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ScienceDirect
Progress in Natural Science 19 (2009) 549–555
Progress in Natural Science
www.elsevier.com/locate/pnsc

Achyranthes bidentata Blume extract promotes neuronal growth in cultured embryonic rat hippocampal neurons

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Received 13 May 2008; received in revised form 10 July 2008; accepted 6 August 2008

Abstract

We have prepared an aqueous extract of *Achyranthes bidentata* Blume, a commonly prescribed Chinese medicinal herb, and reported, in previous studies, that *A. bidentata* extract benefits nerve growth and prevents neuron apoptosis. In this study, we investigated the actions of *A. bidentata* extract on survival and growth of primarily cultured rat hippocampal neurons. The morphological observation revealed that neurite growth from hippocampal neurons was significantly enhanced by *A. bidentata* extract with similar effects to those induced by nerve growth factor (NGF), and the greatest neurite growth appeared on treatment with *A. bidentata* extract at 1 μg/ml for 24 h. DNA microarray analysis indicated that there were 25 upregulated genes and 47 downregulated genes exhibiting significantly differential expression in hippocampal neurons treated with *A. bidentata* extract at 1 μg/ml for 6 h when compared to those in untreated hippocampal neurons. Real-time quantitative RT-PCR and Western blot analysis demonstrated that the expression of growth-associated protein-43 in hippocampal neurons was upregulated at both mRNA and protein levels after treatment with *A. bidentata* extract, and the optimal dosage of the extract was also 1 μg/ml. These data confirm that *A. bidentata* extract could promote *in vitro* hippocampal neuronal growth in a dose- and time-dependent manner.

Keywords: *Achyranthes bidentata* Blume; Aqueous extract; Nerve growth factor; Hippocampal neurons; Neurite outgrowth; Growth-associated protein-43; Gene differential expression

1. Introduction

Since the discovery of nerve growth factor (NGF) and other neurotrophins [1], the understanding of their regulation and action on neurons has led to the use of these factors in clinical trials of human neurological diseases [2–6]. The clinical application of recombinant neurotrophins, however, is limited by the drug delivery problem due to their macromolecular character [7–9]. Considerable efforts have been devoted to either improving the drug delivery system of neurotrophins or developing new drugs with neurotrophic activity as an alternative or supplement to neurotrophins [10,11]. According to traditional medicine in different countries, certain medicinal herbs could probably contribute to these efforts.

Achyranthes bidentata Blume (Chinese name Huai niuxi), belonging to the family *Amaranthaceae*, is one of the commonly prescribed Chinese medicinal herbs with the property of strengthening bones and muscles, and ensuring proper downward flow of blood in terms of the therapeutic theory of traditional Chinese medicine. An aqueous extract from the root of *A. bidentata* Blume has been prepared in our laboratory. In the previous studies, we have shown that the extract enhances nerve growth, prevents neuron apoptosis, and induces neuronal differentiation of PC12 cells through activating the ERK1/2 pathway [12,13]. We also reported that the extract promoted the neurite growth of dorsal root

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doi:10.1016/j.pnsc.2008.08.008

ganglions and the expression of neurofilament-H and growth-associated protein-43 (GAP-43) in dorsal root ganglion neurons, and accelerated peripheral nerve repair in rabbits [14,15]. On the basis of these results, this study was designed to investigate the effects of *A. bidentata* extract on neuronal growth in primarily cultured hippocampal neurons.

2. Materials and methods

2.1. Preparation of herb extracts

The root (Radix) of *A. bidentata* Blume was purchased from a local Chinese pharmacy, and was authenticated by an experienced pharmacognosist Dr. Haoru Zhao at China Pharmaceutical University. The herbs were powdered and decocted two times (2 h each time) with 6-, 8-, or 10-fold amount of water at 80 °C, separately. The extracts were subjected to separation by Sephadex-G50 column chromatography. After eluting with water, the collected fractions were lyophilized and ground to powders. The prepared extract was dissolved in sterilized distilled water at desired concentrations prior to use.

