ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jan; 21 (1): 75 - 79
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

Protective effects of bilobalide on amyloid beta-peptide 25 - 35-induced PC12 cell cytotoxicity[1]

ZHOU Li-Jun, SONG Wei, ZHU Xing-Zu[2], CHEN Zhong-Liang[3], YIN Meng-Long[3], CHENG Xiao-Fang[3]
(*Department of Pharmacology 1*, [3]*Department of Phytochemistry*, *Shanghai Institute of Materia Medica*, *Chinese Academy of Sciences*, *Shanghai 200031*, *China*)

KEY WORDS *Ginkgo biloba*; bilobalide; PC12 cells; amyloid beta-protein; lactate dehydrogenase; lipid peroxidation; superoxide dismutase; catalase; glutathione peroxidase

ABSTRACT

AIM: To study the effect of bilobalide, a terpene extracted from the leaves of *Ginkgo biloba*, on beta-amyloid peptide fragment 25 - 35 (Aβ25 - 35)-induced PC12 cell cytotoxicity. **METHODS**: 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide and lactate dehydrogenase assay were used to measure the viability of PC12 cells. Thiobarbituric acid-reactive substances were measured to determine lipid peroxidation of cells. Antioxidant enzymes in PC12 cells were detected. **RESULTS**: Treatment of PC12 cells with Aβ25 - 35 (100 μmol·L^{-1}) for 24 h caused a great decrease in cell viability ($P < 0.01$ compared with control). Bilobalide 25 - 100 μmol·L^{-1} dose-dependently attenuated the cytotoxic effect of Aβ25 - 35. Bilobalide also inhibited Aβ25 - 35 (100 μmol·L^{-1})-induced elevation of lipid peroxidation and decline of antioxidant enzyme activities. **CONCLUSION**: Bilobalide protected PC12 cells from Aβ25 - 35-induced cytotoxicity.

INTRODUCTION

Among the psychiatric illnesses associated with old age, Alzheimer disease (AD) has gained increasing importance in recent years. Although biochemical disturbances in the neurotransmitter systems and in the glucose metabolism have been detected experimentally and clinically, drug treatment at this level has so far met with limited success. Recently, several placebo-controlled, double-blind, and randomized trials had confirmed that a standardized extract of *Ginkgo biloba* was effective in mild to moderate dementia of the AD patients[1] and capable of stabilizing and improving the cognitive performance and the social functioning of demented patients for 0.5 - 1 year[2]. *Ginkgo biloba* leaves contain a number of flavonoids (eg, aempferol, quercetin, and isorhamnetin derivatives) and terpenes (eg, ginkgolide and bilobalide). At present, it is not known which of the constituents of Ginkgo biloba is/are responsible for its beneficial effects, although attention has focused on the flavonoids[3] and ginkgolides, such as ginkgolide B, a potent platelet-activating factor (PAF) antagonist. Bilobalide is a sesquiterpene isolated from *Ginkgo biloba* leaves. Although this constituent has been well characterized in the chemistry, its pharmacological properties remain unclear.

AD is characterized histologically by selective neuronal loss, neurofibrillary tangles, and extracelluar deposits of insoluble amyloid that form senile plaques. Although the cause of neuronal death in AD is not clear, evidence has put beta-amyloid peptide (Aβ) into the center of current research. The role of Aβ as an essential factor in the degeneration of CNS neurons has been supported by several studies[4-6]. *In vitro*, Aβ25 - 35 has been shown to be directly toxic to neurons[7] and be able to increase the vulnerability of neurons to other insults[8]. It would be very interesting to know the effects of bilobalide on the neurotoxicity of Aβ. In the present study, the effects of bilobalide on Aβ25 - 35-induced cytotoxicity were examined.

