



# Orexin-A protects against oxygen-glucose deprivation/reoxygenation-induced cell damage by inhibiting endoplasmic reticulum stress-mediated apoptosis via the Gi and PI3K signaling pathways



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## ABSTRACT

The neuropeptide orexin-A (OXA) has a neuroprotective effect, acting as an anti-apoptotic factor in response to multiple stimuli. Apoptosis induced by endoplasmic reticulum stress (ERS) underlies oxygen-glucose deprivation and reoxygenation (OGD/R)-induced cell damage, an in vitro model of ischemia/reperfusion injury. However, that OXA inhibits ERS-induced apoptosis in the OGD/R model has not been reported. In the present study, we investigated the neuroprotective effect of OXA (0.1  $\mu$ M) on OGD/R-induced damage in the human neuroblastoma cell line SH-SY5Y. After OXA treatment following 4 h oxygen-glucose deprivation (OGD) and then 4 h reoxygenation (R), cell morphology, viability, and apoptosis were analyzed by histology, Cell Counting Kit-8 assay, and flow cytometry, respectively. Western blotting was used to measure expression levels of ERS- and apoptosis-related proteins. To determine signaling pathways involved in OXA-mediated neuroprotection, the Gi pathway inhibitor pertussis toxin (PTX; 100 ng/mL) and PI3K inhibitor LY294002 (LY; 10  $\mu$ M) were added. In addition, in order to prove the specificity of these characteristics, the OXA antagonist Suvorexant (DORA; Ki of 0.55 nM and 0.35 nM for OX1R and OX2R) was used for intervention. Our results showed that OGD/R induced cell damage, manifested as morphological changes and a significant decrease in viability. Furthermore, Western blotting detected an increase in ERS-related proteins GRP78, p-IRE1 $\alpha$ , p-JNK, and Cleaved caspase-12, as well as apoptosis-related proteins Cleaved caspase-3 and Bax, and a decrease in the anti-apoptosis factor Bcl-2. OXA intervention alleviated the degree of cellular damage, and protein expression was also reversed. In addition, the protective effect of OXA was reduced by adding PTX and LY. Meanwhile, after the use of DORA, changes in the expression of related proteins were detected, and it was found that the protective effect of OXA was weakened. Collectively, our results indicate that OXA has a neuroprotective effect on OGD/R-induced cell damage by inhibiting ERS-induced apoptosis through the combined action of Gi and PI3K signaling pathways. These findings help to clarify the mechanism underlying the neuroprotective action of OXA, which should aid the development of further candidate drugs, and provide a new therapeutic direction for the treatment of ischemic stroke.

## 1. Introduction

Ischemic stroke is a serious global health problem. In recent years, research has focused on various measures to improve cerebral blood circulation, including thrombolytic, antiplatelet, and anticoagulation

therapies, vasodilation, and administration of neuroprotective drugs. At present, the key to alleviating ischemic brain injury is believed to be early recovery of blood flow, and reperfusion therapy such as thrombolysis is currently the most effective clinical treatment for cerebral ischemia. However, reperfusion therapy is limited by the time taken to

**Abbreviations:** CCK-8, Cell Counting Kit-8; DORA, Suvorexant; ERS, endoplasmic reticulum stress; GRP, glucose-regulated protein; HE staining, hematoxylin-eosin staining; I/R, ischemia/reperfusion; LY, LY294002; OXA, Orexin-A; OGD/R, oxygen-glucose deprivation and reoxygenation; OD, optical density; PTX, pertussis toxin

