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Effects of Estradiol, Phytoestrogens, and Ginkgo Biloba Extracts Against 1-Methyl-4-phenyl-pyridine-Induced Oxidative Stress

Benoît Gagné,[1] Sylvie Gélinas,[1,2] Geneviève Bureau,[1] Bruno Lagacé,[1] Charles Ramassamy,[1] Keith Chiasson,[1] Barbara Valastro,[1] and Maria-Grazia Martinoli[1,2]

[1]Department of Biochemistry, Research Group in Neuroscience, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada; and [2]Neuroscience Research Unit, Centre de Recherche de l'Université Laval, Ste-Foy, Québec, Canada

Oxidative stress has been recently considered as a mediator of nerve cell death in several neurodegenerative diseases. We studied the effect of the parkinsonism-inducing toxine 1-methyl-4-phenyl-pyridine (MPP+) on several parameters of cell distress using native and neuronal PC12 cells. Then, since estrogens have been reported to prevent neuronal degeneration caused by oxidative damage, we investigated the ability of 17β-estradiol (E_2); two Ginkgo biloba extracts, EGb 761 and Cp 202; as well as two flavonoids, quercetin and kaempferol, to rescue PC12 cells submitted to MPP+-induced oxidative stress. Our results consistently show that both Ginkgo biloba extracts could prevent cell death in native and neuronal PC12 cells, while in neuronal PC12 cells also quercetin and E_2 could reverse MPP+ neurotoxic effet. Western blot analysis demonstrated that MPP+ injuries might modulate dopamine transporter (DAT) protein expression but not estrogen receptor β (ERβ) protein expression. EGb 761 and Cp 202 also modulate DAT and ERβ protein expression in neuronal cells. From these studies, we outline the importance of testing estrogen-like plant-derived molecules as potent antioxidants and examine their effect on protein expression.

Key Words: Oxidative stress; phytoestrogens; Ginkgo biloba; dopamine transporter; estrogen receptor; 1-methyl-4-phenyl-pyridine.

Introduction

Many factors have been implicated in the pathogenesis of neural cell degeneration. It is likely that a cascade of events involving free radicals, mitochondrial dysfunctions, cerebral amyloidosis, and genetic and environmental factors contributes to the neurodegenerative process *(1–4)*. The central nervous system (CNS) is indeed characterized by a high rate of oxidative metabolic activity; thus, it may be particularly susceptible to oxidative damage *(5)*.

1-Methyl-4-phenyl-pyridine (MPP+) is the active metabolite of the parkinsonism-inducing toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydopyridine (MPTP), which causes dopaminergic cell death through the inhibition of mitochondrial complex I. MPP+ is selectively taken up by dopamine (DA) neurons via its high-affinity dopamine transporter (DAT) *(6)* and it is reported to induce oxidative stress in the cellular environment by increasing free-radical formation *(7,8)*. However, the precise mechanism by which MPP+ or MPTP kills the cells is still under investigation *(9–12)*.

Phytoestrogens have commanded a great deal of investigations owing to their potential for protecting various body systems against free-radical attack (for reviews see refs. *13–15*). Yet, very few studies have examined the role of phytoestrogens against oxidative damage induced by the neurotoxin MPP+ *(16)*. The standardized Ginkgo biloba extract EGb 761 is composed of 22–27% flavonol glycosides (kaempferol, quercetin, and isorhamnetin) and 5–7% terpene lactones (3% bilobalides and 3% ginkgolides) *(17–19)*, while Cp 202 is devoid of terpenes *(20)*. A large body of literature describes Ginkgo biloba extracts as free-radical scavengers both in vitro and in vivo *(17,21,22)*. They are also reported to prevent apoptosis of neural cells *(23–25)*; protect against β-amyloid toxicity *(18)*; help prevent strokes and ischemic attacks *(26)*; and preserve, in part, DA neurons *(27–29)*. However, their possible role as modulators of hormonal receptors is much less studied. The phytoestrogens quercetin and kaempferol are the major flavonoid components of EGb 761 and CP 202 and are also known to bind to the estrogen receptor β (ERβ) with low affinity *(30)*. They are reported to act as potent antioxidants, but whether their antioxidant effect is mediated through a nuclear ER or by a direct mechanism is still a matter of debate *(16,31)*.