[1] Project supported by grants to ZHU Xing-Zu from Chinese Academy of Sciences (KJ952-J1-602), and Shanghai Science and Technology Commission (97XD14018).
[2] Correspondence to Prof ZHU Xing-Zu.
Phn 86-21-6431-1833, ext 309. Fax 86-21-6437-0269.
E-mail xzzhu@server.shcnc.ac.cn
Received 1999-04-20 Accepted 1999-07-03

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jan; 21 (1)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

MATERIALS AND METHODS

Materials Aβ25 – 35 (Sigma Chemical Co) was dissolved in deionized water, and stored in aliquots at – 20 ℃. To obtain the neurotoxic form of Aβ25 – 35, the peptide solution was placed in an incubator at 37 ℃ for 7 d[7]. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and 2-thiobarbituric acid (TBA), were purchased from Sigma Chemical Co. Bilobalide was provided by Prof CHEN Zhong-Liang (Department of Phytochemistry, Shanghai Institute of Materia Medica, Chinese Academy of Sciences). The purity of this compound was 98 % (HPLC), and the electronic impact mass spectrum (EIMS) and ^{1}H-nuclear magnetic resonance spectrum (^{1}H-NMR) spectra were the same as previous report[9]. Bilobalide was dissolved in ethanol before use. The concentration of ethanol in the final culture media was ≤0.1 % that itself had no toxic effect in PC12 cells.

Cell culture and treatment PC12 cells were cultured at 37 ℃ in a humidified CO_2(5 %) incubator in Dulbecoo's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10 %), benzylpenicillin (100 kU·L^{-1}), and streptomycin (100 mg·L^{-1}). Cells (1×10^4 cells in 100 μL culture medium each well) in a 96-well plate were used for MTT assay. For lactate dehydrogenase (LDH) release, lipid peroxidation, and antioxidant enzyme activity measurement, cells (7×10^5 cells in 2 mL culture medium each well) were cultured in 6-well plates. At the time of experiment, cells were first changed into serum free medium, then Aβ25 – 35 (100 μmol·L^{-1}) and different concentrations of bilobalide were added simultaneously for 24 h.

MTT assay After cells were treated with Aβ25 – 35 (100 μmol·L^{-1}) and different concentrations of bilobalide for 24 h, MTT solution (0.5 g·L^{-1}) was added to each culture well. After incubation at 37 ℃ for an additional 4 h, the formazan crystals were dissolved by addition of 100 μL 10 % SDS – 5 % isobutanol – 0.12 % HCl (w:v:v). Plates were incubated at 37 ℃ overnight, and the absorbance was measured at 570 nm using an ELISA plate reader.

LDH release assays LDH activity in the extracellular medium was measured using a commercial kit (Jiancheng Institute of Biotechnology, Nanjing, China), where the colorimetric assay measures the pyruvate-mediated conversion of 2,4-dinitrophenylhydrazine into a visible hydrazone precipitate. Percent of LDH release was expressed as (LDH release in supernatant/maximal release) × 100 %. Where the maximal release was obtained after exposure of untreated culture to 0.2 % Triton X-100 at 37 ℃ for 15 min.

Lipid peroxidation The lipid peroxidation of cells was determined by measuring thiobarbituric acid-reactive substances (TBARS). Cells were lysed with 4 mL fulric acid (0.167 mol·L^{-1}) and 0.5 mL 10 % phosphotungstic acid, then centrifuged at 4000 × *g* for 10 min. The precipitation was resuspended with 1.5 mL distilled water and 0.5 mL TBA reagent [1:1 (v:v) mixture of 0.67 % TBA and acetic acid]. The reaction mixture was heated at 95 ℃ for 1 h. After cooling, 2 mL of *n*-butanol were added, and the mixture was shaken vigorously for 30 s. After centrifugation at 3000 × *g* for 10 min, the *n*-butanol layer was for fluorometric measurement with λ_{ex} 515 nm and λ_{em} 553 nm, using a fluorescence spectrophotometer. The value of fluorescence was calculated by comparing with standards prepared from 1, 1, 3, 3-tetraethoxypropane (TMP).

