Effects of diazoxide on Aβ₁₋₄₂-induced expression of the NR2B subunit in cultured cholinergic neurons

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Abstract. The accumulation of amyloid-β protein (Aβ) is significant in the pathogenesis of Alzheimer's disease. Several previous studies indicate that the NR2B-containing N-methyl-D-aspartate receptors are critically involved in the Aβ-mediated disruption of neuronal function. Diazoxide (DZ), a highly selective drug capable of opening mitochondrial ATP-sensitive potassium channels, has neuroprotective effects against neuronal cell death. However, the mechanism by which DZ protects cholinergic neurons against Aβ-induced cytotoxicity remains to be elucidated. The present study was designed to investigate the effects of DZ pretreatment against Aβ₁₋₄₂-induced expression of NR2B in order to gain novel insights into the neuroprotective mechanisms. Following exposure to Aβ₁₋₄₂ for 24 h, the expression of the NR2B subunit remained unchanged compared with the control group. However, a significant increase in the expression of the NR2B subunit was observed following treatment with Aβ₁₋₄₂ for 72 h (P<0.05); and the upregulation of the expression of the NR2B subunit was reversed by pretreatment with DZ (P<0.05). These results suggested that DZ may counteract Aβ₁₋₄₂-mediated cytotoxicity by alleviating the expression of NR2B.

Introduction

Alzheimer's disease (AD), the most common neurodegenerative disorder, is characterized by the accumulation of the amyloid-β (Aβ) peptide, a hyper-phosphorylation of tau protein and loss of neurons (1). Aβ has a complex role in the molecular events, which lead to the progressive loss of function and eventually to neurodegeneration in this devastating disease (2). Previous studies have confirmed that the activity of N-methyl-D-aspartic acid receptors (NMDARs) may be involved in the cytotoxic mechanism of Aβ (3). NMDARs are widely involved in neuronal excitatory synaptic transmission, synaptic plasticity and long range enhancement. Previous studies indicate that activation of NMDARs by Aβ accumulation may occur at the early stages of the disease and that Aβ evokes an immediate Ca²⁺ rise via the activation of NR2B-containing NMDARs in cultured cortical neurons (4,5). Ronicke et al (6) demonstrated that early neuronal dysfunction induced by Aβ is mediated by an activation of NR2B subunits in primary neuronal cultures and hippocampal slices from rat and mouse. The NR2B subunit is important for the pharmacological features of the NMDAR, which is a potential target for the treatment of NMDAR-associated diseases (7).

Mitochondrial ATP sensitive potassium (mitoKATP) channels are involved in the regulation of mitochondrial volume, ionic homeostasis, pH gradient and membrane potential (8). Pharmacological activation of mitoKATP channels using drugs, including diazoxide (DZ), has been demonstrated to protect neurons against ischemic damage and death (9), and our previous study reported that DZ protects cultured primary cholinergic neurons against Aβ₁₋₄₂-induced cytotoxicity (1,10). Although it has been previously reported that DZ reduces neuronal dysfunction caused by Aβ, the underlying mechanisms remain to be elucidated. Since NR2B subunits have been implicated in increasing neuronal vulnerability, the present study investigated the effect of activation of mitoKATP with DZ on the expression of NR2B in cultured primary rat basal forebrain cholinergic neurons following exposure to Aβ₁₋₄₂.

Materials and methods

Ethics statement. The present study was performed in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Shandong Provincial Qianfoshan Hospital. Female wistar rats (age, 24 h; weight, 300-350 g) were housed under controlled temperature (21°C) and light conditions (12 h light/dark cycle) with free access to water and standard diet.

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Reagents. Aβ1-42, DZ and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO, USA); fetal bovine serum, B27 supplement and Neurabasal were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA); Dulbecco’s modified Eagle’s medium (DMEM) was obtained from HyClone Laboratories, Inc. (Logan, UT, USA); dimethyl sulfoxide (DMSO) was purchased from Shengbo Biotech Co., Ltd. (Shanghai, China). The following antibodies were used: Rabbit anti-NMDAR 2B (cat no. ab65783; 1:100; Abcam, Cambridge, UK), goat anti-choactase (D-16; cat no. sc-19056; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-beta actin antibody (cat no. ab8226; 1:1,000; Abcam). All other chemicals and reagents, unless otherwise specified, were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Aβ1-42 (1 mg) was initially dissolved in DMSO at a concentration of 0.5 mol/l, subsequently diluted with 500 µl phosphate buffered saline (PBS; 0.2 µM; pH 7.4), incubated at 37°C for 36 h and stored at 4°C. Stock solutions were diluted in serum-free medium prior to use in the experiments. The final concentration of DMSO in cell cultures was <0.1%.

