European Review for Medical and Pharmacological Sciences 2018; 22: 6133-6138

Diazoxide induces endoplasmic reticulum stress-related neuroprotection mediated by p38 MAPK against $A\beta_{25-35}$ insults

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Abstract. – OBJECTIVE: The endoplasmic reticulum (ER) -resident caspase-12 was identified as a mediator of Aβ neurotoxicity. Recent evidence indicates that mitochondrial ATP-sensitive potassium (K_{ATP}) channel openers mediate their neuroprotective role by adjusting ER stress pathways, but the molecular details remain largely unknown and have been investigated.

MATERIALS AND METHODS: In this study, the protein expression levels of calreticulin (CRT) and caspase-12 activation and phosphorylated p38 MAPK were observed by immunoblotting in cultured PC12 cells from different groups: treatment with $A\beta_{25-35}$ (group $A\beta_{25-35}$), treatment with diazoxide (group diazoxide), pretreatment with diazoxide and then exposure to $A\beta_{25-35}$ (group diazoxide + $A\beta_{25-35}$), pretreatment with p38 MAPK inhibitor SB 203580 and then exposure to diazoxide and $A\beta_{25-35}$ (group SB 203580 + diazoxide + $A\beta_{25-35}$), and the control (group control).

RESULTS: In response to the treatment with $A\beta_{25-35}$ (10 μM) for 24 h, the protein expression levels of CRT and caspase-12 activation were increased and phosphorylated p38 MAPK was decreased significantly. Diazoxide reduced CRT overexpression and caspase-12 activation and increased the up-regulation of phosphorylated p38 MAPK. When SB 203580 was presented before exposure to diazoxide and $A\beta_{25-35}$, CRT expression was markedly suppressed, and the inhibition effect of diazoxide on caspase-12 activation was almost eliminated.

CONCLUSIONS: We showed that diazoxide induced ERS-related neuroprotection mediated by p38 MAPK against $A\beta_{25-35}$ insults. From the clinical point of view, these results are of considerable importance for the understanding of AD pathogenesis. However, further studies are required to explore more detailed mechanisms of the observed effects.

Key Words:
Diazoxide, Endoplasmic reticulum stress, Neuroprotection, p38 MAPK, $A\beta_{25-35}$.

Introduction

The role of the amyloid β peptide (Aβ) during neurodegeneration has become the focus of studies of the pathogenesis of Alzheimer's disease[1-4]. Previous studies[5,6] have shown that Aβ might result in membrane depolarization and trigger mitochondrial dysfunction, thus leading to cell apoptosis. Based on these findings, mitochondrial ATP-sensitive potassium (mitoK_{ATP}) openers activate potassium channels and have been shown to protect cultured neurons against toxicity induced by Aβ[7]. In addition, current

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research[8] also suggests that Aβ could cause cell death by inducing endoplasmic reticulum (ER) stress. ER stress-induced apoptosis has recently been identified as an important signaling pathway of Aβ toxicity[9]. ER is an organelle that plays an important role in the folding and processing of membrane proteins and in the maintenance of intracellular calcium homeostasis. ER stress induces molecular chaperone such as calreticulin (CRT), which is protective and benefit for cell adaptation[10]. However, when persistent or too tense, ER stress will activate ER proapoptosis factor such as caspase-12. Therefore, the ER stress pathway is characterized by caspase-12 activation[11]. Past research also reported that ER-mitochondria crosstalk is involved in Aβ-induced apoptosis[12]. Our recent investigation[9] has demonstrated that diazoxide, an opener of mito-K_{ATP} channels, mediates its potential neuroprotective role by reducing caspase-12 activation and inhibiting ER stress pathways. The precise mechanisms of these beneficial effects are still obscure. Thus, a fuller understanding of the mechanisms involved in regulating ER stress, caspase-12 activity, and signal transduction pathway in Aβ neurotoxicity would be highly desirable. Here we show that diazoxide induces ER stress-related neuroprotection mediated by p38 MAPK against $A\beta_{25-35}$ insults.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), horse serum, fetal bovine serum (FBS), and 0.25% trypsin were obtained from Gibco (Rockville, MD, USA). Rabbit anti-rat caspase-12 antibody, rabbit anti-rat calreticulin (CRT), rabbit anti-rat phosphorylated p38 MAPK, and diazoxide were from Sigma-Aldrich (St. Louis, MO, USA). $A\beta_{25-35}$ peptide was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in sterile distilled water at 500 μM and incubated at 37°C for 4 days. Rabbit anti-b-actin antibody was obtained from Abcam Ltd. (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody was from Vector Laboratories (Burlingame, CA, USA). The p38 MAPK inhibitor SB 203580 and the chemiluminescence detection system were from Cell Signaling Technology (CST, Danvers, MA, USA; enhanced chemiluminescence detection reagents: 20× LumiGlo reagent and 20× peroxide).

