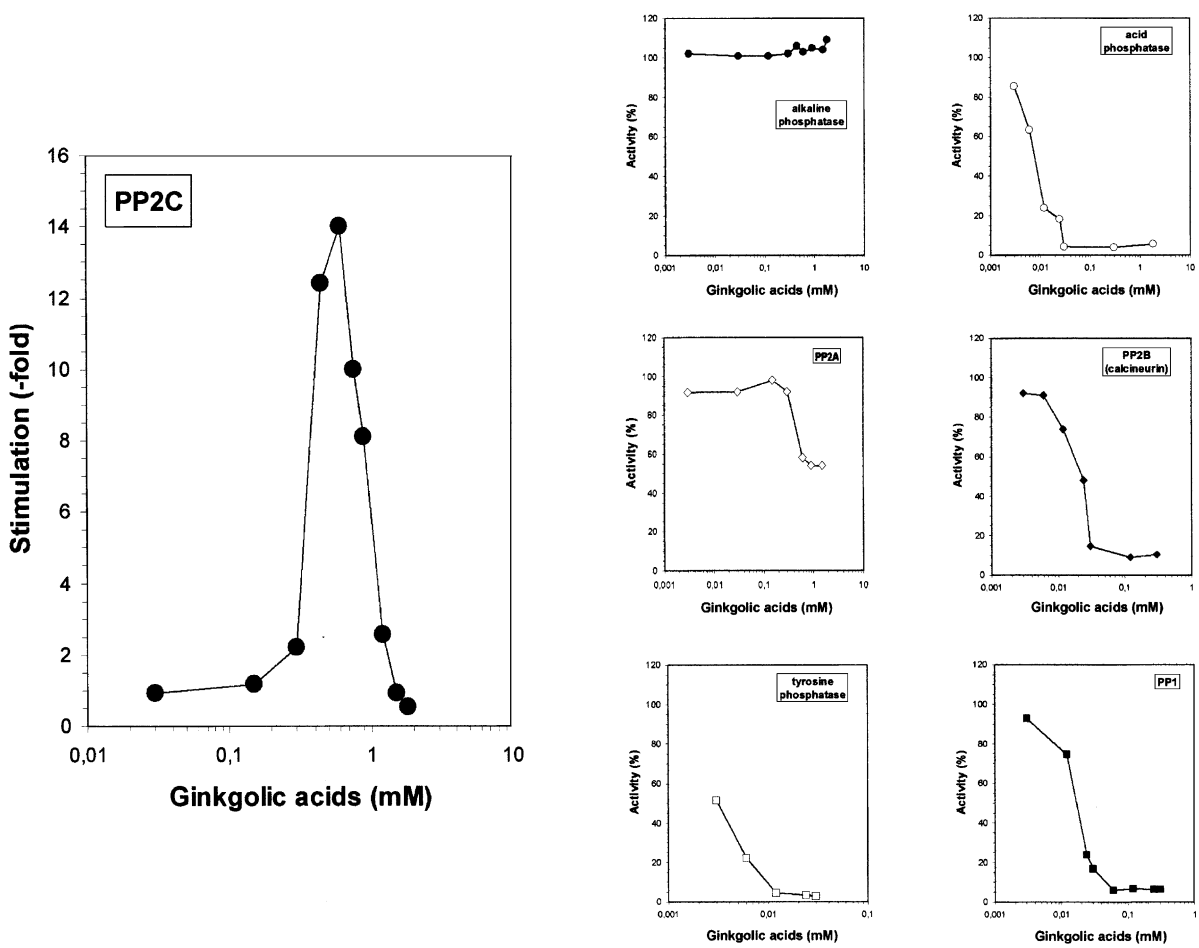

Fig. 5. Ginkgolic acids specifically activate PP2C. Activities of PP2C, PP1, PP2A, PP2B, tyrosine phosphatase as well as unspecific acid-and alkaline phosphatase were determined at concentrations of ginkgolic acids as indicated. Dephosphorylation reactions were performed using [^{32}P]casein as substrate for PP2A and PP2C. Activities of the other phosphatases were monitored using *p*-nitrophenyl phosphate as substrate. Data are given as the mean value of two measurements.

