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Bilobalide promotes expression of glial cell line-derived neurotrophic factor and vascular endothelial growth factor in rat astrocytes[1]

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KEY WORDS *Ginkgo biloba*; neurotrophin; astrocytes; up-regulation (physiology); reverse transcriptase polymerase chain reaction; immunohistochemistry; bilobalide

ABSTRACT

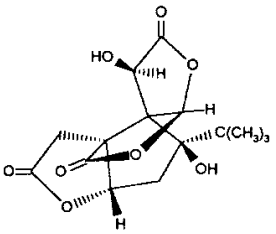
AIM: To study the effects of bilobalide on the expression of glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) in rat astrocytes *in vitro*. METHODS: Semiquantification polymerase chain reaction (SQ-PCR) was used to investigate GDNF and VEGF mRNA expression in the astrocytes after bilobalide (5, 15, 50, 100 $\mu mol \cdot L^{-1}$) treatment. Immunohistochemistry method was used to detect GDNF and VEGF protein expression in cells treated with bilobalide 50 $\mu mol \cdot L^{-1}$ for 24 h. RESULTS: GDNF and VEGF mRNA increased markedly after astrocytes were treated with bilobalide 50 $\mu mol \cdot L^{-1}$ for 12 h. GDNF and VEGF protein were detected in the cytoplasm of astrocytes after the cells were treated with bilobalide 50 $\mu mol \cdot L^{-1}$ for 24 h. CONCLUTION: Bilobalide induced GDNF and VEGF expression in the cultured astrocytes.

INTRODUCTION

Extract of *Ginkgo biloba* leaves (*Gb*E) has been used in the treatment of cardiovascular diseases for many years. *Gb*E may also be useful in the treatment of neurodegenerative disease[1,2]. *Gb*E contains a number of flavonoids (eg, kaempferol, quercetin, and isorhamnetin derivatives) and terpenes (eg, ginkgolide and bilobalide). At present, it is not known which of the constituents is or 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 *Gb*E. Its pharmacological properties remain unclear.

In the present study, we studied the effects of bilobalide on astrocytes and found that bilobalide could induce the expression of glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) in the cultured rat astrocytes.



Bilobalide Bilobalide

MATERIALS AND METHODS

Cell culture Rat cortical type Ⅰ astrocytes were obtained as described previously[4]. The cells were determined by immunostaining for the astrocyte-specific marker for glial fibrillary acidic protein (GFAP) and grown in Dulbecco's modified Eagle medium (DMEM) (Gibco/BRL) supplemented with 10 % fetal calf serum (FCS). Cells (1×10^6) were seeded on 6-well plates for 24 h. Bilobalide was added into the medium in a final concentration of 5, 15, 50 or 100 $\mu mol \cdot L^{-1}$ respectively and cells were cultured at 37 ℃, 5 % CO_2 + 95 % O_2 for another 12, 24, or 48 h.

RNA extraction and reverse transcription Total RNA was isolated from the cells with TRIzol (Gibco/BRL) according to the manufacturer's guidelines. For cDNA synthesis, 20 μL reverse transcription mixture containing 3 μL (1 μg) total RNA temple, 10 μL 2 $mmol \cdot L^{-1}$ each of dNTPs (Forward, China), 1 μL

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(1.6 μg) Oligo $(dT)_{15}$ primer (Sangon, Canada), 1 μL (20 U) RNasin (Promega, USA), 1 μL (20U) M-MuLV reverse transcriptase (MBI, Lithuania) and 4 μL 5× reaction buffer (Tris-HCl 250 mmol·L^{-1}, KCl 250 mmol·L^{-1}, $MgCl_2$ 20 mmol·L^{-1}, DTT 50 mmol·L^{-1}). The mixture was incubated at 37 ℃ for 90 min and then the reverse transcriptase was inactivated by heating the reaction mixture to 95 ℃ for 10 min.

