J. Biochem. 2010;148(4):413–421 doi:10.1093/jb/mvq074

JB THE JOURNAL OF BIOCHEMISTRY

Diazoxide preconditioning alleviates caspase-dependent and caspase-independent apoptosis induced by anoxia-reoxygenation of PC12 cells

Received May 14, 2010; accepted June 30, 2010; published online July 8, 2010

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Although there is increasing evidence that the ATP sensitive potassium channel (K_{ATP}) opener exhibits neuroprotective effects against ischaemic neural damage, little is known about the mechanism. Mitochondria play a key role in apoptosis by releasing many important factors, including cytochrome *c* and apoptosis-inducing factor, which in turn initiate the caspase-dependent and -independent mitochondrial pathway, respectively. In the present study, we sought to determine the locus that K_{ATP} opener uses to mediate this protection in PC12 cells. We found that pre-treatment of PC12 cells with diazoxide (DZX), a mitochondrial ATP sensitive potassium channel (mitoK_{ATP}) opener, dose-dependently increased cell viability under conditions of oxygen glucose deprivation (OGD). The protective effect of this pre-conditioning was attenuated by 5-hydroxydecanoic acid, a selective mitoK_{ATP} blocker. The results showed that DZX inhibits the release of cytochrome *c*, the activation of caspase-3 and the release of AIF evoked by OGD. Taken together, our results demonstrate for the first time that activation of the mitoK_{ATP} channel elicits protective effects against OGD-induced cell apoptosis by caspase-dependent and -independent mitochondrial pathways.

Keywords: apoptosis/apoptosis inducing factor/caspase/cytochrome *c*/diazoxide.

Abbreviations: AIF, apoptosis-inducing factor; Apaf-1, protease-activating factor-1; DMSO, dimethylsulphoxide; DZX, diazoxide; FBS, foetal bovine serum; 5-HD, 5-hydroxydecanoic acid; K_{ATP}, ATP sensitive potassium channel; Kir6.1/Kir6.2, inwardly rectifying potassium subunit6.1/6.2; MAPK, mitogen-activated protein kinases; mitoK_{ATP}, mitochondrial ATP sensitive potassium channel; $\Delta\Psi m$, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OGD, oxygen glucose deprivation; PARP, poly ADP ribose polymerase; PBS, phosphate-buffered saline; SUR, sulphonylurea receptors.

Stroke is one of the leading causes of disability and death in developing countries. Approximately 40% of stroke deaths in developing countries occurred in China, and the majority of survivors are disabled (*1*). However, the therapeutic options for treating strokes are very limited. Currently the only effective treatment is thrombolysis, but its numerous limitations restrict its use to only ~5% of the patients suffering from stroke (*2*). Although recombinant tissue plasminogen activator has been approved by the US Food and Drug Administration as an effective stroke therapy, it is only beneficial to 5–10% of acute ischaemic stroke patients (*3*). This has led to a search for chemicals that might protect neurons against ischaemia by interrupting the biochemical cascade that leads to cell death in the penumbra of strokes. Despite an increasing number of studies focused on the mechanisms of ischaemia, few effective therapeutic drugs have been used in clinical practice (*4*, *5*).

Apoptosis allows multicellular organisms to eliminate unwanted cells, ensuring proper development and maintenance of tissue homoeostasis (*6*). In the past decade, many works have provided additional insight into the regulatory mechanisms of hypoxic response (*7*). Studies of apoptosis have focused on the role of caspases, a class of cysteine proteases (*8*). However, apoptosis can occur when the caspase cascade is blocked, indicating the existence of a caspase-independent pathway (*9*, *10*). The nature of apoptotic signals from mitochondria has been well documented (*11–13*). Cytochrome *c* and apoptosis-inducing factor (AIF) are well known as pro-apoptotic molecules released from the mitochondria (*14*, *15*). Cytochrome *c* is usually released from the mitochondrial intermembrane space into the cytosol because of mitochondrial membrane potential ($\Delta\Psi m$) loss (*14*, *16*, *17*). Cytochrome *c* released from the mitochondria forms a complex with procaspase-9 and apoptotic protease-activating factor-1 (Apaf-1), resulting in activation of procaspase-9. Contrarily, when released into the cytosol, AIF induces chromatin condensation and high-molecular weight (50 kbp) DNA fragmentation. Translocation of AIF to the nucleus occurs during the apoptosis of most mammalian cells (*18*). The proapoptotic effects of AIF are not suppressed by pharmacological caspase inhibitors (z-VAD-fmk or BAF), indicating that AIF triggers apoptosis in a caspase-independent manner (*19*).

ATP-sensitive K^+ channel (K_{ATP}) openers are effective in the prevention of ischaemic neural death (*20–25*). ATP-sensitive K^+ channels, which link cell

metabolism to membrane potential, belong to a class of inwardly rectifying potassium channels that are widely distributed in the brain (*26*, *27*). Functional K_{ATP} channels are hetero-octameric complexes, consisting of discrete pore-forming (inwardly rectifying potassium subunit; Kir6.1/Kir6.2) and regulatory (sulphonylurea receptors; SUR1/SUR2) subunits. The mechanism through which opening of K_{ATP} leads to ischaemic protection involves prevention of ATP loss during ischaemia (*28*, *29*), improved post-ischaemic oxidative phosphorylation (*29*), reduced mitochondrial Ca^{2+} uptake during ischaemia (*28*, *30*, *31*) and decreased generation of mitochondrial reactive oxygen species (*32*, *33*) and their downstream PKC and mitogen-activated protein kinases (MAPK) signaling pathways. K_{ATP} channel openers enhances Bcl-2 expression, and these effects can be reversed by K_{ATP} channel blockers (*34*, *35*). Nevertheless, little is known about the role of regulating PC12 cells' mitoK_{ATP} channels in ischaemic stroke. Based on the protective effect of mitoK_{ATP} channels in ischaemic cell death, we investigated if diazoxide (DZX), a selective mitoK_{ATP} opener (*21*, *36*), can prevent ischaemia reperfusion jnjury-promoted neural damage. Results presented here confirm the efficiency of DZX in preventing OGD-induced PC12 cell death and elucidate the mechanisms of the protective effect of DZX.