Antioxidant enzyme activity assays The activities of antioxidant enzymes in PC12 cells were determined with commercial kits purchased from Jiancheng Institute of Biotechnology (Nanjing, China). The assay for total superoxide dismutase (SOD) was based on its ability to inhibit the oxidation of oxymine by the xanthine-xanthineoxidase system. The red product (nitrite) produced by the oxidation of oxymine had absorbance at 550 nm. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50 %.

The assay of catalase activity was based on its ability to decompose H_2O_2. The absorbance of supernatant at 254 nm changed when the H_2O_2 solution was injected into the cuvette. The change of the absorbance reflected the catalase activity.

The activity of glutathione peroxidase (GSH-PX) was determined by quantifying the rate of H_2O_2-induced oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSH). A yellow product which had absorbance at 412 nm could be formed as GSH reacted with dithiobisnitrobenzoic acid. One unit of GSH-PX was defined as the amount that reduced the level of GSH by 1 μmol·L^{-1} in one minute per mg protein.

Statistical analysis Statistical analysis of the data for multiple comparisons was performed by ANOVA followed by Dunnett's test. For single comparison, the significance of differences between means was determined by t-test.

RESULTS

Effect of bilobalide on Aβ25 – 35-induced cytotoxicity When PC12 cells were treated with Aβ25 – 35 (100 μmol·L^{-1}) for 24 h, an obvious decrease of MTT obsorbance was observed (compared with the control without Aβ25 – 35 treatment, $P < 0.01$). The decrease was attenuated dose-dependently by bilobalide while bilobalide alone at dosages of 12.5 – 100 μmol·L^{-1} did not show any influence on the viability of PC12 cells (Fig 1).

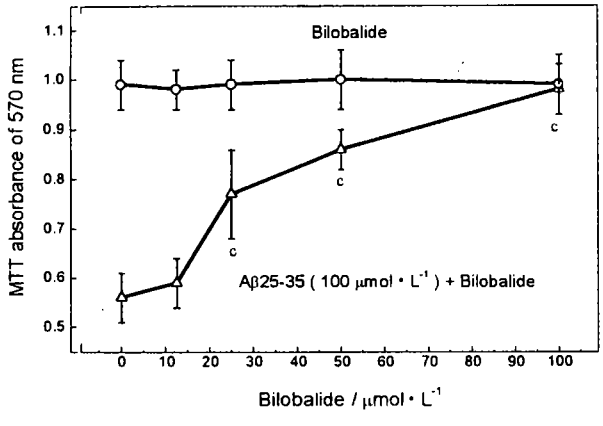

Fig 1. Effect of bilobalide on Aβ25 – 35-induced cytotoxicity in cultured PC12 cells. PC12 cells in serum free medium were exposed to Aβ25 – 35 (100 μmol·L^{-1}) and different concentrations of bilobalide for 24 h. Cell viability was measured by the MTT assay. n = 3 experiments (each done in 3 wells). $\bar{x} \pm s$. $^cP < 0.01$ *vs* respective control at "0" point.

At the concentration of 100 μmol·L^{-1}, Aβ25 – 35 induced an increase of LDH leakage by 3.97 times of control. Treatment of PC12 cells with bilobalide simultaneously resulted in a dose-dependently decline of Aβ25 – 35-induced LDH release (Fig 2).

Effect of bilobalide on oxidative stress Treatment of cells with Aβ25 – 35 100 μmol·L^{-1} caused an obvious elevation of TBARS [(220 ± 10) nmol/g protein, $P < 0.01$ compared with control group (12 ± 4) nmol/g protein]. The elevation was inhibited by bilobalide 12.5 – 100 μmol·L^{-1} (Fig 3).

