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Effects of Gingko Extract (EGb761) on oxidative damage under different conditions of serum supply

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Abstract Standardized Ginkgo biloba extract EGb761 is known to have multivalent properties such as anti-oxidation and anti-apoptosis. In this study, we determined in rat pheochromocytoma (PC12) cells effects of EGb761 treatment on oxidative damage under three different conditions of serum supply: normal growth medium (NGM), serum deprivation (SE) and serum deprivation followed by re-supply (SERS). It was found that, under the condition of serum deprivation, oxidative damage induced less cell death than the condition of serum supply. This appears to be related to inhibition of mitochondrial metabolism. Moreover, after serum deprivation, serum re-supply exacerbated cell necrosis, possibly through enhancement of oxidative damage. EGb761 could attenuate oxidative damage under the condition of serum supply whereas no protective effect on serum-depleted cells was observed. These results suggest that, there is a synergistic effect between trophic factors and EGb761. EGb761 treatment may protect cells from possible oxidative damage induced by the trophic factors. On the other hand, trophic factors appear to strengthen the protective effect of EGb761. To fully understand the synergistic interaction between antioxidants and trophic factors will help to sort out rational use of drugs in clinic practice.

Keywords Oxidative damage · Trophic factor · EGb761

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Introduction

EGb761 is a patented extract from the leaves of the Ginkgo biloba tree. Recently, EGb761 has been reported to have a multitude of beneficial effects on CNS function, from enhancing cognitive function in dementia to facilitating recovery from acute forms of neural damage such as hypoxia/ischemia (Maclennan et al. 2002). Accordingly, neuroprotective effects of EGb761 have been extensively researched. Anti-oxidation and anti-apoptosis are suggested to be two major biochemical activities of EGb761 (Smith et al. 2002). The antioxidant effect of EGb761 can be achieved by either direct attenuation of reactive oxygen species (ROS) (Smith and Luo 2003), chelating prooxidant transitional metal ions (Gohil and Packer 2002), expression of antioxidant proteins such as superoxide dismutase (SOD), and increase in antioxidant metabolites such as glutathione (Gohil and Packer 2002; Oken et al. 1998). Moreover, the flavonoid fraction of this drug appears to be more effective against hydroxyl radicals than the terpene fraction (Bastianetto et al. 2000; Zimmermann et al. 2002). In addition, the anti-apoptotic properties of EGb761 are also multifactoral, and act synergistically upon multiple cellular pathways (Smith et al. 2002). It has been found that EGb761 can protect the pivotal integrity of the mitochondrial membrane thus attenuate the release of Cytochrome c from the mitochondria; up-regulate the transcription of anti-apoptotic Bcl-2-like protein and down-regulate the transcription of pro-apoptotic caspase-12; inhibit the cleavage and activation of caspase-3 and nuclear DNA fragmentation, etc. (Smith et al. 2002). All these properties of EGb761 may contribute to its neuroprotective effects.

Currently, a number of age-associated neurotoxic events, such as high levels of free radicals conducive to oxidative stress and decreased neuronal trophic support by neuro-

trophic factors have been described to induce neuronal impairments that are reportedly associated with aging of the central nervous system or even age-related neurodegenerative diseases such as Alzheimer's disease (AD). The central nervous system is particularly vulnerable to oxidative damage because of its high energy requirements, high oxygen consumption, and high tissue concentration of iron and relatively low levels of some antioxidant systems (Rodríguez-Capote et al. 1998). In addition, the finding that some neuronal impairments during aging or AD occur selectively in nerve growth factor (NGF)-dependent neurons in the basal forebrain and hippocampus of human and animals (Rodríguez-Capote et al. 1998; Hefti and Schneider 1989; Gibbs 1998a, b; Perez-Polo et al. 1990; Salehi et al. 2000), where administration of exogenous NGF can be beneficial (Rodríguez-Capote et al. 1998; Hefti 1994; Jönhagen 2000; Kidd 2005) also suggests a role of decreased neuronal trophic support in age-associated neuronal impairments. It is therefore conceivable that diverse toxic stimuli may co-exist to perturb neuronal physiological homeostasis *in vivo*, suggesting that interventions should be targeted accordingly or comprehensively.

Therefore, in this study, we determined in PC12 cells the cell viability, mitochondrial function and intracellular GSH content in response to oxidative damage and EGb761 treatment under different conditions of serum supply. The results may contribute to better understanding of the neuroprotective effects of EGb761 and interaction between EGb761 and trophic factors.

Methods

Materials

EGb761, a standardized Gingko biloba extract standardized to contain 24% flavonol glycosides, 6% terpene trilactones (of these, 2.9% ginkgolides A, B, C and J and 3.1% bilobalide), and <5 ppm ginkgolic acidesis, were purchased from Dr Willmar Schwabe (German). The aggregate-forming lipohilic dye 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanineiodide (JC-1) was purchased from Sigma (USA). Before use, JC-1 was dissolved in Dimethyl Sulfoxide (DMSO) at 1 mg/ml and stored at −20°C. Unless stated otherwise, all other chemicals were purchased from Sigma (USA).

