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Ginkgo biloba extract regulates differentially the cell death induced by hydrogen peroxide and simvastatin

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Abstract

Several human diseases have been associated with the overproduction of reactive oxygen species (ROS) and subsequently various antioxidants emerged as potential therapeutic agents that scavenge ROS. As an oxidative stress model of human disease, we used hydrogen peroxide (H_2O_2) to study effect of ROS on C6 glioma cells as a surrogate for astrocytes. H_2O_2 induced dose- and time-dependent apoptotic cell death which was preceded by growth arrest, and transiently activated the signalling proteins ATF-2, ERK1/2 and AKT in C6 glioma cells. While several antioxidants failed to block H_2O_2-induced apoptosis of these cells, *Ginkgo biloba* extract (EGb) totally prevented the cell death and growth inhibition induced by H_2O_2. Interestingly, EGb did not prevent the activation of ATF-2, ERK1/2 and AKT induced by H_2O_2 excluding the role of these factors in the pro-apoptotic effect of H_2O_2. We have previously shown that the lipid-lowering drug, simvastatin, causes apoptotic cell death in C6 glioma cells [Koyuturk M, Ersoz M, Altiok N. Simvastatin induces proliferation inhibition and apoptosis in C6 glioma cells via c-jun N-terminal kinase. Neurosci Lett 2004;370(2–3):212–7]. However, in parallel experiments with H_2O_2, EGb was unable to prevent cell death induced by simvastatin suggesting the involvement of separate signalling pathways between H_2O_2 and simvastatin.

Thus, EGb and other plant flavonoids might have potential as protective agents against apoptosis through scavenging ROS upon cerebral or myocardial diseases associated with free radical generation.

Keywords: Apoptosis; ATF-2; ERK; *Ginkgo biloba*; Glioma; Hydrogen peroxide

1. Introduction

Free radical reactions are associated with a range of degenerative diseases and acute ischemic insults affecting neural and other tissues. Oxidative stress, which involves excess accumulation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radical, can damage cells by lipid peroxidation and alteration of protein and nucleic acid structure (reviewed in Berlet and Stadtman, 1997). The biological consequences of this event are mutations, chromosomal breakages, cytotoxicity, carcinogenesis, and cellular degeneration, depending on the concentration of ROS and the type of target cell. However, downstream targets of ROS have remained largely unknown. Recent studies have demonstrated that ligand stimulation, including cytokines and growth factors, results in an increase in intracellular ROS in a wide variety of cell types (Finkel, 2003). Therefore, we reasoned that cellular responses to oxidative stress might be mediated by signal transducing proteins.

The extract of *Ginkgo biloba* leaves has been extensively used as a medicine and nutritional supplement in a variety of cardiovascular and cerebrovascular diseases (Liebgott et al., 2000; De Feudis and Drieu, 2000; Diamond et al., 2000). EGb contains two active constituents preserving antioxidant properties, which include flavanoids and terpenoids (McKenna et al., 2001). The main active ingredients of medicinal ginkgo leaf extract EGb 761® include 22–27% flavone glycosides, 5–7% terpene lactones, and less than 5 mg/g ginkgolic acids. Many researches have been conducted on the role of the extract (Middleton et al., 2000; De Feudis et al., 2003) and *also* other dietary flavonoids (Schroeter et al., 2001) in the treatment of diseases involving free radicals and oxidative damage.

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Although antioxidant action has been attributed to this extract, *the mechanism of action has not been completely established.*

Since free radicals and oxidative damage play a critical role in several cerebrovascular and cardiovascular diseases, we were interested to investigate the signalling mechanisms regulated by H_2O_2 in glial cells and whether EGb and various antioxidants can possibly rescue cells from oxidative damage.

The objective *of the present study was to identify t*he signalling molecules regulated by H_2O_2 and to compare it with the cell death mechanisms induced by simvastatin in C6 glioma cells (Koyuturk et al., 2004). C6 glioma cells grown under serum-containing conditions present mixed astrocyte–oligodendrocyte phenotype (Coyle, 1995). Since astrocytes are the most abundant type of glia in the brain and have key role in normal physiology of the nervous system and also contribute to the response within the CNS to injury we used C6 glioma cells as a model for glial cells in the brain. The characterization of mechanisms underlying growth–survival decisions during oxidative stress may provide some understanding of the resistance of some cells to oxidative stress and may lead to find targets that would be modulated by drugs to prevent cell death under hypoxia in brain.