Cell cultures. Embryos were removed from pregnant Wistar rats at 17-19 days of gestation. The basal forebrain was dissected and digested with 0.125% trypsinogen for 20 min at 37°C. Proliferation growth medium, comprising a 1:9 mixture of fetal bovine serum and DMEM, and 2% B27 supplement was added, and the cells were subsequently dissociated mechanically using a fire-polished pipette. The dispersed cells were seeded into a culture dish or 12-well plate (Falcon, Franklin Lakes, NJ, USA) coated with poly-D-lysine (5 µg/ml), and were maintained at 37°C in 95% air and 5% CO2. The density of the cells was 6.0x10^5 cells/ml in culture dishes. The cells were cultured for 3-5 days in order to inhibit the proliferation of gliocytes (11).

Identification of cholinergic neurons by immunocytochemistry. For the identification of cholinergic neurons, the cells were seeded onto glass coverslips precoated with poly-L-lysine, incubated for 7 days in growth medium and subsequently fixed with 4% paraformaldehyde in PBS for 30 min at 4°C. Choactase was used as a marker protein for identifying cholinergic neurons. Goat anti-Choactase (1:100) was applied over night at 4°C, biotinylated secondary immunoglobulin G (Biostar, Shanghai, China) was subsequently applied for 1 h, followed by streptavidin-Cy3 (Biostar) for 30 min. Antibody binding was visualized using diaminobenzidine substrate and the slides were subsequently restained with hematoxylin prior to observation under a light microscope (IM50; Leica Microsystems, Tokyo, Japan) with 10 random 20x20 fields sampled for microscopic observation. The number of positive cells in each field and total cell numbers were determined. Primary rat fetal neurons were propagated in vitro. Prior work in our laboratory revealed that >90% of these cells were cholinergic neurons (11).

Cell viability measurements. Mitochondrial dehydrogenase cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to determine cell survival in quantitative colorimetric assays. Cultured cells were incubated with MTT (0.5 mg/ml) for 2 h at 37°C and the blue-colored formazan product, produced by dehydrogenase enzyme activity in active mitochondria, was dissolved in DMSO and quantified spectrophotometrically at 570 nm (752N; Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China).

Experimental treatment. The cells were randomly divided into four groups: (i) Control; (ii) Aβ1-42; (iii) DZ; (iv) DZ + Aβ1-42. The cells in the Aβ1-42 group were cultured for 7 days and treated with Aβ1-42 for 24 or 72 h, the cells in the DZ + Aβ1-42 group were pretreated with DZ for 1 h prior to treatment with Aβ1-42 for 24 or 72 h, and the DZ group was pretreated with DZ for 1 h and subsequently treated with equal volumes of PBS rather than Aβ1-42 for 24 or 72 h.

Western blot analysis. The cells were collected and washed with PBS three times and the total cellular proteins were extracted with radioimmunoprecipitation buffer for 20 min on ice. The cell lysates were centrifuged at 1,200 x g for 10 min at 4°C and the protein concentration in the extracts was determined using a bicinechonic acid Protein assay kit. A total of 60 mg samples were loaded per lane for SDS-PAGE on 8% polyacrylamide gels, followed by transfer onto polyvinylidene membranes. The membranes were blocked with 5% dried non-fat milk for 60 min at 25°C and were subsequently probed with primary antibody (1:100) overnight at 4°C. The blots were washed three times in Tris-buffered saline, containing 0.05% Tween-20 and were subsequently incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, followed by washing three times. Signal detection was performed using enhanced chemiluminescence. β-actin was used as loading control, with image analysis software BandScan 5.0 software (Glyko Inc., Novato, CA, USA) being used for integral optical density value analysis.

Statistical analysis. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance followed by a two-tailed Student’s t-test, or multiple comparison tests were performed as appropriate. Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

DZ protects against Aβ1-42-induced neurotoxicity. Aβ1-42 was demonstrated to induce cytotoxicity in cholinergic neurons, as assessed using an MTT assay. The MTT levels were significantly decreased by Aβ1-42 in a concentration-dependent manner, indicating a reduction in the number of viable cells (Fig. 1A). Treatment with 2 µM Aβ1-42 for 72 h was selected for subsequent experiments, since it reduced cell viability to ~48% of the control. The results obtained with the MTT reduction assay revealed that Aβ1-42 (2 µM) induced cytotoxicity and significantly reduced cell viability (P<0.05, 24 h; P<0.001, 72 h), as compared with the control cells. Pretreatment of cholinergic neurons with DZ resulted in a reduction of
Aβ1-42-induced cytotoxicity and significantly increased cell viability (Fig. 1B).

Expression of the NR-2B subunit is induced by different concentrations of Aβ1-42. The neurons were incubated for 72 h with different concentrations of Aβ1-42 (0, 1, 1.5 and 2 µM). Western blot analysis for NR2B revealed that the expression of the NR2B subunit was increased in a concentration-dependent manner (Fig. 2).