PCR procedure Semiquantification PCR (SQ-PCR) was used to observe the effects of bilobalide on GDNF and VEGF mRNAs levels. The forward primers for GDNF and VEGF were 5′-GAAGGATCCATGAAGTTATGGGATGTC-3′ and 5′-CAAGGATCCATGAACTTTCTGCTGTCTTGGG-3′ respectively, and the reverse primers were 5′-GGCCTCGAGTTAGATACATCCACACCG-3′ and 5′-TCTAAGCTTATCACCGCCTCGGCTTGTCACATCT-3′ respectively. To ensure that reverse transcriptase efficiencies were comparable between test groups, β-actin cDNA was also amplified. The forward primer for β-actin was 5′-TTGTAACCAACTGGGACGATA-3′ and the reverse primer was 5′-GATCTTGATCTTCATGGTGCT-3′. VEGF and β-actin PCR were carried out in the same procedure. One μL each of reverse transcription solution was added to PCR mixture containing 5 μL 10× buffer, 2 μL 2 mmol·L^{-1} dNTPs, 1 μL (10 pmol·L^{-1}) each of the primers and 0.75 μL (3.5 U) Taq DNA polymerase (Sangon, Canada) in a total volume of 50 μL. PCR was performed on a thermal cycler PTC-150 (MJ, USA) with the following thermocycle parameters：a 5-min initial denaturation at 98 ℃ followed by 25－35 cycles of 45-s denaturation at 94 ℃, 45-s annealing at 60 ℃, 90-s extension at 72 ℃, and finally a 10-min extension at 72 ℃. The composition for GDNF PCR was the same as that of above, except the primers. After a 6-min initial denaturation at 96 ℃, the reaction of the mixture was then subjected to amplification for 25－35 cycles as follows：95 ℃ 60 s, 50 ℃ 45 s, 72 ℃ 90 s. Then a 10-min extension at 72 ℃ was followed. 5 μL PCR products were separated and visible by silver stained 8 % PAGE. The density of each band was measured by densitometer.

Immunocytochemistry method The streptavidin-biotion-peroxidase complex (SABC) method was performed to detect GDNF and VEGF protein expression in the cells. The cells were fixed in 4 % paraformaldehyde at 4 ℃ for 20 min and then were microwaved in PBS containing 0.1 % Triton X-100 at 95 ℃－98 ℃ for 10 min. After preincubation with 1 % H_2O_2-methanol and normal serum, goat anti-GDNF IgG (R&D System, USA) and rabbit anti-VEGF (IgG) were applied overnight at 4 ℃ in a moist chamber. Secondary biotinylated-antibody was added and then incubated at 37 ℃ for 1.5 h. The streptavidin-biotinylated- peroxidase-complex (SABC) was subsequently added for another 1 h at 37 ℃. The peroxidase activity was made visible with 0.003 % diaminobenzidine (DAB, Sigma, USA) and 0.001 % H_2O_2 in PBS. The nuclei of the cells were counterstained with Hemaeoxylin.

Statistical analysis Data were expressed as $\bar{x} \pm s$, and compared with paired t-test. Each group was performed in triplicate.

RESULTS

PCR was carried out for different numbers of cycles. Once the satisfied bands were visible at the least number of cycles, the optical density of the bands was measured. The $A_{GDNF}/A_{\beta\text{-actin}}$ or $A_{VEGF}/A_{\beta\text{-actin}}$ ratio was used to express the level of GDNF mRNA or VEGF mRNA in astrocytes respectively. No significant change was found in the level of GDNF or VEGF mRNA when cells were treated with 5 μmol·L^{-1} bilobalide. Increases in the level of GDNF or VEGF mRNA were observed when the cells were treated with bilobalide in a final concentration of 15, 50 and 100 μmol·L^{-1}. (Tab 1).

Marked increases in the level of GDNF or VEGF mRNA were detected when the cells were cultured with 50 mol·L^{-1} bilobalide for 24 h (Fig 1).

GDNF or VEGF protein level in the astrocytes was measured by using streptavidin-biotion-peroxidase complex (SABC) method. The untreated-astrocytes showed no signs of positive reaction to GDNF or VEGF antibodies. After the astrocytes were treated with 50 μmol·L^{-1} bilobalide for 24 h, which had a significant effect on GDNF mRNA or VEGF mRNA expression, yellow gains were observed in the cytoplasm of the cells (Fig 2). The results demonstrated that the treatment with bilobalide also induced an increase in GDNF and VEGF protein expression.

