ELSEVIER

Toxicology 148 (2000) 103–110

TOXICOLOGY

www.elsevier.com/locate/toxicol

Mechanisms of apoptosis in rat cerebellar granule cells induced by hydroxyl radicals and the effects of EGb761 and its constituents

Wenjuan Xin *, Taotao Wei, Chang Chen, Yucheng Ni, Baolu Zhao, Jingwu Hou

Institute of Biophysics, Academia Sinica, 15 Datun Road, Chaoyang District, Beijing 100101, People's Republic of China

Accepted 24 July 1999

Abstract

In this study investigation is made on whether oxidative stress produced by treatment with hydroxyl radicals can induce apoptosis in rat cerebellar granule cells. The protective effects of Ginkgo biloba extract (EGb761) and its active constituents against apoptosis are also examined. The results show that hydroxyl radicals generated by the Fenton reaction induced apoptosis in cerebellar granule cells, which was associated with the decrease in the *Bcl-2* mRNA level and the increase in the protein levels of the transcription factors Fos and Jun. Moreover, hydroxyl radicals induced time-dependent lipid peroxidation in cells and caused the changes in the sulfhydryl group binding sites on the membrane proteins. Hydroxyl radicals may induce apoptosis *via* different signaling pathways. EGb761 attenuated these changes and its different constituents showed different effects. The total flavonoid component of EGb761 and a mixture of flavonoids and terpenes protected cerebellar granule cells from oxidative damage and apoptosis induced by hydroxyl radicals. Total terpenes of EGb761 did not protect against apoptosis. Flavonoids and terpenes did not show a synergistic effect in this regard.

Keywords: Hydroxyl radicals; Apoptosis; Cerebellar granule cells; Ginkgo biloba Extract (EGb761); *Bcl-2*; Fos/Jun

1. Introduction

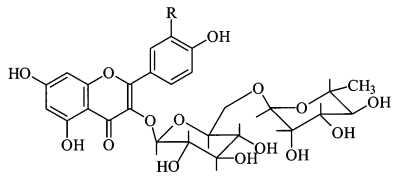
In multicellular organisms homeostasis is maintained by the balance of cell proliferation and cell death. Although much is known about the control of cell proliferation, the control of cell death remains less clear. In the last decade more and more evidence has suggested that reactive oxygen species (ROS) play an important role in the cascade of events leading to neuronal apoptosis (Greenlund et al., 1995). Antioxidants, which inhibit such apoptosis, may provide an exciting preventive and therapeutic prospective for neurodegenerative disorders (Cotman and Anderson, 1995). Ginkgo biloba extract (EGb761), which is standardized to contain 24% flavonoid glycosides

* Corresponding author. Tel.: +86-10-64888576; fax: 86-10-64877837.

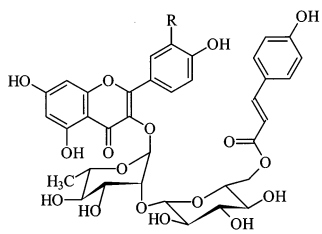
E-mail address: xinwj@sun5.ibp.ac.cn (W. Xin).

0300-483X/00/$ - see front matter
PII: S0300-483X(00)00200-6

(ginkgo-flavone glycosides; Fig. 1) and 6% terpene lactones (ginkgolides, bilobalide; Fig. 2), is widely studied for its effects on the cardiovascular system and the central nervous system (CNS), which are related to its antioxidant properties (DeFeudis, 1991). Recently it was reported that EGb761 caused improvement in about 50% of Alzheimer's patients (Le Bars et al., 1997). Our previous study also showed that EGb761 protected cerebellar granule cells from apoptosis induced by hydroxyl radicals (Ni et al., 1996a). However, it is not clear how the highly reactive and generally nonspecific ROS mediate such highly coordinated and controlled changes that occur during apoptosis. In the present study, the mechanisms of apoptosis induced by hydroxyl radicals were investigated by examining *Bcl-2*, Fos, Jun, lipid peroxidation and the sulfhydryl group binding sites on membrane proteins. The effects of EGb761 and its different constituents in this process were also further studied.