DZ alleviates the expression of NR2B induced by Aβ1-42. Exposure to Aβ1-42 (2 µM) for 24 h compared with the control group revealed no significant difference in the expression of the NR2B subunit in the Aβ1-42 group and Aβ1-42 + DZ group (P>0.05; Fig. 3A). Compared with control group, the expression of the NR2B subunit in the Aβ1-42 group was significantly upregulated for 72 h (P<0.05), and compared with the Aβ1-42 group, the expression of the NR2B subunit in the Aβ1-42 + DZ group was significantly decreased (P<0.05). The NR2B expression in the control and the DZ groups was not significantly different (P>0.05; Fig. 3B).
Discussion

Mitochondrial dysfunction, hyperphosphorylation of Tau protein, apoptosis in neurons, and degradation and disappearance of synapses are all associated with the generation and aggregation of Aβ (12). It can be observed by the toxicology model that Aβ oligomers can lead to extensive damage to the dendritic spines of neurons, reduction of synaptic transmission, weakening of synaptic connections and excitotoxicity of hippocampal neurons (13). Currently, the mechanism of Aβ on synaptic plasticity injury is inconclusive. Previous research indicates that it is closely associated with the system activation of glutamatergic. Aβ can reduce astrocytic glutamate reuptake and influence glutamate transporter-I and glutamate-aspartate transporter activity function, leading to an abnormally high concentration of glutamate in the synaptic cleft and thereby cause the sustained arousal of the NMDA receptor (14). Mattson et al (15) also identified a direct association between Aβ and the NMDA receptors. Aβ directly enhanced NMDA receptor-mediated excitotoxicity. In the hippocampus, when the NMDA receptor was activated, a substantial increase of intracellular Ca^{2+} and the phenomenon of NMDA receptor activation was also observed (15). Therefore, NMDA receptor-mediated glutamate excitotoxicity is vital in Aβ-induced synaptic plasticity injury.

The NMDA receptor is a typical isomer consisting of NR1 and NR2 subunits (NR2A-NR2D). NR1 is a basic subunit of the NMDA receptor, which is necessary for the channel function; NR2 is a regulation subunit, which assists to form the diversified structure of NMDA receptor. NR2B is important for the structure and function of NMDA receptors (16). The in vitro experiments revealed that the NR2B subunit regulated NMDA receptor-mediated excitatory postsynaptic potential and influenced the plasticity in the central nervous system (17). Additionally, NR2B-contained NMDA receptor ion channels revealed a high Ca^{2+} permeability (18). The experiments in the present study revealed that the protein expression of the NR2B subunit was not significantly changed following treatment with Aβ_{1-42} for 24 h in cultured neurons. After 72 h exposure of Aβ_{1-42}, the protein expression levels of the NR2B subunit were significantly increased compared with the control. This indicated that Aβ changed the structure and function of the NMDA receptor complex, particularly the NMDAR1/NMDAR2B type NMDA receptor complex, further activated the NMDA receptor, causing extracellular Ca^{2+} influx augmentation and intracellular calcium overload, and was involved in NMDA-mediated cell excitotoxic damage and destruction process of synaptic plasticity (19).

KATP channels may be crucial in coupling metabolic energy to the membrane potential of cells and potassium channel dysfunction has been indicated in AD. It has been reported that activation of mitoKATP increases cell viability against rotenone-induced cell death (20) and in cultured cerebellar granule neurons subjected to H_{2}O_{2}, DZ can contribute to the stability of mitochondrial membrane potentials (21). A previous study revealed that DZ preconditioning may improve the learning ability and memory of rat models of AD (22), Teshima et al (21) reported that DZ preconditioning may inhibit the release of excitatory glutamate of cerebellar granule cells by activating mitoKATP channels (21). The present study demonstrated that DZ pretreatment diminished the expression of NR2B induced by Aβ_{1-42} in cholinergic neurons, which indicated that DZ may suppress the Aβ_{1-42} induced NMDA-mediated cell excitotoxic damage and destruction process of synaptic plasticity (18), in part, by alleviating the expression of NR2B. However, the exact mechanism of molecule signaling remains to be elucidated.

ATP-sensitive potassium channels may be a trigger and/or effector for a variety of oxidative stress preconditioning in the brain. KATP channel openers as brain protective agents has become a research hotspot (23). The present study demonstrated that KATP opener DZ preconditioning antagonized the upregulation of the expression of the NR2B subunit induced by Aβ_{1-42}, indicating that DZ may affect Aβ_{1-42}-induced cytotoxicity via the NMDA receptor pathway, and may provide a potential insight into the theoretical basis for the prevention of AD.

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