In the present study, we investigated the role of oxidative stress on native (undifferentiated) and neuronal (nerve growth factor [NGF]-differentiated) PC12 cells. We also compared Ginkgo biloba extracts to their flavonoid counterparts and to β-estradiol (E_2) for the ability to revert oxi-

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Author to whom all correspondence and reprint requests should be addressed: Maria-Grazia Martinoli, Department of Biochemistry, C.P. 500, Université du Québec, Trois-Rivières, Québec, G9A 5H7, Canada. E-mail: martinol@uqtr.uquebec.ca

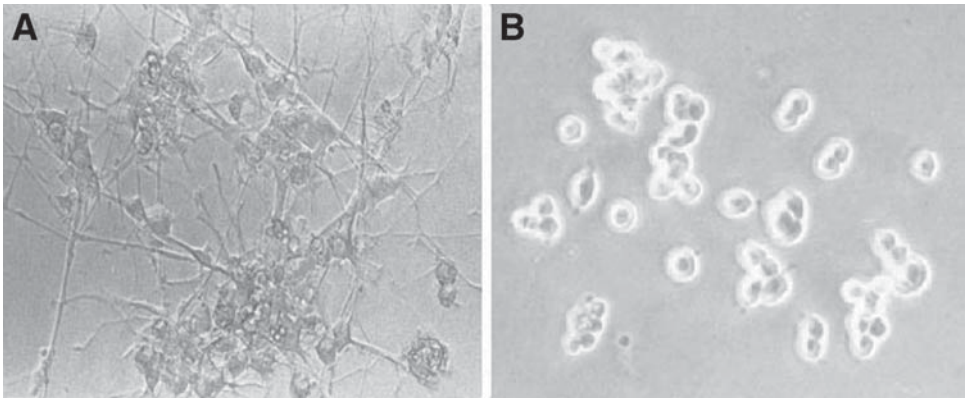

Fig. 1. Photomicroscopy of PC12 treated (**A**) with or (**B**) without (WGF). Cells were induced to differentiate for 12 d with the addition of 50 ng/mL of NGF, as described in Materials and Methods. Magnification: ×200.

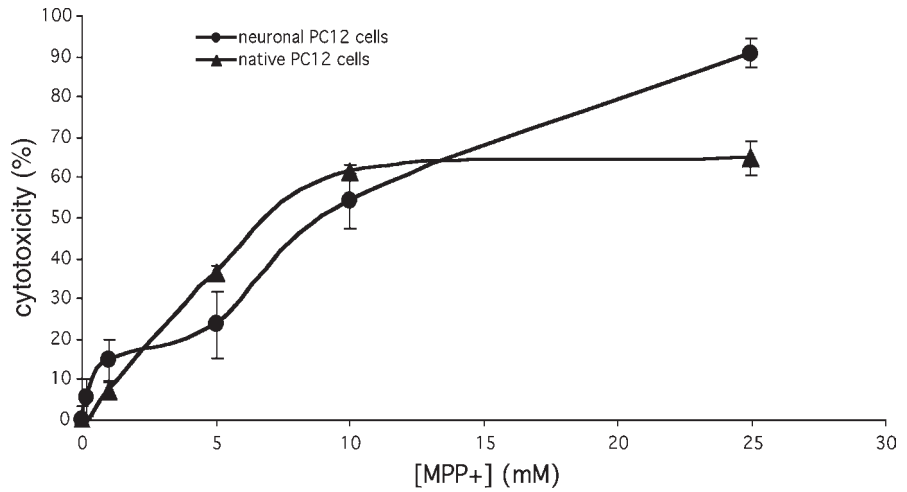

Fig. 2. Dose response curves for MPP+-induced toxicity in (▲) native and (●) neuronal PC12. The experimental conditions are described in Materials and Methods.

dative damages. Our results enlighten the involvement of E_2, Ginkgo biloba extracts, and quercetin to protect against oxidative stress in neuronal cells. Moreover, our data show that treatments with quercetin, EGb 761, or Cp 202 modulates ERβ protein expression as well as DAT protein expression and thus point out the importance of studying the role of plant-derived molecules at the transcriptional level.

Results

MPP+-Induced Cytotoxicity

First we carried out dose response and kinetic studies of MPP+ cytotoxicity in both native and neuronal PC12 (Fig. 1). Cytotoxicity was measured by using lactate dehydrogenase colorimetric analysis and revealed a significant cell death following exposure to 5 m*M* MPP+ for 24 h in both native and neuronal cells (Fig. 2).