1988). Treatment of mice and rats with these terpene lactones significantly reduced the infarct volume after occlusion of the middle cerebral artery (Krieglstein et al., 1995). Furthermore, these constituents diminished glutamate-induced excitotoxic damage of neurons in vitro (Prehn and Krieglstein, 1993; Krieglstein et al., 1995). The neuroprotective effect of *Ginkgo* terpene lactones appeared to be due to an inhibition of apoptosis (Ahlemeyer et al., 1999). Although apoptosis is a physiological event during ontogenetic development of the central nervous system, programmed cell death is suggested to contribute to the pathogenesis of neurodegenerative disorder in adult life, e.g. Parkinson's disease, Alzheimer's disease or amyotropic lateral sclerosis.

Thus, the question arose whether ginkgolic acids, besides their known unfavorable properties, may also exert neurotoxic effects. Indeed, we now demonstrate that ginkgolic acids are able to damage cultured chick neurons. Cell death induced by ginkgolic acids showed features of apoptosis such as chromatin condensation, shrinkage of the nuclei and dependence on protein synthesis. In addition, signs of necrosis were apparent as we neither observed DNA fragmentation nor caspase-3 activation. Although cell death is classically divided into two groups, such as (i) the active, programmed cell death having apoptotic morphology, and (ii) the passive, uncontrolled cell death having necrotic morphology, accumulating in vivo and in vitro findings suggest the existence of exceptions to this classification (Kitanaka and Kuchino, 1999). On the one hand, DNA fragmentation as detected by positive TUNEL-staining or caspase activation can be induced in cells that become necrotic and in such examples, the use of the word apoptosis is misleading (Fujikawa, 2000). Similarly, it has been known for years that in vivo cerebral ischemia and seizures produce shrunken, condensed nuclei that contain irregular chromatin clumps; these features are in vivo evidence of neuronal necrosis (Fujikawa et al., 2000). On the other hand, an apoptotic nuclear morphology without DNA fragmentation has been observed in staurosporine-induced apoptosis in Jurkat cells with a mutant form of the inhibitor of caspase-activated DNAse (Sakahira et al., 1999) and in cerebellar granule neurons after severe oxygen glucose deprivation (Kaasik et al., 1999). In addition, ceramide can induce caspase-independent apoptosis through the translocation of bax to the mitochondrial membrane (Belaud-Rotureau et al., 1999), which mediates death via channel-forming activity, thereby promoting mitochondrial permeability transition (Green and Reed, 1998).

Investigations on the apoptosis-inducing mechanism of action of ginkgolic acids revealed that these alkylphenols activate protein phosphatase 2C, whereas other protein phosphatases remained unchanged or were inhibited. It has been previously shown that oxidizable unsaturated fatty acids with a high lipophilicity stimulate protein phospatase 2C (Klumpp et al., 1998b) and induce neuronal death (Klumpp et al., 2001) in the same concentration range as found for ginkgolic acids. As more than 82% of the ginkgolic acids are alkylphenols with unsaturated fatty acid residues (C15:1, C17:1), we suggest that these side chains are responsible for the observed activation of protein phosphatase 2C. Because the activation of protein phosphatase 2C was found to correlate with neuronal death, although not exactly in the same range of concentration, we suggest that the activation of protein phosphatase 2C contributes to neuronal death caused by ginkgolic acids. Interestingly, the release of free fatty acids from brain phospholipids, which is thought to contribute to cell death, was reduced by EGb 761 during transient cerebral ischemia (Bazan and Rodriguez de Turco, 1992) and hypoxia (Klein et al., 1997). Consequently, EGb 761 may reduce the activation of protein phosphatase 2C during neuronal damage.

Ginkgolic acids have been described to possess weak antimicrobial effects and antitumor activity (Itokawa et al., 1989; Lee et al., 1998) when used at doses of 10–40 mg/kg (30 μM). However, the antitumor activity of ginkgolic acids has been suggested to involve the inhibition of PI-PLCγ1 (Lee et al., 1998). Interestingly, the latter effect could inhibit the breakdown of membrane phospholipids, thereby reducing the release of free fatty acids with a subsequent decrease in the activation of protein phosphatase 2C, suggesting that ginkgolic acids can directly activate and indirectly inhibit the protein phosphatase 2C at different time points. How protein phosphatase 2C activation contributes to neuronal death in our model has to be clarified by further studies.

In the present study, we demonstrated that neuronal death and activation of protein phosphatase 2C were concomitantly induced at high concentrations of ginkgolic acids (around 500 μM). Since the amount of ginkgolic acids in EGb 761 is less than 5 ppm, toxic effects in humans are not expected, although it cannot be excluded that these lipophilic compounds could accumulate in the organism. Therefore, it is reasonable in any case to remove these acids as far as possible from the *G. biloba* extracts used for therapy.

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