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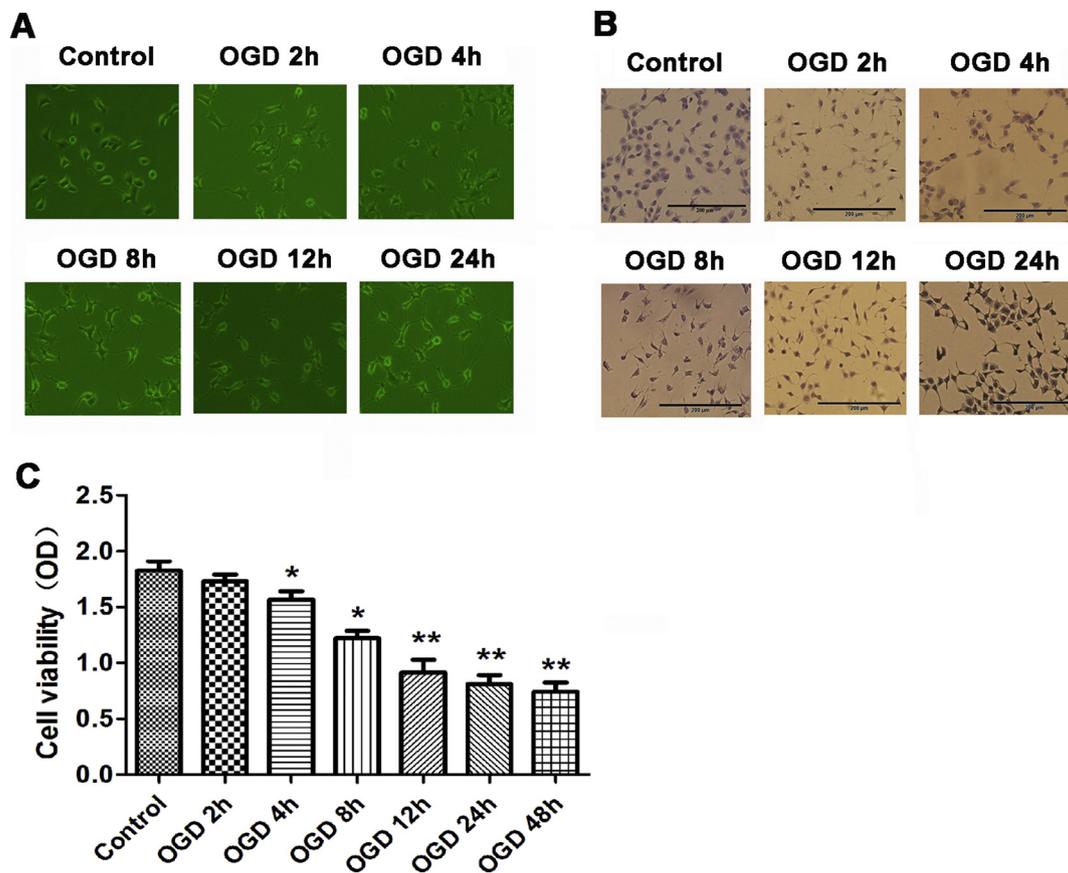
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**Fig. 1.** Morphology and viability of OGD-treated SH-SY5Y cells at different time points. A. Morphological changes of cells treated with OGD were observed under microscope. B. The morphological changes after OGD were observed by HE staining. C. Cell viability was measured at different time points of OGD. Compared with the control group, the cells shrinkage and prominent protuberances and to be spindle shaped or irregular, with little cytoplasm. And the cell viability gradually decreased with increasing duration of OGD. Data are expressed as the mean  $\pm$  standard deviation ( $n = 3$ ). \* $p < .05$ , \*\* $p < .01$  compared with the control group.

reperfusion ischemic tissues and the risk of reperfusion injury [1]. In the treatment of ischemic stroke, the urgent problems to be solved are how best to stimulate endogenous protection and enhance the resistance of nerve cells to ischemia and hypoxia.

Within eukaryotic cells, the endoplasmic reticulum (ER) regulates protein folding,  $Ca^{2+}$  homeostasis, and the stress response, and is thus very sensitive to stress stimuli. ER dysfunction can be induced by ischemia and hypoxia, glucose/nutrient deficiency, ATP depletion, free radicals, and  $Ca^{2+}$  homeostasis disturbance, all of which can ultimately trigger the response known as ER stress (ERS) [2]. ERS is mainly characterized by the up-regulation of glucose-regulated proteins (GRPs), calmodulin, and protein folding enzymes, and activation of apoptosis-promoting factors such as caspase-3 and caspase-12. To a certain extent, ERS can promote the restoration of ER function, and long-term ERS will destroy the stable state of cells, causing tissue damage and apoptosis [3]. The destruction of ER function and activation of apoptosis signaling pathways are the foundation of many neurodegenerative diseases, and one of the mechanisms of ischemia/reperfusion (I/R) injury [4].

