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Adenovirus-mediated transfection with glucose transporter 3 suppresses PC12 cell apoptosis following ischemic injury★

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Abstract

In this study, we investigated the effects of adenovirus-mediated transfection of PC12 cells with glucose transporter 3 after ischemic injury. The results of flow cytometry and TUNEL showed that exogenous glucose transporter 3 significantly suppressed PC12 cell apoptosis induced by ischemic injury. The results of isotopic scintiscan and western blot assays showed that, the glucose uptake rate was significantly increased and nuclear factor kappaB expression was significantly decreased after adenovirus-mediated transfection of ischemic PC12 cells with glucose transporter 3. These results suggest that adenovirus-mediated transfection of cells with glucose transporter 3 elevates the energy metabolism of PC12 cells with ischemic injury, and inhibits cell apoptosis.

Key Words

glucose transporter 3; ischemia; glucose uptake rate; apoptosis; nuclear factor kappaB

Abbreviations

GLUT3, glucose transporter 3; NF-κB, nuclear factor kappa B; GFP, green fluorescent protein

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INTRODUCTION

The mechanism underlying the onset of cerebral ischemia-reperfusion injury is complicated, and therapeutic efficacy is presently not ideal. A previous study showed that disturbances of energy metabolism play an important role in the secondary brain damage induced by ischemia[1]. Energy supply in neuronal cells is mainly derived from the aerobic oxidation of glucose. Glucose in blood entering neuronal cells is mainly mediated by glucose transporter (GLUT) 1 and GLUT3. Under conditions with a low concentration of glucose, GLUT3 transport efficiency is high, and GLUT3 plays an important role in maintaining neuronal function and neuronal survival in the penumbral region[2-3]. Therefore, increasing GLUT3 expression might lessen brain ischemia-induced cell apoptosis[4-7].

In this study, we constructed rat PC12 cell models of ischemic injury, transfected rat PC12 cells using adenovirus, analyzed cell apoptosis using flow cytometry and TUNEL, detected glucose uptake rate and nuclear factor kappaB (NF-κB) expression using isotopic scintiscan and western blot assays, and explored the anti-apoptotic effects of GLUT3.

RESULTS

GLUT3 transfection suppressed PC12 cell apoptosis following ischemic injury

The results of flow cytometry revealed that the rate of apoptosis in GLUT3-transfected

PC12 cells after ischemic injury (4.70 ± 1.75%) was significantly lower than the rate in green fluorescent protein (GFP)-transfected cells (14.25 ± 3.46%) and non-transfected cells (13.10 ± 2.98%) ($P < 0.05$; Figure 1).

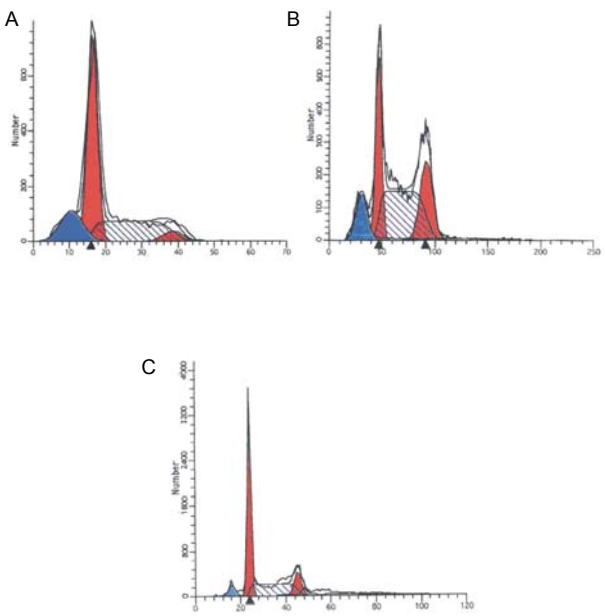

Figure 1 PC12 cell apoptosis following ischemic injury under varied interventions.

Blue represents apoptotic cells, showing that the rates of apoptosis of non-transfected cells (A) and green fluorescent protein-transfected cells (B) were significantly higher than those in glucose transporter 3-transfected cells (C).