DISCUSSION

Reactive astrocytes can synthesize and secrete

Tab 1. Effects of bilobalide on the expression of GDNF mRNA and VEGF mRNA in cultured rat astrocytes. The ratio of $A_{GDNF}/A_{\beta\text{-actin}}$ and $A_{VEGF}/A_{\beta\text{-actin}}$ were used to express the level of GDNF mRNA and VEGF mRNA in astrocytes, respectively. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

Time/h	*n*	Bilobalide/μmol·L^{-1} 0	5	15	50	100
GDNF mRNA						
12	3	0.35±0.04	0.36±0.07	0.39±0.16	0.60±0.06^c	0.66±0.09^c
24	3	0.36±0.07	0.38±0.10	0.49±0.07	0.66±0.10^b	0.67±0.08^c
48	3	0.37±0.07	0.38±0.07	0.51±0.05^b	0.67±0.11^b	0.69±0.05^c
VEGF mRNA						
12	3	0.13±0.06	0.17±0.12	0.30±0.10	0.67±0.16^c	0.80±0.12^c
24	3	0.16±0.06	0.18±0.09	0.42±0.03^c	0.81±0.17^c	0.83±0.22^c
48	3	0.18±0.08	0.18±0.05	0.43±0.12^c	0.82±0.01^c	0.79±0.11^c

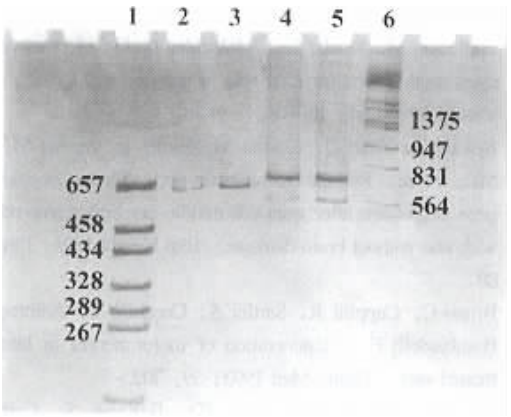

Fig 1. Expression of GDNF and VEGF mRNA in astrocytes treated with bilobalide 50 μmol·L^{-1} for 24 h. RT-PCR was carried out for GDNF, VEGF, and β-actin after 33 PCR cycles. PCR products were run and separated by silver stained 8 % PAGE. Lane 1. Marker pGEM-7Zf (+)/Hae；Lane 2. GDNF expression without bilobalide；Lane 3. GDNF expression with bilobalide；Lane 4. VEGF and β-actin expression without bilobalide；Lane 5. VEGF and β-actin expression with bilobalide；Lane 6. Marker λDNA/EcoR Ⅰ + Hind Ⅲ.

neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which may play an important role in the survival, growth and differentiation of neurons[5,6]. Although evidence has shown that bilobalide exert neuroprotective effect[7], the mechanism remained unknown. Our results that bilobalide induced the expressions of GDNF and VEGF in astrocytes suggest that bilobalide neuroprotective effect in the central nervous system (CNS).

Over the past several years, neurotrophic factors have made considerable progress from the laboratory into the clinic. Evidence from preclinical and clinical studies indicates that it may be possible to use neurotrophic factors to prevent, slow the progression of, or even reverse the effects of a number of neurodegenerative diseases and other types of insults in both the CNS and the peripheral nervous system.

GDNF, originally isolated from the rat glial cell line B49[8], has the ability to promote survival of substantia nigra dopamine neurons. Thus, GDNF may be used in the treatment of Parkinson's disease[9], a disease characterized by the loss of dopaminergic neurons in the substantia nigra with a concomitant loss of dopamine at the axon terminals in the striatum.

VEGF, also known as vascular permeability factor, has the ability to induce vasculogenesis and angiogenesis in many organ systems[10]. Significant angiogenic effects have been found in VEGF-treated adult rat cortical slice explants[11]. VEGF also participates in the CNS response to injury[12].