2.2. Cytotoxicity assays

Human hepatoma (HepG2) cells, obtained from the Cell Bank in Shanghai Institute of Cell Biology (Shanghai, China) and used as a cell model, were sub-cultured into 96-well cell culture plates at a density of 1000 cells/well, and permitted to adhere for 8–12 h at 37 °C. The cells were washed once with sterile phosphate buffered saline (PBS, pH 7.4) and treated with different concentrations of *A. bidentata* extract (0.25, 0.5, 1.0, 2.0, and 4.0 μg/ml) in serum-free DMEM for 24 h at 37 °C. The tetrazolium salt WST-8 (CCK-8, Dojindo, Japan) was then added to the cultures at a final concentration of 500 nM. After incubation for 2 h at 37 °C, the absorbance was measured spectrophotometrically at 450 nm with a reference wavelength at 650 nm. Cell survival determined by WST-8 assays practically reflected the number of viable cells, and cell viability was expressed as the percentage of viable cells relative to untreated cells. All experiments were performed in triplicate on three separate occasions.

2.3. Isolation and characterization of hippocampal neurons

Sprague–Dawley (SD) embryonic rats were obtained from the experimental animal center of Nantong University. All experimental procedures involving animals were conducted as per Institutional Animal Care guidelines and approved ethically by the Administration Committee of Experimental Animals, Jiangsu Province, China.

The isolation of rat hippocampal neurons was conducted according to the published protocols with little modifications [16,17]. In brief, after E18 embryonic rats were sacrificed by cervical dislocation under anesthesia, their brains were quickly removed and the hippocampi were harvested on a cold stage. Hippocampal tissues were mechanically and enzymatically dissociated into cell suspensions which were plated onto poly-lysine-coated plates at the density of 1×10^4 per cm^2. The cells were resuspended in DMEM supplemented with 10% F12 and 10% FBS (Gibco, Grand Island, NY) and incubated for 4 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

For immunocytochemistry characterization, the adhesive cells were fixed with 4% paraformaldehyde for 30 min, and permeabilized and blocked for non-specific sites with PBS containing 10% goat serum and 0.3% Triton X-100 for 60 min at room temperature. A polyclonal antibody of goat anti-GAP-43 (1:200, Santa Cruz, CA) was applied to cells and incubated overnight at 4 °C, followed by reaction with the FITC-labeled secondary antibody (donkey anti-goat IgG, 1:200, Santa Cruz). The cells were also stained with Hoechst 33342 dye (Sigma, St. Louis, MI). Following washes with PBS, the cells were mounted in a fluorescent mounting medium and observed under a confocal laser microscope (Leica, Heidelberg, Germany).

2.4. Culture of hippocampal neurons in the presence of A. bidentata extract

At 4 h after plating, hippocampal neurons attached to the substrate were transferred to a basic DMEM medium and randomly divided into five groups: three *A. bidentata*-treated groups with the extract at low (0.25 μg/ml), middle (0.5 μg/ml), and high (1.0 μg/ml) dosages, respectively, a NGF group with 0.05 μg/ml of NGF (Sigma) added to the medium (serving as a positive control), and a plain medium group without additives (serving as a negative control). After culturing for 6, 12, and 24 h, the neurite growth of hippocampal neurons in different groups was observed and photographed under a confocal laser microscope (Leica). NIH image software (Scion, Frederick, MD) was used to measure the neurite length of neurons and to count the number of neurons.

2.5. DNA microarray analysis

Total RNA was extracted and purified from the hippocampal neurons of the rats. The RNA samples were converted to biotinylated cDNA through reverse transcription, and each sample was hybridized to a separate GeneChip Rat Genome 230 2.0 Array containing 31,099 probes (Affymetrix, Santa Clara, CA). The hybridized cDNA was then stained with a streptavidin–phycoerythrin conjugate and visualized with an array scanner.

2.6. Real-time quantitative RT-PCR

The oligonucleotide primers and probes were designed according to the whole sequences for rat GAP-43 and GAPDH (as an internal control), respectively. Their sequences were as follows: GAP-43-sense (5′-GGAGCCTAAACAA

GCCGATGA-3′); GAP-43-antisense (5′-CTACAGCT TCTTTCTCCTCCTCAG-3′); GAP-43-probe (5′-CTGT CACTGATGCTGCTGCCACCACC-3′); GAPDH-sense (5′-CCTTCATTGACCTCAACTACATG-3′); GAPDH-antisense (5′-CTTCTCCATGGTGGTGGAAAC-3′); GAPDH-probe (5′-CCCATCACCATCTTCCAGGAGC-3′).

