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Full Length Research Paper

Nerve growth factor (NGF) combined with oxygen glucose deprivation OGD induces neural ischemia tolerance in PC12 cells

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Ischemic cerebrovascular disease is a major disease in humans. To better study this disease, a good ischemia model of nerve cells is needed. Nerve growth factor (NGF) could induce PC12 cells to become neurons. Oxygen glucose deprivation (OGD) could lead to hypoxia and neuronal ischemia. In this study, we used NGF and OGD to stimulate PC12 cells and converted them into neurons in order to establish an ischemia model. After stimulation with NGF (100 ng/ml for 6 days), PC12 cells show a neuron - like function as measured by physiology and biochemistry. After 6 days of NGF stimulation, we performed OGD treatment for 16 h to establish an oxygen glucose deprivation model. The results showed that PC12 cells transformed into cells that looked like neurons and that MAP2 was up-regulated in NGF - treated PC12 cells. Cell apoptosis was found to be up-regulated after NGF stimulation and OGD (5% CO_2 and 95% N_2, 1 mmol/l NaS_2O_4 in sugar - free DMEM for 16 h). A western blot analysis showed that OGD treatment increased the expression of HIF - 1. The apoptosis rate after 16 h of OGD was 19.44%. These results postulate that NGF treatment can be combined with OGD to establish an *in vitro* model of acute ischemic brain damage.

Key words: Nerve growth factor, oxygen glucose deprivation, PC12 cells, ischemia tolerance model.

INTRODUCTION

Ischemic cerebrovascular disease, such as cerebral thrombosis (CT), cerebral infaction (CI) and so on, are the most important causes of morbidity, and the third most common cause of death in elderly patients in the world. Effective methods of preventing and controlling ischemic cerebrovascular disease have been a topic of great interest. Ischemic damage of nerve cells leads to a series of complex signaling pathways that produce corresponding biological functions. A better under-standing of the role of the signal transduction mechanisms that underlie brain ischemic injury could identify key targets for neuroprotective substances. Thus, it is very important to establish a stable *in vitro* neuronal ischemia model. Nerve growth factor (NGF) could induce the differentiation of PC12 cells into neuron-like cells. The sympathetic neuron-like cells are characterized by electrical excitability, expression of neuron - specific genes and neurite outgrowth (Dichter, 1977; Greenberg, 1985). PC12 cells were cultured in different oxygen conditions. The metabolic activity of PC12 cells was measured in the final 4 h prior to cellular characterization using an alamar blue assay (Serotec) following the manufacturer's instructions (Hamid, 2004). A neuronal ischemia model is needed to conduct research on the molecular level. PC12 cells can be induced with NGF to become neuron - like in appearance. Neural ischemia tolerance in PC12 cells via the combination of NGF with OGD was little in references. In this study, we combined NGF stimulation and OGD to establish a neuronal ischemia model. We examined the role of ischemic injury and signal transduction mechanisms.

The model might help to establish NGF treatment followed by OGD as an *in vitro* model of acute ischemic brain damage. The model could provide a new tool for the identification of pathways that were involved in cerebral

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ischemia. It provide the reference for further study ActA/smads signaling pathways on acute ischemic brain damage. This cerebral ischemic model could be one example of the cell's broader general-stress response.

Therefore, the present model could be applied to the study of mechanisms that were involved in tolerance to other stressful stimuli, and it is the important step to study reperfusion/reoxygenation after OGD. Cell-based assays with high - throughput capacity could be used as direct screens and models to explore molecular mechanisms that were involved in cellular function and pathology. The model presented in this study will be a new tool to study ischemic cerebrovascular disease.

MATERIALS AND METHODS

Cell culture

PC12 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. The cell line was maintained in DMEM medium supplemented with 10% (v/v) fetal bovine serum and 5% horse serum (FBS, GIBCO), 100 IU/ml streptomycin, 100 IU/ml penicillin, pH 7.0, and detached with 0.25% trypsin (Sigma, USA). PC12 cells were grown at 37°C in 5% CO_2.

Differentiation of PC12 cells by NGF

Cells were grown in 5% horse serum containing media on collagen - coated tissue culture dishes before differentiation. After the cells got attached, they were treated with 100 ng/ml nerve growth factor (NGF 2.5S; Promega, Madison, WI) and cultured with serum - free DMEM for 6 days (Michiyoshi, 2010; Jaehoon, 2010), observed and photographed.