Effect of E_2, Phytoestrogens, and Ginkgo Biloba Extracts Against MPP+-Induced Cytotoxicity

The ability of E_2; the phytoestrogens quercetin and kaempferol; as well as the two Ginkgo biloba extracts, EGb 761 and Cp 202, to reverse MPP+-induced toxicity was investigated using lactate dehydrogenase analysis. A 24-h pretreatment of native PC12 cells with 10^{-7} *M* E_2, quercetin, or kaempferol did not reduce MPP+-induced toxicity in native PC12 (Fig. 3A), whereas, 10 µg of EGb 761 or 10 µg of Cp 202 could significantly reduce MPP+-induced cellular death by 40 and 57%, respectively (Fig. 3A). Notably, treatment of neuronal PC12 with 10^{-7} *M* E_2, quercetin, EGb 761, or Cp 202, but not kaempferol, could indeed reverse neuronal death by 79, 64, 84, and 87%, respectively (Fig. 3B). When 10^{-7} *M* E_2, Ginkgo biloba extracts, quercetin, or kaempferol was used alone for 24 h in native and neuronal PC12, we did not observe any change in lactate dehydrogenase activity compared to control cells treated with vehicle (i.e., RPMI with stripped serum) (data not shown). Moreover, metabolic activity was reduced for native and neuronal PC12 cells treated with MPP+ alone (data not shown), whereas cells treated with E_2, phytoestrogens, or Ginkgo biloba extracts prior to MPP+ administration had a higher metabolic activity, as already demonstrated *(16)*. Treatments for 24 h with the same substances without MPP+ did not induce any statistically significant differences in cellular proliferation.