Initially, investigations focused on recombinant neurotrophic proteins that are identical or highly homologous to the natural human sequence. However, continuous targeted delivery of neurotrophic factors to specific neurons in the central nervous system is a challenge that remains to be met. The blood-brain barrier (BBB), which composed of specialized endothelial cells with tight junction, prevents the passage of large molecules into the brain parenchyma. Problems associated with the use of protein growth factors such as GDNF and VEGF may be bypassed if small molecules are used which can manipulate any step in the synthesis, release or transduction mechanism of endogenous growth factors. Our results suggest that bilobalide, a small molecule with the ability

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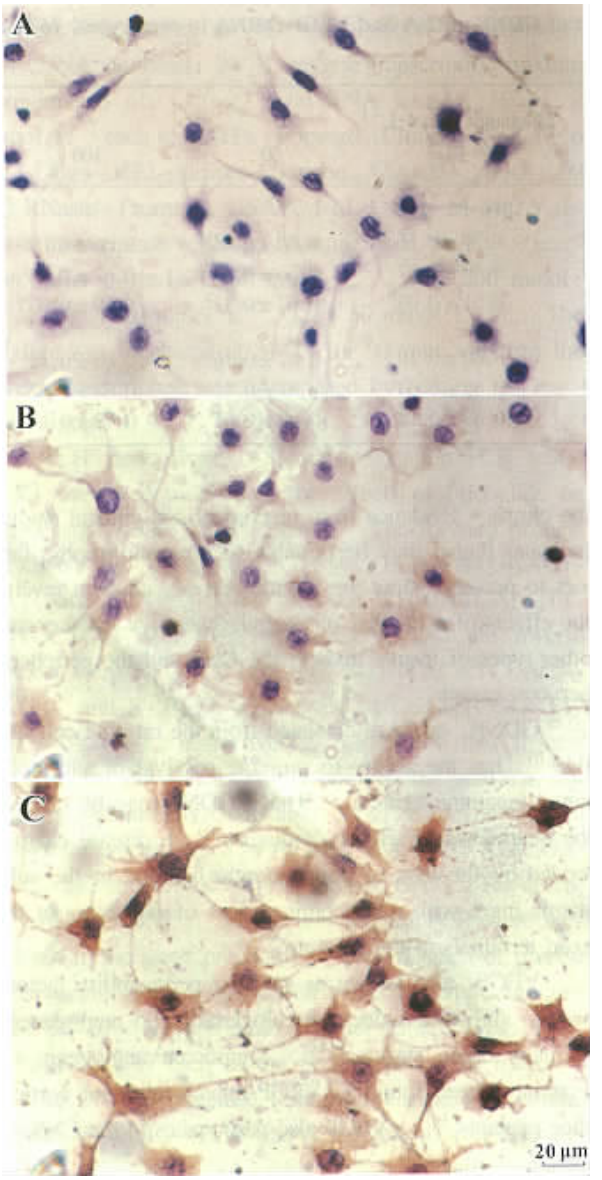

Fig 2. GDNF protein and VEGF protein expression in astrocytes treated with bilobalide 50 μmol·L^{-1} for 24 h. **Yellow gains could only be seen in the cytoplasm of the bilobalide-treated-cells (Immunohistochemistry, SABC, × 400). A: control; B: GDNF; C: VEGF.**

to induce GDNF and VEGF expression in astrocytes, may serve as a useful drug in the treatment of neurodegenerative diseases such as Parkinson's disease.

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白果内酯刺激大鼠星形胶质细胞 GDNF 和 VEGF 表达[1]

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关键词　银杏；神经营养因子；星形胶质细胞；上调（生理学）；逆转录聚合酶链式反应；免疫组织化学；白果内酯

目的：观察白果内酯对大鼠星形胶质细胞 GDNF 和 VEGF 表达的影响. 方法：应用半定量 PCR 了解大鼠星形胶质细胞分别经不同浓度白果内酯（0，5，15，50，100 $\mu mol \cdot L^{-1}$）作用 12、24 和 48 h 后细胞内 GDNF 和 VEGF mRNA 的变化，同时应用免疫组化了解经 50 $\mu mol \cdot L^{-1}$ 作用 24 h 后细胞内 GDNF 和 VEGF 蛋白的变化. 结果：大鼠星形胶质细胞经 50 $\mu mol \cdot L^{-1}$ 白果内酯作用 12 h 后，细胞内 GDNF 和 VEGF mRNA 水平开始显著增高，经 50 $\mu mol \cdot L^{-1}$ 作用 24 h 后细胞胞浆内可见明显的 VEGF 和 GDNF 蛋白表达. 结论：白果内酯可能通过诱导星形胶质细胞 GDNF 和 VEGF 的表达对神经细胞起保护作用.

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