2. Materials and methods

2.1. Materials

EGb 761® was from Dr. Willmar Schwabe Pharmaceuticals, Karlsruhe, Germany, Anti-phospho-ATF2, anti-ATF2, anti-phospho-AKT, anti-phospho-ERK antibodies were from New England Biolabs. Anti-rabbit alkaline phosphatase conjugated antibody was from Santa Cruz Biotechnology Inc. Anti-PCNA monoclonal antibody was from Zymed Laboratories, USA. Anti-rabbit and anti-mouse biotin conjugated antibodies, streptavidin, biotinylated horseradish peroxidase (HRP) and aminoethylcarbazole (AEC) were from DAKO. Cell culture media, antibiotics and all the other reagents were from Sigma Chemical Co.

2.2. Cell culture

C6-glioma cells were grown on tissue culture flasks in Dulbecco's modified Eagle's medium/F12 medium containing 5% heat inactivated fetal bovine serum and antibiotics (100 u/ml penicillin G, 100 μg/ml streptomycin) at 37 °C in 5% CO_2 and 95% air in a humidified incubator.

2.3. Immunocytochemistry

Cells grown on coverslips were incubated with drugs for different time points as indicated. Coverslips were washed with phosphate-buffered saline (PBS) and fixed with methanol for 5 min at −20 °C. In order to avoid nonspecific immunostaining, cells were incubated with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Following PBS washes, primary antibodies specific to the indicated proteins were applied to coverslips overnight at 4 °C. After washing with PBS, biotin-conjugated secondary antibodies, streptavidin and biotinylated HRP were applied. Sections were developed by using aminoethylcarbazole (AEC) as substrate. Cells were photographed through Olympus BX-50 brightfield microscope and BX-FLA fluorescence attachment using UV filter 600× magnification as indicated.

2.4. Cell lysis and immunoprecipitation

After treatments cells grown in culture dishes were washed once with ice-cold PBS, and lysed in a buffer containing 20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, 10 mM NaF, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin. Insoluble material was removed by centrifugation at 13,000 × *g* for 20 min at 4 °C.

2.5. Western blot analysis

An equivalent volume of 2× SDS-sample buffer was added to cell lysates and boiled for 5 min. The supernatants were subjected to electrophoresis on SDS-PAGE gels and transferred to nitrocellulose by using a Bio-Rad apparatus. Membranes were blocked for 1 h at room temperature in PBS containing 3% non-fat dried milk and probed overnight at 4 °C with primary antibodies. The immobilized proteins were detected by using alkaline phosphatase conjugated secondary antibodies in PBS-3% milk. After washing with PBS, bands were visualized by using BCIP/NBT as substrates.

2.6. Detection of apoptotic cells

Cells grown on coverslips were fixed with methanol for 5 min at −20 °C and then washed with PBS. Then cells were stained with 4, 6-diamidino 2-phenylindole (DAPI) (0.1 mg/ml) for 15 min at room temperature to allow visualization of the nuclei. Cells were washed and mounted for fluorescence microscopy and photographed through UV filter.

3. Results

3.1. H_2O_2 induced growth arrest and apoptosis of C6 glioma cells

C6 glioma cells were treated with several doses of H_2O_2 (10–1000 μM) for 48 h and viable cell numbers were determined by trypan blue exclusion assay. As shown in Fig. 1, concentration-dependent studies with H_2O_2 showed that the number of cells in H_2O_2-treated plates was reduced by almost 100% relative to controls by 48 h after treatment with an IC_{50} value of 50 μM. Treatment with H_2O_2 significantly induced apoptosis in a concentration-dependent manner. The characteristics of apoptosis such as, chromatin condensation, nuclear fragmentation, and shrinkage of cytoplasm, induced by H_2O_2 (100 μM) after 48 h of treatment was shown by DAPI staining of the nuclear DNA of the cells (Fig. 2C).