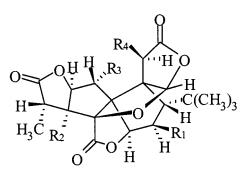


R = H: kaempferol-3-*O*-rutinoside
R = OH: quercetin-3-*O*-rutinoside (rutin)
R = OCH_3: isorhamnetin-3-*O*-rutinoside



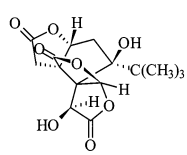
R = H: kaempferol-3-*O*-(6'''-trans-*p*-coumaroyl-2''-glucosyl)rhamnoside
R = OH: quercetin-3-*O*-(6'''-trsns-*p*-coumaroyl-2''-glucosyl)rhamnoside

Fig. 1. Chemical structures of typical flavonoids in EGb761.



Ginkgolides

	R^1	R^2	R^3	R^4
Ginkgolide A (GA)	H	OH	H	OH
Ginkgolide B (GB)	H	OH	OH	OH
Ginkgolide C (GC)	OH	OH	OH	OH
Ginkgolide J (GJ)	OH	OH	H	OH



Bilobalide

Fig. 2. Chemical structures of typical terpenes in EGb761.

2. Materials and methods

2.1. Materials

EGb761 was a generous gift of Lester Packer (University of California, Berkeley). The total flavonoids and total terpenes were isolated from EGb761 according to the method described in reference (Kwak et al., 1990). The constituents of EGb761 were selected as follows: total terpenes (T), total flavonoids (F), and a mixture of total flavonoids and total terpenes in a ratio of 4:1 (The same as that in EGb761) (F + T). Dulbecco's modified Eagle medium (DMEM), trypsin (1:250), and proteinase K were products of Gibco BRL, USA. Poly-L-lysine, guanidine isothiocyanate, HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)], MOPS [3-(*N*-morpholino)propanesulfonic acid], and spin label 3-maleimido-proxyl (3-mal) were purchased from Sigma, USA. Culture dishes were purchased from Nunc, Denmark. Random Primer Labeling Kit was from Promega, USA. *Bcl-2* probe was supplied by Sino-American Biotechnology, China. Fos and Jun antisera were kindly provided by Ziff (Medical Center, New York University). Other reagents made in China were of analytical grade. Wistar rats aged 7 days were purchased from Beijing Medical University, China.

2.2. Cell culture

Primary cultures of rat cerebellar granule cells were prepared following the procedures described previously (Ni et al., 1996a). Briefly, cerebella of Wistar rats were collected, rinsed with Hanks' Balanced Salt Solution (HBSS), and dissociated by mild trypsinization. Cells were plated on six-well multidishes (2×10^6 cells/ml, 2 ml/well) or 24-well multidishes (2.5×10^6 cells/ml, 0.4 ml/well) previously coated with poly-L-lysine in DMEM supplemented with KCl (19.6 mmol/l), L-glutamine (2 mmol/l), HEPES (10 mmol/l) and fetal calf serum (10%, v/v). Cells were maintained at 37°C in humidified 5% CO_2-95% air atmosphere. 48 h after plating, cells were exposed to hydroxyl radicals by the addition of 100 μM $FeSO_4$ and 50 μM H_2O_2. Antioxidants were added to cells 15 min before treatment with hydroxyl radicals. Unpublished results from our laboratory indicate that the addition of $FeSO_4$ (100 μM) alone does not induce apoptosis.

2.3. Determination of cell injury

Cell injury was assessed by measuring the lactate dehydrogenase (LDH) released from the lysed cells into the culture medium (Koh and Choi, 1987) using an LDH clinical diagnosis kit (Zhongsheng Hightech Bioengineering Co., Beijing, China). The percentage of LDH released into the medium was defined as the ratio of LDH activity in the culture medium to the total LDH activity, where the total LDH activity represents the LDH activity in the cells and the medium. The total LDH activity was measured in sister cultures by freezing/thawing cells rapidly (Papadopoulos et al., 1997).

2.4. DNA fragmentation assay

Cytosolic DNA fragments were quantified by a cell death detection ELISA^{PLUS} kit (Boehringer Mannheim, Cat. No. 1 774 425) as previously described (Sandoval et al., 1997), which is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. An enrichment factor, which represented the enrichment of nucleosomes in the cytoplasm of cells, was used as an index of DNA fragmentation.