By TUNEL, the rate of apoptosis in GLUT3-transfected PC12 cells after ischemic injury (7.7 ± 3.31%) was significantly lower than that in GFP-transfected cells (17.1 ± 3.10%) and non-transfected cells (15.2 ± 2.70%) ($P < 0.05$; Figure 2).

GLUT3 transfection elevated the glucose uptake rate in PC12 cells following ischemic injury

The results of isotopic scintiscans revealed that the glucose uptake rate (d/min per mg) in GLUT3-transfected PC12 cells (603.0 ± 13.1) was significantly higher than that in GFP-transfected cells (300.0 ± 8.8) and non-transfected cells (319.0 ± 11.2) ($P < 0.05$).

GLUT3 transfection inhibited NF-κB expression in PC12 cells following ischemic injury

The results of western blot assays showed that the level of NF-κB in GLUT3-transfected PC12 cells (0.92 ± 0.17) relative to ß-actin was significantly lower than the level in GFP-transfected cells (1.02 ± 0.24) and non-transfected cells (1.06 ± 0.21) ($P < 0.05$; Figure 3).

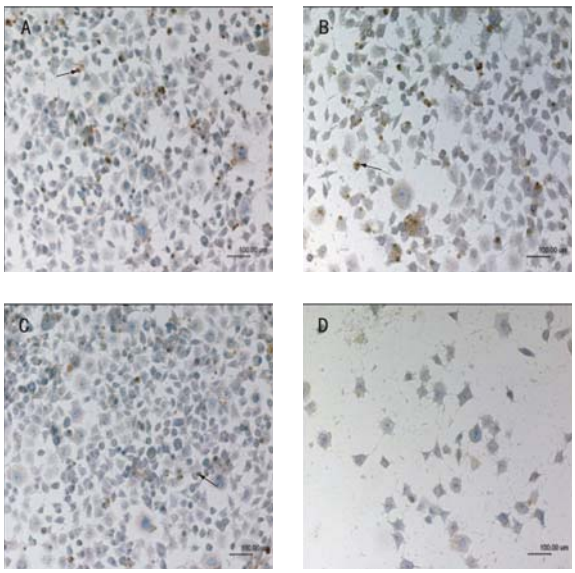

Figure 2 PC12 cell apoptosis following ischemic injury (TUNEL, bars: 100 μm).

Arrows show apoptotic cells; (A) non-transfected cells; (B) green fluorescent protein-transfected cells; (C) glucose transporter 3-transfected cells; (D) negative control.

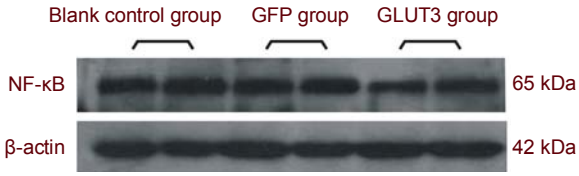

Figure 3 Nuclear factor kappaB (NF-κB) expression in PC12 cells after ischemic injury (western blot assay).

Blank control group: non-transfected PC12 cells; green fluorescent protein (GFP) group: GFP-transfected PC12 cells; glucose transporter 3 (GLUT3) group: GLUT3-transfected PC12 cells.

DISCUSSION

Cell apoptosis is an important form of cell death during ischemic injury. Energy metabolism can affect cell apoptosis. Glucose metabolism is a major form of metabolism providing energy to brain cells. A decrease in the level of glucose transporters led to a reduction in glucose metabolism and cell apoptosis, possibly *via* the following three pathways: (1) reduced adenosine triphosphate levels resulted in abundant mitochondrial death; (2) oxidative stress response; (3) activation of hypoxia-inducible factor-1α[7]. A previous study confirmed

that oxidation leads to a decrease in GLUT3 expression and neuronal cell and vascular endothelial cell apoptosis[8]. Another study showed that during cerebral ischemia/hypoxia-induced oxidation, Ad-GLUT1 intervention suppressed cell apoptosis, and reduced infarct volume[9]. These experimental results confirmed that PC12 cell apoptosis was obviously decreased following GLUT3 transfection.