In the present study, we first investigated whether activation of mitoK_{ATP} channels in PC12 cells confers protection against OGD-induced cell apoptosis. Because OGD-induced cell apoptosis is regarded as a model for the pathogenesis of ischaemic stroke, the results suggest that activation of mitoK_{ATP} channel signalling could have potential beneficial effects in ischaemic disease. Further understanding of the mechanisms that underlie this interesting phenomenon may lead to the opening of novel therapeutic avenues for the treatment of ischaemic stroke in which mitochondrial dysfunctions play pivotal aetiological roles.

Materials and Methods

Materials

DZX and 5-hydroxydecanoic acid (5-HD), a selective mitoK_{ATP} channel blocker, were purchased from Beyotime Institute of Biotechnology, Inc. (China), and were dissolved in dimethylsulphoxide (DMSO) purchased from Sigma (St Louis, MO, USA). The final concentration of DMSO in cell cultures did not exceed 0.1%. DMSO at this concentration has no harmful effect on the cells. All other chemicals were dissolved in tissue culture grade water.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Hoechst 33342 were purchased from Sigma (St Louis). DMEM and foetal bovine serum (FBS) were purchased from Hyclone. Antibodies against cleaved-caspase-3, AIF and Cyt-C were purchased from Santa Cruz Biotechnology, Inc. (USA). For the protein isolation of nuclear and mitochondria from cytosol, we use Keygen Nuclear-Cytosol Protein Extraction Kit and Keygen mitochondria-Cytosol Protein Extraction Kit (Nanjing KeyGen Biotech, Co. Ltd, China). All other chemicals and reagents, unless specified otherwise, were purchased from Sigma (St Louis).

Cell culture and treatment

PC12 cells were cultured as described earlier (*37*). Briefly, PC12 cells were cultured in 75 ml vented culture flasks using high glucose DMEM with pyruvate and supplemented with 5% (v/v) FBS, 10% (v/v) horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in a humidified incubator at 37°C and 5% CO_2. The medium was changed every 3 days. After 5–6 days of incubation in the flask, cells reached 60–70% confluence. After being cultured for 5–6 days *in vitro*, the PC12 cells were randomized into six groups: control, OGD group, OGD + DZX (1 μmol/l), OGD + DZX (10 μmol/l), OGD + DZX (100 μmol/l) and OGD + DZX (100 μmol/l) + 5-HD (100 μmol/l). DZX and 5-HD were both prepared as 500× stocks in DMSO. Prior to anoxia, PC12 cells, with the exception of those in the control group and OGD group, were treated with different concentration of DZX for 1 h per day for 3 days following transient oxygen–glucose deprivation (OGD). Mixed cultures were subjected to a transient OGD as described by Goldberg and Choi (*38*) with minor modification. Cells were placed in an anaerobic chamber (HERA cell 150, partial oxygen pressure was maintained <2 mmHg) and the medium was replaced with a pre-warmed (37°C) glucose-free balanced salt solution (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 0.025 mM phenol red and 20 mM sucrose) that had been bubbled with an anaerobic gas mix (95% N_2, 5% CO_2) for 30 min to remove residual oxygen. Cell cultures subjected to OGD were called OGD cells and were incubated in this solution at 37°C for a 4 h period to produce lethal oxygen deprivation and then reoxygenated (returned to the normal aerobic environment). Experimental parameters were assayed at various times after reoxygenation. OGD was terminated by removing cultures from the chamber, replacing the exposure solution with oxygenated minimum essential media with Earle salts (L-glutamine–free) supplemented with 20 mM glucose, and returning the multi well plates to the incubator under normoxic conditions. DZX was added at the start of OGD and was removed by washing during the reoxygenation process. The control cell cultures, which were not subjected to OGD, were cultured as described earlier.

Cell viability assay

Cell viability was quantitatively evaluated at different times (6, 12, 24 and 48 h) of reoxygenation and with different concentration of DZX (1 μmol/l, 5 μmol/l, 10 μmol/l, 50 μmol/l, 100 μmol/l and 200 μmol/l) and 5-HD (100 μmol/l) using the MTT reduction test described by Mosmann (*39*). This test quantifies the formation of a dark blue formazan produced by the reduction of the tetrazolium ring of MTT by the mitochondrial succinate dehydrogenase in living cells. Briefly, cells in 96-well plates were rinsed with phosphate-buffered saline (PBS), MTT (0.5 mg/ml) was added to each well, and cells were incubated for 4 h at 37°C. After the medium with MTT was removed, cells and dye crystals were solubilized with 200 ml DMSO. Cell viability corresponded to the value of the optical density at 570 nm. Background subtraction was analysed at 630 nm using a spectrophotometer (ELX 800; Biotek Instruments, Winooski, VT, USA). Results were expressed as a percentage of the optical density measured in control group cells. Each experimental condition was analysed in triplicate and was replicated in a minimum of four plates per culture using cells obtained from six independent cultures.