2.5. RNA extraction and Northern blot analysis

The *Bcl-2* mRNA expression level in rat cerebellar granule cells was examined by Northern blot analysis. *Bcl-2* mRNA level was measured 1.5 h after treatment with hydroxyl radicals. Briefly, the total RNA was isolated by the guanidinium isothiocyanate method (Chomczyski and Sacchi, 1987). RNA was denatured and electrophoresed in 1% agarose-formaldehyde gel, subsequently transferred to nylon membranes and fixed by UV cross-linking. ^{32}P-labeled *Bcl-2* and *β-actin* cDNA probes were prepared using the random priming method. Northern blots were prehybridized at 42°C for 4 h in the solution consisting of 50% formamide, 5 × SSPE, 2 × Denhardt's solution, 0.1% SDS and 1% denatured salmon sperm DNA, and hybridized with the labeled probes in the same buffer overnight. After hybridization, the blots were washed twice in 2 × SSPE/0.1% SDS at room temperature, and washed twice in 0.1 × SSPE/0.1% SDS at 37°C, then exposed to X-ray films. Densitometric analysis of films was performed with Bio-Rad GS 670. *β-actin* was used as an internal standard.

2.6. Western blot analysis

The Fos/Jun protein expression levels in rat cerebellar granule cells were examined by Western blot analysis. Cell extracts were prepared by lysing the cells in 1 × SDS gel loading buffer as previously described (Sambrook et al., 1989). Protein concentrations were determined using a Bio-Rad kit according to the instruction of the manufacturer. Proteins were subjected to 10% SDS-PAGE and transferred electrophoretically to nitro-cellulose membranes. The membranes were blocked in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween20) containing 5% skim milk and then immunoblotted with the indicated antibodies in TBS-T containing 1% skim milk at 4°C for 2 h. After being washed with TBS-T, the membranes were incubated with anti-rabbit im-

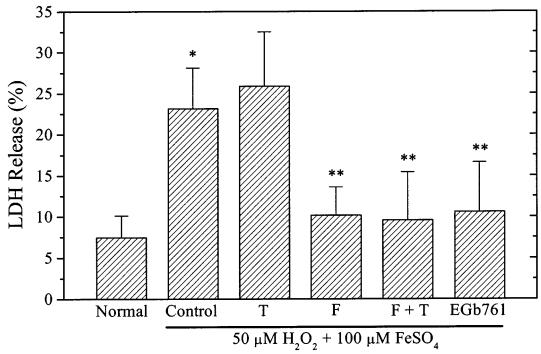


Fig. 3. Cytotoxicity of hydroxyl radicals. Cerebellar granule cells were treated with Ginkgo terpenes (T), Ginkgo flavonoids (F), mixture of Ginkgo terpenes and Ginkgo flavonoids (F + T) or EGb761 at doses of 100 μg/ml for 15 min followed by exposed to 50 μM H_2O_2 plus 100 μM $FeSO_4$ for 8 h. Cell injury was determined by LDH efflux assay. The data are means ± S.E.M. for four experiments. * $P < 0.05$ in comparison with normal cells; ** $P < 0.05$ in comparison with control cells.

munoglobulin G conjugated with alkaline phosphatase. Protein bands were visualized by an alkaline phosphatase substrate kit.

2.7. TBARS measurement

Thiobarbituric acid-reactive substances (TBARS) were measured with minor modification as previously described (Ohkawa et al., 1979). Briefly, cells were treated with 8.1% SDS, 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 0.8%TBA, then incubated at 95°C for 1 h. The solution of *n*-butanol and pyridine (15:1, v/v) was added and the mixture was shaken vigorously. After centrifugation, the absorption of the organic layer at 532 nm was measured.

2.8. Spin labeling and ESR measurement

Cell membrane was labeled with 3-mal as previously described (Ni et al., 1996b). Briefly, cells were washed with PBS, mixed with spin label 3-mal (100 μM) and incubated at 37°C for 3 h. The labeled cells were then washed four times with PBS, and transferred into quartz capillaries for ESR measurement. The ESR spectra were recorded at room temperature (298 K) by a Varian E-109 spectrometer with measurement conditions as: X-band, central magnetic field 325 mT, sweep width 20 mT, microwave power 20 mW, frequency 100 kHz, modulation amplitude 0.2 mT, time constant 0.128 s. The methods of calculation for the ratio of strongly immobilized component to the weakly immobilized component (S/W) were the same as previously described (Xin et al., 1984).