MAP2 immunocytochemical analysis

The cells were fixed with 4% paraformaldehyde/PBS and were permeabilized with 0.1% Triton X - 100 in PBS for 10 min. The cells were then incubated in 5% goat serum/PBS for 1 h at room temperature (20 to 25°C). Cells were washed again then incubated at 4°C overnight (14 to 16 h) in the presence of anti- MAP2 (1:1,000 dilution, Santa SC - 20172). After washing twice with PBS, the cells were incubated with fluorescently labeled secondary FITC-goat anti-rabbit (Santa SC - 3839) for 1 h at room temperature (Kumar, 2006). The results were observed by a fluorescence microscope equipped with a photomicrograph system.

OGD model of PC12 cells after NGF treatment

PC12 cells were treated with NGF (100 ng/ml) for 6 days. Cells were then washed 3 times with DMEM, and the cells were cultured with DMEM without sugar and 1 mmol/L NaS_2O_4 in hypoxic conditions (37°C, 5% CO_2 and 95% N_2) for 3 , 6 , 9 , 12 , 16 , or 24 h respectively, (Larsena, 2007; Damian, 2010).

Flow cytometry analysis

Cytometry was used to quantitatively assess the apoptosis detection kit, Annexin V - FITC and PI double-staining followed. Cells (1×10^5) were harvested and stained with Annexin V-FITC and PI using a double staining kit (Kaiji Bio Co., Nanjing, China) according to the manufacturer's instructions(Beckman coulter, USA). The cells were then immediately analyzed by flow cytometry. Signals from apoptotic cells were localized in the lower right quadrant of the resulting dot-plot graph.

MAP2 and HIT-1 western blot detection

PC12 cells were treated with NGF for 1, 2, 3, 4, 5, or 6 days. Samples were washed twice with cold PBS and then 1×10^6 cells were lysed with RIPA buffer (50 mmol/l Tris (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate) containing protease inhibitors (1% Cocktail and 1 mmol/L PMSF). Total proteins were separated with 15% SDS-PAGE and transferred to PVDF membranes. The membrane was blocked for with Tris-buffered saline with 0.1% Tween 20 (pH 7.6, TBST) for 1 h at room temperature and then immunoblotted with the primary antibody (1:1000) at 4°C overnight. After washing twice with TBST, the membrane was incubated with HRP-labeled secondary antibody (Santa SC-2073) for 1 h at room temperature and was washed three times with TBST. Final detection was performed with enhanced chemiluminescence (ECL) western blotting reagents (Amersham Biosciences, Piscataway, NJ), and the membranes were exposed to Lumi-Film Chemiluminescent Detection Film (Roche). Differences of loading were normalized using a monoclonal ß-actin antibody. The antibodies used in the study included anti-MAP2 (1:1000 dilution, Santa SC-20172) and anti-HIT-1 (1:1000 dilution, Santa SC-101907). After the PC12 cells were treated with NGF for 6 days, all the samples were treated with OGD for 3, 6, 9, 12, 16, or 24 h,respectively. HIT-1 protein expression was assessed at various times as described previously.

Statistical analysis

SPSS softwareseries and origin were used for statistical analyses, and values are presented as means ± SD. An ANOVA was used to compare the mean valueswithin or between samples. P values less than 0.05 indicated that the results showed statistically significant differences.

RESULTS

Morphological changes of PC12 cells

The results show that samples treated with NGF (100 ng/ml) stimulates neuron-like differentiation of PC12 cells as seen under the microscope. PC12 cells changed into neurons after one day of NGF treatment and followed by the formation of synapses. Synapses extended up the length of the cell after 3 days of treatment. The synaptic length increased 6 to 8 fold after 6 days of treatment (Figure 1). PC12 cells obtained from four cell depositories yielded essentially the same results. PC12 cells were exposed to the indicated concentrations of NGF for 3 days, and the differentiated cells were counted. Significant differences were detected. PC12 cells were treated NGF 6 days. The cultures were photographed under a phase contrast microscope (Olmpus, Japan) (scale bars = 20 um), Values are means ± S.D. of results from four microscopic fields. Asterisks indicate the

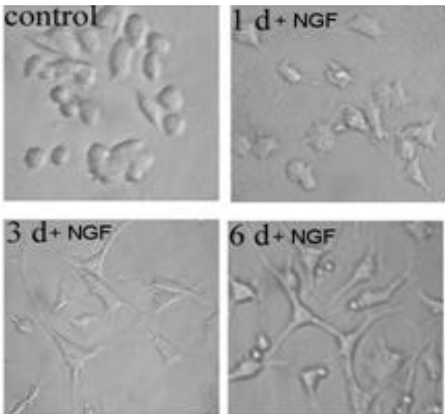

Figure 1. The morphological changes of PC12 cells(×200).PC12 cells were pretreated with 100 ng/mINGF for 1, 3 or 6 days. The differentiated cells were photographed under a phase contrast microscope.

