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Original Article

Brain-derived neurotrophic factor protects rat cerebral cortical neurons from intermittent hypoxia *in vitro*

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Abstract: Brain-derived neurotrophic factor (BDNF) plays an important role in neuronal survival and growth and participates in neuronal plasticity. BDNF binds to its high affinity receptor TrkB and activates signal transduction cascades crucial for the production of cyclic adenosine mono phosphate (cAMP)-response element binding protein (CREB), which contributes to neurotrophin-mediated survival in the central nervous system. This is accomplished by activating antiapoptotic gene expression and CREB Serine-133 (Ser133) phosphorylation, which is required for the activation of CREB-regulated transcription by neurotrophins. However, the specific mechanisms of BDNF-mediated neuroprotection *in vitro* remain not well understood. The aim of this study was to determine whether BDNF's neuroprotective effects on embryonic rat cortical neurons exposed to intermittent hypoxia involved CREB phosphorylation. We constructed an adenoviral vector encoding rat BDNF (Ad-BDNF) and performed an enzyme linked immunosorbent assay (ELISA) to measure the levels of BDNF and CREB. We also measured the viability of neurons transfected with BDNF in hypoxic culture conditions. We found that the levels of BDNF were significantly higher in neurons compared to control ($P < 0.01$). The neurons in the experimental groups transfected with BDNF were much more viable than those cultured under the hypoxic control conditions without BDNF. Hypoxia and BDNF both induced the phosphorylation of CREB in embryonic cortical neurons. This study suggests that BDNF may promote neuronal survival in intermittently hypoxic environments. Furthermore, the neuroprotective action of BDNF on embryonic rat cortical neurons exposed to intermittent hypoxia is partly mediated via CREB phosphorylation.

Keywords: Intermittent hypoxia, obstructive sleep apnea, cortical neurons, brain-derived neurotrophic factor

Introduction

Obstructive sleep apnea (OSA) is a major and widely prevalent clinical disorder characterized by repetitive episodes of apnea or hypopnea due to the obstruction of the upper airway during sleep and results in oxygen desaturation and arousal. Intermittent hypoxia (IH) resulting from OSA may cause structural neuronal damage and dysfunction in the central nervous system (CNS) [1, 2]. IH apparently plays a major role in causing many of the abnormalities associated with OSA. Regrettably, compared to the existing knowledge on acute and chronic sustained hypoxia, our understanding about IH remains limited.

Recent studies support the idea that the reduced expression of brain-derived neurotrophic factor (BDNF) could cause impairments in long-term synaptic plasticity and neurocognitive functions during chronic IH [3]. BDNF is a member of the nerve growth factor family, which promotes the survival and differentiation of neuronal tissues by acting on receptor kinases [4]. Studies have shown that BDNF can protect neurons from injuries and death [5-7]. A recent complementary approach for the treatment of neurological disorders is to use neurotrophic factors to reduce progressive neuronal loss [8-10].

However, the hypothesized mechanisms underlying BDNF protection against neuronal hypoxic injury are controversial and the actual mechanisms of BDNF-mediated neuroprotection *in vitro* remain unknown. Recent work suggests that cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) contributes to neurotrophin-mediated survival in

the central nervous system by activating anti-apoptotic gene expression. Furthermore, it is reported that CREB Serine-133 (Ser133) phosphorylation is required for the activation of CREB-regulated transcription by neurotrophins [11, 12].

Adenoviral delivery has great potential for use as a routine transfection method. In this study, we constructed an adenoviral vector encoding rat BDNF (Ad-BDNF). We attempted to better understand the signaling mechanism underlying the neuroprotective effect of BDNF against intermittent hypoxia. We also investigated whether the neuroprotective effects of BDNF on embryonic rat cortical neurons subjected to intermittent hypoxia involved CREB phosphorylation.

Materials and methods

Construction of the recombinant BDNF adenoviral vector (Ad-BDNF)

Primary cultures of dissociated cortical astrocytes were obtained from the brains of 1- to 3-day-old neonatal rats. Brains were triturated repeatedly in 8 ml of Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco, USA) medium supplemented with 7% heat-inactivated fetal bovine serum and 10 mM Na_2HCO_3 using a fire-polished Pasteur pipette to yield a mixture of single cells. The cells were then plated onto poly-D-lysine-coated 75 cm^3 tissue culture plates and incubated in a humidified atmosphere of 5% CO_2/95% O_2 at 37°C.