Cell Cultures

Rat PC12 pheochromocytoma cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% horse serum, 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% carbon dioxide in air. Neuron-like differentiation of PC12 was induced by 7-day treatment with a nerve growth factor (100 ng/ml). Aggregated $A\beta_{25-35}$ peptides were applied to the cells for 24 h. Diazoxide was prepared in a stock solution of 50 mg/ml in dimethyl sulfoxide (DMSO, final concentration of 0.08%) and added to the medium to obtain a final concentration of 1 mM.

Drug Treatments

The cells were randomly divided into five groups: treatment with $A\beta_{25-35}$ (group $A\beta_{25-35}$), treatment with diazoxide (group diazoxide), pretreatment with diazoxide and then exposure to $A\beta_{25-35}$ (group diazoxide + $A\beta_{25-35}$), pretreatment with p38 MAPK inhibitor SB 203580 and then exposure to diazoxide and $A\beta_{25-35}$ (group SB 203580 + diazoxide + $A\beta_{25-35}$), and the control (group control). The cells were treated with $A\beta_{25-35}$ (10 mM) for 24 h. The diazoxide group was treated with diazoxide (1 mM) for 1 h. The diazoxide+$A\beta_{25-35}$ group was pretreated with diazoxide (1 mM) for 1 h and then exposed to $A\beta_{25-35}$ for 24 h. The SB 203580 + diazoxide + $A\beta_{25-35}$ group was pretreated with SB 203580 for 10 min, and treated with 1 mM of diazoxide for 1 h and then exposed to $A\beta_{25-35}$ for 24 h, whereas the control group was treated with equal volumes of PBS instead of $A\beta_{25-35}$ for 24 h. Thereafter, Western blot analyses were carried out.

Western Blots

Cells were collected and washed by ice-cold phosphate-buffered saline (PBS) and lysed in 250 μL lysis buffer (50 mmol/L Tris–HCl pH 8.0, 150 mmol/L NaCl, 0.02% sodium vanadate, 0.1% SDS, 0.5% deoxycholic acid, 100 μg/mL PMSF, 0.2 μg/mL leupeptin, 1% NP-40) per dish. After incubation for 20 min on ice, cell lysates were centrifuged (10,000g for 10 min at 4°C) and the protein concentration in the extracts was determined by BCA Protein Assay Kit. Samples were re-suspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiled for 5 min. Twenty microliter of solubilized total cell lysate (50 μg protein)

were loaded per lane for SDS-PAGE on a 10% polyacrylamide gel followed by transfer onto a polyvinylidene difluoride (PVDF) membrane at 120 V for 120 min at 4°C by a Mini Trans-Blot Cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked for 60 min at room temperature (25°C) with 5% dried milk in Tris-buffered saline (TBS, 10 mM Tris–HCl, 150 mM NaCl, pH 7.4), then probed with primary antibody (1:400) overnight at 4°C. The blots were washed three times in Tris-buffered saline (TBS-T) [with 0.05% Tween-20] at room temperature and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted in TBS-T (1:3,000) for 1 h at room temperature followed by washing four times. Signal detection was performed with a chemiluminescence kit. The blots were then removed from the working solution, and exposed to Kodak Biomax MS-1 films for 0.5-3 min. Quantitative analysis of Western blots was performed by calculating the relative density of the immunoreactive bands after acquisition of the blot image with a Nikon CCD video camera module and analysis with NIH Image (1.34). Values of each band of sample are compared with that of b-actin.

Statistical Analysis

Statistical analyses were carried out using SPSS18 software (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance, followed by a two-tailed Student *t*-test or a multiple comparison test where appropriate. A *p*-value less than 0.05 was considered significant for all analyses.

Results

Changes of CRT Expression

CRT protein was expressed weakly in the control group. In the diazoxide group, the level of CRT expression increased at 1 h after exposure to diazoxide compared with the control group ($p < 0.01$). In the $A\beta_{25-35}$ group, the level of CRT expression increased significantly at 24 h after exposure to $A\beta_{25-35}$ compared with the diazoxide group ($p < 0.01$). The expression of CRT expression in the diazoxide + $A\beta_{25-35}$ group was significantly less than that in the $A\beta_{25-35}$ group ($p < 0.01$). When p38 MAPK inhibitor SB 203580 was presented before exposure to diazoxide and $A\beta_{25-35}$, CRT expression was markedly suppressed (Figure 1).