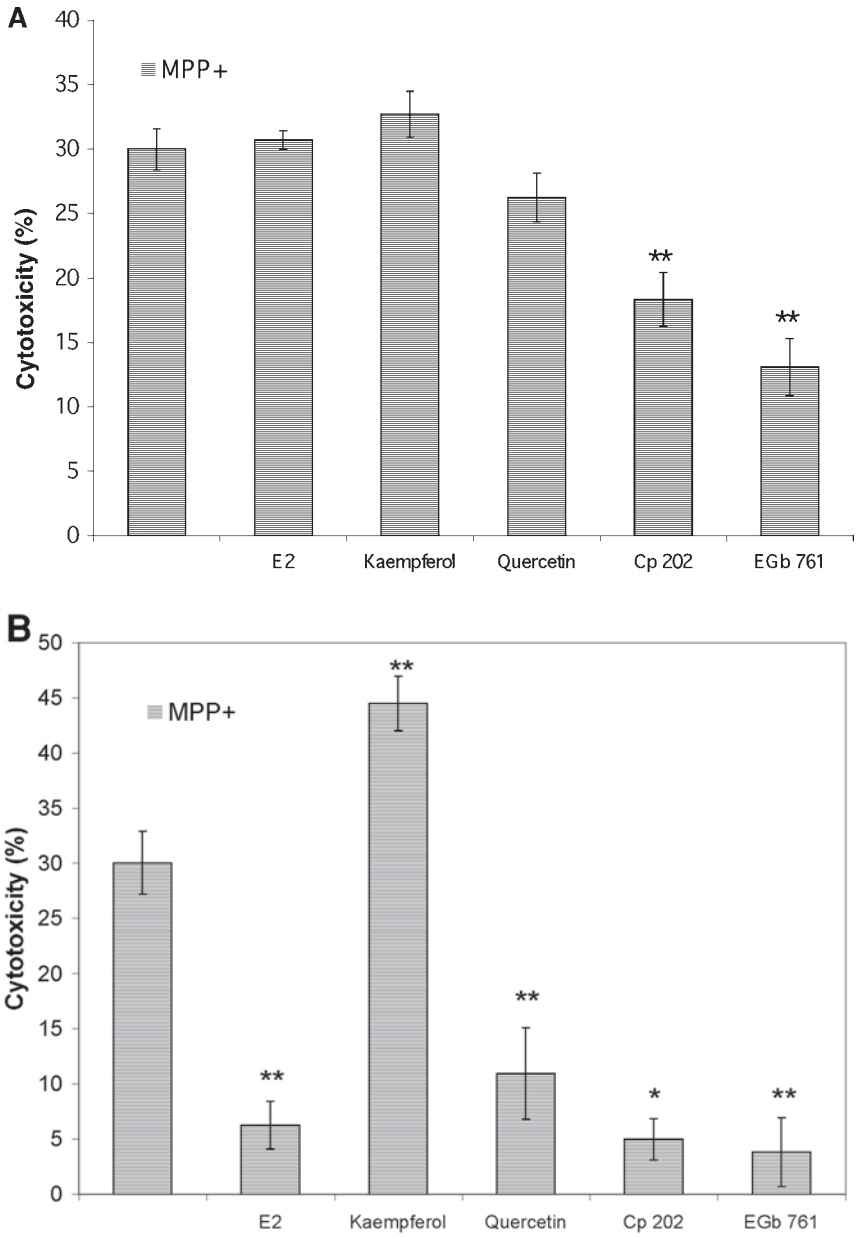

Fig. 3. (**A**) Effect of E_2; phytoestrogens quercetin and kaempferol; and Ginkgo biloba extracts, EGb 761 and Cp 202, on MPP+-induced toxicity in native PC12. The absorbance value obtained for the control (background) was subtracted from all other values. Control: vehicle (RPMI with stripped serum). Only pretreatment with Cp 202 or EGb 761 prior to MPP+ administration could partially reverse cellular death. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparison test, in which $n = 6$. $^{**}p < 0.01$ vs MPP+ alone. **(B)** Effect of E_2; phytoestrogens quercetin and kaempferol; and Ginkgo biloba extracts, EGb 761 and Cp 202, on MPP+-induced toxicity in neuronal PC12. The absorbance value obtained for the control (background) was subtracted from all other values. Control: vehicle (RPMI with stripped serum). Pretreatments with E_2, quercetin, Cp 202, or EGb 761 prior to MPP+ administration could reverse cell death. Statistical analysis was carried out using one-way ANOVA followed by the Dunnett multiple comparison test, in which $n = 6$. $^{**}p < 0.01$ vs MPP+ alone.

Effect of E_2, Phytoestrogens, and Ginkgo Biloba Extracts on ERβ and DAT Protein Expression

To determine whether E_2, phytoestrogens, or Ginkgo biloba extracts could modulate ERβ and/or DAT protein expression in neuronal PC12, we performed Western blot analysis as already described *(16)*. Figure 4A shows that MPP+ treatment did not change the relative levels of ERβ protein expression yet decreased DAT protein expression (Fig. 4B). When we administered E_2, quercetin, or kaempferol prior to oxidation, we did not detect any change in ERβ expression; however, EGb 761 and Cp 202 could modulate ERβ by decreasing its relative levels (Fig. 4A). In particular, administration of Cp 202 prior to MPP+ significantly decreased ERβ protein expression while treatment with EGb