Cell culture

The rat pheochromocytoma cell line (PC12 CRL-2266, ATCC, US) was maintained in F-12K medium (Sigma, USA) supplemented with 10% (v/v) heat-inactivated horse serum (Gibco, USA), 5% (v/v) fetal calf serum (Gibco, USA), 100 IU/ml penicillin, and 100 g/ml streptomycin (Invitrogen, Grand Island, NY). Monolayer cultures at a density of 10^4 cells/cm^2 were incubated in plastic flasks precoated with 0.03% poly-L-ornithine in a 95% air, 5% carbon dioxide humidified atmosphere at 37°C. The culture medium was changed every 48 h.

Oxidative damage under different conditions of serum supply and EGb761 treatment

Oxidative damage was produced by treating PC12 cells with 25 μM hydrogen peroxide (H_2O_2) for 24 h and lowered intracellular GSH content to about 50%. After rinsing three times with PBS, cells were cultured under three different conditions: (a). normal growth medium for 4 h in presence or absence of 200 μg/ml EGb761; (b). serum deprivation for 4 h with or without 200 μg/ml EGb761; (c). serum deprivation for 2 h followed by a 2 h serum re-supply in presence or absence of 200 μg/ml EGb761.

MTT assay

Before and after exposure cells to different serum supply conditions, cell viability was determined by a colorimetric 3–4,5-dimethyithiazol–2-yl–2,5-diphenyl-tetrazolium bromide (MTT) assay according to Kobayashi et al (2007). Briefly, cells were plated at 1×10^4 cells/well into 96-well plates. After cells were treated as described above, 10 μl 5 mg/ml MTT solution was added to each well of the 96-well plates and incubated for 4 h at 37°C. Then, the medium containing MTT was aspirated off and 200 μl of DMSO was added to each well. With DMSO as the blank, the absorbance was then read with the Victor-2 Multilabel counter (EG&G wallac) at a wavelength of 490 nm.

Annexin V (AV)/ Propidium iodide (PI) flow cytometric analysis

Annexin V/Propidium iodide flow cytometric analysis was performed according to the manufacturer's protocol (Vybrant apoptosis assay kit #2, Invitrogen, USA). Briefly, an aliquot of cells (about 5×10^5cells) was harvested and washed twice in cold PBS at 400×g, 3 min. The supernatant was aspirated and the pellets were resuspended in 100 μl of annexin-binding buffer. After the addition of 5 μl of AV and 1 μl of PI, cell suspension was then incubated at room temperature for 15 min in the dark. After the incubation period, to each sample, 400 μl annexin-binding buffer was added, gently mixed and kept on ice. The stained cells were analyzed by Beckman Coulter flow cytometry (Coulter Epics AltraTM, USA) with PMT value of the detector in FL1 (Florescence Channel 1) set at 525 BP (Band Pass Filter) for AV detection and the FL2 PMT set at 610 BP for PI detection. The percentages of AV

positive (+) cells and PI positive (+) cells were quantified by Expo32TM software supplied in the instrument.

Flow cytometric analysis of mitochondrial membrane potential using JC-1

Cells plated at a density of 10^6 per well in a 6-well plate were loaded with JC-1 (at the final concentration of 5 μg/ml) directly in 6-well plates at 37°C for 20 min and then washed twice with PBS. Cells were then scraped from the bottom of the wells and, after centrifugation at 400×g for 3 min, were resuspended in 0.5 ml PBS. Flow cytometric analysis was then performed within 10 min by using the flow cytometry described earlier in this paper. Green and red fluorescence were analyzed on the FL1 (525 nm BP) and FL2 (575 nm BP) channels, respectively. Data were analyzed with Expo32TM software to quantify the percentages of red and green fluorescence.

Measurement of cytochrome oxidase (COX) activity

The isolation of mitochondria and measurement of activity of COX was performed using a commercially available mitochondria isolation kit (Sigma, USA) and cytochrome c oxidase assay kit (Sigma, USA).

Protein concentration of mitochondrial pellets was determined by the method of Bradford (Shi et al. 2008) with the Victor-2 Multilabel counter (EG&G wallac) at a wavelength of 595 nm, using protein assay dye reagent concentrate (Bio-Rad, USA) and protein standard (bovine serum albumin, Bio-Rad, USA) to plot the standard curve.

Measurement of the rate of mitochondrial Adenosine Triphosphate (ATP) synthesis by a reversed phase high performance liquid chromatography (HPLC)-based method

The rate of mitochondrial ATP synthesis was measured as previously described (Shi et al. 2008). Isolated mitochondrial pellets (1.0 mg/ml) were suspended in M-medium consisting of 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mM ethylene glycol tetraacetic acid, 1.6 mM MgCl2, 5 mM succinate, 1 mg/ml bovine serum albumin (fatty acid-free), 0.5 μg/ml rotenone and 2.5 mM potassium phosphate buffer (pH 7.4). Ten seconds after addition of adenosine diphoshate (at a final concentration of 100 μM), the reaction was terminated by adding ice-cold 1.6 M perchloric acid. The mixture was incubated on ice for 30 min and then centrifuged at 15,000 rpm for 5 min. The resulting supernatant was neutralized by adding an equal volume of saturated potassium hydroxide solution and then centrifuged at 15,000 rpm for 5 min. Supernatants were stored at −80 °C until high performance liquid chromatograph assay (Waters, USA) was performed.