Orexin-A (OXA) is a neuropeptide with neuroprotective effects against multiple stimuli. Intracerebroventricular injection of OXA into an experimental middle cerebral artery occlusion in rats can significantly reduce the volume of cerebral infarction [5]. Our laboratory has preliminarily confirmed that OXA has a neuroprotective effect against I/R injury in vivo [6] and against  $H_2O_2$  damage in vitro [7]. We also found that ERS participates in engendering I/R injury [6,8], but that the neuroprotective effect of OXA occurs by inhibiting ERS has not been shown. Therefore, in the present study the oxygen-glucose deprivation and reoxygenation (OGD/R) of SH-SY5Y neuroblastoma cells

was used as in vitro model of I/R injury to evaluate the protective effect of OXA on ERS-induced damage and the possible signaling pathways involved. Our aim was to explore the mechanism of the neuroprotective effect of OXA, and to provide the experimental basis for a new therapeutic approach for the clinical treatment of ischemic stroke.

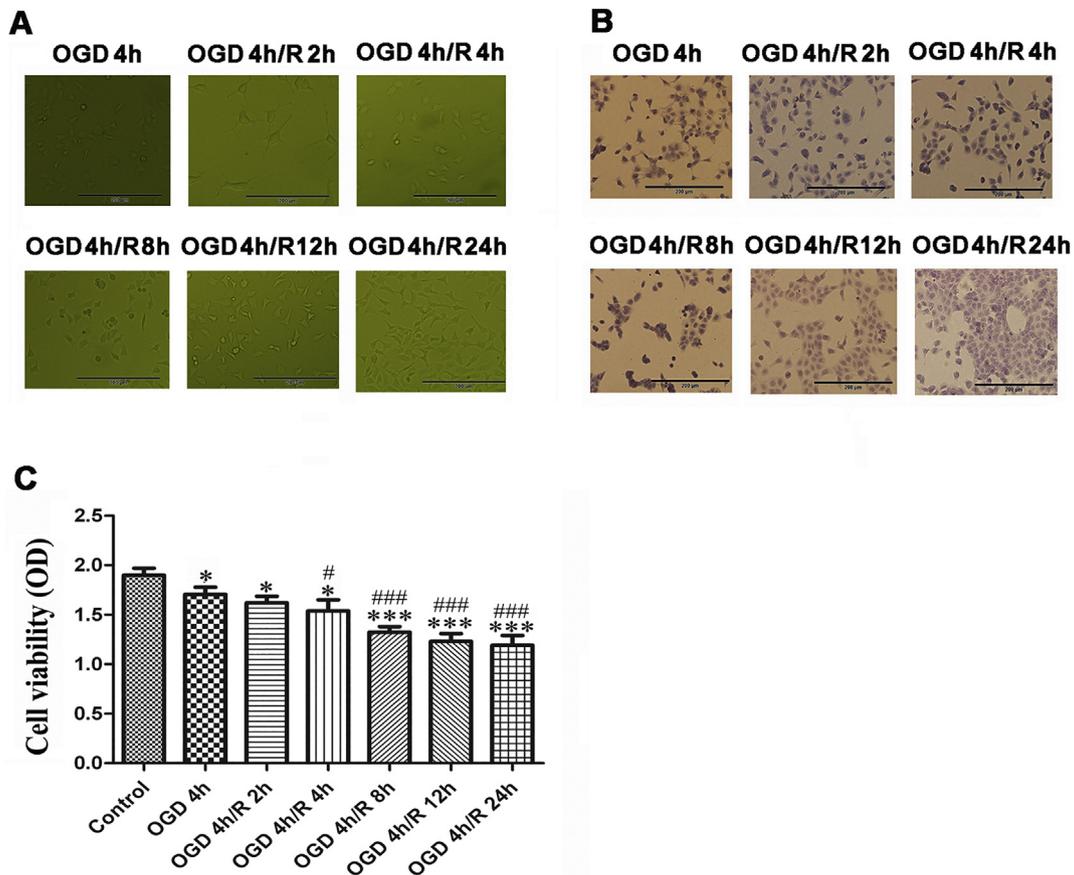
## 2. Materials and methods

### 2.1. Chemicals and reagents

Human OXA was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Primary antibodies against GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-3, Cleaved caspase-12, Bcl-2, and Bax were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti- $\beta$ -actin antibody was obtained from BZSGB Technology (Beijing, China). Suvarson (DORA), PI3K inhibitor LY294002 (LY) and Gi inhibitor pertussis toxin (PTX) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Life Technologies (Grand Island, NY, USA).