After 2 weeks of astrocyte culture, total RNA was extracted using the guanidinium isothiocyanate extraction procedure, with the addition of phenol-chloroform-isoamylalcohol to increase RNA purity. The prepared total RNA sample was analyzed by reverse transcription polymerase chain reaction (RT-PCR) and cDNA synthesis was performed. Two primers were used for BDNF PCR as follows: forward, 5'-GGGAATTCCATATGCACTCCGACCCCGCCCG-3'; reverse, 5'-CCGCTCGAGCTATCTTCCCCTTTTAATG-3'. PCR was performed with 2 μl of cDNA in a 50 μl reaction mixture containing 10 pM of each primer, 5 U of ExTaq polymerase, 2.5 mM of dNTP and 5 μl of 10× buffer. The reaction was performed in a Biometra thermal cycler. Thirty cycles of PCR amplification were carried out as follows: denaturation at 94°C for 40 s, annealing at 69°C for 40 s, and extension at 72°C for 2 min. PCR products were subjected to electrophoresis on 0.8% agarose mini-gels, stained with ethidium bromide, and visualized under UV light.

The Ad-CMV5-BDNF adenoviral vector was constructed by the insertion of the NdeI/XhoI fragment of BDNF, which was isolated from normal astrocytes, in between the single BglII and PmeI sites of Ad-CMV5 downstream of the CMV promoter (Quantum, USA). In order to generate a recombinant adenovirus, 5 μM of linearized Ad-CMV5-BDNF plasmid DNA was co-transfected with 5 μM of the large ClaI fragment of Ad-CMV5 DNA into transformed human kidney cell line 293 using Transfection Reagent (Roche, Germany) according to manufacturer instructions. After 10 days of incubation, clumps of the 293 cells routinely formed plaques and detached from the monolayer. The propagation and purification steps of the recombinant adenovirus were conducted using sequential centrifugation in CsCl gradients and dialysis against 10% glycerol in phosphate-buffered saline (PBS). Virus titer was determined using the Tissue Culture Infectious Dose 50 (TCID50) method.

Neuron cultures

Neurons were prepared as described previously, with modifications [13]. Briefly, cortical tissue from E17 Wistar rat embryos was trypsinized in Dulbecco's minimum essential medium (Whittaker Bioproducts) containing 0.027% trypsin at 4°C for 20 min. The resulting mixture was then triturated in media consisting of Dulbecco's minimum essential medium supplemented with 10% bovine calf serum (Hyclone), 10% Ham's F12 with glutamine (Whittaker Bioproducts), and 50 U/mL penicillin-streptomycin (Sigma). Dissociated cells were plated on poly-L-lysine-coated glass coverslips in 35 mm Petri dishes and cultured at 37°C in a humidified 5% CO_2 incubator. Dissociated cells were plated at a density of 100,000 cells/mL in serum-free Neurobasal medium (Gibco) supplemented by B27 (Gibco). All procedures involving animals were approved by the Animal Care and Use Committee of Shandong University.

Cortical neurons were co-transfected with Ad-CMV5-BDNF, Ad-RC, and p-Helper using the

calcium phosphate method. To generate hypoxic stress conditions, cells were placed in an airtight chamber with inflow and outflow valves infused with a gas mixture. Cell cultures were exposed to alternating cycles of hypoxia (1.5% O_2, 15 s) and normoxia (21% O_2, 4 min) in a humidified Lucite chamber at 37°C, as previously described [14]. We studied embryonic cortical neurons for 6 h after exposure to the hypoxic environment.