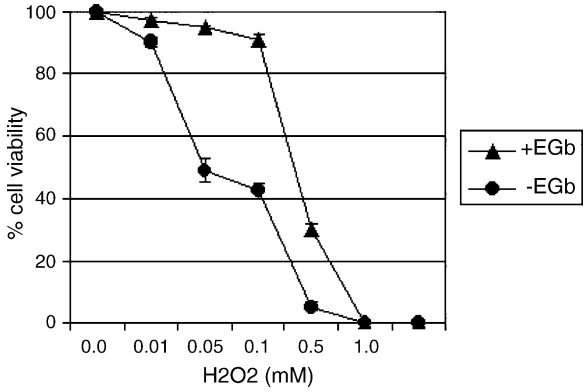

Fig. 1. Protective effect of EGb in C6 glioma cells exposed to increasing concentrations of H_2O_2. C6 glioma cells were pretreated with 200 μg/ml EGb or vehicle for 30 min prior to treatment with H_2O_2 (10–1000 μM) for 48 h. Viable cells were detected by trypan blue exclusion assay. Data points are average of results obtained from three separate experiments.

We further examined the effect of H_2O_2 on growth of C6 glioma cells by detecting the expression of the proliferating cell nuclear antigen (PCNA) by immunocytochemical method. PCNA functions as a cofactor of DNA-polymerase and an important marker for evaluating the proliferation of various cancers. As shown in Fig. 2D, H_2O_2 (100 μM) effectively inhibited the expression of PCNA in the nucleus of these cells within 24 h indicating the cell growth inhibition.

3.2. EGb protected C6 glioma cells against H_2O_2-induced apoptosis and growth inhibition

We have tested effects of EGb and other antioxidants on cell death and growth inhibition induced by H_2O_2 in C6 glioma cells. Cells were cultured with H_2O_2 (100 μM) either alone or in combination with EGb (200 μg/ml) for 48 h. EGb abolished the ability of H_2O_2, at concentrations lower than 1 mM, to induce apoptosis in C6 glioma cells as analysed by trypan blue