Intracellular GSH content measurement

Before and after exposure of cells to different serum supply conditions, intracellular GSH content was measured by using a commercially available Glutathione Assay kit (Sigma, USA). Measurements were carried out in a cell extract preparations from 1×10^7 cells with the Victor-2 Multilabel counter (EG&G wallac) at an excitation wavelength of 390 nm and emission wavelength of 478 nm. Protein concentration of a cell extract was determined as previously described.

Statistics

Statistical analysis was done by SPSS 15.0 software (Statistical Package for the Social Sciences). After analyzed by the Kolmogovor-Smirnov test for normal distribution and Levene's test of Equal Variances for variance homogeneity, all data were submitted to Two-Way Univariate analysis of variance (ANOVA) followed by the Least Significant Difference t test (LSD). For skewed data with variance heterogeneity, square root transformation was used.

Results

Decreasing of cell viability under different conditions of serum supply and protective effect of EGb761

After 24 h H_2O_2 treatment, the viability of cells under all three conditions of serum supply decreased ($p<0.01$, Fig. 1). The cell viability of SD group still maintained the level immediately after H_2O_2 was removed (Fig. 1). But the cell viability of the NGM or SDRS group was lower than this level ($p<0.05$, Fig. 1). Moreover, the cell viability of the SDRS group was also lower than the NGM group ($p<0.01$, Fig. 1).

Although no significant differences of cell viability between the SD group and its corresponding EGb761-treated group were found (Fig. 1), the cell viability of the SDRS group and NGM group was significantly lower than their corresponding EGb761-treated groups ($p<0.01$, Fig. 1). The difference was EGb761 treatment could maintain the cell viability of the NGM group at the level immediately after H_2O_2 was removed (Fig. 1) whereas the cell viability of the EGb761-treated SDRS group still below this level ($p<0.01$, Fig. 1).

It seemed that, cell death induced by oxidative damage under the condition of serum deprivation was not so severe as under normal serum supply or serum deprivation and re-supply; EGb761 treatment showed no protective effects on cell viability under the condition of serum deprivation but alleviated cell death under other two conditions.

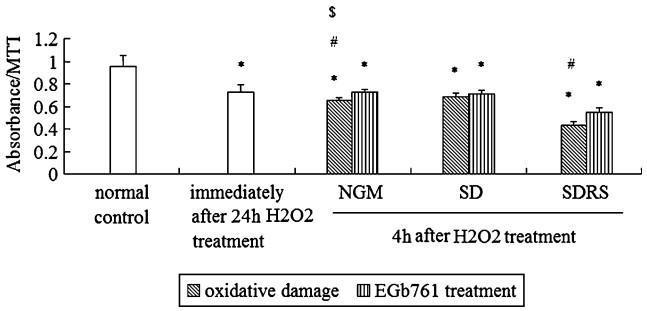

Fig. 1 Effects of EGb761 on oxidative damage-induced decrease of cell viability under the three different conditions of serum supply. Cells were treated as described in "Methods" section. Then, cell viability was determined by MTT assay. *: compared with control, $p<0.01$; #: compared with the level immediately after 24 h H_2O_2 treatment, $p<0.05$; $: compared with SDRS group, $p<0.01$

Cell apoptosis/necrosis under different conditions of serum supply and protective effect of EGb761

Annexin V (AV) and propidium iodide (PI) double staining detected by flow cytometry were used as criteria for distinguishing cycling (AV(−)/PI(−)), early apoptotic (AV(+)/PI(−)), early necrotic (AV(−)/PI(+)), and late apoptotic/necrotic cells (AV(+)/PI(+)). Exposure of proliferating PC12 cells to oxidative damage under three conditions of serum supply increased the binding of AV and PI (Figs. 2 and 3). In this study, AV(+) cells, the sum of AV(+)/PI(−) and AV(+)/PI(+) cells, and PI(+) cells, the sum of AV(−)/PI(+) and AV(+)/PI(+) cells, were considered as apoptotic and necrotic cells respectively. As shown in Fig. 2a, for serum-depleted cells, increase of AV uptake was more apparent than increase of PI uptake (44% vs. 20%, $p<0.01$), suggesting that the mode of cell death under serum deprivation might mainly be the apoptotic cell death; for cells treated with serum deprivation and serum re-supply, increase of PI uptake was more obvious than AV uptake (55% vs. 22%, $p<0.01$), indicating that the mode of cell death might mainly be the necrotic cell death; for cells exposed to normal growth medium, increase of AV uptake was almost equal to increase of PI uptake (28% vs. 31%), suggesting that exposed to normal growth medium, both apoptosis and necrosis might be induced.

EGb761 treatment decreased the binding of AV and PI to cells exposed to normal growth medium or serum deprivation and re-supply ($p<0.01$, Fig. 2b, c; Fig. 3), but showed no such effects on serum depleted cells.