Immunofluorescence analysis and cell counts

Cells were fixed with 10% neutral buffered formalin (10 min at room temperature [RT]), permeabilized with Tris-buffered saline (TBS) containing 0.3% Triton (TBST), blocked in 5% goat or donkey serum in TBS, and exposed to the primary antibody overnight at 4°C. They were then washed and incubated with the corresponding secondary antibody conjugated to fluorescin, washed, and inverted onto glass slides using Vectashield mounting media containing DAPI (Vector Laboratories) to identify nuclei. The cultured embryonic cerebral cortical neurons were identified using the immunofluorescin histochemical method. Neurons were identified by immunostaining with rabbit polyclonal anti-MAP2 (1:500, Santa Cruz). The primary antibody was incubated and then washed with TBS and FITC-conjugated anti-rabbit IgG (Sigma-Aldrich) which was used as a secondary antibody. The specificity of staining was confirmed by omitting the primary antibody in each case. Digital images in each channel were acquired with a Nikon microscope, processed using ImagePro software, and pseudocolored appropriately. Manual cell counts were performed by two observers on digital images of 4-10 randomly-selected, nonoverlapping single-channel 10× fields. To calculate the overall percent transduction, the number of MAP-expressing cells was expressed as a percentage of nuclei stained with DAPI (MAP+/DAPI+).

Assessment of neuronal injury

MTT was added to the cells to a final concentration of 250 mM and the cells were incubated for 1 h to reduce MTT to a dark blue formazan product. The media was removed and the cells were dissolved in dimethylsulphoxide. The formation of the formazan reaction product was assessed by measuring the absorbance change at 595 nm using a microplate reader (BioRad Laboratories, CA, USA). Cell viability results were expressed as a percentage of the absorbance measured in untreated cells.

ELISA assay

We used an ELISA to measure BDNF concentration. In brief, 96 hole plates were coated with antigen in a carbonate coating buffer (pH 8.2) overnight at 4°C and blocked for 2 h at room temperature. The samples and standards were applied to the plates and the plates were incubated for 6 h at room temperature, with agitation. The wells were washed, rabbit polyclonal anti-BDNF antibody (1:1000; Santa Cruz Biochemical, CA, USA) was added, and the plates were incubated for 2 h at room temperature. After another washing step, anti-rabbit IgG-HRP conjugate was added and the plates were agitated at room temperature for 2 h. Following a final wash, TMB and peroxidase substrates were applied to the wells. The reaction was stopped with 1 M H_2SO_4 following a 15 min incubation at room temperature and the absorbance was measured at 450 nm using a spectrophotometer. The standard curve was linear in the observed detection range.

Preparation of total cell extracts and immunoblot analysis

To prepare whole-cell extracts, cells were washed with PBS containing zinc ion (1 mM) and suspended in lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinin, 170 μg/ml leupeptin, 100 μg/ml PMSF; pH 7.5). After mixing for 30 min at 4°C, the mixtures were centrifuged (10,000× g) for 10 min and the supernatants were collected as whole-cell extracts. Protein content was determined using the Bio-Rad protein assay reagent with bovine serum albumin as a standard. Enhanced chemiluminescent (ECL) Western blotting was performed as follows. Equal protein amounts from total cell lysates from control and luteolin-treated samples were resolved on 10-12% SDS-PAGE gels along with a prestained protein molecular weight standard (Bio-Rad). Proteins were then blotted onto a nitrocellulose (NC) membrane and incubated with the primary antibodies (anti-BDNF, anti-CREB, anti-phospho-CREB, and anti-bactin as an internal control). The secondary antibody was a peroxidase-conjugated goat anti-mouse

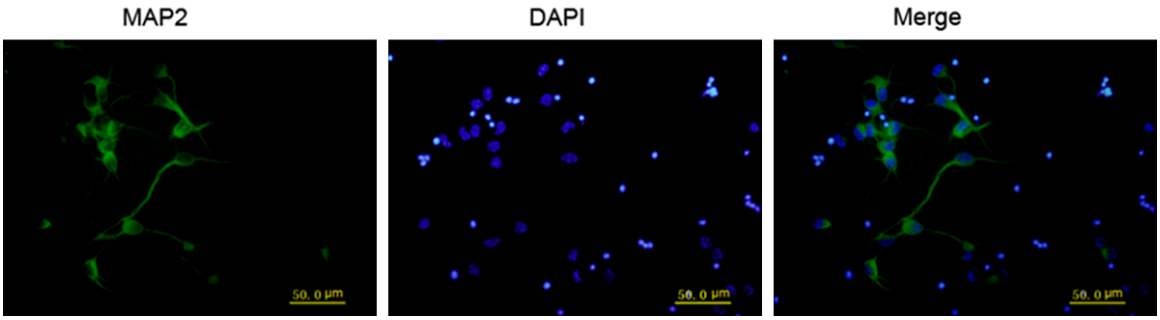

Figure 1. The MAP2 positive neuron.