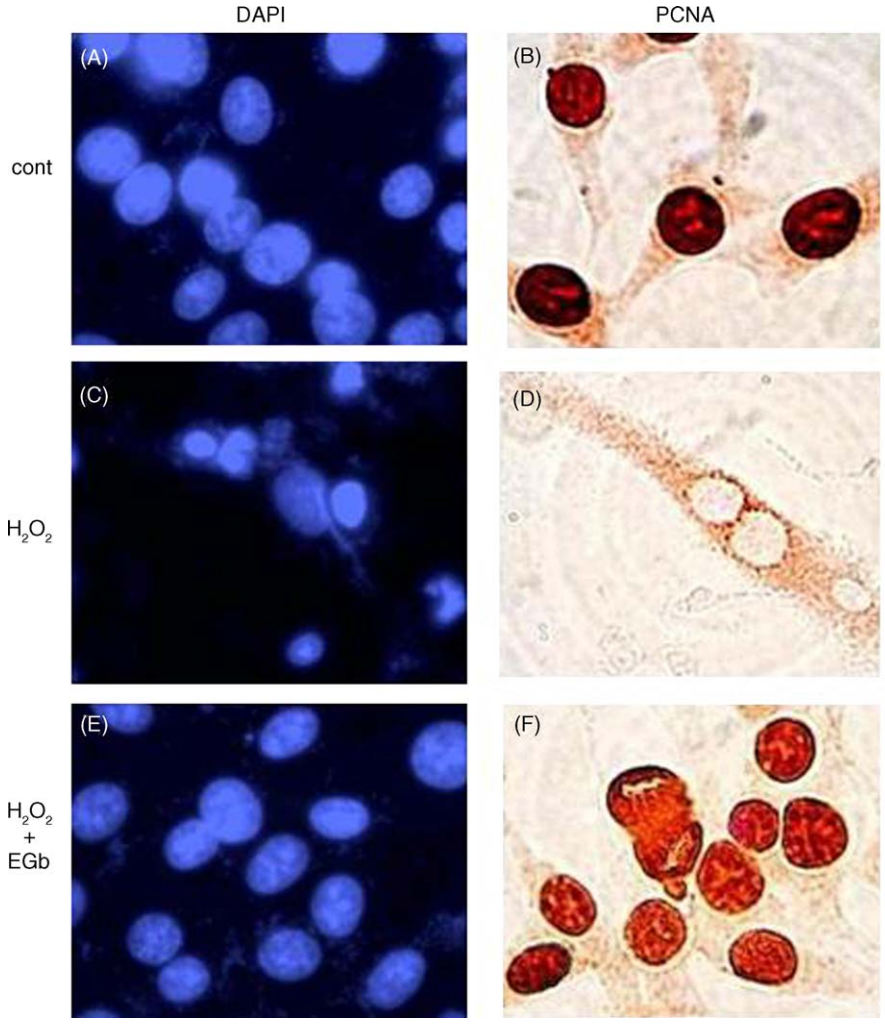

Fig. 2. EGb prevents apoptotic cell death and growth arrest induced by H_2O_2. C6 glioma cells were pretreated with EGb (E and F) or vehicle (A–D) for 30 min and then incubated with H_2O_2 (100 μM) as indicated for 24 h (D and F) and 48 h (C and E). Then they were fixed and stained with DAPI for nuclear visualization and photographed with fluorescence microscope using UV filter under 600× magnification (A, C and E), or stained with a monoclonal antibody against PCNA, then visualized by using biotinylated secondary antibody, streptavidin/HRP and AEC chromogen and photographed through brightfield microscope under 600× magnification (B, D and F).

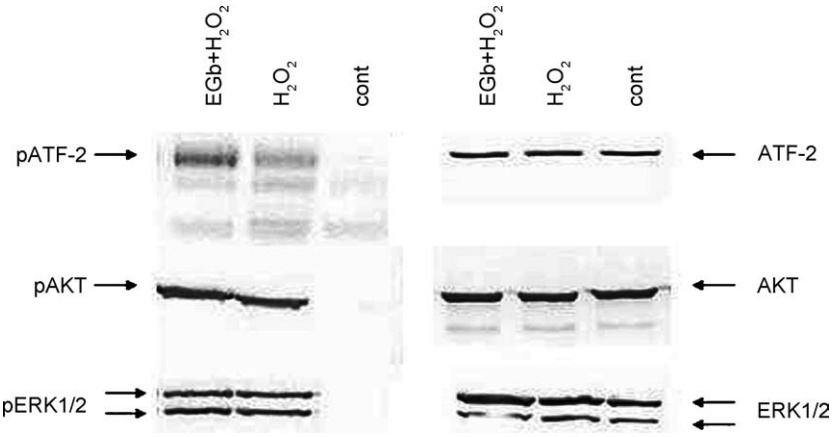

Fig. 3. The stimulation of phosphorylation of ATF-2, AKT and ERK1/2 by H_2O_2. C6 glioma cells were pretreated with 200 μg/ml EGb or vehicle for 30 min prior to treatment with H_2O_2 (0.5 mM) for 10 min as indicated. Cell lysates were separated by 10% SDS-PAGE, western blotted with polyclonal antibodies against phosphorylated forms of ATF-2 (pATF-2) (left upper panel), AKT (pAKT) (left, middle panel), and ERK1/2 (pERK1/2) (left, lower panel). Their corresponding native proteins were blotted in separate membranes and shown in the right panel. Then visualized by using alkaline phosphatase conjugated secondary antibodies and BCIP/NBT as substrates.

exclusion assay (Fig. 1). As shown in Fig. 2E by DAPI staining, the characteristics of apoptosis such as, chromatin condensation, nuclear fragmentation, and shrinkage of cytoplasm induced by H_2O_2 were completely blocked by EGb treatment during 48 h of incubation. EGb (50–300 μg/ml), had no effect on cell survival or morphology before H_2O_2 treatment, but had a specific dose-dependent protective effect (EC50 value of 100 μg/ml) when added together with H_2O_2. EGb (200 μg/ml) treatment also prevented cell growth inhibition caused by H_2O_2 (100 μM) (Fig. 2F), as shown by the nuclear staining of PCNA in C6 glioma cells. Interestingly, the antioxidants *N*-acetyl-L-cysteine (NAC) (20 mM), α-tocopherol (200 μM), deferoxamine (50 μM), selenium (20 μM) or L-NAME (50 μM) failed to block H_2O_2-induced toxicity in these cells. The above effects of EGb and the other antioxidants were not altered if present before or after the oxidative treatment.

