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CIC-3 chloride channel in hippocampal neuronal apoptosis

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Research Highlights

(1) Current studies addressing ion disturbance mainly concentrate on the influence of cation channels on neuronal apoptosis following ischemic brain injury. However, little evidence is available on how the anion chloride channel might affect ischemic injury. The CIC-3 chloride channel is an anion channel that is expressed in normal hippocampal neurons. Its potential role in hippocampal neuronal apoptosis after ischemic brain injury remains unclear.

(2) Our findings showed that the increased activities of the CIC-3 chloride channel may be involved in hippocampal neuronal apoptosis induced by nitric oxide, providing theoretical evidence for understanding the role of chloride channel on neuronal apoptosis following ischemic brain injury.

Abstract

Over-production of nitric oxide is pathogenic for neuronal apoptosis around the ischemic area following ischemic brain injury. In this study, an apoptotic model in rat hippocampal neurons was established by 0.5 mmol/L 3-morpholinosyndnomine (SIN-1), a nitric oxide donor. The models were then cultured with 0.1 mmol/L of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS; the chloride channel blocker) for 18 hours. Neuronal survival was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and apoptosis was assayed by Hoechst 33342-labeled neuronal DNA fluorescence staining. Western blot analysis and immunohistochemistry staining were applied to determine the changes of activated caspase-3 and CIC-3 channel proteins. Real-time PCR was used to detect the mRNA expression of CIC-3. The results showed that SIN-1 reduced the neuronal survival rate, induced neuronal apoptosis, and promoted CIC-3 chloride channel protein and mRNA expression in the apoptotic neurons. DIDS reversed the effect of SIN-1. Our findings indicate that the increased activities of the CIC-3 chloride channel may be involved in hippocampal neuronal apoptosis induced by nitric oxide.

Key Words

neural regeneration; brain injury; nitric oxide; CIC-3 chloride channel; 3-morpholinosyndnomine; 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; hippocampal neurons; apoptosis; grants-supported paper; neuroregeneration

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Chang QZ and Zhang SL designed and created the experimental concepts. Xu LJ and Fan HL performed the experiments and wrote the manuscript. Zhong ZC, Li X, and Jin XX were responsible for data analysis and processing. All authors approved the final version of the manuscript.

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INTRODUCTION

Within 1 hour after stroke onset, focal cerebral ischemia causes irreversible brain damage at the center, while neuronal damage at the ischemic penumbra is delayed. Apoptosis is the main type of neuronal death and is a program-controlled active death process. Appropriate intervention measures can prevent neuronal apoptosis^[1-5].

The mechanism of neuronal apoptosis following ischemic brain injury is controversial. Some scholars have proposed that excessive production of nitric oxide and an imbalance of intramembrane and extramembrane ions contribute to neuronal apoptosis^[6-11]. Current studies addressing ion disturbance mainly concentrate on the influence of cation channels on neuronal apoptosis following ischemic brain injury. However, little evidence is available on how the anion chloride channel might affect ischemic injury. The CIC-3 chloride channel is an anion channel that is expressed in normal hippocampal neurons. Its potential role in hippocampal neuronal apoptosis after ischemic brain injury remains unclear.

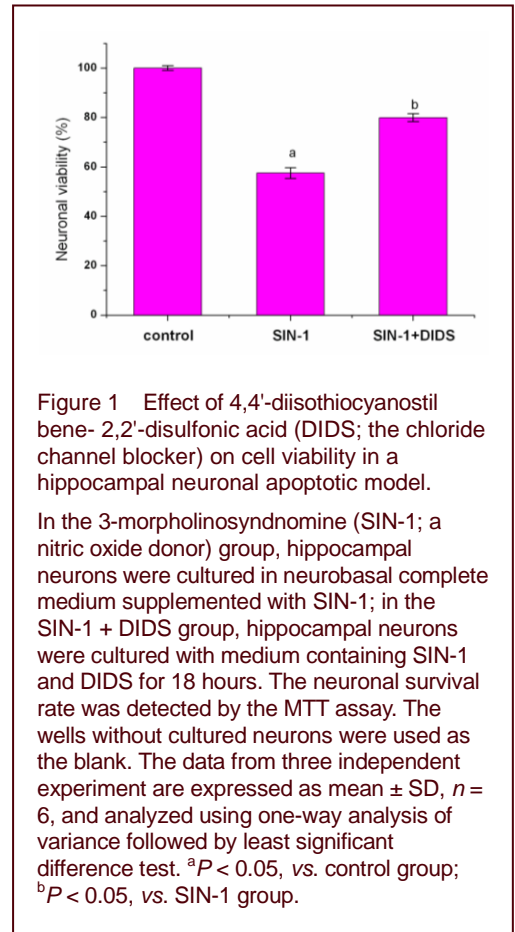
In this study, we established a rat model of hippocampal neuronal apoptosis by using 3-morpholinosyndnomine (SIN-1), a nitric oxide donor. The models were then administered the chloride channel blocker, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), in a broader attempt to observe CIC-3 chloride channel expression in the neuronal apoptosis process and to investigate the correlation between chloride channel activity and ischemia-sensitive neuronal apoptosis.

