Protective effects of bilobalide on amyloid beta-peptide 25–35-induced PC12 cell cytotoxicity

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**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 extract from the leaves of Ginkgo biloba, on beta-amyloid peptide fragment 25–35 (A\(_\beta\)25–35)-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 A\(_\beta\)25–35 (100 \(\mu\)mol \(\cdot\) L\(^{-1}\)) for 24 h caused a great decrease in cell viability (\(P < 0.01\) compared with control). Bilobalide 25–100 \(\mu\)mol \(\cdot\) L\(^{-1}\) dose-dependently attenuated the cytotoxic effect of A\(_\beta\)25–35. Bilobalide also inhibited A\(_\beta\)25–35 (100 \(\mu\)mol \(\cdot\) L\(^{-1}\))-induced elevation of lipid peroxidation and decline of antioxidant enzyme activities. **CONCLUSION**: Bilobalide protected PC12 cells from A\(_\beta\)25–35-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 have 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 extracellular 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 (A\(_\beta\)) into the center of current research. The role of A\(_\beta\) as an essential factor in the degeneration of CNS neurons has been supported by several studies\(^4\text{–}^6\). In vitro, A\(_\beta\)25–35 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 A\(_\beta\). In the present study, the effects of bilobalide on A\(_\beta\)25–35-induced cytotoxicity were examined.

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MATERIALS AND METHODS

Materials A(25 - 35 (Sigma Chemical Co) was dissolved in deionized water, and stored in aliquots at -20 ºC. To obtain the neurotoxic form of A(25 - 35, the peptide solution was placed in an incubator at 37 ºC 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 ¹H-nuclear magnetic resonance spectrum (¹H-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 ºC in a humidified CO₂ (5%) incubator in Dulbeco'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 × 10⁶ 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 × 10⁶ 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 A(25 - 35 (100 µmol·L⁻¹) and different concentrations of bilobalide were added simultaneously for 24 h.

MTT assay After cells were treated with A(25 - 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 ºC 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 ºC 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 ºC 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 ºC 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 λₐₘ 515 nm and λₐₘ 533 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 H₂O₂. The absorbance of supernatant at 254 nm changed when the H₂O₂ 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 H₂O₂-induced oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). 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 absorbance 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). \( x \pm s \). \( ^{*}P < 0.01 \) 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 \([220 \pm 10] \) nmol/g protein, \( P < 0.01 \) compared with control group \([12 \pm 4] \) 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). \( x \pm s \). \( ^{*}P < 0.01 \) 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). \( x \pm s \). \( ^{*}P < 0.05 \), \( ^{*}P < 0.01 \) 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.
( 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 agree-
ment with previous studies, our results indicated
that Aβ25 – 35 caused a great decrease of the reduc-
tion 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 in-
duced by Aβ play a very important role in Aβ neurotox-
icity. Aβ has also been shown to increase lipid
peroxidation in rat primary cortica, hippocampal
cultures and rat neuronal cell lines. In the pres-
ent study, we found that bilobalide inhibited Aβ25 –
35-induced elevation of TBARS. The results suggest
that superoxide scavenging effect 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, although studies in
AD patients have not shown a consistent result. 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 bilob-
alide suggest that the ability of bilobalide to preserve the
antioxidant enzyme activities may contribute its neuro-
protective effect. This hypothesis was supported by the
finding 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. Al-
though 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.

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Tab 1. Effect of bilobalide on Aβ25 – 35-induced decrease of antioxidant enzyme activities in cultured PC12 cells.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD/ kU·g⁻¹(protein)</th>
<th>Catalase/ kU·g⁻¹(protein)</th>
<th>GSH-PX/ kU·g⁻¹(protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>164 ± 15⁰</td>
<td>20.9 ± 3.3⁰</td>
<td>37 ± 9⁰</td>
</tr>
<tr>
<td>Aβ25 – 35 (100 μmol·L⁻¹)</td>
<td>71 ± 16</td>
<td>5.0 ± 1.7</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>Aβ25 – 35 + Bilobalide (12.5 μmol·L⁻¹)</td>
<td>73 ± 16</td>
<td>5.0 ± 1.6</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>Aβ25 – 35 + Bilobalide (25 μmol·L⁻¹)</td>
<td>100 ± 10⁶</td>
<td>9.4 ± 3.2⁰</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>Aβ25 – 35 + Bilobalide (50 μmol·L⁻¹)</td>
<td>145 ± 16⁰</td>
<td>15.1 ± 3.1⁰</td>
<td>29 ± 9⁰</td>
</tr>
<tr>
<td>Aβ25 – 35 + Bilobalide (100 μmol·L⁻¹)</td>
<td>163 ± 14⁰</td>
<td>21.2 ± 4.6²</td>
<td>35 ± 8⁰</td>
</tr>
</tbody>
</table>

⁰ P < 0.05, ⁶ P < 0.01 vs group treated with Aβ25 – 35 alone.

白果内酯对抗淀粉样β-肽25-35所致PC12细胞毒作用

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关键词：银杏；白果内酯；PC12细胞；淀粉样β-肽蛋白；乳酸脱氢酶；脂质过氧化；超氧化物歧化酶；抗氧化酶；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）μmol·L⁻¹剂量依赖性地抑制Aβ25-35（100 μmol·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。

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