Hoechst 33342 staining

To observe nuclear changes occurring during apoptosis, the chromatin-specific dye Hoechst 33342 was used. Cells were harvested and fixed with 4% paraformaldyhyde for 30 min at room temperature, then washed with pre-chilled PBS three times and exposed to 10 mg/ml Hoechst 33342 at room temperature in the dark for 10 min. Samples were observed under a fluorescence microscope (*40*, *41*). Apoptotic cells were identified by the presence of chromatin condensation and nuclear fragmentation. More than 500 nuclei from random fields were analysed for each data point.

Apoptotic rate assay by flow cytometry

Annexin V, an FITC conjugate of the PS-binding protein, is capable of detecting PS externalization in early apoptotic cells. PC12 cell apoptosis was estimated using the Annexin-V Fluorescein (FITC) apoptosis staining kit (Nanjing KeyGen Biotech. Co. Ltd, China) according to the kit instructions. The cell samples were analysed in a flow cytometry apparatus (BD Co., USA). Annexin V binds to phosphatidylserine that is translocated during apoptosis from the inner to the outer leaflet of the plasma membrane. Normal cells with intact membranes are distinguished by their ability to exclude propidium iodide (PI), which readily penetrates dead or damaged cells. Dual analysis was introduced using a quadrant dot plot, in which early

apoptotic cells were annexin V-FITC-positive only, necrotic cells and late apoptotic cells were recognized as double-positive for annexin V-FITC and PI. Cells that stained negative for both annexin V-FITC and PI were classified as normal cells. Finally, the number of cells in each category was expressed as a percentage of the total number of stained cells counted. Each experiment was repeated three times.

Immunofluorescence

PC12 cells cultured on poly-L-lysine-coated glass coverslips in the six-wells plates were washed once in PBS and fixed with 1% paraformaldehyde for 15 min. Coverslips were then washed once in PBS and briefly immersed in 0.1% saponin in PBS as described earlier (*42*). Cells were counterstained for the detection of AIF (1/100) revealed by a goat anti-mouse IgG-FITC conjugate and DNA (Hoechst 33342; 1 μM; Sigma). Preparations were observed using a Nikon Eclipse TE800 microscope, and analysed using Nikon NIS-Element 2.3 software.

Western blot analysis

For the protein isolation from the nucleus, mitochondria and cytosol we used the Keygen Nuclear-Cytosol Protein Extraction and Keygen mitochondria-Cytosol Protein Extraction Kits from Nanjing KeyGen Biotech. Co. Ltd. (China). After the samples were collected, SDS polyacrylamide gel electrophoresis (SDS–PAGE) was performed. The separated blots were electrophoretically transferred to nitrocellulose membrane and blocked with 5% non-fat milk for 2 h at 4°C. Immunoblotting and immunodetection of AIF (1/1000; mouse mAb from Santa Cruz, revealed by a goat anti-mouse IgG-HRP conjugate; Sigma) and cytochrome *c* (1/1000; Santa Cruz, revealed by a goat anti-mouse IgG-HRP conjugate) were performed as described earlier (*43*). After further washing with PBS, the membrane was analysed by the ECL method (*44*).

Statistics and data analysis

Data are presented as the mean ± SEM for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by a *t*-test; $P > 0.05$ were considered significant.

Results

Activation of mitochondrial K_{ATP} channels increases cell viability

First, we established that exposure of PC12 cells to OGD for 4 h and reoxygenation for 6 h, 12 h, 24 h and 48 h resulted in a significant decrease in cell viability. It was obvious that OGD and reoxygenation induced a time-dependent viability loss in PC12 cells. Cells exposed to OGD for 4 h and reoxygenation for 12 h had a viability that was 60.9 ± 1.7% of the control value (Fig. 1). Next, to investigate a role for mitochondrial K_{ATP} channels in protection, PC12 cells were preconditioned by exposure to 1–200 μmol/l DZX for 1 h per day for 3 days. Both control (untreated) and DZX-treated PC12 cells were exposed to OGD for 4 h and reoxygenated for 12 h. The percentage of OGD-induced cell death was assessed by MTT after reoxygenation for 12 h. DZX (1–200 μmol/l) increased cell viability from 61.9 ± 1.1 to 82.4 ± 2.3% in a dose-dependent manner (Figs 1 and 2). To determine whether this observed increase in cell viability with DZX preconditioning was the result of mitoK_{ATP} activation, PC12 cells were pre-conditioned with 1–100 μmol/l DZX in the presence of 100 μmol/l 5-HD, a mitochondrial K_{ATP} channel blocker, for 1 h per day for 3 days. The protective effect of DZX was attenuated by 5-HD, as the cell viability decreased from 61.9–82.4% to 61.8–73.64% after exposure to