### 2.2. Cell culture

The human neuroblastoma cell line SH-SY5Y, obtained from the Cell Resource Center Chinese Academy of Sciences (Shanghai, China), was cultured in DMEM with 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin at 37  $^{\circ}$ C in a humid atmosphere containing 5%  $CO_2$  and 95% air. SH-SY5Y cells were incubated in 96- or 6-well plates, and used in subsequent experiments once the cells had grown to the appropriate concentration.



**Fig. 2.** Morphology and viability of SH-SY5Y cells at different time points of reperfusion after OGD 4 h. A. Morphological changes of cells treated with OGD/R were observed under microscope. B. The morphological changes after OGD/R were observed by HE staining. C. Cell viability was measured at different time points of reperfusion. Compared with the OGD 4 h group, the cell morphology had recovered to normal, the number of cells had increased, and the boundary had blurred, and the cell viability decreased more obviously as the reoxygenation time was increased. Data are expressed as the mean  $\pm$  standard deviation ( $n = 3$ ). \* $p < .05$ , \*\*\* $p < .001$  compared with the control group; # $p < .05$ , ### $p < .001$  compared with the OGD 4 h group.

### 2.3. OGD/R injury and experimental design

To achieve I/R injury-like conditions in vitro, SH-SY5Y cells were treated with OGD/R [9]. Briefly, the cells were exposed to OGD conditions comprising culture in glucose-free medium in an atmosphere of  $< 0.1\%$   $O_2$  in  $5\%$   $CO_2$  and  $95\%$   $N_2$  for several hours, after which they were returned to glucose-containing medium and cultured under the standard conditions of  $95\%$   $O_2$  and  $5\%$   $CO_2$  for several hours. To determine the appropriate degree of cell damage, the state of cells exposed to OGD followed by reoxygenation (R) conditions for different periods of time was evaluated. Treatment of SH-SY5Y cells with OGD for 4 h followed by reoxygenation for 4 h was determined to cause an appropriate I/R injury-like state, after which cells were treated with OXA. Studies [10,11] have shown that OXA acts via the G protein subunit  $G_i$  signaling pathway and PI3K signaling pathway; the effect of adding the inhibitor PTX or LY 30 min before OXA intervention was, therefore, also investigated. Each treatment group consisted of at least three replicates.

### 2.4. Hematoxylin-eosin (HE) staining

HE staining was used to observe morphology after fixation of cultured SH-SY5Y cells. The covered slides with cells were washed thrice with phosphate-buffered saline (PBS), fixed in  $95\%$  alcohol for 15 min, and then washed twice in PBS for 1 min. Slides were stained with hematoxylin dye for 10 min then rinsed with running water, stained with differentiated solution for 30 s then rinsed with running water, and finally stained with eosin dye for 5 min then rinsed with running water.

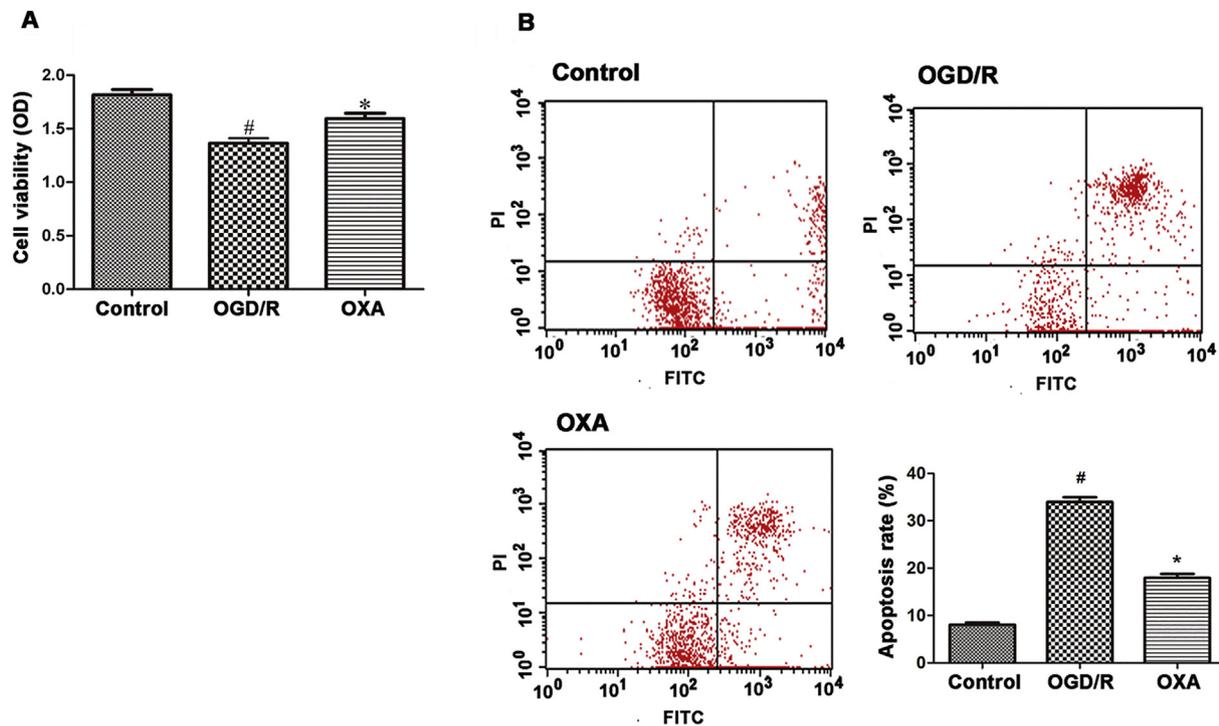
After staining, the cell climbing were dehydrated and transparent in sequence, and finally residence was dripped and fixed on the glass slide.