Table 1. The O.D. of BDNF in culture media and cell lysates at different days after transfected with BDNF-vector

Time	O.D. of BDNF	
	Cell lysates	Culture media
0	0	0
2 days	0.50±0.05	0.49±0.03
1 week	0.62±0.04	0.56±0.02
2 weeks	0.72±0.06	0.66±0.07
4 weeks	0.75±0.07	0.83±0.05

antibody. After secondary antibody binding, the bands were revealed using a commercial ECL kit. The data presented are representative of three independent experiments.

Statistical analysis

All quantitative data are presented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was used to compare the groups and was followed by a Fisher protected least significant difference test as appropriate. $P < 0.05$ was accepted as statistically significant.

Results

Production of BDNF in cultured cells

Neurons were transduced with either the BDNF vector or the control vector. Three days later, culture media was collected and cell lysates were prepared (**Figure 1**). The levels of BDNF were assayed by ELISA. The cells transduced with the control vector did not produce detectable levels of BDNF, similar to mock-transduced cultures. BDNF was detected in both the media and the cell lysates from cells transduced with the BDNF vector. The levels of BDNF in these cells were significantly higher than that detected in the control cells ($P < 0.01$) (**Table 1**).

Viability of BDNF-transfected neurons after hypoxic culture

The neurons transfected with BDNF were much more viable than the neurons in the hypoxic-cultured control group (without BDNF) 6 h after hypoxic culture (**Table 2**). The percentage of apoptotic neurons in the experimental group was lower than that in the hypoxic-cultured control group (**Figure 2**; **Table 3**).

CREB levels in neurons treated with BDNF after hypoxic culture

Hypoxia and BDNF both induced the phosphorylation of CREB in embryonic cortical neurons. CREB phosphorylation levels were much higher in the experimental group (BDNF-treated) than in the hypoxic control group at the same time points ($P < 0.01$) (**Figure 3**). While the levels of phosphorylated CREB decreased slowly over time, they remained at a higher level for a much longer time in the experimental group than in the control group. The total amount of CREB in embryonic cortical neurons during the first 3 h after exposure to hypoxia was the same in both the experimental and the hypoxic control groups. CREB levels decreased more quickly in the hypoxic control group 5-6 h after hypoxia, compared to the experimental group (**Figure 3**).

Discussion

Human adenoviral vectors have successfully been used to express a wide variety of viral and cellular genes in mammalian cells [15]. Adenoviruses transfer genes to a broad spectrum of cell types, and gene transfer is not dependent on active cell division. Additionally,

Table 2. Viability of neurons hypoxic-cultured in different time and different group

Group	Time after hypoxic culture						
	0	1	2	3	4	5	6
Control	100	84.3±1.68	82.5±2.26	75.4±2.21	71.5±1.47	65.5±2.58	60.44±8.50
BDNF-transfected	114.7±9.02	102.5±4.30	96.7±3.32	90.4±4.45	88.3±2.22	80.1±6.55	76.9±4.23

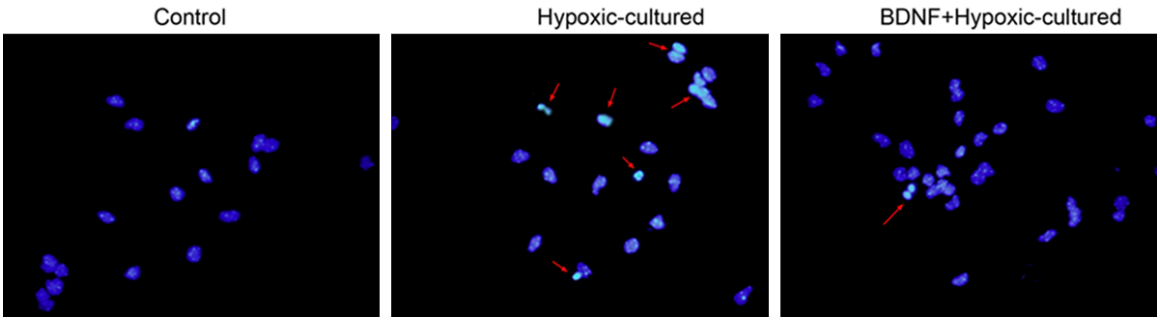

Figure 2. Apoptosis of neurons under fluorescence microscope by DAPI stain after 3 hours of hypoxic culture.