Depolarization of mitochondrial membrane potential under different conditions of serum supply and protective effect of EGb761

Mitochondrial membrane potential (MMP) could be used as an indicator for mitochondrial integrity and bioenergetic function. Changes in MMP were assessed by flow cytometry using the lipohilic dye JC-1. The monomeric form of the dye shows a green fluorescence, whereas within the mitochondrial matrix at high membrane potential, JC-1 forms aggregates which emit red fluorescence. Therefore, cells stained with JC-1 exhibit a heterogeneous distribution of polarized (red fluorescing JC-1 aggregates) and depolarized (green fluorescing monomers) mitochondria. The

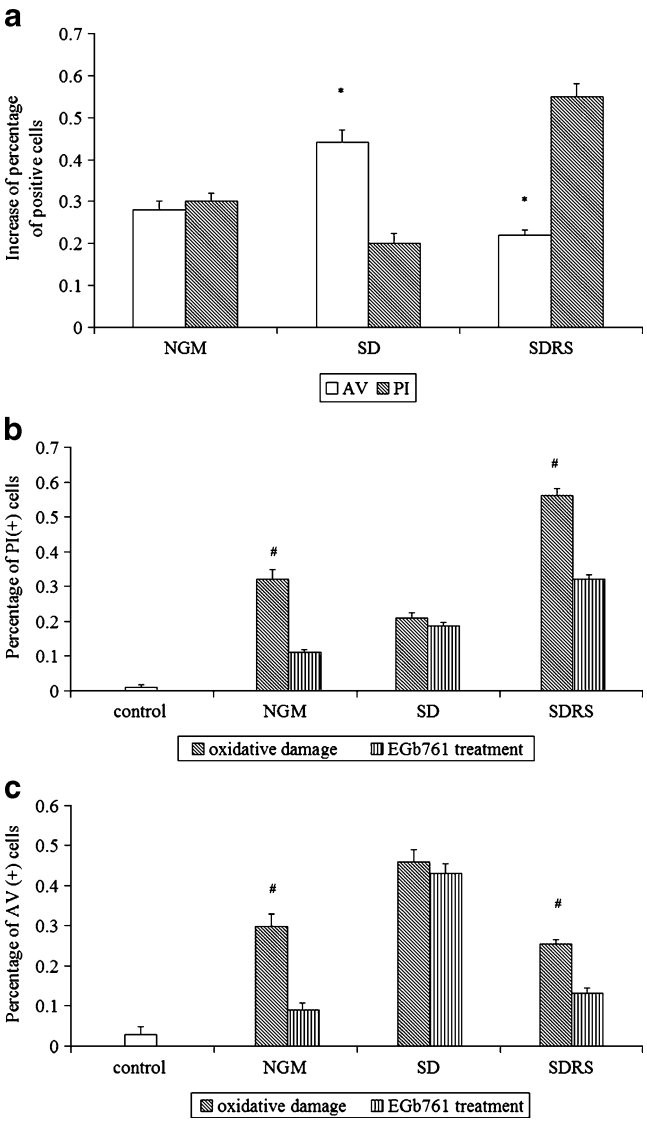

Fig. 2 Effects of EGb761 on oxidative damage-induced cell death under different conditions of serum supply. Cells were treated as described in "Methods" section. Oxidative damage-induced cell death was then determined with AV/PI double staining **a** oxidative damage-induced increase of AV and PI uptake in PC12 cells under three different conditions of serum supply; **b** effects of EGb761 on AV uptake under three different conditions of serum supply; **c** effects of EGb761 on PI uptake under three different conditions of serum supply. *: compared with increase of percentage of PI positive cells, $p<0.01$; #: compared with corresponding EGb761-treated group, $p<0.01$