We have demonstrated previously that C6 glioma cells undergo concentration dependent apoptosis when exposed to simvastatin (Koyuturk et al., 2004). In the present study, we have compared the effect of EGb on both simvastatin- and H_2O_2-induced cell death. While EGb protected cell death induced by H_2O_2, it did not affect the cell death induced by simvastatin up to 5 μM of concentration (data not shown).

3.3. H_2O_2 activated ATF-2, AKT, and ERK1/2 in C6 glioma cells

We examined next the effect of H_2O_2 on signal transduction pathways known to be involved in cell survival decisions in a variety of cells (reviewed in Martindale and Holbrook, 2002). To characterize the changes in the activation states of the signalling proteins induced by the H_2O_2 treatment of C6 glioma cells, we utilized phosphospecific antibodies, recognizing only the phosphorylated residues of extracellular signal regulated kinase 1 and 2 (ERK1/2), activating transcription factor–2 (ATF-2), and AKT/protein kinase B in western blot analysis. The immunoblotting results indicated that the phospho-Thr-71 ATF-2, phospho-Thr-308 AKT, phospho-Thr-202/Tyr 204 ERK1/2 were undetectable in unstimulated cells, were strongly activated in response to H_2O_2 (0.5 mM) within 10 min, with a rapidly reversible effect, which disappeared within 1 h (Fig. 3, left panel). In summary, our results indicate that H_2O_2 can induce the phosphorylation of ERK1/2, AKT/protein kinase B and ATF-2 in C6 glioma cells.

The increase in the phosphorylation of ERK1/2, ATF2, and AKT was not due to the increase of their protein levels because in western blot analysis with antibodies recognizing these proteins independently from their phosphorylation status showed that the expression levels of these proteins are similar in all treatments (Fig. 3, right panel).

We have further examined whether the inhibitors of MEK-1/ERK, JNK, p38 or PI3K/AKT would be able to reverse the apoptotic effect of H_2O_2. Trypan blue and DAPI staining examinations revealed that, a MEK-1 inhibitor PD98059, JNK inhibitor SP600125, p38 kinase inhibitor SB203580 or PI3K/AKT inhibitor wortmanin were unable to block the toxic effect of H_2O_2 from 0.05 to 5 mM concentrations, eventhough they diminished the phosphorylation of the respective kinases under identical culture conditions of C6 glioma cells (data not shown).

3.4. H_2O_2 stimulated and changed the localization of ATF-2, AKT and ERK1/2 in C6 glioma cells

To investigate the cellular localization of these signalling proteins activated by H_2O_2, we next performed immunocytochemistry experiments with the antibodies that we have used in western blot studies. When cells were exposed to 0.5 mM H_2O_2 for 30 min, we observed that ERK1/2, ATF2, and AKT all were phosphorylated and showed different localization patterns. In unstimulated cells phosphorylated ERK1/2 (Fig. 4C) and ATF2 (Fig. 4A) were hardly detectable, whereas phosphorylated AKT (Fig. 4B) was found in the cytoplasm. After stimulation with H_2O_2, phosphorylated ERK1/2 (Fig. 4F)