After 6, 12, and 24 h of culturing, the hippocampal neurons from five groups were homogenized in 1 ml Trizol, and total RNA was extracted and used in a reverse transcription reaction according to the protocol described in the kit (Gibco), followed by PCR amplification. PCR products, purified from agarose gels using gel purification kit, were cloned into the pGEM-T vector (Promega, Madison, WI) according to the manual. Plasmid DNA was extracted and identified by sequencing. After being quantified, the serially diluted plasmids were used as quantitative standards.

For real-time PCR, the initial 3 min incubation at 93 °C was performed, followed by 40 cycles of denaturation at 93 °C for 20 s and annealing/extension at 60 °C for 55 s. Fluorescent detection was performed after each annealing/extension step. Each assay was carried out in a separate microcentrifuge tube containing 1 μl of cDNA template and 19 μl of quantitative PCR solution, the composition of which contained PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM each of GAP-43 or GAPDH primers, 0.4 μM each of GAP-43 or GAPDH fluorescence probes, and 1 U *Taq* DNA polymerase. The negative control and plasmid standards were also amplified simultaneously. The mRNA quantity was automatically calculated based on the calibration curves generated by serially diluted plasmid preparations.

2.7. Western blot analysis

After 24 h of culturing, protein was extracted from the hippocampal neurons of five groups with a buffer containing 1% SDS, 100 mM Tris–HCl, 1 mM PMSF, and 0.1 mM β-mercaptoethanol. Protein concentration of each specimen was detected by the Bradford method to maintain the same loads. Protein extracts were heat denatured at 95 °C for 5 min, electrophoretically separated on 12% SDS–PAGE, and transferred to PVDF membranes. The membranes were subjected to the reaction with a 1:1000 dilution of goat anti-GAP-43 polyclonal antibody in TBS buffer at 4 °C overnight, followed by a reaction with a 1:2000 dilution of mouse anti-goat antibodies conjugated with horseradish peroxidase (HRP) at room temperature for 2 h. After the membrane was washed, the HRP activity was detected using an ECL kit. The image was scanned with a GS800 Densitometer Scanner (Bio-Rad), and the data were analyzed using PDQuest 7.2.0 software (Bio-Rad). GAPDH (1:300) was used as an internal control.

2.8. Statistics

At least three repetitive assessments were performed. All data were given as the means ± SEM. One-way ANOVA and post hoc Scheffe test were used to compare differences between groups. All statistical analyses were conducted with a STATA 7.0 software package (Stata Corp, College Station, TX), and significance levels were set at $p < 0.05$.

3. Results

3.1. Cytotoxicity of A. bidentata extract

To assess the toxicity of *A. bidentata* extract, HepG2 cells were treated with the extract at different concentrations for 24 h, and the cell viability was measured by WST-8 assay. The dose–response toxicity curve for the HepG2 cells indicated that no cytotoxicity of the extract to this model cells was within the dose window used (Fig. 1).

3.2. Effect of the extract on neurite outgrowth of hippocampal neurons in vitro

Before being cultured in different mediums, over 90% of the cell population derived from embryonic rat hippocampi were identified to be neurons by GAP-43 immunocytochemistry.

Visual inspection under a confocal laser microscope revealed the morphological changes of the hippocampal neurons in different groups after they were cultured for 24 h. Shortly after plating, the hippocampal neurons on the substrate formed lamellipodia and then established neurites. Within 24 h, neurons showed a transition from the unpolarized to polarized state, and axons elongated out of the immature neurites rapidly. In the plain medium group (the negative control), the short, tapering neurites outgrew very slowly from hippocampal neurons. In contrast, the long, thick neurites outgrew rapidly from hippocampal neurons in either *A. bidentata* extract high dosage (1.0 μg/ml) or the NGF group.