GLUT3 is a major glucose transporter in adult brain parenchymal cells[10-12]. Normally, the stable internal environment of cells is retained by regulation of the distribution of GLUT3 protein in the cell membrane and cytoplasm[11-13]. Energy metabolism in brain cells is completed by aerobic oxidation of glucose. Glucose mainly enters neuronal cells through transmembrane transport of GLUT3 protein. A previous study confirmed that glucose transport is critical for neuronal survival under conditions of hypoxia, and that increased glucose transport may be an important mechanism underlying neuronal hypoxia tolerance[14]. The decrease in GLUT3 level was not only a result of cell apoptosis, but also played an important role in cell apoptosis[15-16]. GLUT is a significant regulatory factor to initiate the apoptosis cascade[17-20]. The apoptosis cascade is started by many cytokines and growth factors affecting glucose transport or energy metabolism[17-20]. In this study, PC12 cells were transfected with GLUT3 following ischemic injury. The results suggested that the rate of apoptosis in GLUT3-transfected PC12 cells was significantly decreased, and that glucose uptake rate was significantly increased. These findings indicated that an increase in glucose uptake achieved *via* an exogenous increase in GLUT3 protein expression in PC12 cells could elevate cell energy metabolism and suppress cell apoptosis, consistent with the results of previous studies[13, 16]. However, Morissette *et al* [13] demonstrated that GLUT1-transfected myocardial cells effectively inhibited myocardial cell hypertrophy and suppressed myocardial cell apoptosis, while the glucose uptake rate was not elevated. The above-mentioned results suggest that GLUT3 does more than merely elevate cell energy metabolism, and inhibits cell apoptosis by suppressing NF-κB activation in oxidative stress reaction.

NF-κB can combine with the nucleotide sequence of the target gene promoter and initiate gene transcription. Under non-activation conditions, NF-κB strongly combines with its inhibitory protein IκB in the cytoplasm and exists in the inactive state. NF-κB can be activated by numerous stimulating factors. Activated NF-κB detaches from IκB, moves into nuclei and combines with target gene NF-κB motifs, resulting in enhanced target gene expression. NF-κB exerts a crucial effect on cell apoptosis and proliferation[21]. Oxidative stress leads to NF-κB activation and induces cell apoptosis[22]. Therefore, it is presumed that GLUT3 transfection in PC12 cells increases cell energy metabolism, decreases hypoxia-inducible factor-1α protein expression, lessens ischemic injury, suppresses NF-κB activation, and inhibits cell apoptosis. The results of this study also confirm that NF-κB activation is markedly decreased after GLUT3 transfection.

In summary, transfection of PC12 cells with GLUT3 effectively elevated the glucose uptake rate and suppressed ischemic injury-induced PC12 cell apoptosis, providing an important experimental basis for gene therapy for cerebral ischemia-reperfusion injury.

MATERIALS AND METHODS

Design

Cytological *in vitro* comparative observation study.

Time and setting

Experiments were performed at the Central Laboratory of Sun Yat-sen University, China in 2008.

Materials

The PC12 cell line was stored in Pharmacy College of Sun Yat-sen University. HEK-293 cells were supplied by Tumor Treatment and Prevention Center of Sun Yat-sen University in China.

Methods

Construction and intervention of rat PC12 cell models of ischemia

PC12 cells have been widely used in studies of neural cell differentiation and function. In a study describing the mechanisms underlying cell sensitivity to oxygen, we utilized PC12 cells to simulate neuronal cells. PC12 cells were gifted by School of Pharmaceutical Sciences, Sun Yat-sen University, China. Cell models of hypoxia were constructed by cobalt chloride-evoked chemical method[23].