3. Results

3.1. Cell injury

The loss of membrane integrity that characterizes cell injury was examined by measuring the release of LDH. LDH released into the culture supernatant increased after exposure to hydroxyl radicals, which indicated the increase of lysed cells. In the group pre-treated with flavonoid component of EGb761, a mixture of flavonoids and terpenes or EGb761, the LDH release induced by hydroxyl radicals was inhibited markedly, which indicated that these constituents effectively protected cells from death. Pre-treatment with Ginkgo terpenes did not have a protective effect on cells (Fig. 3).

3.2. DNA fragmentation

Cerebellar granule cells treated with hydroxyl radicals showed characteristics of apoptosis. Quantitative detection of DNA fragmentation by ELISA indicated that exposure to hydroxyl radicals caused a marked increase in histone-associated DNA fragments (mono- and oligonucleosomes) (Fig. 4), which is a well-known characteristic of apoptosis. Cells pre-treated with flavonoid component of EGb761, a mixture of flavonoids and terpenes, and EGb761 were protected from apoptosis. Cells pre-treated with Ginkgo terpenes still underwent apoptosis.

3.3. Effect of hydroxyl radicals on Bcl-2 mRNA

Since *Bcl-2* is an important regulator of apoptosis, it is useful to test the possibility that hy-

droxyl radicals induce apoptosis by antagonizing *Bcl-2* activities. As shown in Fig. 5, *Bcl-2* mRNA declines in cells treated with hydroxyl radicals. On the contrary, EGb761 inhibited the decrease in *Bcl-2* mRNA.

3.4. Effect of hydroxyl radicals on Fos and Jun

The transcription factor AP-1, consisting of heterodimer of Fos and Jun, is involved in distinct

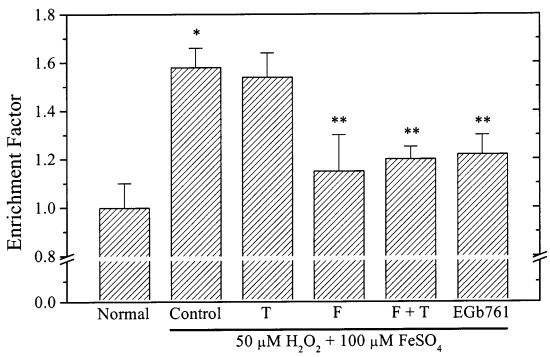


Fig. 4. DNA fragmentation induced by hydroxyl radicals. Cerebellar granule cells were treated with Ginkgo terpenes (T), Ginkgo flavonoids (F), mixture of Ginkgo terpenes and Ginkgo flavonoids (F + T) or EGb761 at doses of 100 μg/ml for 15 min followed by exposed to 50 μM H_2O_2 plus 100 μM $FeSO_4$ for 8 h. DNA nucleosomal fragmentation was determined by ELISA. The data are means ± S.E.M. for three experiments. * $P < 0.01$ in comparison with normal cells; ** $P < 0.05$ in comparison with control cells.

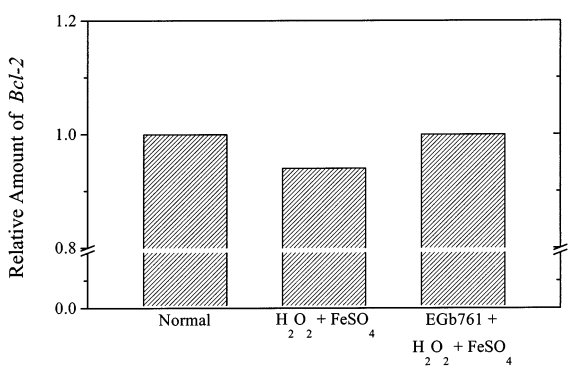


Fig. 5. The regulation of *Bcl-2* mRNA by hydroxyl radicals. Cerebellar granule cells were treated with EGb761 (100 μg/ml) for 15 min followed by exposure to 50 μM H_2O_2 and 100 μM $FeSO_4$ for 1.5 h. The amount of *Bcl-2* mRNA was analyzed by Northern blot. *β-actin* was used as an internal standard.