### 2.5. Cell viability measurement

The cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay (KeyGEN BioTECH Corp., Nanjing, China) according to the manufacturer's instructions. SH-SY5Y cells were cultured in 96-well plates at an initial density of  $10 \times 10^4$  per well. The incubation and treatment of cells were carried out according to the experimental requirements. CCK-8 reagent ( $10 \mu\text{L}$ ) was added to each well and incubated for 2 h, after which the optical density (OD) at  $450 \text{ nm}$  wavelength was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). All assays were repeated at least three times.

### 2.6. Cell apoptosis measurement

Apoptosis of SH-SY5Y cells was detected by flow cytometry with an Annexin V- FITC Apoptosis Detection Kit (KeyGEN BioTECH Corp.). According to the manufacturer's instructions, the cells were repeatedly blown after being cleaved by trypsin and dispersed into single cells. After centrifugation, the cells were collected and washed twice with PBS, and suspended in Binding Buffer, and then  $5 \mu\text{L}$  Annexin V-FITC and  $5 \mu\text{L}$  propidium iodide were added and reacted in the dark for 15 min. After reaction, fluorescence was measured by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). All assays were repeated at least three times.



**Fig. 3.** The effects of OXA on cell viability and apoptosis after OGD/R. A. Cell viability of the OGD/R and OXA intervention group was measured. B. Apoptosis was detected by flow cytometry. OXA could indeed alleviate the decline in cell viability induced by OGD/R and inhibit apoptosis induced by OGD/R damage. Data are expressed as the mean  $\pm$  standard deviation ( $n = 3$ ). #  $p < .05$  compared with the control group; \*  $p < .05$  compared with the OGD/R group.

## 2.7. Western blotting assay

Protein extracted from the SH-SY5Y cell lysate was separated by SDS-PAGE, then transferred to a polyvinylidene fluoride membrane. After blocking with 5% milk for 1 h at room temperature, and washing with Tris Buffered saline Tween (TBST), the membrane was incubated with primary antibody at 4 °C overnight. Following washing, the membrane was incubated at room temperature for 1 h with the corresponding secondary antibody labeled with horseradish peroxidase. Bands were revealed using an ECL kit according to the manufacturer's instructions. Image processing and analysis were carried out with ImageJ2x software.

## 2.8. RT-PCR

Total RNA of SH-SY5Y cells from different treatment groups were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of RNA was determined by A BioSpec-nano spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Then, the cDNA was reverse transcribed using FastQuant cDNA first-strand synthesis kit (TIANGEN Biotechnology, Beijing, China) for PCR amplification. Amplification primers were synthesized by Biological Technology Co., Ltd. (Shanghai, China). Primer sequences were as follows:  $\beta$ -actin (human): forward: 5'-acactgtgccatctacagggg-3', reverse: 5'-atgatggagttgaaggtatgttctggat-3'; OX1R (human): forward: 5'-ttaagagggtgttcgggatg-3', reverse: 5'-actagggccttcagagagc-3'. OX2R (human): forward: 5'-atttgggatgtttgccata-3', reverse: 5'-atgttgaactccaaggcaac-3'. The PCR reaction procedure was pre-denaturation at 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 55 °C for 40 s, and 72 °C for 40 s, followed by 72 °C for 10 min and 4 °C preservation. The productions were detected by 1% agarose gel electrophoresis. Image processing and analysis were carried out with ImageJ2x software.