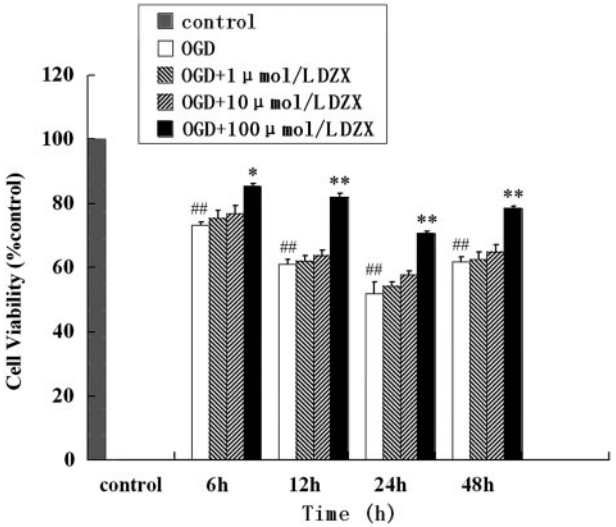

Fig. 1 DZX inhibited OGD-induced injury in PC12 cells. Cells were treated with OGD reoxygenation for indicated times (6 h, 12 h, 24 h and 48 h) in the presence or absence of DZX (with different concentration of DZX). After treatment, cell viability was estimated using the MTT method. These results were expressed as the percentage of optical density observed in the control (untreated PC12 cells). Data were expressed as the mean ± SEM of six independent experiments. $^{\#\#}P < 0.01$, compared to the control group. $^{**}P < 0.01$, $^{*}P < 0.05$ compared to the OGD group.

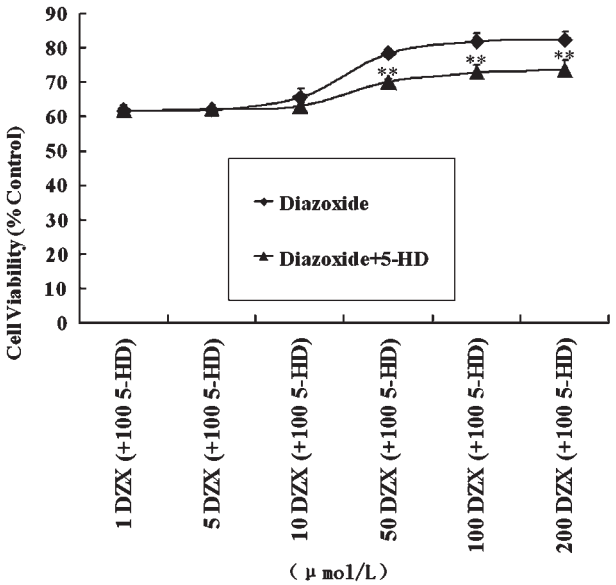

Fig. 2 Activation of mitoK_{ATP} channels confers protective effect against OGD-induced PC12 cell viability decrease. Dose-response curves of DZX (1–200 μmol/l) on cell viability in the absence (filled rhombus) or presence of 100 μmol/l 5-HD (filled triangle). Each point represents the mean ± SEM of four independent experiments. $^{**}P < 0.01$, compared to the group with DZX preconditioning in the absence of 5-HD.

OGD for 4 h and reoxygenation for 12 h (Fig. 2), suggesting the protective effect resulted from activation of mitoK_{ATP} channels. We found that a lower dose of DZX (50 μmol/l) did not confer effective neuroprotection. The results showed that DZX protected cells against OGD and reoxygenation-induced cytotoxicity in a dose-dependent manner.

Effect of DZX on OGD-induced PC12 cell apoptosis by Hoechst 33342 staining and flow cytometry

Apoptotic cells undergo chromatin condensation, which can be visualized using the DNA-binding fluorescent dye Hoechst 33342 (Fig. 3). Nuclei of control cells appeared round to oval, with a separate pattern of blue fluorescence. After treatment by OGD for 4 h and reoxygenation for 12 h, cell nuclei became increasingly bright, decreased in size and fragmented into apoptotic bodies. In contrast, cells pre-incubated with DZX (100 μmol/l) appeared remarkably preserved and apoptosis were significantly attenuated. The protective effect of DZX was attenuated by 5-HD.

Flow cytometry showed that the apoptotic frequency in PC12 cells in the control group was 8.8%. When cells were subjected to OGD and reoxygenation for 12 h, the apoptotic rates in the DZX 0 μmol/l, DZX 10 μmol/l, DZX 100 μmol/l and DZX 100 μmol/l + 5-HD 100 μmol/l groups were elevated to 34.3 ± 4%, 33.9 ± 6%, 11.8 ± 3% and 31.3 ± 9%, respectively. DZX of 100 μmol/l reduced the frequency of apoptosis to ~22%. However, this reduction was reversed by 5-HD. No significant differences were detected in cells exposed to other concentrations of DZX (Fig. 4).

Effect of DZX on OGD-induced PC12 cells apoptosis in caspase-dependent way

To study the role of DZX on cell apoptosis, we performed western blot analysis studies that revealed that OGD for 4 h and reoxygenation for 12 h caused the release of mitochondrial pro-apoptotic proteins cytochrome c (Fig. 5A) and the expression of active (cleaved) caspase-3 (Fig. 5B) in PC12 cells and this effect was significantly attenuated with the pre-treatment of DZX with a concentration of 100 μmol/l. Equivalent amounts of proteins were subjected to immunoblot analysis in order to determine the presence/absence of cytochrome *c* or caspase-3 in the cytosolic fraction.

Effect of DZX on OGD-induced PC12 cells apoptosis in caspase-independent way

Immunofluorescence (Fig. 6A) and western blot (Fig. 6B) analysis revealed that OGD for 4 h and reoxygenation for 12 h caused the mitochondrial levels of AIF to decrease and the nuclear levels of AIF to increase. The translocation effect of AIF during apoptosis can be attenuated by pretreatment of DZX (100 μmol/l). In contrast, DZX at a concentration of 1 μmol/l or 10 μmol/l could not block the effect of AIF translocation during apoptosis induced by OGD. We also found that this reduction effect of DZX could be reversed by 5-HD.