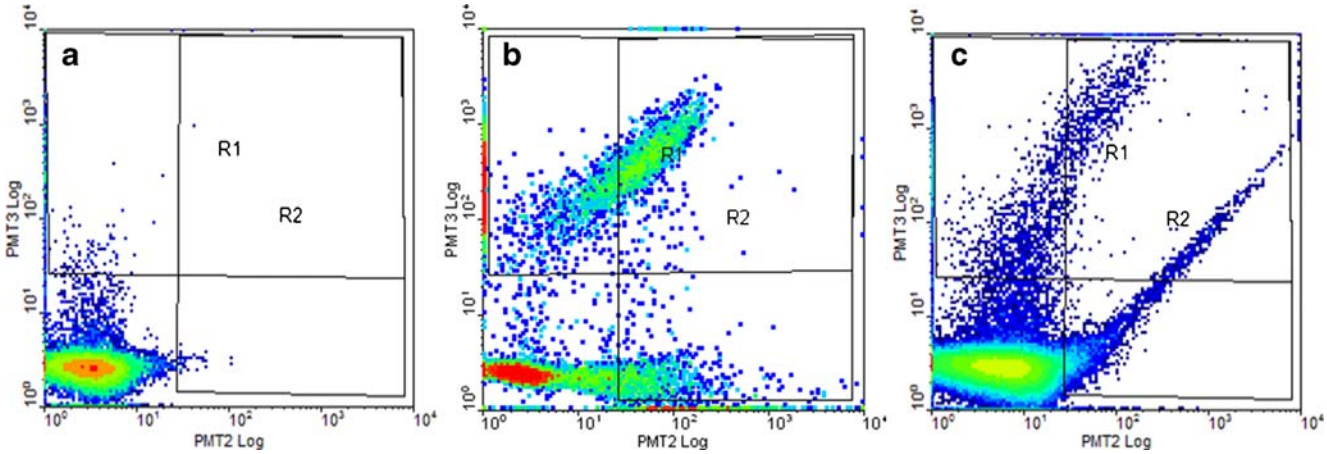

Fig. 3 Representative flow cytometry histogram showing AV and PI double staining of PC12 cells. Cells stained with AV are shown in R1 region and cells stained with PI are shown in R2 region. It can be seen that, compared with the pattern of untreated PC12 cells (**a**), PC12 cells treated with serum deprivation and re-supply after 24 h H_2O_2 treatment show an increase in the binding of both AV and PI (the number of cells in both R1 and R2 regions was raised) (**b**). But after EGb761 treatment, this change was partially reversed (**c**)

change in the proportions of these mitochondrial subpopulations reflects mitochondrial functional states. Specifically, fluorescence in PMT3 (red) decreased with a corresponding increase in PMT2 (green) fluorescence suggesting a depolarization enhancement of mitochondria (Fig. 4a, b, c).

Exposure of cells to oxidative damage under three conditions of serum supply all decreased red/green JC-1 fluorescence ratio ($p<0.01$, Fig. 5). But the red/green JC-1 fluorescence ratio of the SD group was significantly higher than the NGM group and SDRS group ($p<0.01$, Fig. 5). Moreover, the red/green JC-1 fluorescence ratio of the NGM group was also higher than the SDRS group ($p<0.01$, Fig. 5).

The red/green JC-1 fluorescence ratio of the NGM and the SDRS groups was lower than their corresponding EGb761-treated groups ($p<0.01$, Fig. 5). But no significant difference of red/green JC-1 fluorescence ratio between the SD group and its corresponding EGb761-treated group was found (Fig. 4). In addition, the red/green JC-1 fluorescence ratio of all the EGb761-treated groups was still lower than the control group ($p<0.01$, Fig. 5).

These results suggested that, oxidative damage under the three conditions of serum supply decreased the MMP to different extent and MMP depolarization of cells exposed to serum deprivation was not so severe as cells under the other two conditions. EGb761 treatment showed no protective effects on MMP under the condition of serum deprivation but ameliorated MMP depolarization under the other two conditions.

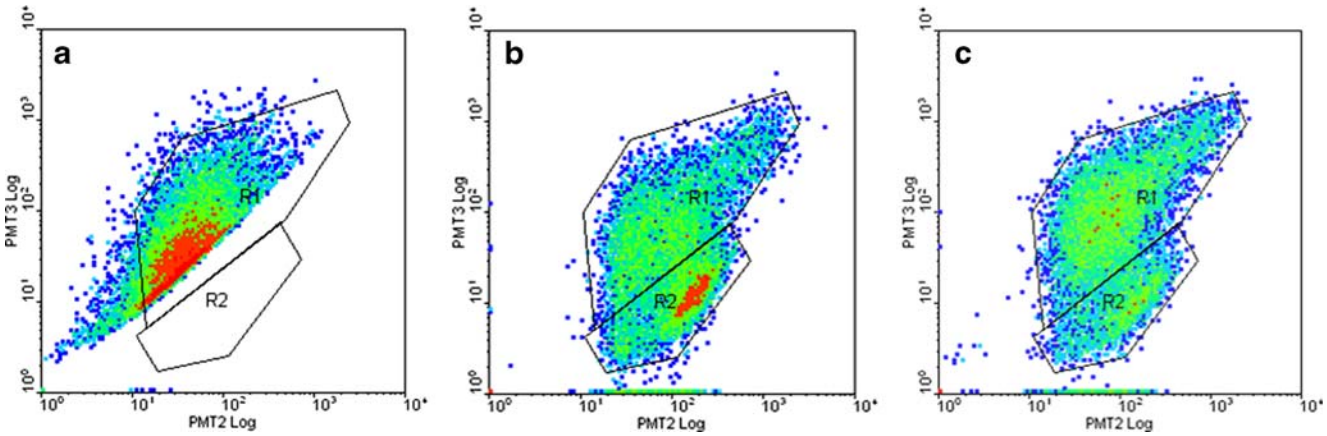

Fig. 4 Representative flow cytometry dot plots showing JC-1 staining of PC12 cells. **a** shows untreated PC12 cells. Approximately 80 percent of the cell events express red J-aggreregions (PMT3, as shown in the R1 region) and 0.08 percent contains green monomer fluorescence (PMT2, as shown in the R2 region); **b** shows the PC12 cells exposed to normal growth medium after 24 h H_2O_2 treatment. Approximately 46 percent of the cell events express red J-aggreregions (PMT3, as shown in the R1 region) and 51 percent contains green monomer fluorescence (PMT2, as shown in the R2 region); **c** shows EGb761-treated PC12 cells which were exposed to normal growth medium after 24 h H_2O_2 treatment. Approximately 72 percent of the cell events express red J-aggreregions (PMT3, as shown in the R1 region) and 25 percent contains green monomer fluorescence (PMT2, as shown in the R2 region)