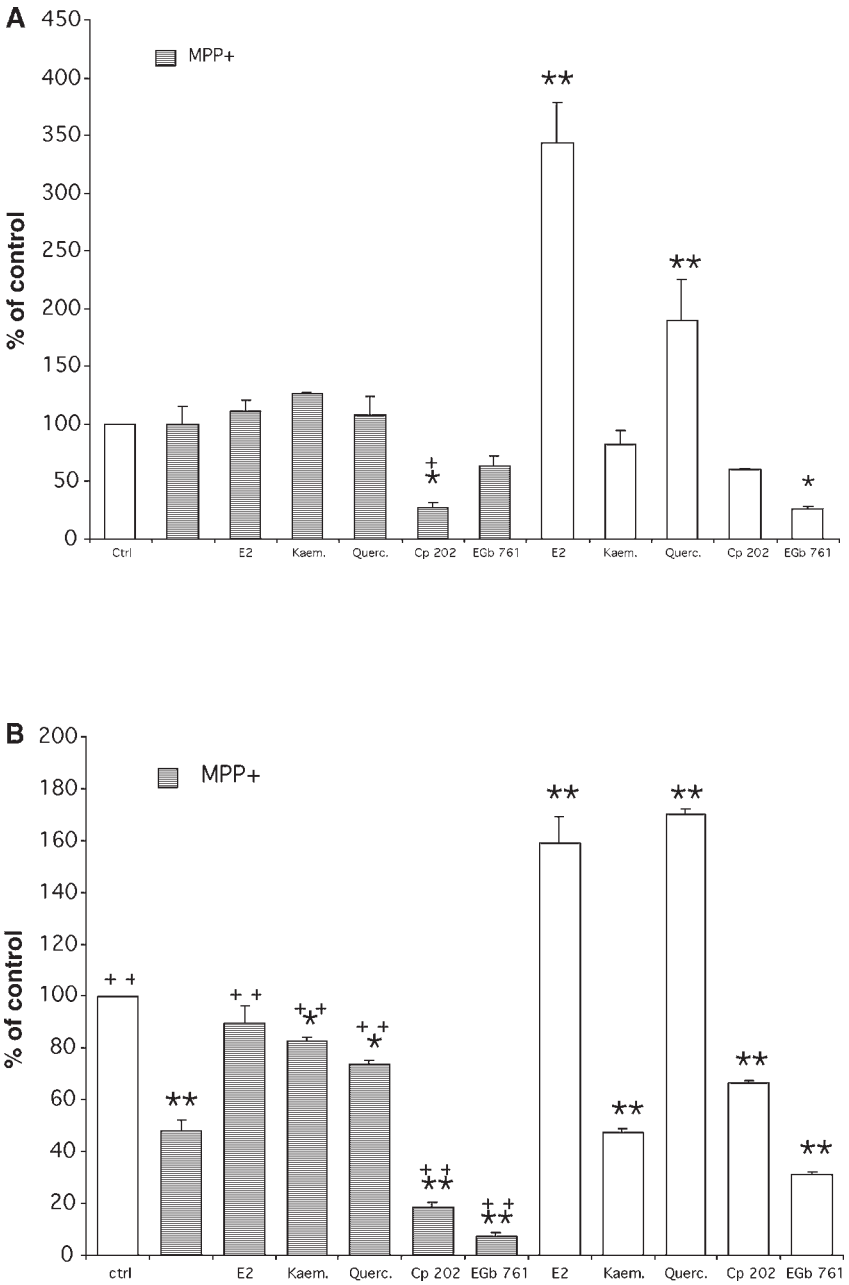

Fig. 4. (**A**) Semiquantitative immunoblot analysis showing ERβ protein expression in neuronal PC12 following treatments with E_2, kaempferol, quercetin, EGb 761, or Cp 202 on MPP+-induced cell death. Control: vehicle (RPMI with stripped serum). Western blots were performed as described in Materials and Methods. Autoradiograms were digitized, transferred into a personal computer, and analyzed with the E.D.A.S. Image analysis system (Kodak). Histograms are representative of at least three separate experiments. (☐) Vehicle (Ctrl) or the indicated compounds were added without MPP+. (▤) The indicated compounds were added 24 h prior to MPP+ treatment, as described in Materials and Methods. *$p < 0.05$ vs ctrl; **$p < 0.01$ vs ctrl; +$p < 0.05$ vs MPP+ alone. (**B**) Semiquantitative immunoblot analysis showing DAT protein expression in neuronal PC12 following treatments with E_2, kaempferol, quercetin, EGb 761, or Cp 202 on MPP+-induced cell death. Control: vehicle (RPMI with stripped serum). Western blots were performed as described in Materials and Methods. Autoradiograms were digitized, transferred into a personal computer, and analyzed with the E.D.A.S. Image analysis system (Kodak). Histograms are representative of at least three separate experiments. (☐) Vehicle (ctrl) or the indicated compounds were added without MPP+. (▤) The indicated compounds were added 24 h prior to MPP+ treatment, as described in Materials and Methods. *$p < 0.05$ vs ctrl; **$p < 0.01$ vs ctrl; +$p < 0.05$ vs MPP+ alone; ++$p < 0.01$ vs MPP+ alone.

761 prior to MPP+ showed an ERβ protein reduction that is actually not statistically significant (Fig. 4A). E_2 or quercetin, when used alone, increased ERβ expression in our cellular paradigm, whereas kaempferol or Cp 202 did not modulate ERβ and EGb 761 reduced it.

Figure 3B shows that MPP+ is responsible for an important decrease in DAT protein levels. Treatments with kaempferol or quercetin prior to oxidative stress reverted DAT expression to almost control levels, while the two Ginkgo biloba extracts reduced it further. In addition, kaempferol,

Cp 202, or EGb 761 decreased DAT protein expression when used alone, suggesting that they could prevent or otherwise hamper MPP+ toxic effects by acting on DAT transcription. Administration of E_2 or quercetin prior to MPP+ induced an important increase in DAT expression (Fig. 4B).