We compared the neurite length of hippocampal neurons in different groups after 24 h of culturing (Fig. 2(a)). *A. bidentata* extract-treated groups at high dosages (0.5 or 1.0 μg/ml) as well as the NGF-treated group exhibited a longer neurite length of hippocampal neurons than the plain medium group (the negative control) with statistically

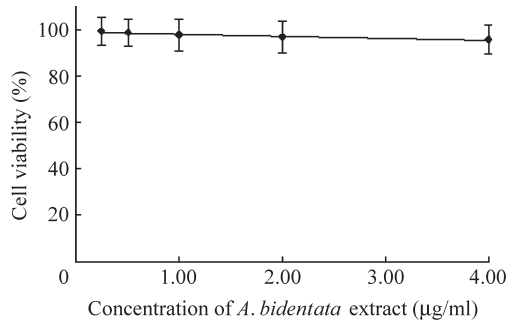

Fig. 1. The dose–response toxicity curves of HepG2 hepatocytes treated with *Achyranthes bidentata* extract ($n = 3$).

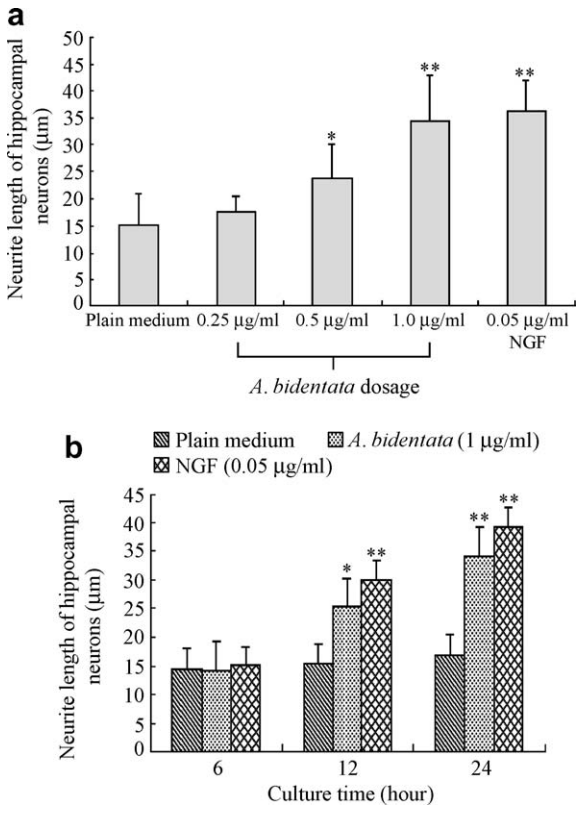

Fig. 2. The graphs showing neurite length of rat hippocampal neurons in five different groups after culturing for 24 h (a) and the time-dependent changes (b). In both (a) and (b), $^{*}p < 0.05$ and $^{**}p < 0.01$, vs. plain medium group (negative control) at the same time point.

significant differences. The neurite length of hippocampal neurons was increased with the concentration of *A. bidentata* extract added to the medium, and the greatest neurite length was observed in the high dosage (1.0 μg/ml) group, which was similar to that of the NGF group (the positive control) without significant difference ($p > 0.05$).

Fig. 2(b) presents the time-dependent changes in the neurite length of hippocampal neurons of the *A. bidentata* extract-treated group at a high dosage (1.0 μg/ml) and also the comparison to those of NGF and plain medium groups. After 12 or 24 h of culturing, this *A. bidentata* extract-treated group exhibited a similar neurite length to that of the NGF group (the positive control) without significant difference ($p > 0.05$), and it has a significantly longer neurite length than that of the plain medium group (the negative control) with statistically significant difference. However, at 6 h after culturing no significant difference was observed between the *A. bidentata* extract-treated group (1.0 μg/ml) or NGF-treated group and the plain medium group.

3.3. Gene microarray analysis

DNA microarray analysis showed that after 6 h of culturing, of the total 15,866 genes examined, 248 were upregulated and 316 were downregulated in rat hippocampal neurons treated with a high dosage (1.0 μg/ml) of *A. bidentata* extract as compared to the negative control. Among them, 25 upregulated genes and 47 downregulated genes were significantly differentially expressed, including genes for G-protein coupled receptors, signal transduction molecules, cell growth and metabolism regulating factors, cellular structure molecules, enzymes, immuno-globulins and apoptosis signaling molecules. Fig. 3 presents the microarray heat maps of the genes from rat hippocampal neurons.