## 2.9. Statistical analysis

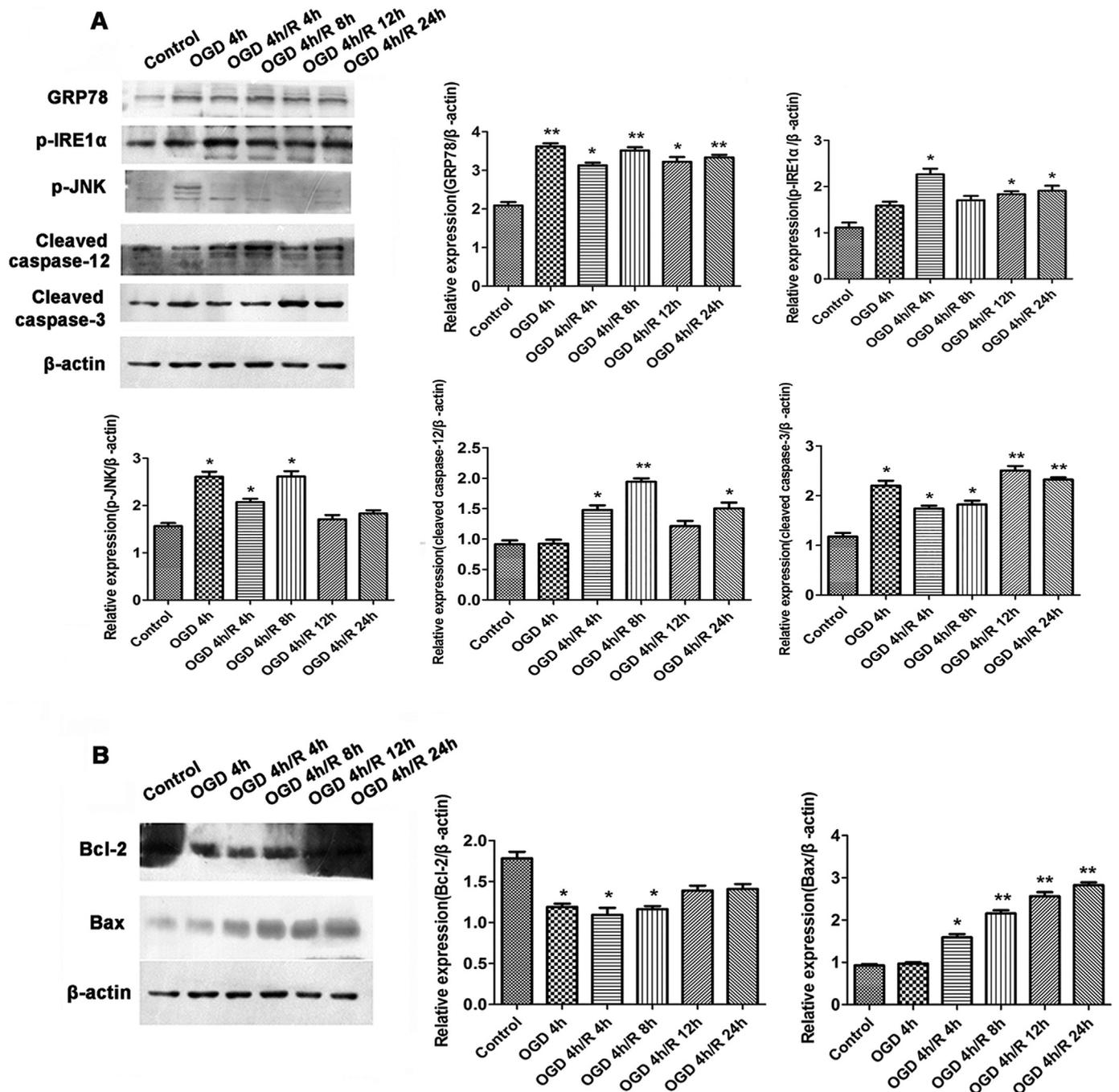
The experimental data were analyzed using GraphPad Prism 5 software, and the results are expressed as mean  $\pm$  standard deviation. All data shown are representative of at least three independent experiments. Comparisons between two experimental groups were made by *t*-tests, and differences between groups were tested using a one-way analysis of variance (ANOVA). Multiple comparison between the groups was performed using S-N-K method;  $p < .05$  was considered significant.