Discussion

OGD in the PC12 cell line has been used as a rapid and sensitive *in vitro* model of ischaemic stroke for the development of potential neuroprotective agents. To mimic cerebral ischaemia–reperfusion injury, PC12 cells are first subjected to a short period of OGD (ischaemia) followed by a prolonged period of reoxygenation and return to normal culture conditions (reperfusion). Thus, this model is believed to mimic the pathological conditions of stroke. The DZX pre-conditioning means were utilized according to the methods that Kis *et al.* (*45*) has reported. Mimicking

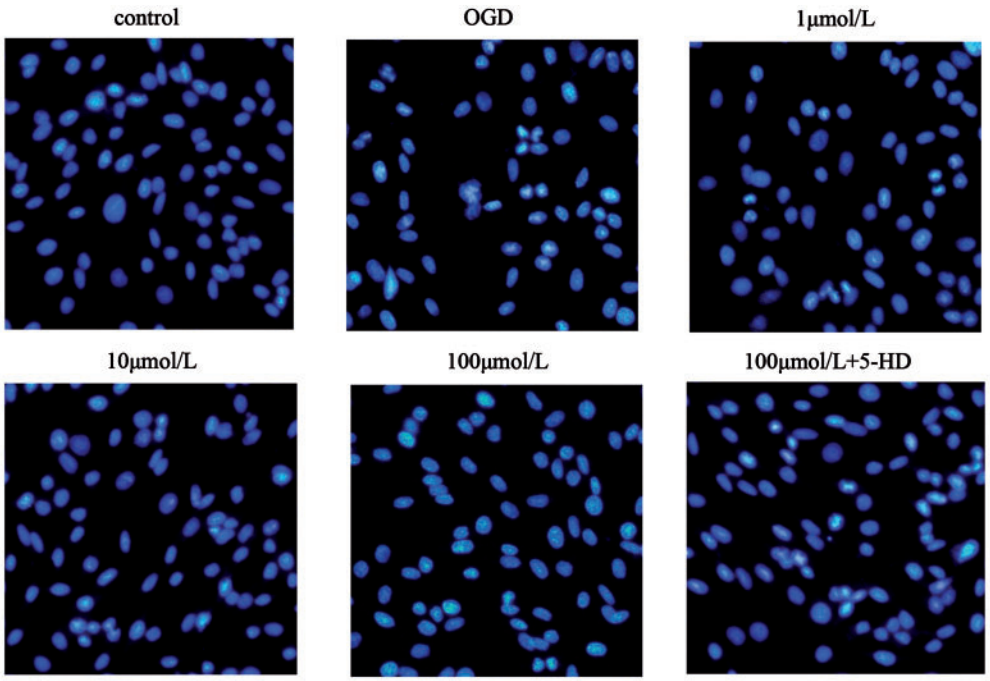

Fig. 3 DZX inhibits OGD-induced PC12 cells apoptosis. The morphological features of apoptosis were monitored by fluorescence microscopy after staining with Hoechst-33342. Cells that exhibited reduced nuclear size, chromatin condensation, intense fluorescence and nuclear fragmentation were considered apoptotic. DZX (100 μmol/l) can effectively inhibit the damage induced by OGD.