3.4. Effect of A. bidentata extract on the expression of GAP-43 mRNA in hippocampal neurons

The mRNA levels of GAP-43, expressed as the relative values to those of GAPDH (internal control), in the hippocampal neurons were examined by real-time quantitative RT-PCR. After 6 h of culturing, for NGF-treated and three of *A. bidentata* extract-treated groups, the mRNA levels of GAP-43 in the hippocampal neurons were all higher than those of the plain medium group with a significant difference. Moreover, there was no significant difference in the GAP-43 mRNA expression between NGF- and *A. bidentata* extract-treated (1.0 μg/ml) groups (Fig. 4(a)). The time-dependent changes in the GAP-43 mRNA level of these groups are also shown in Fig. 4(b).

3.5. Effect of A. bidentata extract on the expression of GAP-43 protein in hippocampal neurons

Western blot analysis showed that the treatment with *A. bidentata* extract at each dosage increased the expression level of GAP-43 protein in the hippocampal neurons, in a dose-dependent manner. The highest expression level appeared at the dosage of 1.0 μg/ml, which induced a similar protein expression level to that induced by NGF (Fig. 5). There was a significant difference observed between the *A. bidentata* extract-treated group (1.0 μg/ml) or NGF-treated group and the plain medium group.

4. Discussion

Although *A. bidentata* is not a toxic plant often used as a prescription ingredient or health-care food as recorded in the classical Chinese herbal texts, we performed a test with the HepG2 cells to evaluate its cytotoxicity, which indicated that this medicinal herb has no *in vitro* toxicity to the HepG2 cells within the dose range of 0.25–4.0 μg/ml, and suggested that its desired biological activity could be elicited without inducing *in vivo* toxicity [18].

The neuroactive property of *A. bidentata* extract has been examined by a series of studies [13,14]. In this study, we further investigated its effects on the primarily cultured hippocampal neurons. As is known, hippocampus, an important part of the limbic system, plays a major role in learning, memory, and emotional regulation, and the pathological changes in hippocampus are involved in many neurological diseases, such as Alzheimer's disease, demen-