RESULTS

Survival rate of hippocampal neurons

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay results showed that hippocampal neurons cultured in normal neurobasal complete medium (control group) had significantly decreased survival rates

after application of SIN-1 (SIN-1 group) ($P < 0.05$). The survival rate in SIN-1 + DIDS group was higher than that in SIN-1 group ($P < 0.05$; Figure 1).



Hippocampal neuronal apoptosis and CIC-3 expression

Changes to the nuclear morphology were observed with Hoechst 33342 staining using a DNA fluorescent reagent. Anti-NeuN antibody was used for neuronal specific staining, and anti-CIC-3 antibody was applied to detect CIC-3 chloride channel protein expression (Figure 2).

Hoechst 33342 staining showed that a large number of neurons in the SIN-1 group exhibited small nuclei. In the SIN-1 + DIDS group, these fluorescent neurons were significantly reduced in quantity. The neuronal apoptosis rate in the SIN-1 group significantly increased compared with the control group ($54.38 \pm 1.71\%$ vs. $8.11 \pm 2.01\%$; $P < 0.05$), and the SIN-1 + DIDS group showed a significantly lower rate ($31.74 \pm 1.44\%$) than the SIN-1 group ($P < 0.05$).

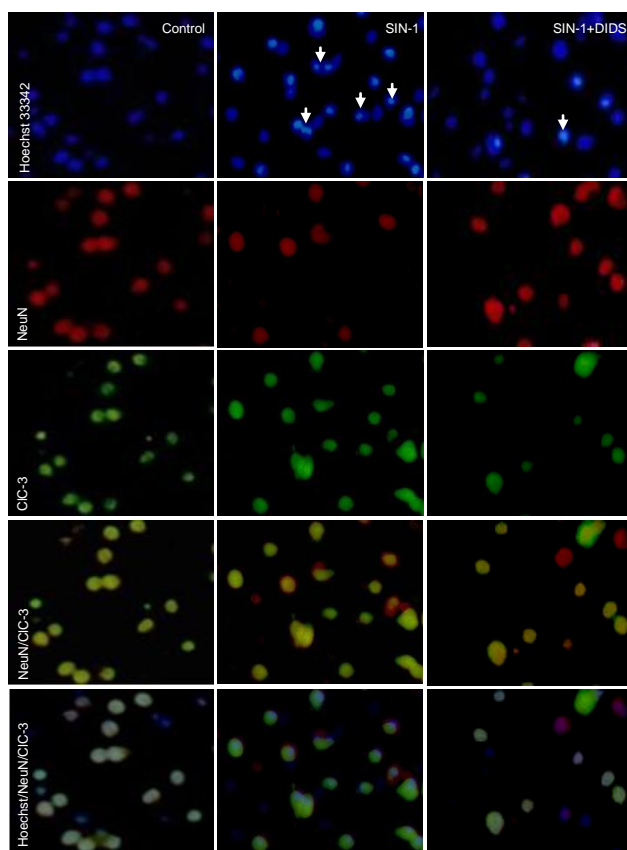


Figure 2 CIC-3 immunoreactivity in a hippocampal neuronal apoptotic model ($\times 400$).