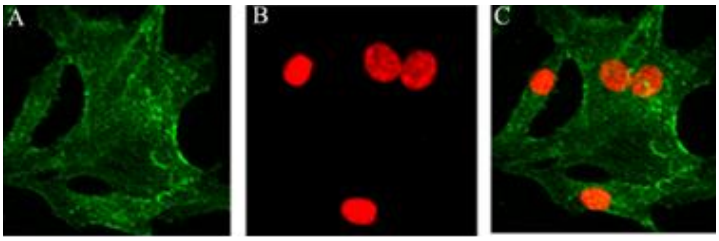

Figure 2. Morphological changes captured with fluorescence microscopy following immunofluorescence staining (×200). PC12 cells were cultured with NGF for 6 days and were then assessed with MAP2 immunofluorescence staining. (A) Anti- MAP2 and FITC-labeled IgG staining under the blue excitation, (B) nuclear counter staining of PI under purple excitation, (C) merged image of the FITC and PI staining.

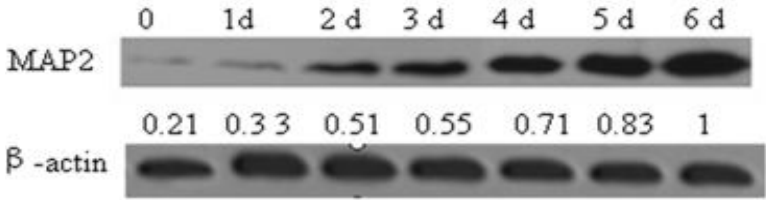

Figure 3. Analysis of western blot with samples treated with NGF. The results shown are representative of three repeated experiments.

statistical significance as determined by unpaired t-tests ($P < 0.01$).

Immunofluorescence analysis

The results showed that PC12 cells cultured with NGF for 6 days showed characteristic MAP2 immunofluorescence staining (Figure 2). Application plus pro 6.0 software to add image fusion after confirm green fluorescent were for PC12 cells transformation of neurons appearance cell. MAP2 immunofluorescence stain was strong positive expression. PBS control was negative fluorescence.

MAP2 protein expression increased in PC12 cells after NGF treatment

Expression of MAP2 protein of NGF treatment after 1, 2,

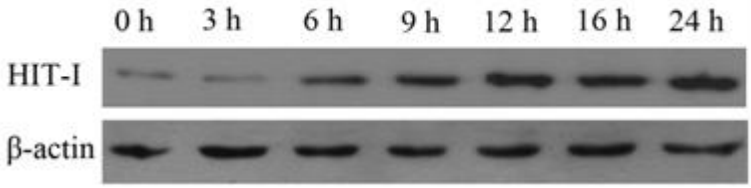

Figure 4. Analysis of western blot with samples treated with OGD after NGF stimulation. The results shown are representative of three repeated experiments.

3, 4, 5, or 6 days (Figure 3) showed that NGF couldincrease MAP2 protein expression. MAP2 expression
wasdetermined by western blotting after different treatment time periods. The results shown are representative of three repeated experiments. NIH imaging indicated that the protein signal densities were increased in cells treated with NGF for 3, 4, 5, or 6 days compared with control cells (no treatment).

OGD increased expression of HIT-1 in PC12 cells

After the PC12 cells were treated with NGF for 6 days, samples were treated with OGD. Western blot analysis was used to study the effect of OGD treatment on HIT-1 expression after 0, 3, 6, 9, 12, 16, or 24 h (Figure 4). The results showed that OGD could increase HIT-1 protein expression. The expression of HIT-1 protein was significantly increased by OGD. The results shown are representative of three repeated experiments. NIH imaging indicated that the protein signal densities were increased in cells treated with OGD for 6 h compared with control cells (no treatment).