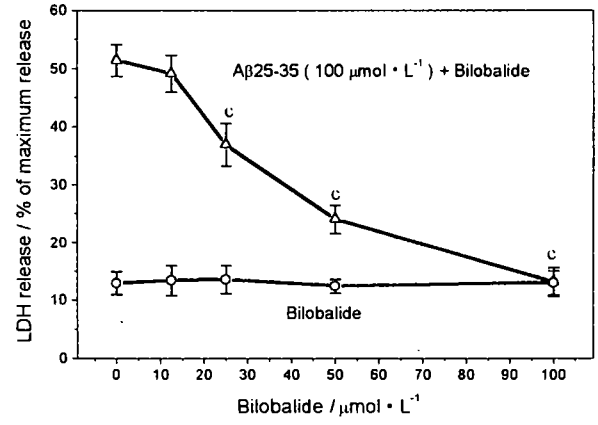

Fig 2. Effect of bilobalide on Aβ25 – 35-induced LDH release in cultured PC12 cells. PC12 cells in serum free medium were exposed to Aβ25 – 35 (100 μmol·L^{-1}) and different concentrations of bilobalide for 24 h before LDH activity in the culture medium was measured. n = 3 experiments (each done in 3 wells). $\bar{x} \pm s$. $^cP < 0.01$ *vs* respective control at "0" point.

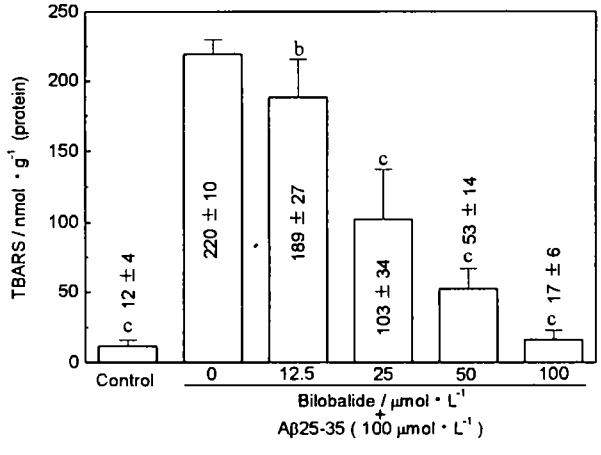

Fig 3. Effect of bilobalide on Aβ25 – 35-induced lipid peroxidation in cultured PC12 cells. After 24-h treatment with Aβ25 – 35 (100 μmol·L^{-1}) and different concentrations of bilobalide, the lipid peroxidation of cells was determined by measuring thiobarbituric acid-reactive substances (TBARS). n = 3 experiments (each done in 3 wells). $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* group treated with Aβ25 – 35 alone.

Effect of bilobalide on antioxidant enzymes After PC12 cells were incubated with Aβ25 – 35 (100 μmol·L^{-1}) for 24 h, obvious decreases of antioxidant enzymes, SOD, catalase, and GSH-PX were observed

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jan; 21 (1)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

($P<0.01$ compared with corresponding control groups). Bilobalide dose-dependently attenuated the decrease of SOD, catalase, and GSH-PX (Tab 1).

DISCUSSION

In our study, MTT and LDH release assay were used to measure the viability of PC12 cells. In agreement with previous studies[10,11], our results indicated that Aβ25 – 35 caused a great decrease of the reduction of MTT by mitochondria. Our results showed that bilobalide could protect PC12 cells from the insult of mitochondria. The results were further confirmed by the assay of LDH release.

Strong evidence has shown that free radicals induced by Aβ play a very important role in Aβ neurotoxicity[6,12]. Aβ has also been shown to increase lipid peroxidation in rat primary cortical[13], hippocampal cultures[14], and rat neuronal cell lines[13]. In the present study, we found that bilobalide inhibited Aβ25 – 35-induced elevation of TBARS. The results suggest that superoxide scavenging effect[15,16] may underlie the protective effect of bilobalide.