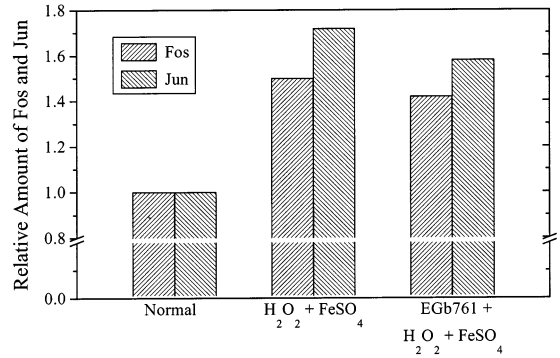


Fig. 6. The regulation of Fos and Jun by hydroxyl radicals. Cerebellar granule cells were treated with EGb761 (100 μg/ml) for 15 min followed by exposure to 50 μM H_2O_2 and 100 μM $FeSO_4$ for 2 h. The amount of Fos and Jun protein was analyzed by Western blot.

cellular processes including cell proliferation, differentiation and apoptosis (Lo and Cruz, 1995; Preston et al., 1996). To test whether AP-1 is involved in the apoptotic pathway induced by hydroxyl radicals, Western blot analysis was carried out. Results in Fig. 6 showed that Fos/Jun protein increased about 1.5 times in cells exposed to hydroxyl radicals. Pretreatment with EGb761 didn't show significant effect on the increase in Fos and Jun.

3.5. Effects of hydroxyl radicals on lipid peroxidation

Since the metabolite of lipid peroxidation is implicated in apoptosis (Buttke and Sandstrom, 1994), hydroxyl radicals may induce apoptosis through lipid peroxidation. To test this possibility and examine whether hydroxyl radicals and EGb 761 act on the same signaling pathway involving lipid peroxidation, we analyzed the lipid peroxidation in the presence of hydroxyl radicals and EGb 761. After treatment with 50 μM H_2O_2 and 100 μM $FeSO_4$, lipid peroxidation increased significantly with the exposure time (Fig. 7). By 8-h exposure, TBARS increased about 1.24 times to the control. EGb761 showed dose-dependent inhibition on lipid peroxidation induced by hydroxyl radicals (Fig. 8).

3.6. Change of sulfhydryl group binding sites

There are two types of sulfhydryl group binding sites on cell membrane proteins. One is on the surface of the proteins. When maleimide spin labels are bound to SH group site on the protein surface, the nitroxides of spin labels have greater freedom of movement. Hence the ESR spectra are weakly immobilized. The other type of binding site (type two) is within the deep layers of protein tertiary structure. When maleimide spin labels are bound to the internal sites, the movement of nitroxide is limited. Hence these labels give rise to strongly immobilized spectra (Xin et al., 1984). The change value of S/W indicates the change of suflhydryl group binding sites within the membrane protein. As shown in Fig. 9, the increase of S/W in apoptotic cells indicated the increase of the type two suflhydryl group binding sites. EGb761 attenuated such changes.

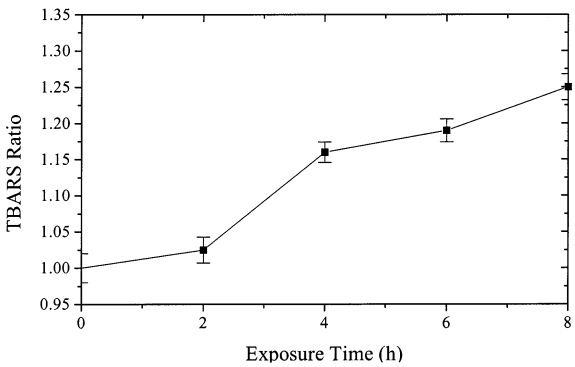


Fig. 7. Kinetics of lipid peroxidation induced by hydroxyl radicals. Cerebellar granule cells were treated with 50 μM H_2O_2 and 100 μM $FeSO_4$ for indicated time. The level of lipid peroxidation in cells was determined by TBA method. The data are means ± S.E.M. for three to four experiments.