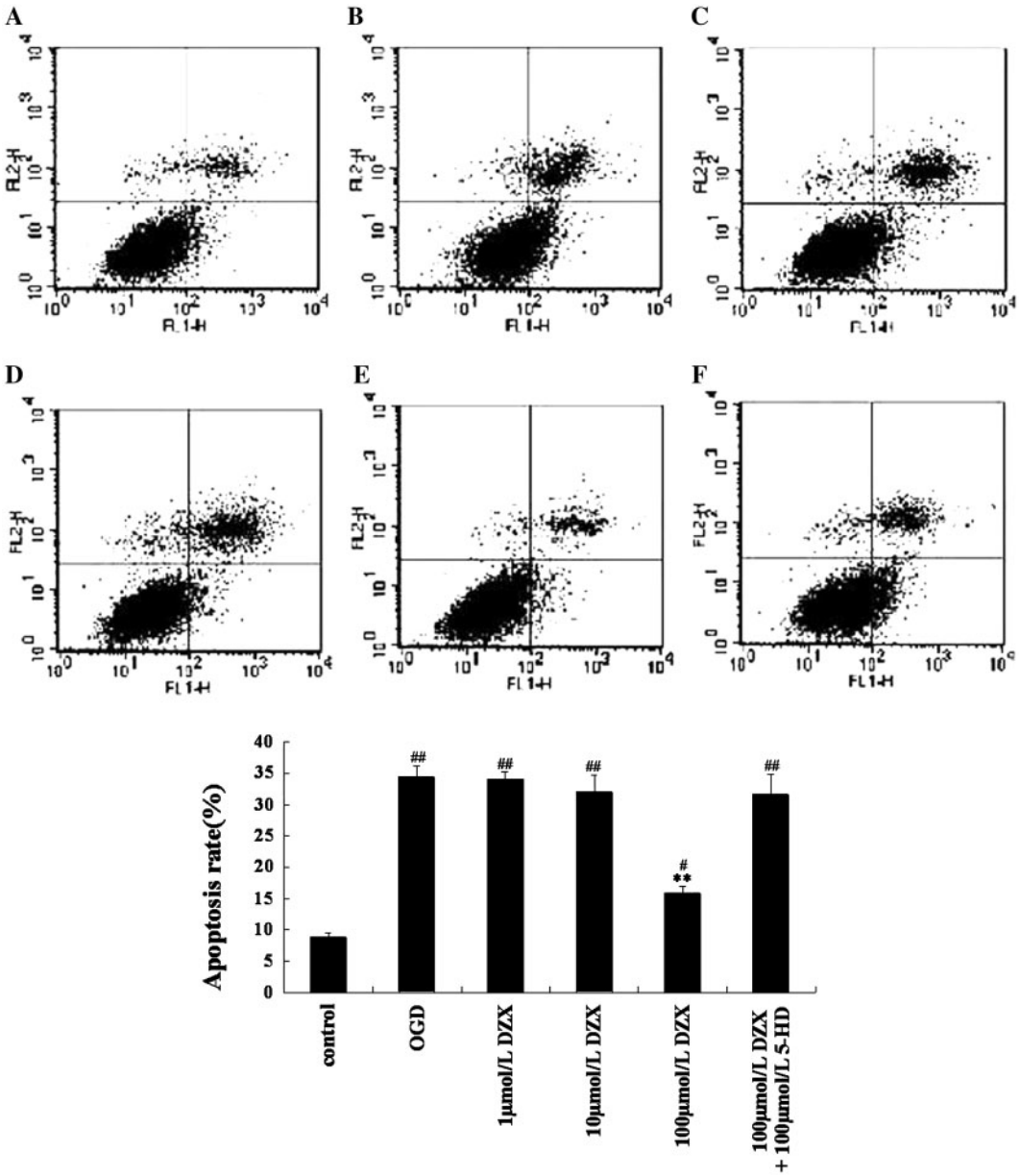

Fig. 4 Annexin V-FITC staining for PC12 cells exposed to 4 h of OGD and 12 h of reoxygenation. The different labeling regions in this quadrant represented the different cell populations. Cells in the lower-left quadrant, unstained for both Annexin V-FITC and PI, are defined as normal cells. Cells in the lower-right quadrant, stained for Annexin V-FITC but negative for PI, are defined as early-medium apoptotic cells. Cells in the upper-right quadrant, positive for both Annexin V-FITC and PI, are defined as late apoptotic and necrotic populations. (A) Lower apoptotic rate was shown in the control group. (B) PC12 cells were insulted by anoxia-reoxygenation without DZX pretreatment. (C) PC12 cells were treated by 1 μmol/l DZX before anoxia. (D) PC12 cells were treated by 10 μmol/l DZX before anoxia. (E) PC12 cells were treated by 100 μmol/l DZX before anoxia. (F) PC12 cells were treated by 100 μmol/l DZX plus 100 μmol/l 5-HD before oxygen deprivation. As the center of the whole cell population in E shifted left compared with those in (B), (C), (D) and (F), it was shown that apoptosis was depressed in the DZX 100 μmol/l group. No significant differences were detected among (B), (C), (D) and (E). The lower panel of the figure shows the results of quantitative determination of apoptosis rate by flow cytometry. Similar results were obtained in three independent experiments. Data are expressed as the mean ± SEM of three independent experiments. $^{\#\#}P<0.01$, $^{\#}P<0.05$ as compared to the control group. $^{**}P<0.01$, compared to the OGD group.

the multiple cycled methods of ischaemic preconditioning *in vivo*, we treated the PC12 cells once a day for 3 days before oxygenation deprivation because a single use of DZX was unable to induce preconditioning against ischaemia -induced damage.

K_{ATP} openers, especially mitoK_{ATP} openers, can provide protective effects for neurons and neuroblasts against cell damage induced by ischaemia, trauma and toxic reagents (such as rotenone and MPP^+) (*46–48*). Moreover, Bajgar demonstrated that the number of mitoK_{ATP} channels in brain cells is at least 6-fold higher than that in heart cells (*49*), indicating that mitoK_{ATP} channels play an essential role in the functions of the central nervous system. Thus, mitoK_{ATP} channels may be an important molecular target in neuronal cell function.