Changes of Caspase-12 Activation

Caspase-12 protein was weakly active in the control group. There were no differences of caspase-12 activation between the control group and the diazoxide group. The active caspase-12 protein level in the $A\beta_{25-35}$ groups was significantly higher than that in the control group ($p<0.01$). However, when cultured, cells were pretreated with diazoxide for 1 h and then exposed to $A\beta_{25-35}$ for 24 h. The active caspase-12 protein level was significantly less than that in cells exposed to $A\beta_{25-35}$ alone. This effect of diazoxide was negated by p38 MAPK inhibitor SB 203580 (Figure 1).

Changes of Phosphorylated p38 MAPK

Phosphorylated p38 MAPK was expressed steadily in the control group. In the diazoxide group, the level of phosphorylated p38 MAPK expression increased at 1 h after exposure to diazoxide compared with the control group ($p < 0.01$). In the $A\beta_{25-35}$ group, the level of phosphorylated p38 MAPK decreased at 24 h after exposure to $A\beta_{25-35}$ compared

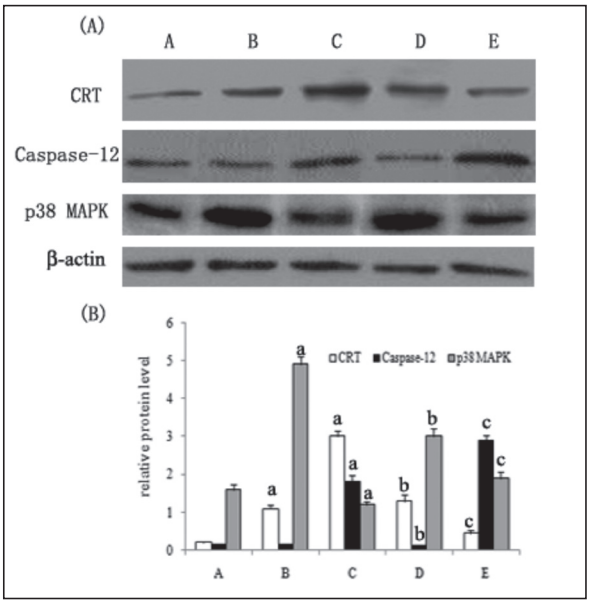

Figure 1. Overexpression and Caspase-12 Activation Induced by $A\beta_{25-35}$ in PC12 cells**.** Cultured cells were treated with $A\beta_{25-35}$ or diazoxide or diazoxide + $A\beta_{25-35}$ or SB 203580 + diazoxide+$A\beta_{25-35}$. (**A**) Western blot analysis of CRT, caspase-12 and p38 MAPK protein is compared at different groups. (**B**) Bands corresponding to CRT, caspase-12, p38 MAPK and b-actin protein Western blot were scanned and their optical density quantified by densitometry. A = control, B = diazoxide, C = $A\beta_{25-35}$, D = diazoxide+$A\beta_{25-35}$, E = SB 203580 + diazoxide+$A\beta_{25-35}$. $^{a}p < 0.05$ denote *vs.* control, $^{b}p < 0.05$ denote *vs.* $A\beta_{25-35}$, $^{c}p < 0.05$ denote *vs.* diazoxide + $A\beta_{25-35}$.

with the control group ($p < 0.01$). After cells were cultured, they were pretreated with diazoxide for 1 h and exposed to $A\beta_{25-35}$ for 24 h. The phosphorylated p38 MAPK protein level was significantly more than that in cells exposed to $A\beta_{25-35}$ alone. When SB 203580 was presented before exposure to diazoxide and $A\beta_{25-35}$, the phosphorylated p38 MAPK expression was markedly suppressed (Figure 1).