OGD induced apoptosis in PC12 cells

To assess the apoptosis of differentiated PC12 cells treated with OGD, the cells were analyzed using a dual-laser FACSVantage SE flow cytometer (Becton Dickinson, Mountain View, CA, USA). Annexin V-FITC and PI signals were excited using a 488 nm laser light and their emissions captured using bandpass filters set at 530 ± 30 and 613 ± 20 nm, respectively. OGD induced apoptosis in PC12 cells. Cells were treated with OGD and cultured for 3, 6, 9, 12, 16, or 24 h. Signals from each group of cells were located in the lower right quadrant of the dot-plot graph. The cells were then analyzed with flow cytometry following AnnexinV-FITC/PI staining. The results are shown in Figure 5. Compared with the control transfected but untreated cells, the proportions of apoptotic cells treated with OGD after 3, 6, 9, 12, 16 and 24 h were 0.25, 0.38, 4.62, 10.54, 19.44 and 23.18%, respectively. After 9 h of OGD, most cells (95%) were viable with the remaining being either early apoptotic (2%) or necrotic (3%). After 16 h of OGD, a pattern ofincreased AnnexinV-positive, PI-negative apoptotic cells. Quantitative analysis showed that 16 h of OGD produced a 20-fold increase in the number of apoptotic cells compared to controls. Necrotic cells also increased significantly after 16 h of OGD, but less so (2 to 3-fold). Furthermore, preexposure of PC12 cells to 9 h of OGD, attenuated cell death induced by 16 h of OGD 1 d later by significantly decreasing the number of apoptotic, but not necrotic cells (Figure 5). These resultscould help to provide the reference for further study ActA/smads signaling pathways on acute ischemic brain damage by the model.

DISCUSSION

Rat adrenal pheochromocytomas have been made into PC12 cell lines. They have been the object of intense study in neurobiology for the investigation of signal transduction mechanisms (Tischler, 2002). For example, studies of cell differentiation and survival (Agell et al., 2002; Michiyoshi, 2010; Jaehoon, 2010), Ca^{2+} signaling (Ghosh et al., 1994), apoptosis (Macdonald et al., 2003), Parkinson's disease (Ryu et al., 2002) and Huntington's disease (Peters et al., 2002) have been conducted in PC12 cell model systems. Primary neuronal cells came from the animals, which could express neurons tissue damage directly. But it was limited for the purity and number to the experiments. And primary neuronal cells were non-regeneration cells. Because of spontaneous apoptosis *in vitro* the survival time was short. So it was hard to uniform the initial condition of the experiment. In this study, we chose PC12 cells as the experiment model.

MAP2 is a neuron specific protein. It is present in PC12 cells during differentiation. There is a direct correlation between the rate of microtubule assembly and the increase of neurite length (Koji, 2006; Kumar, 2006). Microtubules stabilizing are critical for dendrite development and neurite outgrowth. MAP2 plays a critical role in neurite outgrowth (Dehmelt, 2003). It is a helpful diagnostic and prognostic feature in various neurological disorders. In the present study, we examined MAP2 expression by immunoblotting and western blot analysis.