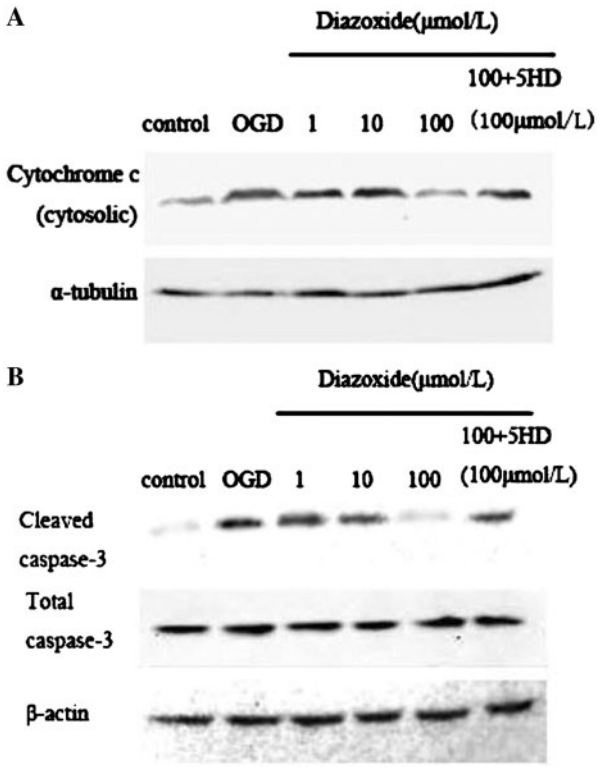

Fig. 5 DZX (100 μmol/l) inhibited OGD-induced caspase-dependent apoptosis in PC12 cells. Before exposure to OGD, cells were incubated with the indicated concentrations of DZX. Cell proteins were obtained and analysed with anti-cytochrome *c* and anti-cleaved-caspase-3 antibodies by western blot analysis of the cytosolic fraction and the total cell lysate. (A) DZX (100 μmol/l) significantly inhibited the release of cytochrome *c* in OGD-induced PC12 cells. Pre-treatment with 5-HD (100 μmol/l) suppressed the effect of DZX (100 μmol/l). (B) DZX(100μmol/l) inhibited caspase-3 activation. The release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol and the activation of caspase-3 are accepted as markers of caspase-dependent apoptosis. Tubulin was used as a cytosolic marker protein. Actin expression levels were assayed as a control for protein loading. Each observation was repeated four times.

In the present study, we found that the classic mitoK_{ATP} opener DZX possessed the protective effects on OGD-induced PC12 cells apoptosis *in vitro*. The protective effect of DZX is dose-dependent; 100 μmol/l DZX protects PC12 cells, whereas 1 μmol/l or 10 μmol/l DZX offers no protection. These results suggest that DZX protects neurons from anoxia-reoxygenation injury, but this protection is correlated with the dose of DZX. Because of the difficulty in keeping DZX in water-based solution, we should note here that if the concentration of DZX in medium was >500 μmol/l, the DZX would form crystals. Hence, the concentrations of DZX in our study were all <500 μmol/l. It would be intriguing to know the outcome if concentrations of DZX over 500 μmol/l could be used in future studies. Because 5-HD, a selective blocker of mitoK_{ATP} channels, eliminated the protective effect, it is likely that DZX alters apoptosis protection by regulating mitoK_{ATP} channels. Additionally, DZX can protect against OGD-induced cell apoptosis by inhibiting the activation of caspase-3, suppressing the translocation of the pro-apoptotic factors cytochrome *c* and AIF, and subsequently preventing nuclear fragmentation.

Mitochondrial dysfunction is a prominent feature of apoptosis. The translocation of pro-apoptotic proteins (*e.g.* cytochrome *c*, AIF, and Endo G, etc.) from the mitochondrial intermembrane space is a critical event that occurs during apoptosis (*50*). Both cytochrome *c* and AIF are required for cell viability when they are located in mitochondria, but when either is released from the mitochondria, cell death programs are activated. Mitochondrial release of cytochrome *c* into the cytoplasm induces the formation of an oligomeric complex containing cytochrome *c* and Apaf-1. This complex, called the apoptosome, activates a downstream caspase program. Activated caspases can also affect the function of mitochondria. Caspases can be activated by Apaf-1/cytochrome *c* or cell surface death receptors. Caspases (*e.g.* caspase-3) are activated by two cleavage events that occur between the prodomain and the large subunit (p17) and between the large subunit and the small subunit (p12). The activated caspase, composed of two large and two small subunits, cleaves death substrates (*e.g.* PARP) and ultimately leads to cell death (*51*). AIF primarily induces caspase-independent cell death. Following AIF translocation from the mitochondria to the nucleus, classic apoptotic features, such as phosphatidylserine exposure, partial chromatin condensation, and nuclear condensation occur in the absence of caspase activation. AIF appears to play an important role in the acute neurotoxicity induced by trauma, hypoglycemia, transient ischaemia, and chronic neurodegenerative diseases (*52–54*). Therefore, therapeutic strategies targeting both caspase-dependent and caspase-independent pathways may be more protective against toxic insults. The present study demonstrated that DZX significantly inhibited both OGD-induced apoptosis and the release of proapoptotic proteins (cytochrome *c* and AIF) from the mitochondria of PC12 cells. These findings suggest that DZX may protect PC12 cells from apoptosis by inhibiting both caspase-dependent and caspase-independent apoptosis.

DZX may have potential as a treatment for stroke. In 2001, the Japanese government approved Edaravone for the treatment of acute ischaemic stroke, and its use was recommended by the Japanese Guidelines for the Management of Stroke in 2004. The protective effect of Edaravone has been documented in the PC12 cells using the *in vitro* OGD model (*55*). In the OGD model, maximum protection of Edaravone was ~25% at 0.1 μmol/l, with no further protection achieved at higher doses (*55*). DZX reduced OGD-induced PC12 cell death by 8 to 22% at concentrations between 100 μmol/l and 200 μmol/l. Therefore, the beneficial effects of DZX are comparable to Edaravone in the PC12 cell model of stroke.