It has been shown that under damaging conditions such as ischemia or oxidative stress, the activities of antioxidant enzymes increased[17], although studies in AD patients have not shown a consistent result[18]. In our study, we found that the activities of antioxidant enzymes (total SOD, catalase, GSH-PX) in cultured PC12 cells were reduced remarkably by 24-h treatment with Aβ. Our results that the inhibitory effect of Aβ on antioxidant enzymes could be attenuated by bilobalide suggest that the ability of bilobalide to preserve the antioxidant enzyme activities may contribute its neuroprotective effect. This hypothesis was supported by the finding[19] that clonal cell lines with greater antioxidant enzyme levels possessed greater resistance to Aβ toxicity than the normal cell lines did.

In summary, our results demonstrated that Aβ25 – 35 induced a decrease in cell viability, an elevation of lipid peroxidation, and a decline of antioxidant enzyme activities. Bilobalide inhibited the Aβ25 – 35-induced decrease in cell viability, elevation of lipid peroxidation, and decline of antioxidant enzyme activities. Although the exact mechanism by which bilobalide acts remains unknown, our results suggest that the effects of bilobalide on mitochondria and antioxidant enzymes are involved in its neuroprotective effects.

REFERENCES

1 Maurer K, Ihl R, Dierks T, Frolich L. Clinical efficacy of *GINKGO BILOBA* special extract EGb 761 in dementia of the Alzherimer type. J Psychiatr Res 1997; 31: 645 – 55.
2 Le Bars PL, Karz MM, Berman N, Itil TM, Freedman AM, Schatzberg AF. A placebo-controlled, double-blind, randomized trial of an extract of *Ginkgo biloba* for dementia, North American EGb Study Group. JAMA 1997; 278: 1327 – 32.
3 Tighilet B, Lacour M. Pharmacological activity of the *Ginkgo biloba* extract (EGb 761) on equilibrium function recovery in the unilateral vestibular neurectomized cat. J Vestib Res 1995; 5: 187 – 200.
4 Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, *et al*. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 1991; 349: 704 – 6.

Tab 1. Effect of bilobalide on Aβ25 – 35-induced decrease of antioxidant enzyme activities in cultured PC12 cells. PC12 cells in serum free medium were exposed to Aβ25 – 35 (100 μmol·L^{-1}) and different concentrations of bilobalide for 24 h before antioxidant enzyme activities in the cells was measured. $n = 3$ experiments (each done in 3 wells). $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* group treated with Aβ25 – 35 alone.

Groups	SOD/ kNU·g^{-1}(protein)	Catalase/ kU·g^{-1}(protein)	GSH-PX/ kU·g^{-1}(protein)
Control	164 ± 15^c	20.9 ± 3.3^c	37 ± 9^c
Aβ25 – 35 (100 μmol·L^{-1})	71 ± 16	5.0 ± 1.7	14 ± 8
Aβ25 – 35 + Bilobalide (12.5 μmol·L^{-1})	73 ± 16	5.0 ± 1.6	14 ± 8
Aβ25 – 35 + Bilobalide (25 μmol·L^{-1})	100 ± 10^b	9.4 ± 3.2^b	20 ± 6
Aβ25 – 35 + Bilobalide (50 μmol·L^{-1})	145 ± 16^c	15.1 ± 3.1^c	29 ± 9^b
Aβ25 – 35 + Bilobalide (100μmol·L^{-1})	163 ± 14^c	21.2 ± 4.6^c	35 ± 8^c