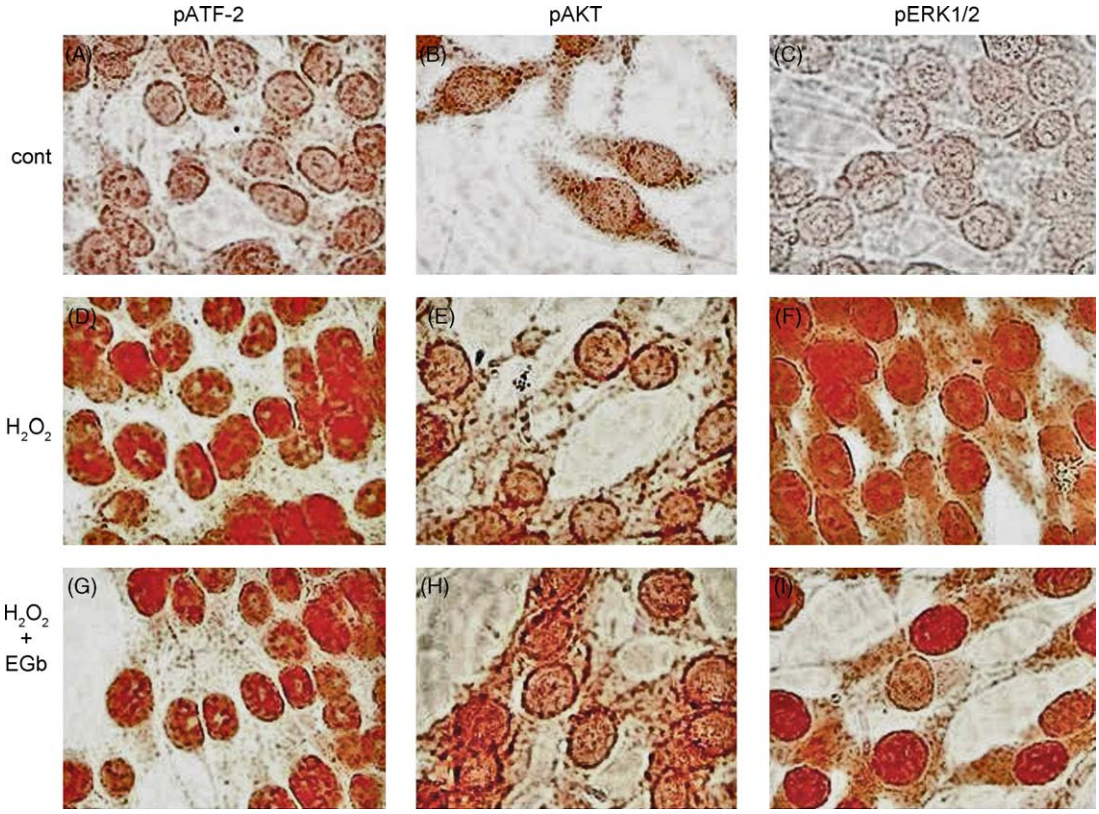

Fig. 4. H_2O_2 activated and changed the localization of ATF-2, AKT and ERK1/2 in C6 glioma cells. Cells were preincubated for 30 min with EGb (G, H and I) or vehicle (A–F) then stimulated with (D–I) or without (A–C) H_2O_2 (0.5 mM) for 30 min as indicated. Cells were fixed, stained with polyclonal antibodies against phosphorylated forms of ATF-2 (A, D and G) and AKT (B, E and H) and ERK1/2 (C, F and I). Cells were visualized by using biotinylated secondary antibody, streptavidin/HRP and AEC chromogen. Cells were photographed through brightfield microscope under 600× magnification.

and phosphorylated ATF2 (Fig. 4D) were detected strongly in the nucleus and phosphorylated AKT was detected predominantly in the perinuclear membrane and nucleus (Fig. 4E).

We have previously shown that activation of JNK is involved in the apoptotic cell death induced by simvastatin in these cells (Koyuturk et al., 2004). This study presented that in C6 glioma cells apoptotic cell death caused by H_2O_2 and simvastatin activated different signalling pathways. While simvastatin induced sustained activation of JNK/ATF2 (Koyuturk et al., 2004), H_2O_2 transiently stimulated ATF2, ERKs and AKT.

3.5. EGb did not block the activation of ATF-2, AKT and ERK1/2 in C6 glioma cells

We explored whether the signalling proteins ERK1/2, ATF2, and AKT that were activated by H_2O_2 were involved in the protective effect of EGb in C6 glioma cells by using western blot and immunocytochemical analysis. As shown in Figs. 3 and 4(G–I), in the presence of EGb (200 μg/ml) the phosphorylation of ERK1/2, ATF2, and AKT by H_2O_2 (100 μM) was not affected. Since pharmacological inhibitors of these kinases also did not protect C6 glioma cells from apoptotic cell death induced by H_2O_2, we concluded that anti-apoptotic effect of EGb in these cells is independent of these signalling proteins.

4. Discussion

In the present study, we showed that H_2O_2 causes growth inhibition and apoptotic cell death which is prevented by EGb in C6 glioma cells. Oxidative stress has been implicated in the pathophysiology of several human diseases, including atherosclerosis, cancer, neurodegenerative diseases and aging. These reports suggest that ROS produced during oxidative stress can damage various cell components or activate specific physiological signalling pathways, with the relative effects determined by ROS concentration (reviewed in Berlet and Stadtman, 1997).