Discussion

The neuroprotective effects of diazoxide, an opener of mitoK_{ATP} channels, have been demonstrated against various neurotoxic agents, including Aβ. Previous studies [13,14] have shown that the diazoxide significantly inhibits neuronal apoptosis induced by Aβ and decreases the expression levels of caspase-3, cleaved caspase-3, and cytochrome *c* proteins by regulating mitochondrial signaling pathways. However, its protective mechanism is still unclear. Previous research [12] also reported that ER-mitochondria crosstalk is involved in Aβ-induced apoptosis. Our recent research has demonstrated that diazoxide mediates its potential neuroprotective role by reducing caspase-12 activation and inhibiting ER stress pathways[9]. ER is exquisitely sensitive to alterations in homeostasis. Stimuli in the course of Aβ insults can affect ER function, namely ER stress[15,16]. Caspase-12 is an ER-specific caspase[11,17]. Accumulation of unfolded proteins in ER may cause ER stress, which will activate caspase-12 and subsequent caspases. The ER stress pathway is characterized by caspase-12 activation. In our study, exposure of PC12 cells to $A\beta_{25-35}$ significantly elevated caspase-12 activation. The results indicated that the ER stress pathway took part in the regulation of neuronal apoptosis following $A\beta_{25-35}$ insults. Our study also showed that the K_{ATP} opener diazoxide could significantly reduce caspase-12 activation following exposure to $A\beta_{25-35}$, indicating that the K_{ATP} opener protected PC12 cells against apoptosis following exposure to $A\beta_{25-35}$ by intervening with the ER stress pathway. The current research further supports ER-mitochondria crosstalk is involved in Aβ-induced apoptosis. Next, we continued to explore the mechanisms involved in diazoxide regulating ER stress. CRT is an important Ca^{2+}-binding molecular protein chaperone in the ER, which is responsible for proteins folding and calcium homeostasis adjusting[10]. However, when persistent or too tense, ER stress will activate ER proapoptosis factor such as caspase-12. In our study, exposure of PC12 cells to diazoxide pretreatment increased CRT expression. Exposure of PC12 cells to $A\beta_{25-35}$ significantly increased CRT expression. However, the K_{ATP} opener diazoxide could significantly reduce CRT overexpression following exposure to $A\beta_{25-35}$. We speculated that moderate stress on the ER would trigger many rescuer responses; in contrast, excessive or long-term exposure to ER stress would induce tissue damage. The possible mechanisms were as follows: $A\beta_{25-35}$ induced CRT overexpression, which could bind Ca^{2+} overload and promote Ca^{2+}-ATPase2a inactivation and thus lead to perturbation of intracellular calcium homeostasis and excessive ER stress[18]. Diazoxide resulted in moderate CRT expression, which could upregulate the capacity of Ca^{2+}-binding in the ER, and inhibit the intracellular Ca^{2+} release from the ER, and reduce intracellular Ca^{2+} overload[19], and thus benefit for cell adaptation. However, a fuller understanding of the mechanisms involved in regulating ER stress-related signal transduction pathway in Aβ neurotoxicity would still be highly desirable. It is well known that the mitogen-activated protein kinases (MAPKs) are the family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, including stress. Three subfamilies of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38-MAPKs. It has been shown that ERKs are important for cell survival, whereas JNKs and p38-MAPKs were deemed stress responsive and thus involved in apoptosis. MAPKs are serine/threonine kinases that, upon stimulation, phosphorylate their specific substrates at serine and/or threonine residues. Such phosphorylation events can either positively or negatively regulate substrate, and thus entire signaling cascade activity. Thus, the MAPK signaling pathways modulate stress protein gene expression, mitosis, proliferation, motility, metabolism, and programmed death "apoptosis"[20,21]. The p38-MAPKs is called stress kinases and involved in regulating several endoplasmic reticulum chaperones. CRT is one such chaperone[22]. The p38 MAPK inhibitor, SB203580, has been reported[23] to inhibit the up-regulation of the CRT expression level following an ER stress. In our study, exposure of PC12 cells to diazoxide pretreatment increased phosphorylated p38 MAPK and CRT expression. When SB 203580 was presented before exposure to diazoxide and $A\beta_{25-35}$, the phosphorylated p38

MAPK and CRT expression was markedly suppressed, and the inhibition effect of diazoxide on caspase-12 activation was almost eliminated. The results indicated that p38 MAPK signaling pathway played an important role in regulating ER stress and CRT expression up-regulation. It was possible that diazoxide induced ER chaperone CRT expression up-regulation via activating p38 MAPK signaling pathway and relieve cellular stress state, and thus reduced CRT overexpression and caspase-12 activation induced by $A\beta_{25-35}$.

Conclusions

We found that diazoxide induced ERS-related neuroprotection mediated by p38 MAPK against $A\beta_{25-35}$ insults. These results are of considerable importance for the understanding of AD pathogenesis. Further researches are required to explore more detailed mechanisms of the observed effects.

Acknowledgements

This study was funded by Shandong Provincial Key Research and Development Project (Grant No. 2017GSF218043), National Natural Science Foundation of China (Grant No. 81501185), Yantai Science and Technology Planning Project (Grant No. 2016WS017), Medicine and Health Science and Technology Development Plan Project of Shandong Province (Grant No. 2017WS388).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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