Discussion

Our results show that Ginkgo biloba extracts are powerful molecules to revert free-radical damage in both native and neuronal PC12. Our data also show that EGb 761 and Cp 202 regulate ERβ and DAT protein expression.

PC12 cells are well-known catecholamine-secreting cells that, following NGF treatment, differentiate in a neuronal-like phenotype that secretes a high level of DA, expressing neurofilaments and DAT proteins *(32,33)*. In addition, PC12 cells produce detectable levels of ERβ *(34)*, and they have already successfully been used to study oxidative stress *(16, 19,24)*. Within our experiments, exposure of native or neuronal PC12 cells to the neurotoxin MPP+ resulted in a concentration-dependent increase in cell death, with approx 30% cell death with 5 m*M* MPP+ over a 24-h time period. This relatively high dose of MPP+ was required in our cellular system as well as in others *(16,35,36)* to reach significant cell death over a short period.

Recent clinical data have reported the benefits of estrogen replacement therapy (ERT) on various CNS functions. For example, estrogens may play an important role in the etiology of Alzheimer disease by preventing amyloid plaque formation, oxidative stress, and decline of the cholinergic system (for a review see refs. *37–40*). In addition, estrogens have been shown to maintain the integrity of DA neurons and thus might be a potential therapy for Parkinson disease *(16,41–43)* and be beneficial in preserving cognitive function in both humans *(44)* and animals *(45)*. On the other hand, ERT may be detrimental on other target tissues. There is in fact a serious concern that ERT may increase the incidence of breast cancer (reviewed in ref. *40*).

Recently, phytoestrogens have been studied as a possible alternative treatment to ERT *(13,40,46)*. Interestingly, the neuroprotective potential of certain phytoestrogens appears rooted in their antioxidant properties and may be independent of their estrogenic effects *(31,47–50)*. With respect to quercetin, the major flavonoid component of the Ginkgo biloba extracts EGb 761 and Cp 202, it binds to both ERα and ERβ with low affinity *(30)*, and it is reported to be a potent antioxidant *(31)*. In our experiments, quercetin effectively reduced cell death when administered prior to MPP+ to neuronal PC12 but not when given to native PC12 cells. Since MPP+ enters the dopaminergic neuron by using DAT, our results showing that quercetin modulates DAT protein expression in neuronal PC12 when it is used alone and also prior to oxidative damage are original and of particular importance. They also suggest that quercetin may exert its neuroprotective role by regulating the plasticity of the tips of PC12 cells, where DAT is localized *(32,33)*. Quercetin also moderately increases ERβ protein expression when used alone. Kaempferol, the other flavonoid component of Ginkgo biloba extracts, could not revert cell death in both native and neuronal PC12; rather, it was found to be toxic, as already reported *(16,51)*. Additionally, it did not wield any effect on ERβ expression but it decreased DAT protein levels. Our results also show that E_2 and quercetin could decrease cell death only when used in neuronal PC12, suggesting that they play a role in the complex celluler pathway between estrogenic compounds and NGF *(52)*.

Ginkgo biloba extracts are widely studied for their antioxidative effects in neurons and other tissues. However, few reports have detailed their possible effect on protein expression in the nervous system *(17,19)*. Watanabe et al. *(17)* have recently pointed out that EGb 761 may regulate a variety of genes in the CNS. We now confirm a clear role of Ginkgo biloba extracts EGb 761 and Cp 202 as modulator of ERβ and DAT protein expression. Altogether, these studies reveal that diet supplements with Ginkgo biloba extracts, marketed as therapeutic for a variety of disorders, may actually have an important neuromodulatory effect and as such should be carefully analyzed. Note also that several data enlighten the antioxidative role of the terpenoid fraction of Ginkgo biloba extracts *(24,53)*. In our study, both EGb 761 and Cp 202 were able to rescue native as well as neuronal PC12 cells from MPP+-induced cell death. Even if the flavonoid constituents of Ginkgo biloba extracts have been found to be active in a variety of assays, in our opinion, the putative therapeutic benefits of Ginkgo biloba may reside in the synergistic effect of all its components.