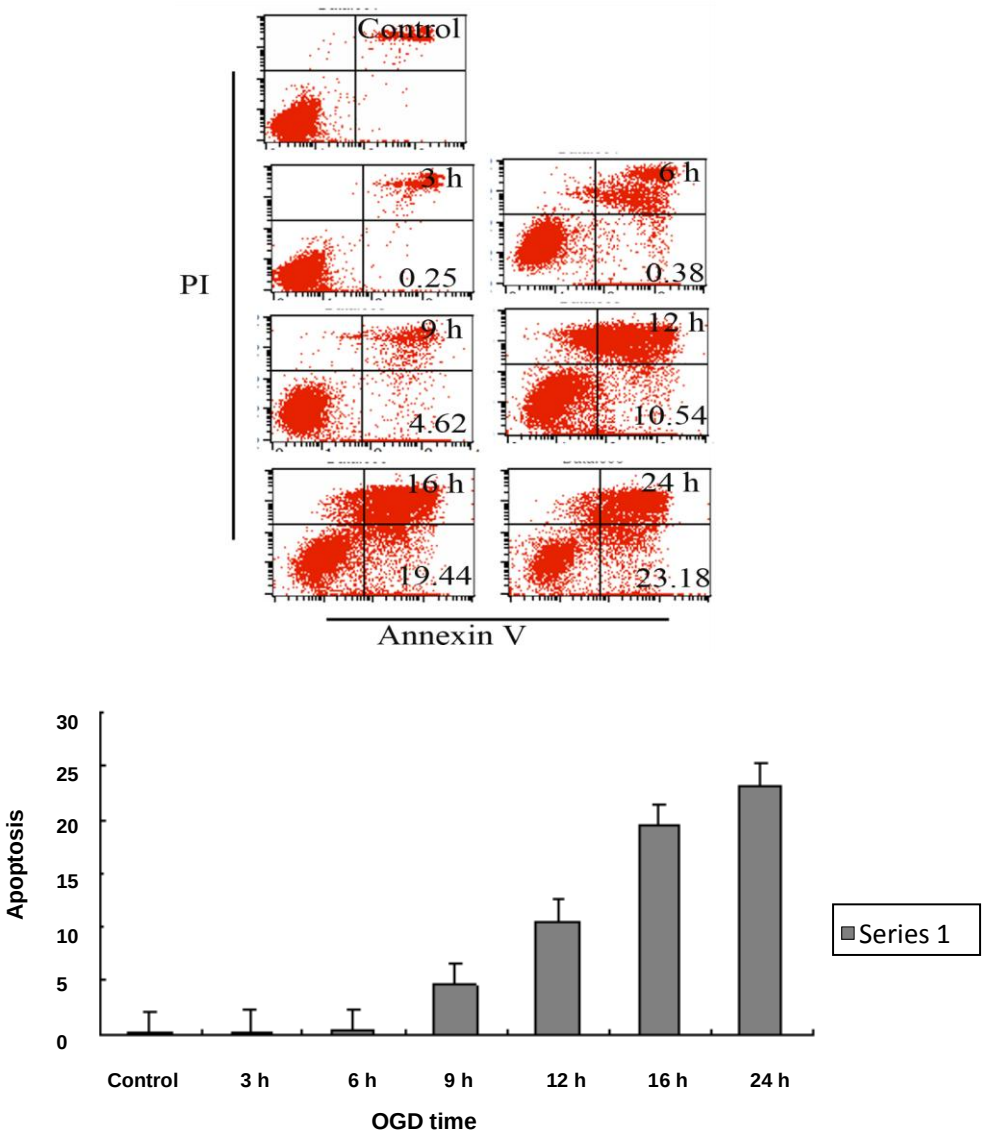

Figure 5. Apoptosis of differentiated PC12 cells treated with OGD was analyzed with Annexin V-FITC and PI double-staining flow cytometry.

Our results showed that MAP2 is differentially expressed in PC12 cells exposed to NGF for different time periods.

Protein signal densities were increased in cells treated with NGF for 3, 4, 5, or 6 days compared with control cells. HIF-1(Hypoxia-inducible factor-1 alpha) is critical mediators of physiological responses to acute and chronic hypoxia. HIF-1 plays an essential role in cellular and systemic homeostatic responses to hypoxia (Semenza, 2000). HIF-1α protein is stabilized and translocation into the nucleus is increased under hypoxia. Tolerance to ischemia and hypoxia can be modeled *in vitro* and has been described in cultured PC12 cells (Larsena, 2007; Damian, 2010). Our results showed thatHIF-1 is differentially expressed in PC12 cells that have been exposed to OGD for different time periods. These resultscould help to provide the reference for furtherstudy ActA/smads signaling pathways on acute ischemic brain damage by the model.

In this study, physical and chemical methods were usedto establishOGD conditions, and sugar-free culture medium and NaS_2O_4 were used to establish a liquid environment lacking oxygen and sugar. HIF-1 protein expression was assessed to confirm the effects of OGD (Damian, 2010). NGF-stimulated PC12 cells differentiate into sympathetic neurons *in vitro* and have been shown to have neuronal characteristics both physiologically and biochemically. The circulation of oxygen and glucose is necessary to maintain neurons' normal physiologyfunction

and survival. During the ischemic damage process, neurons' blood supply is disrupted. Oxygen glucose deprivation (OGD) is a model of oxygen and glucose shortage. We used the OGD on neurons to simulate the ischemic brain damage process that occurs in the body. NGF combined with OGD to set up an ischemia tolerance model. Our study shows that PC12 cells treated with NGF form cells that are neuron-like in appearance. These resultssuggested establishing NGF treatment followed by OGD as an *in vitro* model of acute ischemic brain damage. The model provides a new tool for the identification of pathways that are involved in cerebral ischemia. This model is one example of the cerebral ischemic response. Therefore, the present model could be applied to the study of mechanisms involving in tolerance to other stressful stimuli. Cell-based assays with high-throughput capacity can be used as models and direct screens to explore molecular mechanisms that are involved in pathology and cellular function.

Conclusion

In conclusion, we describe here an *in vitro* model of acute ischemic brain damage in the PC12 cell line established NGF treatment followed by OGD. The model provides a new tool for the identification of pathways involved in ischemic brain damage. The model can be one example of a more widely, general-stress response of the cell. In neurology, the model presented in this study will be a new tool to study ischemic cerebrovascular disease.

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