In the 3-morpholinosynndomine (SIN-1; a nitric oxide donor) group, hippocampal neurons were cultured in neurobasal complete medium supplemented with SIN-1; in the SIN-1 + 4,4'-diisothiocyanostil bene-2,2'-disulfonic acid (DIDS; the chloride channel blocker) group, hippocampal neurons were cultured with medium containing SIN-1 and DIDS for 18 hours. Shown are images of the immunofluorescent staining of nuclei (blue, arrows indicate apoptotic neurons), of neurons with NeuN (red) and for chloride channels with CIC-3 (green). The apoptotic neurons and CIC-3 positive neurons in the SIN-1 group were significantly increased compared with the control group, and were significantly decreased in the SIN-1 + DIDS group compared with the SIN-1 group.

NeuN antibody staining showed that the cultured cells in control group were mainly neurons. CIC-3 antibody staining revealed that CIC-3 co-expressed with NeuN in neurons. After SIN-1 application, CIC-3 expression on the membrane of apoptotic neurons was up-regulated. After SIN-1 + DIDS application for 18 hours, CIC-3 expression decreased, suggesting that DIDS attenuated the SIN-1-induced CIC-3 expression in apoptotic hippocampal neurons.

Changes of caspase-3 protein in hippocampal neurons

Western blot analysis showed that caspase-3 expression was significantly up-regulated after hippocampal neurons were cultured with SIN-1 for 18 hours ($P < 0.01$). However, the expression level significantly decreased after DIDS intervention ($P < 0.01$; Figure 3).

Changes in CIC-3 chloride channel proteins in hippocampal neurons

Western blot analysis showed that after hippocampal neurons were cultured with SIN-1 for 18 hours, CIC-3 protein expression significantly increased compared with the control group ($P < 0.01$). After neurons were cultured with DIDS in the SIN-1 + DIDS group, CIC-3 protein expression significantly decreased compared

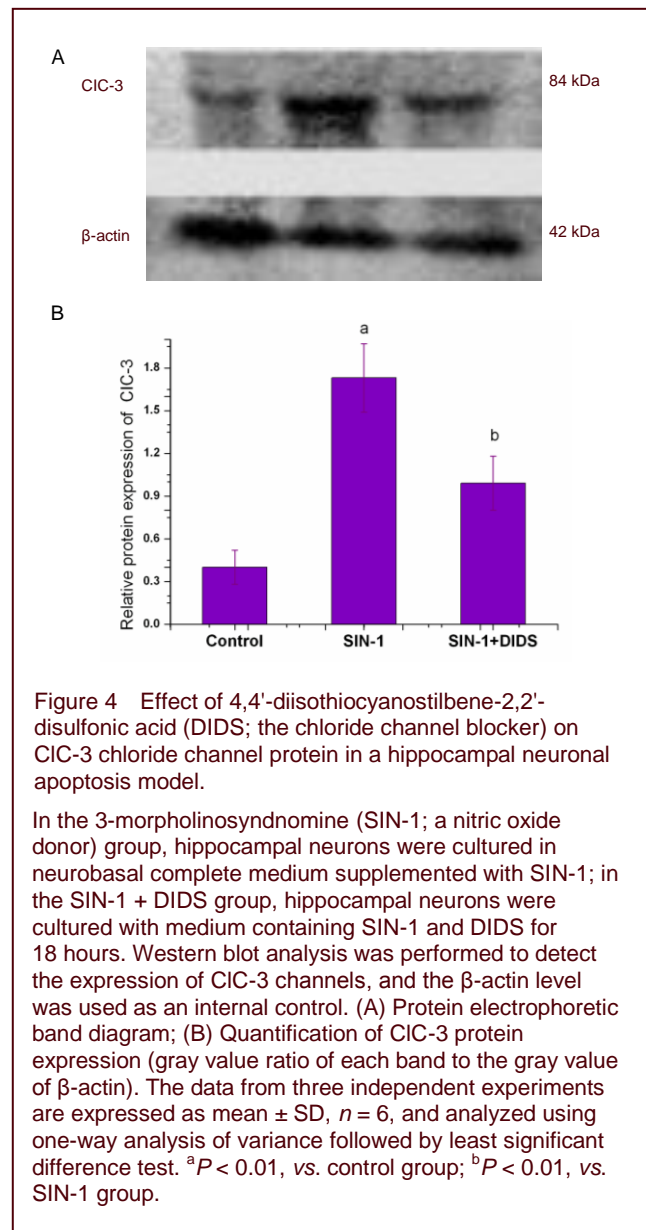
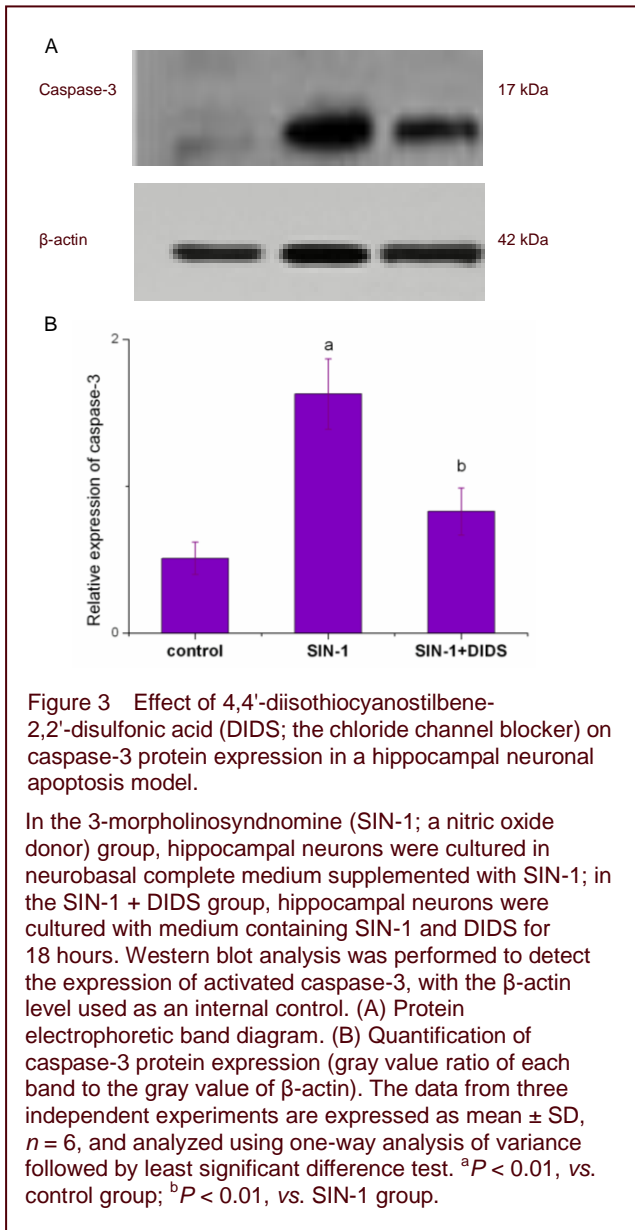
with the SIN-1 group ($P < 0.01$; Figure 4).