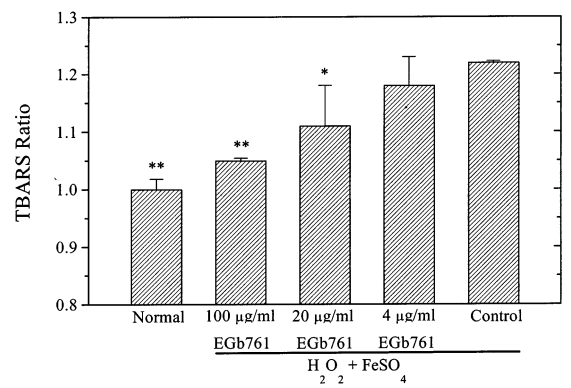


Fig. 8. Inhibitory effect of EGb761 on lipid peroxidation. Cerebellar granule cells were treated with different doses of EGb761 for 15 min followed by exposure to 50 μM H_2O_2 and 100 μM $FeSO_4$ for 8 h. The level of lipid peroxidation in cells was determined by TBA method. The data are means ± S.E.M. for three to four experiments. * $P < 0.05$ in comparison with control cells; ** $P < 0.01$ in comparison with control cells.

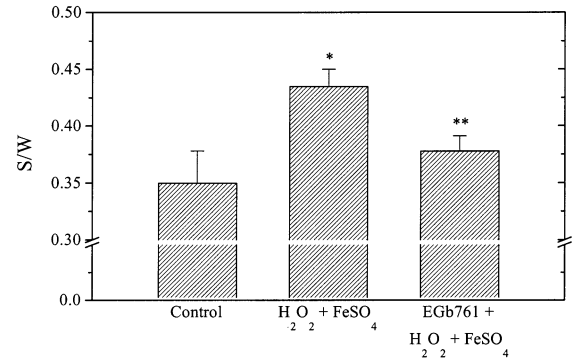


Fig. 9. Change of membrane proteins induced by hydroxyl radicals. Cerebellar granule cells were treated with EGb761 (100 μg/ml) for 15 min followed by exposure to 50 μM H_2O_2 and 100 μM $FeSO_4$ for 8 h. The ratio of strongly immobilized to weakly immobilized components (S/W) was measured by spin labeling. The data are means ± S.E.M. for three experiments. * $P < 0.05$ in comparison with normal cells; ** $P < 0.01$ in comparison with control cells.

4. Discussion

According to the above results, we conclude that hydroxyl radicals generated by Fenton reaction induced apoptosis in cerebellar granule cells, which was associated with the decrease in *Bcl-2* mRNA and increase in the protein levels of the transcription factors Fos and Jun. Moreover, hydroxyl radicals induced time-dependent lipid peroxidation and caused changes in the sulfhydryl group binding sites on membrane proteins. Hydroxyl radicals may induce apoptosis *via* different signaling pathways that can be shown in Fig. 10. Regulating the expression of *Bcl-2*, Fos and Jun, initiating lipid peroxidation whose metabolite might mediate particular signals derived from cell

surface receptors, or inducing membrane damage will all lead to apoptosis. We hypothesize that these multiple signaling pathways constitute an internet in cells and interact each other *via* ROS or ambient redox reaction. EGb761 attenuated these changes and its different constituents showed different effects. The total flavonoid component of Egb761 and a mixture of flavonoids and terpenes protected cerebellar granule cells from oxidative damage and apoptosis induced by hydroxyl radicals. Our previous data (Chen et al., 1999) showed that the IC_{50} of the Ginkgo flavonoids for scavenging hydroxyl radicals was almost the same as that of EGb761, even though flavonoids make up only 24% of EGb761, implying that other constituents of EGb761 besides flavonoids can also scavenge hydroxyl radicals. Total terpenes of EGb761 did not protect against apoptosis. The mixture of flavonoids and terpenes did not exhibit a greater effect than the sum of the effects of flavonoids and terpenes tested separately under the same conditions. Therefore, flavonoids and terpenes did not show a synergistic effect in this regard. Terpenes did not scavenge hydroxyl radicals directly, which might be related to their 'cage-like' structures.

To our knowledge this is the first demonstration that apoptosis in cerebellar granule cells occurred *via* different signaling pathways. These results might be helpful in understanding the mechanism of EGb761 in protecting neuronal cells from apoptosis, and may suggest other application of EGb761 in the future.