5 Kowall NW, Mckee AC, Yankner BA, Beal MF. *In vivo* neurotoxicity of β-amyloid [β (1 – 40)] and the β (25 – 35) fragment. Neurobiol Aging 1992; 13: 537 – 42.
6 Smith MA, Hirai K, Hsiao K, Pappolla MA, Harris PLR, Siedlak SL, *et al*. Amyloid-β deposition in Alzheimer transgenic mice is associated with oxidative stress. J Neurochem 1998; 70: 2212 – 5.
7 Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, Cotman CW. Neurodegeneration induced by β-amyloid peptides *in vitro*: the role of peptide assembly state. J Neurosci 1993; 13: 1676 – 87.
8 Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. β-amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci 1992; 12: 376 – 89.
9 Nakanishi K, Habaguchi K, Nakadaira Y, Woods MC, Maruyama M, Major RT, *et al*. Structure of bilobalide, a rare tert-butyl containing sesquiterpenoid related to the C_{20}-ginkgolides. J Am Chem Soc 1971, 93: 3544 – 6.
10 Schearman MS, Ragan CI, Iversen LI. Inhibition of PC12 cell redox activity is a specific, early indicator of the mechanism β-amyloid-mediated cell death. Proc Natl Acad Sci USA 1994; 91: 1470 – 4.
11 Zhou Y, Gopalakrishnan V, Richardson JS. Action of neurotoxic β-amyloid on calcium homeostasis and viability of PC12 cells are blocked by antioxidants but not by calcium channel antagonists. J Neurochem 1996; 67: 1419 – 25.
12 Butterfield DA. β-Amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease. Chem Res Toxicol 1997; 10: 495 – 506.
13 Behl C, Davis JB, Lesley R, Schubert D. Hydrogen peroxide mediates amyloid β protein toxicity. Cell 1994; 77: 817 – 27.
14 Goodman Y, Steiner MR, Steiner SM, Mattson MP. Nordihydroguaiaretic acid protects hippocampal neurons against amyloid β-peptide toxicity, and attenuates free radical and calcium accumulation. Brain Res 1994; 654: 171 – 6.
15 Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Gardès-Albert M. Antioxidant action of Ginkgo biloba extract EGb 761. Methods Enzymol 1994; 234: 462 – 75.
16 Scholtyssek H, Damerau W, Wessel R, Schminke I. Antioxidative activity of ginkgolides against superoxide in an aprotic environment. Chem Biol Interact 1997; 106: 183 – 90.
17 Liu X, Zhu XZ, Ji XQ. Effect of basic fibroblast growth factor on focal ischemic injury and antioxidant enzyme activities. Acta Pharmacol Sin 1999; 20: 227 – 31.
18 Markesbery WR. Oxidative stress hypothesis in Alzheimer's disease. Free Radic Biol Med 1997; 23: 134 – 47.
19 Sagara Y, Dargusch R, Klier FG, Schubert D, Behl C. Increased antioxidant enzyme activity in amyloid β protein-resistant cells. J Neurosci 1996; 16: 497 – 505.

白果内酯对抗淀粉样 β-肽 25 – 35 所致 PC12 细胞毒作用[1]

周利军，宋 伟，朱兴族[2]，陈仲良[3]，殷梦龙[3]，程晓芳[3]（中国科学院上海药物研究所药理一室，[3]植化室，上海 200031，中国）

关键词 银杏；白果内酯；PC12 细胞；淀粉样 β-蛋白；乳酸脱氢酶；脂质过氧化；超氧化物歧化酶；过氧化氢酶；谷胱甘肽过氧化酶

目的：观察白果内酯对 β – 淀粉样蛋白片段 25 – 35 (Aβ25 – 35)所致 PC12 细胞毒性的影响. **方法**：用噻唑兰(MTT)及乳酸脱氢酶法检测 PC12 细胞的存活率；硫代巴比妥酸法测定细胞脂质过氧化；并同时检测了细胞内抗氧化酶活性. **结果**：白果内酯 (25 – 100) $\mu mol \cdot L^{-1}$剂量依赖性地抑制 Aβ25 – 35 (100 $\mu mol \cdot L^{-1}$)引起的细胞存活率下降，脂质过氧化及抗氧化酶活性下降. **结论**：白果内酯具有对抗 Aβ25 – 35 引起的 PC12 细胞毒性的作用.

（责任编辑 刘俊娥）