CIC-3 mRNA expression in hippocampal neurons

Real-time PCR results showed that after hippocampal neurons were cultured with SIN-1 for 18 hours, CIC-3 mRNA expression was significantly up-regulated ($P < 0.01$). After neurons were cultured with DIDS for 18 hours in the SIN-1 + DIDS group, CIC-3 mRNA expression was significantly lower than in the SIN-1 group ($P < 0.01$; Figure 5).

DISCUSSION

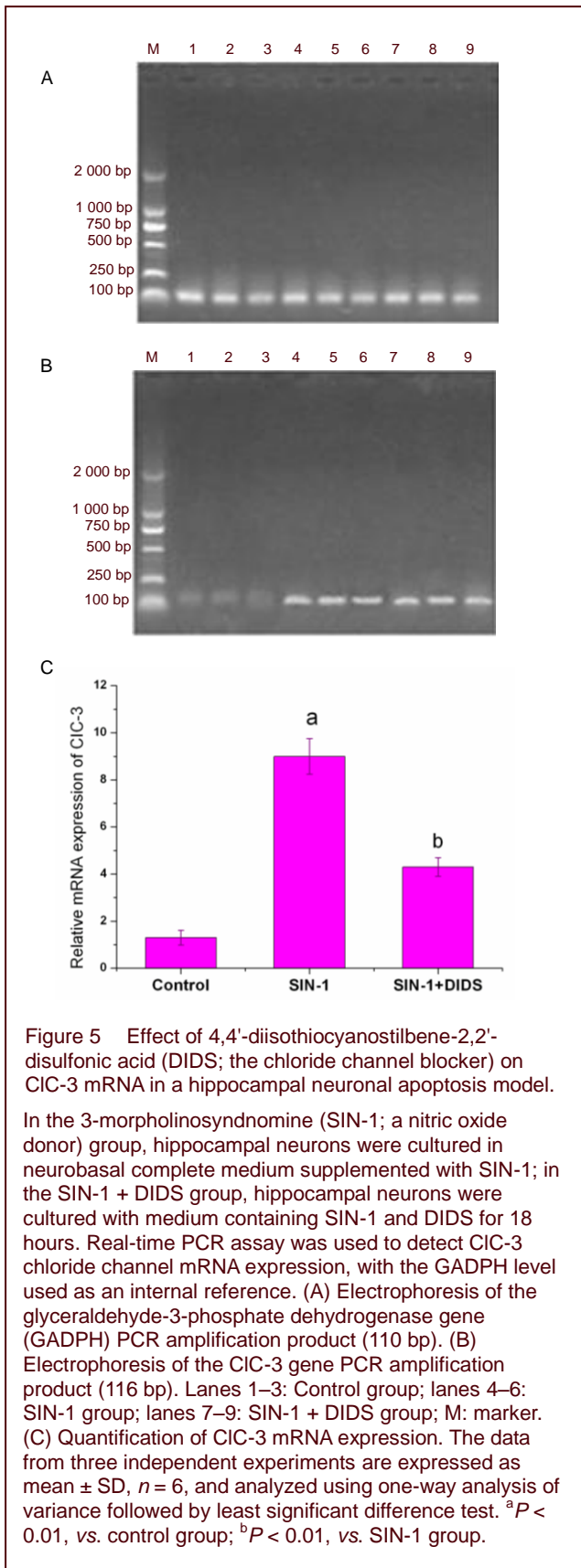
Excessive production of nitric oxide is a pathogenic mechanism underlying neuronal apoptosis at the ischemic penumbra following ischemic brain injury. SIN-1 is the main donor of nitric oxide, and can be decomposed to produce nitric oxide and O^{2-} , as well as form $OONO^-$ in aqueous solution. Nitric oxide and $OONO^-$ at physiological concentrations cannot induce apoptosis, although excessive nitric oxide and $OONO^-$ produce toxic effects on neurons and trigger neuronal apoptosis^[12]. Therefore we applied SIN-1 to induce neuronal apoptosis in a dose dependent manner^[13].



Our study found that 0.5 mmol/L SIN-1 induced apoptosis in 54% of the cells, suggesting that our apoptotic model is reliable. As for the role of chloride channels in non-neuronal apoptosis, Takahashi *et al* [14] found that DIDS and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) inhibited the staurosporine-induced apoptosis of ischemic myocardial cells in transgenic mice. Zuo *et al* [15] showed that the chloride channel blocker (NPPB) could inhibit H₂O₂-activated chloride channel currents and prevent the reduction of apoptotic volume, thus preventing apoptosis in PC12 cells. Multiple studies have investigated the role of chloride channels in nerve cell apoptosis. For example, Chang *et al* [16-17] observed whole-cell current density variations in neuron chloride channels before and after application of SIN-1 and/or chloride channel blockers in the SIN-1-induced rat hippocampal neuronal apoptosis model, using the whole-cell voltage clamp mode.

They found that chloride channel currents were voltage-gated, showing outward rectification characteristics. Their findings, from an electrophysiological viewpoint, supported the correlation between chloride channel activity and SIN-1-induced neuronal apoptosis. Similar results were also obtained in our experiments, observing the effect of DIDS on cell survival and apoptosis.

The CIC-3 chloride channel is a member of the CIC-type chloride channel family, which is widely present in various cells, and is located mainly in the cell membrane, cell organelles and synaptic vesicles in the rat brain hippocampus, olfactory bulb and cerebellum [18-21]. The NeuN antibody is a specific antibody expressed in mature neurons. Immunofluorescence staining in this study found that rat hippocampal cells *in vitro* cultured for 12 days were mainly neurons and CIC-3 positive expression was observed.