## 3. Results

### 3.1. Determination of the optimal time point for OGD

To determine the optimal duration of oxygen and glucose deprivation for modeling ischemic injury in vitro, SH-SY5Y cells were deprived of oxygen and glucose for 2 h (OGD 2 h), 4 h (OGD 4 h), 8 h (OGD 8 h), 12 h (OGD 12 h), and 24 h (OGD 24 h). Microscopy and HE staining were used to observe cell morphology. As shown in Fig. 1, SH-SY5Y cells cultured under normal conditions had clearly defined round or oval shaped nuclei, and abundant cytoplasm. After OGD treatment, the morphology changed, showing cell shrinkage and prominent protuberances (Fig. 1A). HE staining revealed the cells to be spindle shaped or irregular, with little cytoplasm (Fig. 1B). Moreover, as the duration of OGD increased, the changes in cell morphology became more obvious.

To determine the optimal time point for the OGD model, a CCK-8 assay was used to measure cell viability at the different time points. As shown in Fig. 1C, compared with the control group, the cell viability gradually decreased with increasing duration of OGD. Moreover, the cell viability of SH-SY5Y cells was significantly decreased at OGD 4 h ( $p < .05$ ), with a reduction of 17.7%, and the cell viability had decreased by 29.7% at OGD 8 h ( $p < .05$ ). Even by OGD 48 h, the cell survival rate had decreased by 56.5% ( $p < .01$ ). As a starting point for



**Fig. 4.** Expression of ERS-related and apoptosis-related proteins detected by Western blotting. **A.** ERS-related proteins GRP78, p-IRE1α, p-JNK, Cleaved caspase-12, and Cleaved caspase-3. **B.** Apoptosis-related proteins Bcl-2 and Bax. Compared with the control group, the expression of GRP78, p-IRE1α, p-JNK, Cleaved caspase-12, Cleaved caspase-3 and Bax increased after OGD/R treatment, and the expression of Bcl-2 decreased. Data are expressed as the mean ± standard deviation (n = 3). \*p < .05, \*\*p < .01 compared with the control group.

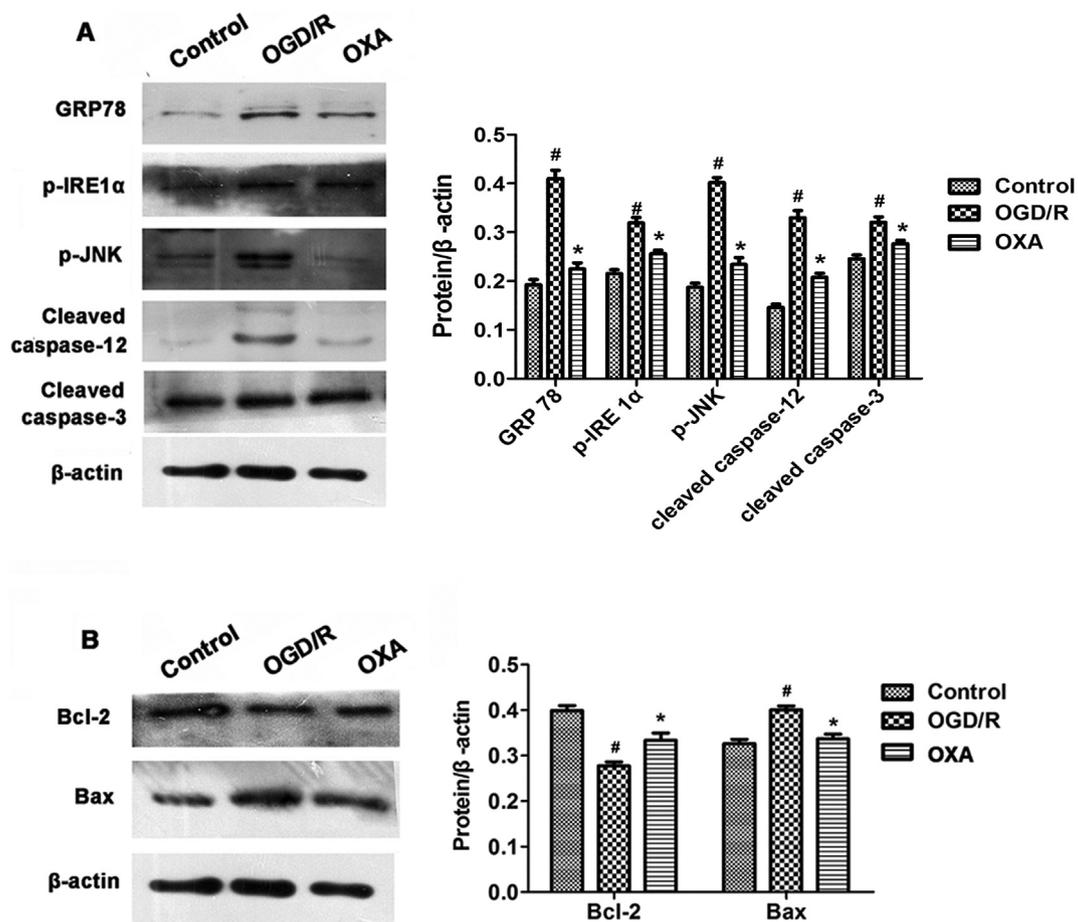
modeling reperfusion injury, and considering the injury effect of reperfusion, the optimal time point of OGD was determined as 4 h.