Table 3. Percentages of apoptosis neurons detected by DAPI stain (% n=5)

Group	Time after hypoxic culture						
	0	1	2	3	4	5	6
Control	100.00	6.8±3.30	9.55±4.19	11.23±3.21	33.5±9.74	41.3±6.59	45.6±8.57
BDNF-transfected	100.00	2.2±1.2	4.87±3.85	7.85±4.33	13.3±3.29	22.1±6.99	26.1±7.25

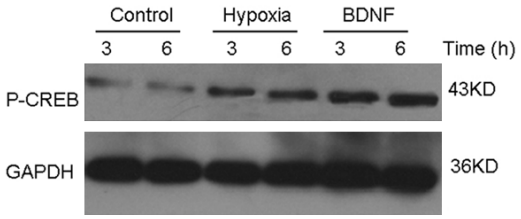

Figure 3. Expression of phosphorylation of CREB in different time and different group.

high virus titers and high transgene expression levels can generally be obtained using adenoviruses [16].

Recombinant adeno-associated virus vectors are capable of delivering a wide variety of useful cassettes for the overexpression or downregulation of genes, including many reporter genes, cell-type-specific cDNAs, or drug-regulated promoters. Previous reports have shown that some adeno-associated virus serotypes mediate highly efficient, nontoxic, and stable long-term gene expression in cultured cortical and hippocampal neurons *in vitro* [17]. In the present study, we successfully constructed an adeno-associated virus containing the BDNF gene, which was effectively expressed in cortical neurons.

It is known that OSA causes pathological intermittent nocturnal hypoxia, hypercarbia, sleep fragmentation, and exaggerated negative intrathoracic pressure swings. These physiological changes can further lead to multiple cardiovascular [18-20], cerebrovascular [21], neurocognitive, and metabolic pathophysiological abnormalities [22, 23].

The molecular mechanisms underlying the adaptive responses to continuous hypoxia have been extensively investigated, and the activation of specific genes and the resulting de novo protein synthesis are considered important for triggering these adaptive responses [24]. It is uncertain whether IH would cause molecular changes similar to those seen with continuous hypoxia. Some data indicate that intermittent hypoxia-induced systemic and cellular responses are different from those observed in response to continuous hypoxia. For example, although IH produces a significant stimulation

of c-fos, continuous hypoxia fails to activate c-fos [25]. Some data indicate that chronic intermittent hypoxia mediated mitochondrial oxidative stress may play a major role in the neuronal cell loss and neurocognitive dysfunction associated with OSA [26].

It has been shown that BDNF is associated with spinal respiratory plasticity following intermittent hypoxia [27]. The effects of BDNF on cortical neurons after intermittent hypoxia are still unknown. BDNF has been found to attenuate the lesion-induced loss of neurons in animal models of Parkinson's disease and hypoxia [10]. BDNF exerts its protective effects on neurons through its high-affinity receptor TrkB and the low-affinity receptor p75 [28]. Upon binding to these receptors, BDNF elicits its survival-promoting effects by activating various intracellular signaling cascades [29]. Previous studies have shown that BDNF protects cultured cortical neurons from hypoxic injury via the activation of both ERK and PI-3-K pathways [30].

We examined whether neuroprotection by BDNF was associated with the activation of the transcription factor CREB. In neurons, a wide range of extracellular stimuli are capable of activating CREB, resulting in its subsequent binding to DNA and gene transcription [31]. Many recent reports support the idea that the survival effects of phosphorylated CREB in neuronal cells are mediated by the regulation of downstream target genes [12]. In our study, we found that CREB phosphorylation was up-regulated in the intermittent hypoxia and BDNF groups. These results suggest that both intermittent hypoxia and BDNF can induce the activation of CREB.

Most importantly, we found that BDNF could promote neuronal survival in an intermittently hypoxic environment. The neuroprotective effects of BDNF on embryonic rat cortical neurons subjected to intermittent hypoxia is partly mediated via CREB phosphorylation.

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Disclosure of conflict of interest

None.

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