CIC-3 chloride channel expression was up-regulated after SIN-1 (0.5 mmol/L) application to induce apoptosis in hippocampal neurons, but CIC-3 expression signifi-

cantly decreased in the SIN-1 + DIDS group. Thus, DIDS decreased CIC-3 chloride channel expression in the SIN-1-induced apoptosis of hippocampal neurons. We speculate that CIC-3 may be involved in SIN-1-induced neuronal apoptosis. Furthermore, western blot analysis and real-time PCR analysis showed that the CIC-3 chloride channel protein and mRNA expression were significantly up-regulated after hippocampal neurons became apoptotic under the induction of SIN-1, and DIDS reversed the increase in expression. We speculate that the CIC-3 chloride channel may be involved in SIN-1-induced apoptosis. Caspase-3 is the final execution protein in the caspase-dependent apoptosis pathway. Caspase-3 is inactive under normal circumstances, but is activated with apoptosis to produce 17 kDa and 11 kDa fragments^[22-24]. In this study, western blot analysis detected the 17 kDa caspase-3 active fragment, which showed that DIDS attenuates neuronal apoptosis.

The mechanism of CIC-3 chloride channel activity involved in the SIN-1-induced apoptosis in rat hippocampal neurons remains elusive. The CIC-3 chloride channel has outwardly rectifying properties^[25-28]. Based on our experimental results, we speculate that after hippocampal neuronal injury, CIC-3 chloride channel expression becomes upregulated. Simultaneously, Cl⁻ and K⁺ efflux lead to massive outflow of intracellular water, which reduces the cell volume and then triggers neuronal apoptosis. Apoptosis is a cascade of events involving a variety of pathways. Interestingly, the reduction of cell volume occurred at the early apoptosis stage. Whether the CIC-3 chloride channel is involved in apoptosis needs further study. The experimental results of this study provide evidence for understanding the role of chloride channels in ischemic brain injury.

MATERIALS AND METHODS

Design

In vitro cell experiments.

Time and setting

Experiments were performed from August 2011 to May 2012 in the Center Laboratory, Zhuhai Campus of Zunyi Medical College, China.

Materials

Newborn Sprague-Dawley rats within 1 day after birth, males or females, were provided by the Experimental Animal Center of Guangdong Medical College (license No. SCXK (Yue) 2008-0002).

Methods

Culture of hippocampal neurons

The hippocampal neurons were cultured *in vitro* as previously described^[29-32], with minor modifications. In brief, neonatal rats were killed by decapitation. The brains were removed aseptically, and bilateral hippocampi were isolated in cold D-Hank's solution. Hippocampal tissue was minced into pieces, digested with 0.25% trypsin at 37°C for 15 minutes, and incubated in DMEM/F12 complete medium (Gibco, Carlsbad, CA, USA). Then the cell suspension was filtered using 400-mesh and centrifuged at 1 000 r/min for 10 minutes; the centrifugal radius was 280 mm. After the supernatant was discarded, the cells were incubated with DMEM/F12 complete medium by pipetting, and cultured on polylysine-coated culture wells (Sigma, St. Louis, MO, USA) in 95% air and 5% CO₂ at 37°C in saturated humidity. After 48 hours of culture, the medium was replaced with cytarabine (1 × 10⁻⁵ mol/L; Sigma) for additional 48 hours to prevent the proliferation of glial cells. Afterwards, the culture medium was replenished with neurobasal medium (Gibco; containing B27). The neurons were identified using anti-NeuN antibody (Sigma).

SIN-1 and DIDS for neuronal culture

As previously reported^[12-13], after hippocampal neurons were cultured for 12 days, those cells in the control group were cultured with neurobasal complete culture medium for 18 hours. The neurons in the SIN-1 and the SIN-1 + DIDS groups were respectively cultured with neurobasal complete medium containing SIN-1 (Sigma) or SIN-1 plus DIDS (Sigma), respectively, for 18 hours. The final concentration of SIN-1 was 0.5 mmol/L and the final concentration was 0.1 mmol/L for DIDS.

MTT assay for neuronal survival rate

Hippocampal neurons in each group were incubated with 20 μL MTT (500 μg/mL; Sigma) in an incubator for 4 hours, as described previously^[33-35]. Culture fluid was aspirated carefully, 150 μL DMSO was added to each well and then triturated for 10 minutes to completely dissolve the purple crystalline particles. The absorbance value at 490 nm was measured using a microplate reader (ELX-800; Leica, Somme, Germany), and culture wells without neurons were used as the blank control. Cell viability (%) = (absorbance of experimental group – absorbance of blank well)/(absorbance of control group – absorbance of blank well) × 100%.

Immunofluorescence staining

Apoptosis was determined using immunofluorescence staining according to previously described methods^[36-38].