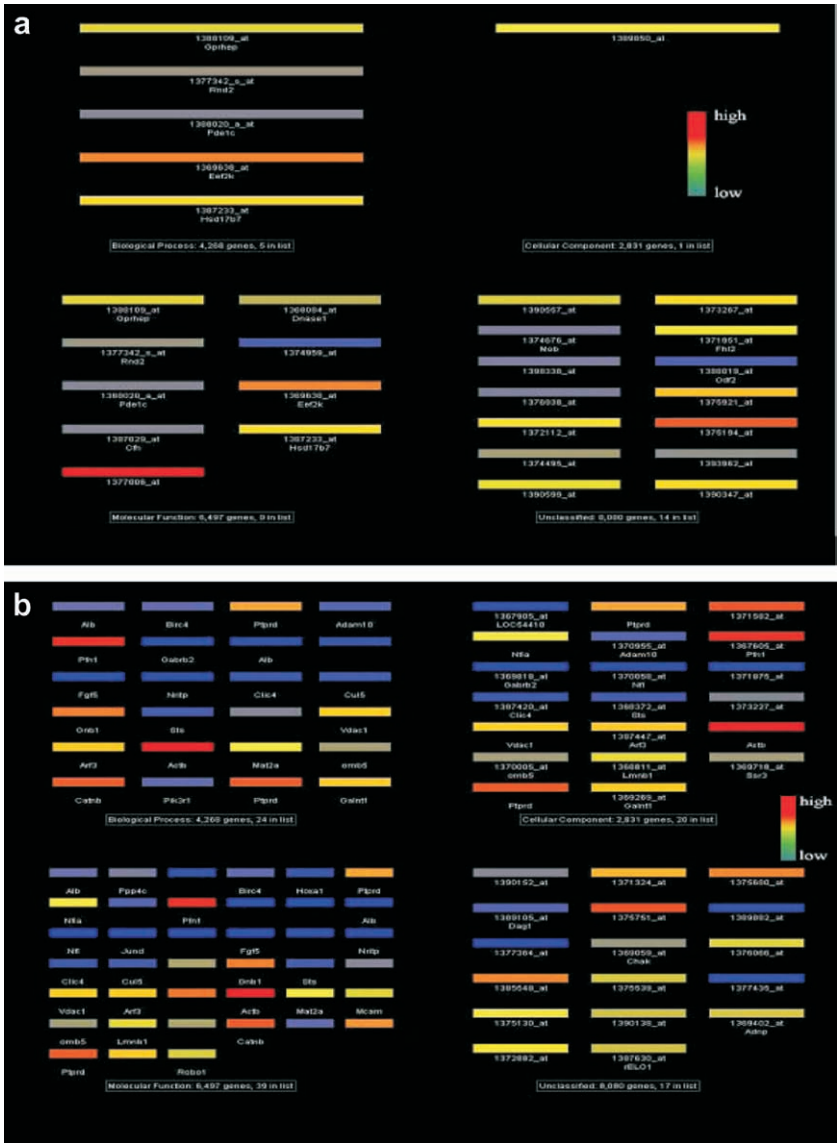

Fig. 3. Heat maps of genes from cultured rat hippocampal neurons. In maps, the color codes represent the signal strength, digits designate the ID of gene chip probes and letters represent the gene symbols. The upregulated and downregulated genes are shown in (a) and (b), respectively.

tias, Parkinson's disease and epilepsy. As a result, the primarily cultured hippocampal neurons usually serve as a well-characterized cell model for seeking neuroactive compounds [19].

In our experiment, the hippocampal neurons were cultured for 4 h and then transferred to different culture media for an additional 6, 12, and 24 h for the treatments. We found that a relatively short-term culture was suitable for the neurite outgrowth induced by *A. bidentata* extract and was more easily investigated at the early stages of neuronal development; while the long-term culture might result in much more elaborate neurite extension so as to render the accurate measurement of neurite length which becomes difficult or even impossible [20,21].

Our experimental results revealed that treatment with *A. bidentata* extract for 24 h at a dosage of 1.0 μg/ml enhanced the neurite outgrowth from hippocampal neurons, and this effect was also induced by NGF at 0.05 μg/ml dosage. The morphometric measurements using NIH Scion Image software yielded a quantitative comparison of neuronal growth in different culture mediums, offering evidence that *A. bidentata* extract could be a new type of neurotrophic agent with a function similar to NGF's function. In addition, morphological observations also showed that *A. bidentata* extract enhanced the neuronal growth in a dose- and time-dependent manner.

GAP-43 is a neuron-specific phosphoprotein whose expression is associated with neural development and synaptic plasticity. Accordingly we investigated the GAP-43 expression in the cultured hippocampal neurons at both the transcript (mRNA) and the protein levels after treatment with *A. bidentata* extract. The higher expression of GAP-43 was induced by a high dosage (1.0 μg/ml) of *A. bidentata* extract when compared to negative control, indicating that *A. bidentata* extract, like NGF, could upregulate the GAP-43 expression.

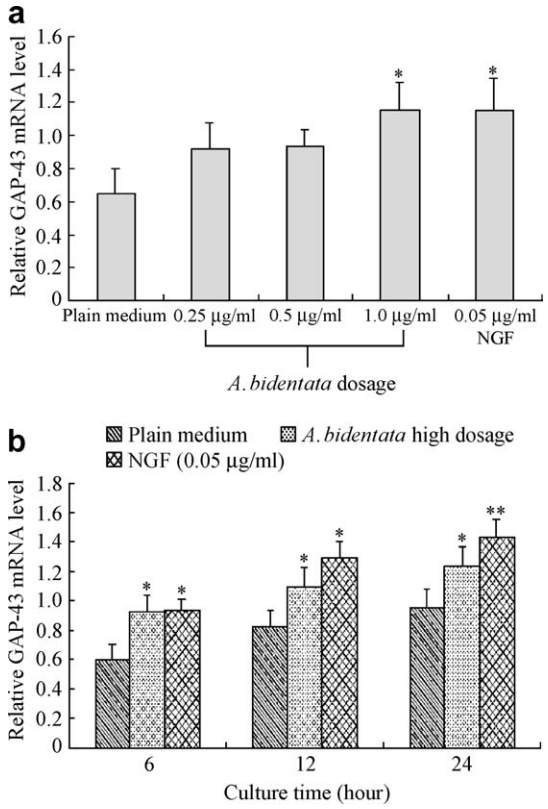

Fig. 4. The mRNA levels of GAP-43 expressed by rat hippocampal neurons in five different groups after culturing for 6 h (a), (b) shows the time-dependent GAP-43 expression. $^{*}p < 0.05$ and $^{**}p < 0.01$, vs. the plain medium group (negative control) at the same time point.