In summary, 100 μmol/l DZX could prevent cultured PC12 cells from undergoing apoptosis induced by anoxia-reoxygenation in part by inhibiting mitochondrial caspase-dependent and caspase-independent apoptosis. Accepting the complexity of

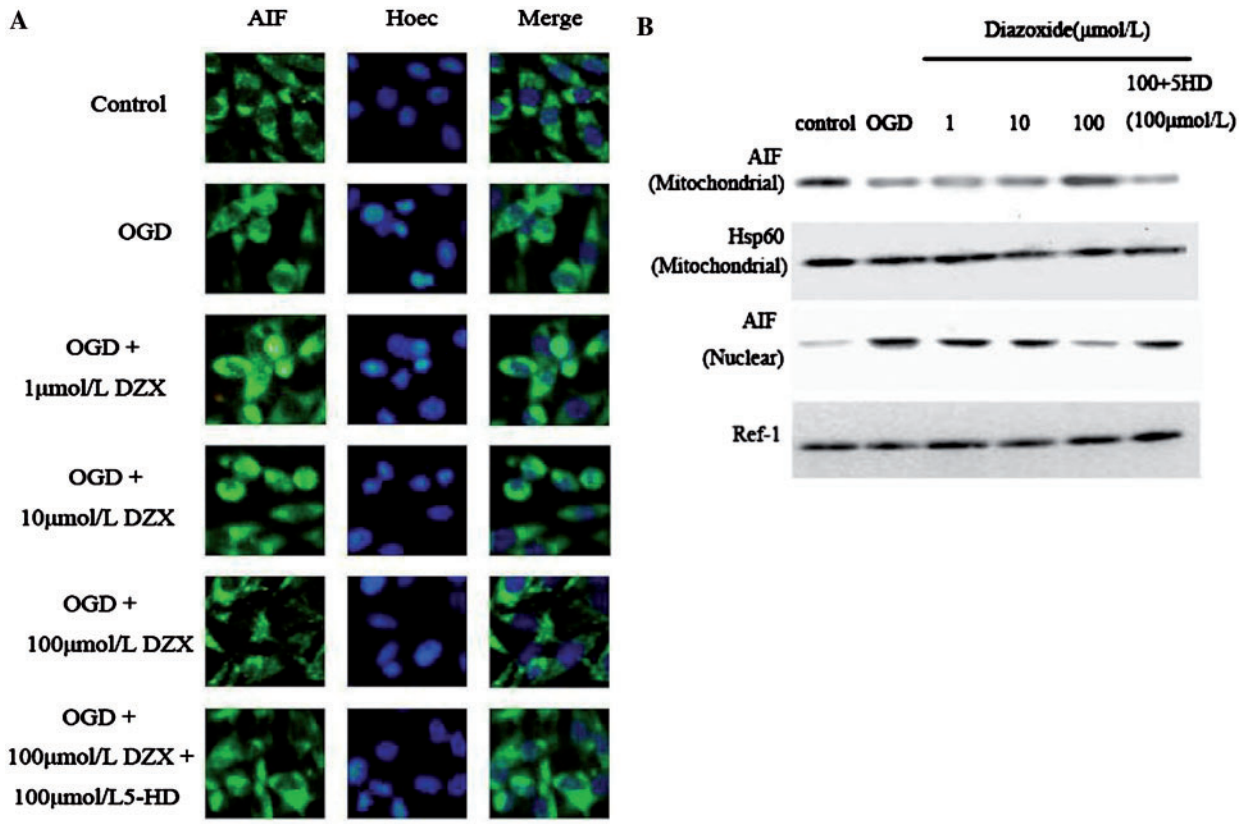

Fig. 6 DZX (100 μmol/l) prevents OGD-induced caspase-independent apoptosis in PC12 cells. (A) DZX (100 μmol/l) prevented OGD-induced nuclear condensation and cell death in an AIF-dependent (caspase-independent) manner. Representative immunofluorescence for translocation of AIF to the nucleus and nuclear condensation in OGD-induced PC12 cells is shown. The nuclear translocation of AIF is shown by the overlap of AIF (green) and nuclear staining (blue). Morphological analysis of nuclear chromatin by Hoechst 33342 is shown. AIF is detected in the cytoplasm of control cells (control). Under the condition of OGD and reoxygenation for 12 h, the intensity of AIF staining increases in the cytoplasm and around the nucleus. With the pretreatment of (1 and 10 μmol/l) nuclear morphology is not conspicuously altered, as compared with the OGD group. Most cells showing nuclear AIF staining appear shrunken with serrated nuclei, indicating irreversible nuclear damage. DZX (100 μmol/l) can inhibit the damage effect induced by OGD in a caspase-independent manner. However, this protective effect can be blocked with 100 μmol/l 5-HD. (B) Analysis of AIF translocation by subcellular separation. Subcellular separation was performed with untreated PC12 cells (control group) and cells treated by OGD for 4 h and reoxygenated for 12 h (OGD group). Cells pretreated with DZX (1 μmol/l, 10 μmol/l, 100 μmol/l) and the cells pre-treated with a mixture of DZX and 5-HD (100 μmol/l) were analysed by western blot of the mitochondrial (*M*) and nuclear (*N*) fractions. Heat shock protein 60 (*HSP60*) and Ref-1 were used as mitochondrial and nuclear marker proteins, respectively.

the mechanism by which DZX promoted cell protection, we assume that there are different mechanisms that directly or indirectly inhibit cell apoptosis; however, our results suggest that the mitochondria are at the core of the anti-apoptotic effects. DZX may be a hopeful therapeutic drug for treatment of ischaemic and anoxic cerebral injury.

Acknowledgements

We would like to thank Rong Yan for providing PC12 cells. We are also grateful to Zhengwei Yuan and Dongyan Liu for technical assistance.

Funding

The Natural Science Foundation of LiaoNing province (20052097), China.

Conflict of interest

None declared.

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