In brief, hippocampal neurons were rinsed with 0.1 mol/L PBS three times for 5 minutes, fixed in 4% paraformaldehyde for 30 minutes, rinsed with 0.1 mol/L PBS, and blocked with goat serum for 1 hour. Hippocampal neurons were incubated with rabbit anti-mouse CIC-3 antibody (1:1 000; Sigma) at 4°C overnight. Cells were rinsed with 0.1 mol/L PBS five times for 10 minutes each, blocked with goat serum for 1 hour, and fixed in 4% paraformaldehyde for 30 minutes. Then neurons were incubated with mouse anti-rat NeuN antibody (1:800; Sigma) at 4°C overnight, followed by 0.1 mol/L PBS washes and 4% paraformaldehyde fixation for 30 minutes. Finally, the neurons were incubated with goat anti-rabbit IgG (1:800; Sigma) or goat anti-mouse IgG (1:800; Sigma) at room temperature for 1 hour. After 0.1 mol/L PBS washes, 4% paraformaldehyde fixation, and Hoechst 33342 staining (final concentration 20 mg/L; Sigma) for 15 minutes, hippocampal neurons were observed under an inverted fluorescent microscope (Olympus 2X71; Olympus, Tokyo, Japan). Three fields of vision were randomly selected to count 200 cells in each, and the percentage of apoptotic cells was calculated.

Western blot analysis for caspase-3 and CIC-3 protein expression

Caspase-3 and CIC-3 protein expression was determined as previously described^[39-41]. In brief, hippocampal neurons were rinsed with ice-cold 0.1 mol/L PBS three times for 5 minutes each before cell lysate was added. Samples were centrifuged at 12 000 r/min for 15 minutes, with a centrifugal radius of 250 mm. The protein concentration of samples in each group was detected using the BCA method^[42]. Ten μL per well was loaded onto a sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, the proteins were transferred to polyvinylidene chloride film, and blocked with 5% skim milk at room temperature for 2 hours. The samples were incubated with rabbit anti-caspase-3 polyclonal antibody (1:300; Sigma), rabbit anti-mouse CIC-3 antibody (1:300; Boster, Wuhan, Hubei Province, China) and rabbit polyclonal β-actin antibody (1:500; Boster) at 4°C overnight. After washing with TBST buffer, samples were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5 000; Boster) at 37°C for 1 hour, and rinsed with TBST buffer. The images were developed using enhanced chemiluminescence (Boster) and exposed. The gray value of electrophoretic bands was analyzed using Image J software (Super D, Beijing, China), with high gray values indicating lower protein content. The results represent the ratio of each band gray value to β-actin.

Real-time PCR quantitative determination for CIC-3 mRNA expression

The target gene mRNA sequence was screened in GenBank (<http://www.ncbi.nlm.nih.gov/genbank>). Specific CIC-3 primers were designed in the coding sequence region and synthesized at Guangzhou Jetway Biotechnology Co., Ltd. (Guangzhou, Guangdong Province, China). The primer sequences are as follows: CIC-3 upstream primer 5'-CTA CTA CCA CCA CGA CTG-3', reverse primer 5'-TTG TCA CAC CAC CTA AGC-3', to amplify a 116 bp product. For controls, the GAPDH forward primer 5'-CTC CCA TTC CTC CAC CTT TG-3', downstream primer 5'-CCA CCA CCC TGT TGC TGT AG-3' were used to amplify a 110 bp product.

According to previously published methods^[43-45], the total RNA of neurons was extracted, detected by 1.0% agarose gel electrophoresis, and reverse transcribed. Quantitative analysis was performed with SYBR Green I: The PCR cycling conditions were: 93°C predenaturation for 2 minutes, then 40 cycles of 93°C denaturation for 30 seconds, 55°C annealing for 45 seconds, and 72°C extension for 30 seconds. After the PCR reaction was completed, the purity of the amplification product was analyzed using a melting curve at 72–95°C, with temperature intervals of 0.5°C for 6.0 seconds; and 30°C for 30 seconds. The results were analyzed using an image analysis system (ABI PRISM 7000 Sequence Detection System; Life Technologies, Shanghai, China) with the $2^{-\Delta\Delta Ct}$ relative quantification method^[46]. GAPDH served as the reference. To eliminate differences, each sample was tested three times and the average value was calculated.

Statistical analysis

The data are expressed as mean \pm SD and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) by one-way analysis of variance followed by least significant difference test. A value of $P < 0.05$ was considered significantly different.

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