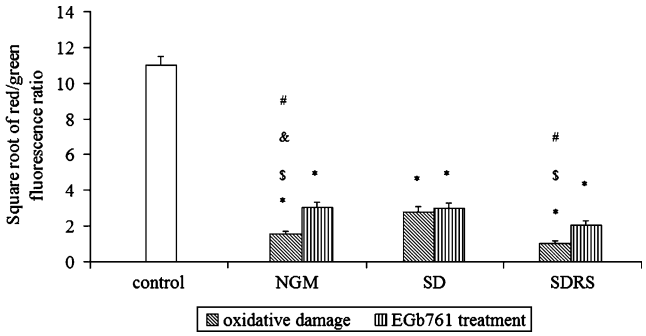

Fig. 5 Effects of EGb761 on oxidative damage-induced decrease of mitochondrial membrane potential under three conditions of serum supply. Cells were treated as described in "Methods" section. Oxidative damage-induced cell death was then determined with JC-1 staining. *: compared with control, $p<0.01$; $: compared with SD group, $p<0.01$; &: compared with SDRS group, $p<0.01$; #: compared with corresponding EGb761-treated group, $p<0.01$

Reduction of COX activity under different conditions of serum supply and protective effect of EGb761

COX activity of cells under three conditions of serum supply was lowered ($p<0.01$, Fig. 6). Furthermore, the COX activity of the SD group was significantly lower than the NGM group and SDRS group ($p<0.01$, Fig. 6). But no significant difference of the COX activity between the NGM group and SDRS group was found (Fig. 6).

COX activity of NGM and SDRS groups was lower than their corresponding EGb761-treated groups ($p<0.01$, Fig. 6). But no significant difference of COX activity between SD group and its corresponding EGb761-treated group was found (Fig. 6). In addition, COX activity of all the EGb761-treated groups was still lower than control group ($p<0.01$, Fig. 6).

It appeared that, COX activity reduction of serum-depleted cells was the most obvious compared with cells exposed to normal growth medium or serum deprivation and re-supply; EGb761 treatment showed no protective effects on COX activity under the condition of serum deprivation but attenuated decrease of COX activity under the other two conditions.

Decrease of mitochondrial ATP synthesis under different conditions of serum supply and protective effect of EGb761

Similarly, the rate of ATP synthesis of cells under all three conditions of serum supply were lowered as well ($p<0.01$, Fig. 7). Moreover, the rate of ATP synthesis of the SD group was significantly lower than the NGM group and SDRS group ($p<0.01$, Fig. 7). But no significant difference of the rate of ATP synthesis between the NGM group and SDRS group was found (Fig. 7)

The rate of ATP synthesis of the NGM and SDRS groups was lower than their corresponding EGb761-treated groups ($p<0.01$, Fig. 7) whilst no significant difference of the rate of ATP synthesis between the SD group and its corresponding EGb761-treated group was found (Fig. 7). In addition, the rate of ATP synthesis of all the EGb761-treated groups was still lower than the control group ($p<0.01$, Fig. 7).

It seemed that, the decrease of the rate of ATP synthesis of serum-depleted cells was the most obvious when compared with cells exposed to normal growth medium or serum deprivation and re-supply; EGb761 treatment showed no protective effects on ATP synthesis under the condition of serum deprivation but ameliorated reduction of ATP synthesis under other two conditions.

Intracellular GSH content before and after exposure to three different conditions of serum supply and protective effect of EGb761

As shown in Fig. 8, after 24 h H_2O_2 treatment, intracellular GSH content was reduced by half. After exposure of cells

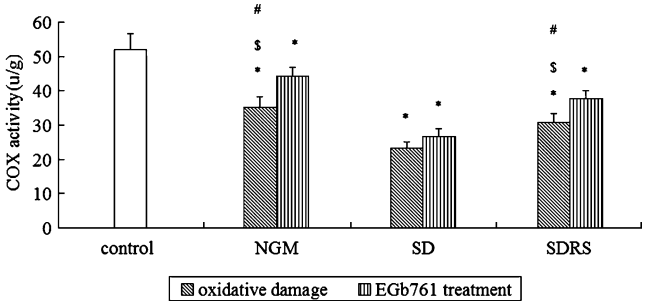

Fig. 6 Effects of EGb761 on oxidative damage-induced COX activity reduction under the three different conditions of serum supply. Cells were treated as described in "Methods" section. Intracellular GSH content was then determined using Cytochrome c oxidase assay kit. *: compared with control, $p<0.01$; $: compared with SD group, $p<0.01$; #: compared with corresponding EGb761-treated group, $p<0.01$