Several components of the ERK1/2 and PI3K/AKT signalling pathways have been associated with survival signals in mammalian cells (Downward, 1998), whereas JNK and p38 kinases have been implicated in apoptosis during oxidant injury (reviewed in Martindale and Holbrook, 2002). The transactivation capacities of the N-terminal domains of ATF-2 and c-jun can be enhanced through phosphorylation at Thr-69 and Thr-71 residues by p38 kinase and JNK (Davis, 2000; Chang and Karin, 2001). In our studies, the substrate of these kinases, ATF-2, was markedly phosphorylated and localised to nucleus in response to H_2O_2, but the pharmacological inhibitors of these kinases were unable to block the pro-apoptotic effect of H_2O_2 in C6 glioma cells. However, although EGb did not inhibit the phosphorylation of ATF-2 by H_2O_2, it protected cells from H_2O_2-induced toxicity.

Therefore, we concluded that a kinase other than JNK and p38 kinase may regulate the toxic effect of H_2O_2.

ERK1/2 and AKT were also phosphorylated and activated following H_2O_2 treatment, however, neither EGb blocked the activation of these kinases induced by H_2O_2, nor the inhibitors of these kinases protected cells from H_2O_2-induced cell death. It has been suggested that ROS activates contradictory signalling pathways and that the balance between these pathways may be important in determining whether a cell survives or undergoes apoptosis (reviewed in Martindale and Holbrook, 2002). Many studies have identified the concomitant activation of both pro- and anti-apoptotic responses to ROS (Park et al., 2001; Kim et al., 2002). Similarly, our study implies that C6 glioma cells might respond to H_2O_2-induced apoptosis by activating pro-survival signalling proteins ERK1/2 and AKT.

EGb has a broad spectrum of pharmacological activities on the central nervous system, e.g. in memory enhancing properties and in the regulation of cerebral glucose/energy metabolism (Hoyer et al., 1999; De Feudis and Drieu, 2000). Prevention of neuronal cell damage by EGb against oxidative injury via hydroxyl radical scavenging capacity has been reported (Middleton et al., 2000; Guidetti et al., 2001; Bastianetto and Quirion, 2002). However, the mechanisms underlying its neuroprotective ability remain to be fully established. In acute injuries to the brain, such as by hypoxia/ischemia, there is evidence that apoptosis as well as necrosis occur. The family of cysteine proteases known as caspases has been shown to be required for the execution of certain forms of apoptosis (Schwartz and Milligan, 1996). We found that oxidative stress caused by H_2O_2 triggered cell death in C6 glioma cells which showed features of apoptosis but a cell permeable inhibitor of caspases, DEVD-CHO, was not protective (not shown). These data suggest that the protective ability of EGb in C6 glioma cells may be attributable to its free radical scavenging antioxidant property.

We have reported recently that simvastatin induces apoptosis and increases the phosphorylation of ATF-2 and c-jun in a concentration- and time-dependent manner in C6 glioma cells. In contrast to H_2O_2, EGb did not rescue C6 glioma cells from simvastatin-induced apoptosis (not shown). This may suggest that ROS are not involved in the pro-apoptotic effect of simvastatin and different signalling pathways are used by H_2O_2 and simvastatin to regulate apoptosis in these cells. The results indicated a clear difference between the H_2O_2-dependent activation of ATF-2 and that of simvastatin, showing that H_2O_2-induced ATF-2 phosphorylation was transient, with an almost complete decline within 1 h, while simvastatin-induced ATF-2 activity exhibited a sustained kinetics, with the phosphorylated protein fully detectable more than 24 h after the induction. Also, H_2O_2 caused transient activation of ERK1/2 and AKT, which we have not observed previously with simvastatin in C6 glioma cells (Koyuturk et al., 2004).

Taken together, our findings indicate the protective role of EGb under oxidative stress in glial cells and underscore the beneficial effect of EGb in Alzheimer's disease, stroke, and aging, where oxidative stress have been implicated in their etiology. Furthermore, differential pro-apoptotic effect between H_2O_2 and simvastatin via induction of distinct signalling pathways was also delineated.

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