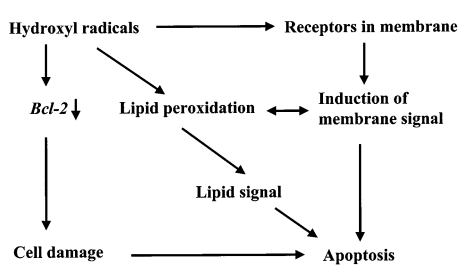


Fig. 10. Schematic representation of signal pathways involved in apoptosis induced by hydroxyl radicals.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China.

References

Buttke, T.M., Sandstrom, A., 1994. Oxidative stress as a mediator of apoptosis. Immunol. Today 15, 7–10.

Chen, C., Wei, T.T., Gao, Z.H., Zhao, B.L., Hou, J.W., Xu, H.B., Xin, W.J., Packer, L., 1999. Different effects of the constituents of EGb761 on apoptosis in rat cerebellar granule cells induced by hydroxyl radicals. Biochem. Mol. Biol. Int. 47, 397–405.

Chomczyski, P., Sacchi, N., 1987. Single step method of RNA isolation by acid guanidine thiocyanate phenol chloroform extraction. Anal. Biochem. 162, 156–159.

Cotman, C.W., Anderson, A.J., 1995. A potential role for apoptosis in neurodegeneraotion and Alzheimer's disease. Mol. Neurobiol. 10, 19–45.

DeFeudis, F.V., 1991. Ginkgo Biloba Extract (EGb761): Pharmacological Activities and Clinical Applications. Elsevier, Paris.

Greenlund, L.J.S., Deckwerth, T.L., Johnson, E.M. Jr, 1995. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed cell death. Neuron 14, 303–315.

Koh, J.Y., Choi, D.W., 1987. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J. Neurosci. Methods 20, 83–90.

Kwak, W.J., Park, H.K., Oh, K.B., 1990. A method of isolating ginkgolides from the leaves of the Ginkgo tree and purifying them, EP 0 402 925 A2.

Le Bars, P.L., Katz, M.M., Berman, N., Itil, T.M., Freedman, A.M., Schatzberg, A.F., 1997. A placebo-controlled, double-blind, randomized trial of an extract of Ginkgo biloba for dementia. J. Am. Med. Assoc. 278, 1327–1332.

Lo, Y.Y.C., Cruz, T.F., 1995. Involvement of reactive oxygen species in cytokine and growth factor induction of Fos expression in chondrocytes. J. Biol. Chem. 270, 11727–11730.

Ni, Y.C., Zhao, B.L., Hou, J.W., Xin, W.J., 1996. Preventive effect of Ginkgo biloba extract on apoptosis in neuronal cells induced by hydroxyl radicals. Neurosci. Lett. 214, 115–118.

Ni, Y.C., Zhao, B.L., Hou, J.W., Xin, W.J., 1996. Ginkgo biloba extract protection of brain neurons from damage induced by free radicals. In: Packer, L., Traber, M.G., Xin, W.J. (Eds.), Proceedings of the International Symposium on Natural Antioxidants Molecular Mechanisms and Health Effects. AOCS Press, Champaign, IL, pp. 506–512.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Papadopoulos, M.C., Koumenis, I.L., Dugan, L.L., Giffard, R.G., 1997. Vulnerability to glucose deprivation injury correlates with glutathione levels in astrocytes. Brain Res. 748, 151–156.

Preston, G.A., Lyon, T.T., Yin, Y., Lang, J.E., Solomon, G., Annab, L., Srinivasan, D.G., Alxorta, D.A., Barrett, J.C., 1996. Induction of apoptosis by Fos protein. Mol. Cell. Biol. 16, 211–218.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandoval, M., Zhang, X.J., Liu, X.P., Mannick, E.E., Clark, D.A., Miller, M.J.S., 1997. Peroxynitrite-induced apoptosis in T84 and RAW 264.7 cells: attenuation by L-ascorbic acid. Free Radic. Biol. Med. 22, 489–495.

Xin, W.J., Zhao, B.L., Zhang, J.Z., 1984. Studies on the property of sulfhydryl binding site on the lung normal and cancer cell membrane of Chinese hamster with maleimide spin labels. Scientia Sinica (Series B) 27, 1008–1014.