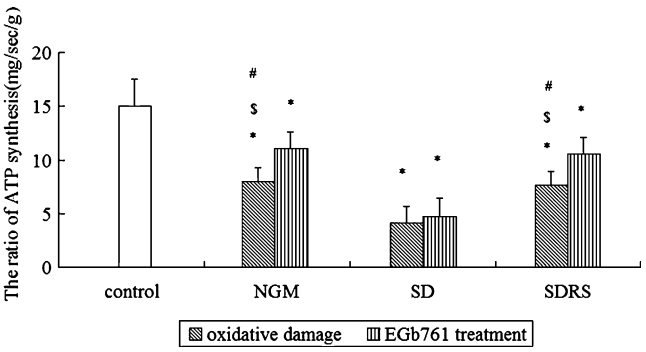

Fig. 7 Effects of EGb761 on oxidative damage-induced decrease of mitochondrial ATP synthesis under the three different conditions of serum supply. Cells were treated as described in "Methods" section. The rate of ATP synthesis was then determined using a HPLC-based method. *: compared with control, $p<0.01$; $: compared with SD group, $p<0.01$; #: compared with corresponding EGb761-treated group, $p<0.01$

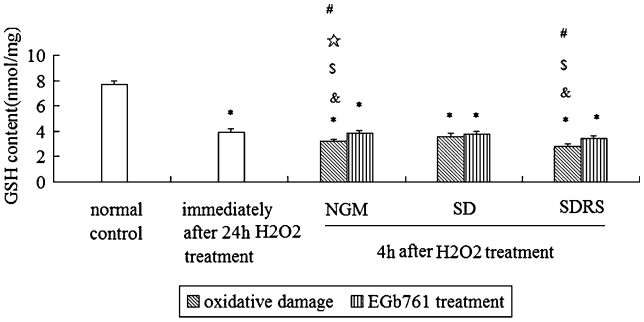

Fig. 8 Effects of EGb761 on oxidative damage-induced decrease of intracellular GSH content under the three different conditions of serum supply. Cells were treated as described in "Methods" section. Intracellular GSH content was then determined using Glutathione Assay kit. *: compared with control, $p<0.01$; &: compared with the level immediately after 24 h H_2O_2 treatment, $p<0.01$; $: compared with SD group, $p<0.05$; ☆: compared with SDRS group, $p<0.01$; #: compared with corresponding EGb761-treated group, $p<0.01$

to three different conditions of serum supply for 4 h, intracellular GSH content decreased to lower than the level immediately after H_2O_2 was removed ($p<0.01$, Fig. 8). In addition, intracellular GSH content under the condition of serum deprivation was significantly higher than under the other two conditions ($p<0.05$, Fig. 8). Moreover, intracellular GSH content of the NGM group was also higher than the SDRS group ($p<0.01$; Fig. 8).

Although no significant difference of intracellular GSH content between SD group and its corresponding EGb761-treated group was found (Fig. 8), the GSH content of EGb761-treated SDRS and NGM groups was significantly higher than their corresponding insult groups ($p<0.01$) and maintained the GSH content at the level immediately after H_2O_2 was removed.

It appeared that, after exposure of cells to three different conditions of serum supply, intracellular GSH content continued to decline. But the decrease of intracellular GSH content was the least under the condition of serum deprivation. EGb761 treatment showed no protective effects on intracellular GSH content under the condition of serum deprivation but attenuated reduction of intracellular GSH content under the other two conditions.

Discussion

According to the free radical theory of aging, free radicals react with biomolecules, such as proteins and lipid membranes, which are responsible for the functional deterioration related to aging (Harman 2003). This theory was later refined to include the concept that mitochondria play a key role in aging acting as the major source and target of oxidants (Miquel et al. 2004). Oxidative damage to mitochondria lead to an increased generation of reactive oxygen species (ROS), which in turn leads to increased oxidation eventually resulting in mitochondrial dysfunction. This positive feedback of oxidative damage has been proposed to lead to an accumulation of dysfunctional mitochondria as well as an increased accumulation of oxidative damage in the cytosol and nuclei (Van Remmen and Richardson 2001). But cellular fate is controlled by a number of factors within the cell, including the defenses against free radicals generated both endogenously and exogenously. GSH is a ubiquitous tripeptide (gamma-glutamyl-cysteinyl-glycine), a main function of which is to protect the cell against damage induced by ROS. GSH is normally present in a cell 98 to 99% in the reduced form, with the remainder largely as glutathione disulfide (GSSG) or as mixed disulfides. Small variations in intracellular GSH in response to an oxidative stress will have a large impact on GSSG and can cause profound effects on thiol redox-dependent cell signaling (Kern and Kehrer 2005). It is found that, upon aging, oxidation of GSH has been observed in whole cells (Garcı´a de la Asuncio´n et al. 1996). On the basis of these, a cell damage model in this study was established by incubating PC12 cells with H_2O_2 for 24 h to reduce intracellular GSH content by half. After H_2O_2 was removed, intracellular GSH content kept on declining, suggesting a continuing intracellular oxidative damage. Oxidative damage under three different conditions of serum supply all induced mitochondrial dysfunction and cell death. However, it appeared that, cell loss was the least under the condition of serum deprivation. Actually, similar results have been obtained by previous studies. Ratan et al. (1996) have reported that, serum deprivation diminishes glutathione depletion-induced death of cortical neurons as compared to cultures treated with growth factors or serum. Furthermore, they resolve that, under the condition of serum deprivation, mitochondrial metabolism may be inhibited and thereby decrease the net amount of free radicals produced unavoidably as a result of the basic cell functions but they did not give more evidence for this presumption (Halliwell 1992). Our study further verified this by showing that, compared with cells exposed to normal growth medium or serum deprivation and re-supply, serum-depleted cells had lower COX activity and rate of ATP synthesis but maintained relatively higher mitochondrial membrane potential which was indicative of a inhibition of mitochondrial activities. In addition, after exposed to serum deprivation, no further decrease in intracellular GSH content was observed, possibly because serum deprivation may result in phosphorylation of the alpha subunit of the eukaryotic initiation factor (eIF-2α) and thereby lead to inhibition of protein synthesis (Montine and Henshaw 1989; Hu and Wieloch 1993) and inhibition of protein synthesis may subsequently result in shunting of cysteine from protein into the formation of GSH (Ratan 1994).