Materials and Methods

Chemicals

Culture medium RPMI-1640, fetal calf serum (FCS), and horse serum were obtained from Life Technologies (Ontario, Canada). Penicillin, streptomycin, and nerve growth factor 7S (NGF), were purchased from Sigma (Ontario, Canada). A cytotoxicity detection kit (lactate dehydrogenase) and peroxidase (POD)-conjugated secondary antibody were purchased from Roche (Quebec, Canada). Polyclonal anti-DAT antibody (no. AB1591P) was purchased from Chemicon (Temecula, CA), and polyclonal anti-ERβ antibody (no. PC168) was purchased from Calbiochem-Novabiochem (San Diego, CA). Kaempferol, quercetin, E_2, MPP+, and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetra-zolium-5-carboxyanilide (XTT) were from Sigma. Ginkgo biloba extract EGb 761 (Tanakan; Beaufour-IPSEN, Paris, France) contains 24% flavonoid glycosides and 6% terpenoids while Cp 202 corresponds to the EGb 761 extract devoid of terpenic substances *(20)*.

Cell Culture and Treatments

PC12 cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in RPMI-1640 medium, without phenol red, supplemented with 5% FCS

and 10% horse serum. Cells were cultured on Primaria 96-well plates (BD Falcon, Ontario, Canada) for lactate dehydrogenase and XTT experiments and on 6-well plates for immunoblot analysis. They were fed with fresh medium every 3 d and maintained at 37ºC in a humidified atmosphere containing 5% CO_2. PC12 neuronal phenotype (Fig. 1) was induced by adding NGF (50 ng/mL) in fresh RPMI containing 1% fetal bovine charcoal-treated serum to remove steroids from the medium (stripped serum) *(54)*. After 12 d, cells were fed with fresh medium and pretreated for 24 h with 10^{-7} *M* E_2, kaempferol, quercetin, 10 µg/mL of EGb 761, or 10 µg/mL of CP 202 *(16,55)*. All of these experimental conditions were selected after time course (data not shown) and dose response studies (Fig. 1). After pretreatment, 5 m*M* MPP+ was added and cells were incubated for 24 h *(16, 35,36)*. Cell density was constant at 20,000 cells/cm^2. The same experimental conditions were used for native PC12 cells without adding NGF. All controls were treated with vehicle alone (i.e., RPMI medium with stripped serum).

Cytotoxicity and Metabolic Activity

MPP+ cytotoxicity was evaluated by colorimetric assay for the quantification of cell death based on the measurement of lactate dehydrogenase activity released from the cytosol of damaged cells into the supernatant, as already described *(16)*. Briefly, media were collected and centrifuged, and 100 µL of cell-free supernatant was used to determine lactate dehydrogenase activity by measuring the absorbance at a wavelength of 490 nm on an enzyme-linked immunosorbent assay plate reader (model 450; Bio-Rad, Ontario, Canada). Total cellular lactate dehydrogenase was determined by lysing cells with 1% Triton X-100. In parallel with cytotoxicity evaluation, we assessed cellular proliferation by measuring total metabolic activity using the XTT test *(56)*. XTT is a soluble tetrazolium salt that is converted in formazan by cell dehydrogenases. Absorbance was measured directly in 96-well plates at 490 nm (with a reference wavelength of 690 nm) 20 h after the addition of XTT to cell culture medium at a concentration of 1 mg/mL in the presence of 1% phenazine methosulfate as a coupling agent.

Electrophoresis and Immunoblot Analysis

Total cellular protein was extracted by TRI Reagent (Sigma). Proteins were solubilized in 50 µL of 100 m*M* phosphate-buffered saline (pH 8.0), and their concentrations were determined by a protein assay (Bio-Rad). Equal amounts of protein were loaded onto a 10% sodium dodecyl sulfate gel. After electrophoretic separation, polyacrylamide gels were transferred onto polyvinyl difluoride membranes (0.2-µm pore size; Bio-Rad) at 180 V for 1 h. Then membranes were blocked with 5% nonfat powder milk for 1 h. Immunoblotting was probed with the primary antibody for 1 h. Dilution of anti-DAT and anti-ERβ was 1:1000. POD-conjugated secondary antibody was added for 30 min. Immunoblots were developed with an enhanced chemiluminescence method (Roche).

Data Analysis and Statistics

Autoradiograms were digitized, transferred onto a personal computer, and analyzed with the EDAS image analysis system (Kodak, Rochester, NY). Statistical significance was assessed using one-way ANOVA followed by Dunnett multiple comparison test. Data are expressed as mean ± SEM.

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