To acquire some clues relating to the mechanism that underlies the neurotrophic action of *A. bidentata* extract, the gene microarray analysis was accomplished for the cultured hippocampal neurons in NGF high dosage (1.0 μg/ml) and plain medium groups after 6 h of culturing [22–24]. In the sample treated with *A. bidentata* extract, we identified 25 significantly upregulated genes including GAP-43, G-protein coupled receptor (GPCR) 116, phosphodiesterase 1C (PDE1C), NAD(P)H dehydrogenase quinone 2, and eukaryotic elongation factor 2 (EEF2). Moreover, 47 significantly downregulated genes including BCL2-like 11 (apoptosis facilitator), fibroblast growth factor 5 (FGF-5) phosphatidylinositol-3 kinase (PI3K), and calcium calmodulin-dependent protein kinase IV (CaMK IV) were also identified.

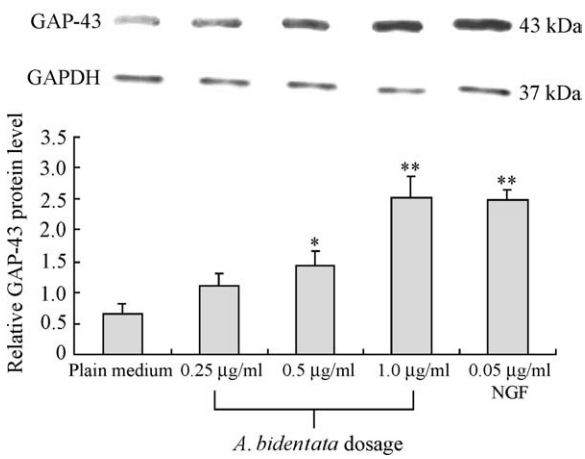

Fig. 5. The protein levels of GAP-43 expressed by rat hippocampal neurons in five different groups after culturing for 24 h. Also shown are the representative Western blots of GAP-43 and GAPDH proteins. $^{*}p < 0.05$ and $^{**}p < 0.01$, vs. plain medium group (negative control).

Since the DNA microarray analysis revealed the upregulation of GAP-43 gene induced by *A. bidentata* extract, a real-time quantitative PCR was further performed to confirm the changes in GAP-43 abundance after being treated with *A. bidentata* extract. Also, as revealed by microarray analysis, both GPCR and PDE1C showed high expressions in the presence of *A. bidentata* extract, implying the activation of the GPCR signaling cascade for mediating neuronal growth cone guidance [25,26]. The upregulation of NAD(P)H dehydrogenase by *A. bidentata* extract is associated with cell metabolism and growth. In addition, an increased expression of EEF2, as revealed by microarray analysis, contributes to neuronal growth because EEF2 is a highly conserved protein kinase in the calmodulin-mediated signaling pathway and links the activation of cell surface receptors to cell division.

Also as indicated by microarray analysis, BCL2-like 11 (apoptosis facilitator) was downregulated by *A. bidentata* extract, resulting in the reduction of its proapoptotic function. FGF-5 belongs to a group of mitogenic and angiogenic heparin binding growth factors and its decreased expression is beneficial to cell differentiation rather than proliferation. PI3K and CaMK IV, as two most important regulatory proteins, are involved in different signaling pathways and controlling of cell functions. Their downregulation is thus associated with a series of neuronal events.

Despite new mechanism clues that were provided by microarray data, and despite ERK1/2 phosphorylation cascade that was previously proposed as a signaling pathway through which *A. bidentata* extract induced neuronal differentiation of PC12 cells [13], the molecular mechanisms for the enhancement of *A. bidentata* extract on hippocampal neuronal growth remain to be determined. Further studies are being done to explore the signaling molecules involved in the mechanisms and to identify what compound(s) in *A. bidentata* extract is(are) responsible for its actions, although the extract has been known to include ketosteroids, polysaccharides, and polypeptides in its composition.

Acknowledgements

This work was supported by the National Basic Research Program of China (Grant No. 2003CB515306). We thank Prof. Jie Liu for assistance in the preparation of the manuscript.

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