Our results also demonstrated that, compared with serum-depleted cells, after cells were exposed to serum deprivation followed by re-supply, necrosis might be enhanced. Damage to cells cultured in normal growth medium fell in between. Similarly, Koh et al. (1995) showed that Brain-derived neurotrophic factor (BDNF) reduced apoptotic death but enhanced necrotic cell death of cortical neurons after an excitotoxic insult. The possible reason is, after removal of trophic support, trophic factors re-supply may activate specific radical generating enzyme such as nitric oxide synthase, thus increase the formation of free radicals, particularly the nitric oxide, which enhanced N-methyl-D-aspartic acid neurotoxicity and finally exacerbate cell damage (Koh et al. 1995; Ko et al. 2000; Klocker et al. 1998; Samdani et al. 1997; Kalb and Agostini, 1993). This can be supported also by our results showing that intracellular GSH level of cells exposed to serum deprivation and re-supply is lower than cells cultured in normal growth medium and serum-free medium. Increasing of production of free radicals induced by serum re-supply possibly increases intracellular GSH consumption.

Analysis of responses of cells to oxidative damage under three conditions of serum supply helps to better understand the protective effects of EGb761. As mentioned above, EGb761 possesses multivalent properties (such as anti-oxidation and anti-apoptosis) and helps regulate the general physiological status of the cells in response to stress posed by both intracellular and extracellular conditions (Smith and Luo 2004). In our study, when cells exposed to normal culture medium or serum deprivation and serum re-supply, EGb761 decreased oxidative damage-induced cell death to a certain extent and prevented decrease of intracellular GSH content, which supported the known antioxidant effect of EGb761. In addition, EGb761 attenuated oxidative damage-induced mitochondrial membrane potential depolarization, COX activity reduction and decreasing mitochondrial ATP synthesis, which further suggests that, mitochondria may serve as one important target for EGb761 to protect cells from oxidative damage. Similarly, previous studies also have shown that, EGb761 is able to decrease ROS levels and ROS-induced apoptosis in lymphocytes from aged mice after *in vivo* administration and protect mitochondria of PC12 cells from the attack of H_2O_2, antimycin and Abeta (Eckert et al. 2003). In another study, EGb761 improved sodium nitroprusside (nitric oxide donor)-induced decrease of mitochondrial membrane potential and ATP production in PC12 cells, dissociated brain cells and isolated brain mitochondria (Eckert et al. 2005). In addition, EGb761 specially protects mitochondrial ATP synthesis against anoxia/reoxygenation injury by scavenging the superoxide anion generated by mitochondria (Du et al. 1999). These findings together with our results, all suggest the beneficial effect of EGb761 on oxidative damage-induced mitochondrial dysfunction and cell death.

However, our study also showed that, no obvious protective effect of EGb761 on serum-depleted cells was observed during the process of observation. This finding can also be supported by previous *in vitro* and *in vivo* studies showing that the neuroprotective effects of serum or nerve growth factors can be potentiated by the addition of antioxidants, and an enhancement of excitotoxicity by trophic factors seems to be the most prudent explanation for this better survival effect of the combined treatment (Ratan et al. 1996; Klocker et al. 1998; Mayer and Noble 1994).

In summary, we have demonstrated that serum deprivation antagonizes oxidative damage-induced cell death by inhibition of mitochondrial activities; after serum deprivation, serum re-supply may exacerbate cell necrosis possibly through enhancement of oxidative damage. In addition, EGb761 treatment seemed more effective at promoting cell survival under the condition of serum supply than serum deprivation. These results support previous studies suggesting that antioxidant in combination with trophic factors are superior over either agent alone in promoting cell survival. EGb761 treatment may protect cells from possible oxidative damage induced by the trophic factors. On the other hand, trophic factors strengthen the protective effect of EGb761.

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