PC12 cells were seeded onto 6-well plates at a density of 1×10^6/well in 2 mL of DMEM/F12 (1:1; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, 5% horse serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, Zhejiang Province, China). These cells were incubated at 37°C in a 5% CO_2 incubator for 8 hours. The supernatant was then removed. Cells were transfected with GLUT3 (constructed in our previous study) using adenovirus-mediated transfection, rinsed twice in PBS, and incubated in a 2-mL mixture of 150 μM cobalt chloride (Sigma, St. Louis, MO, USA) and serum-free low-glucose DMEM (Sigma) in a 37°C 5% CO_2 incubator

for 4 hours. After the removal of supernatant, the specimen was washed twice in PBS. Then, 2 mL of serum-free low-glucose DMEM (Sigma) was added and cells were incubated in a 37°C 5% CO_2 incubator for 24 hours. GFP-transfected cells and non-transfected cells served as controls.

Flow cytometry for assessment of cell apoptosis

Following removal of supernatant, cells were digested in 0.25% trypsin (Invitrogen), washed three times in PBS, and fixed in 70% ice-cold alcohol at −20 °C overnight. Cell apoptosis was determined by flow cytometry (Becton Dickinson, San Jose, CA, USA).

TUNEL for assessment of cell apoptosis

Cells were fixed in 4% paraformaldehyde. Cell apoptosis was detected using an *in situ* cell death detection kit (Roche Diagnostics, Penzberg, Germany). In accordance with the instructions of the kit, the precise procedures are as follows: (1) cells were fixed in 4% paraformaldehyde at room temperature for 1 hour, and washed in PBS; (2) they were then incubated in 3% H_2O_2 methanol at room temperature for 10 minutes, and washed in PBS; (3) cells were treated with 0.1% sodium citrate solution containing 0.1% Triton X-100 at room temperature for 2 minutes, and rinsed in PBS; (4) 50 μL of TUNEL reaction solution or No.2 liquid from the kit (negative control) was added, cells were covered with coverslips in a wet box at 37°C for 60 minutes, followed by a wash in PBS; (5) 50 μL of Converter 2 POD solution was added, and cells were covered by coverslips in a wet box at 37°C for 30 minutes, followed by a wash in PBS; (6) cells were developed with 50 μL of diaminobenzidine substrate solution at room temperature for 2 minutes. The reaction was terminated by adding PBS. Negative nuclei were counterstained using hematoxylin. Visual fields were randomly selected from each sample. Five hundred cells were counted under an optical microscope (Olympus, Tokyo, Japan) to calculate the cell apoptotic index.

Isotopic scintiscan of glucose uptake rate in cells

PC12 cells were rinsed three times in 1 mL potassium phosphate buffer, after removal of medium in a 24-well plate. Cells were incubated in 1 mL of potassium phosphate buffer containing 0.5 μCi/mL[3H] glucose (Invitrogen) at 37°C in water bath for 10 minutes, followed by three washes in 1 mL ice-cold PBS containing 10 mM glucose, to terminate the reaction. Then, 0.4 mL of 0.1 M NaOH fluid was added at room temperature for 2 hours to lyse cells. Lysis buffer (0.3 mL) was obtained from each well to detect disintegration per minute utilizing a liquid scintillation counter (HetticH, Kirchlengern, Germany). Lysis buffer (0.1 mL) was also obtained to measure protein concentration using the bicinchoninic acid assay. Glucose uptake rate was equal to the ratio of disintegration per minute (d/min per mL) to protein concentration (μg/μL).

Western blot assay of NF-κB expression

The above-mentioned cells were lysed to extract cellular protein. Cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In accordance with the instructions in the kit, following transmembrane, cells were blocked in blocking buffer at room temperature for 3 hours. Cells were incubated in rabbit anti-rat NF-κB monoclonal antibody (1:200; Boster, Wuhan, China) at 4°C overnight, and then in goat anti-rabbit IgG (1:1 000; Boster) at room temperature for 1 hour, in a dark room. Chemiluminescence reagents (Boster) working fluid was added for exposure and development. Films were scanned using the Image J (National Institutes of Health, Bethesda, Maryland, USA) image analysis system to measure absorbance in the photosensitive zone. β-actin served as an internal reference. Experiments were repeated five times using different cells.

Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA), and were expressed as mean ± SD. Differences between groups were compared using *t*-tests. A value of $P < 0.05$ was considered to represent statistical significance.

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Author contributions: Fangcheng Li designed this study. All authors performed the experiments.

Conflicts of interest: None declared.

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