**3.2. Determination of the optimal time point for reoxygenation after OGD**

To determine the optimal time point for reoxygenation after OGD to model I/R injury in vitro, The SH-SY5Y cells were re-exposed to oxygen and glucose for 2 h (R 2h), 4 h (R 4h), 8 h (R 8h), 12 h (R 12h), and 24 h (R 24h). Microscopy and HE staining were used to observe cell morphology. After oxygen and glucose were restored, fluorescence microscopy demonstrated a change in cell morphology: the slender protrusions gradually disappeared, and the cell shape became blunt or

tapered (Fig. 2A). HE staining showed that the cell morphology had recovered to normal, the number of cells had increased, and the boundary had blurred (Fig. 2B).

The CCK-8 assay was also used to measure cell viability after restoring oxygen and glucose for different lengths of time after OGD 4 h. As shown in Fig. 2C, after OGD 4 h, compared with the control group, the cell viability had decreased by 14.7% (p < .05), and by 25.1% at OGD 4 h/R 4 h (p < .05). The cell viability decreased more obviously as the reoxygenation time was increased. At R 24 h, the cell viability had decreased by 41.4% (p < .001). Microscopy revealed that the number of cells increased during reoxygenation. After comprehensive consideration, the optimal timings for OGD/R were determined to be



**Fig. 5.** Effect of OXA on the expression of ERS-related proteins detected by Western blotting. A. The expression levels of GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-12, and cleaved caspase-3. B. The expression levels of Bcl-2 and Bax. Compared with cells treated with OGD/R alone, intervention with OXA significantly decreased the expression of GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-3, Cleaved caspase-12 and Bax, and Bcl-2 increased. Data are expressed as the mean  $\pm$  standard deviation ( $n = 3$ ). <sup>#</sup> $p < .05$  compared with the control group; <sup>\*</sup> $p < .05$  compared with the OGD/R group.

OGD 4 h/R 4 h, and these, denoted OGD/R hereafter, were used in all subsequent experiments.

### 3.3. OXA protects against OGD/R injury

To determine whether OXA could alleviate the reduction in cell viability induced by OGD/R, the CCK-8 assay was used to detect viability in OGD/R-treated cells subsequently treated with OXA (OXA intervention group) or not. Compared with untreated cells (control group), the viability of SH-SY5Y cells was significantly decreased after OGD/R treatment ( $p < .05$ ), by about 22%. After OXA intervention, the cell viability increased significantly by 18.6% ( $p < .05$ ), compared with that of the OGD/R only-treated group (Fig. 3A). The results showed that OXA could indeed alleviate the decline in cell viability induced by OGD/R.

To further clarify the protective effect of OXA on OGD/R-induced SH-SY5Y cell damage, flow cytometry was used to detect apoptosis. Compared with the control group, the apoptosis rate in the OGD/R group significantly increased by about 26.1% ( $p < .05$ ), while the apoptosis rate in the OXA intervention group decreased by about 18.2% ( $p < .05$ ), compared with the OGD/R only-treated group (Fig. 3B). OXA is therefore able to inhibit apoptosis induced by OGD/R damage.

### 3.4. OXA inhibits ERS via Gi and PI3K signaling pathways

#### 3.4.1. OGD/R triggers ERS

To evaluate whether ERS is involved in OGD/R-induced cell

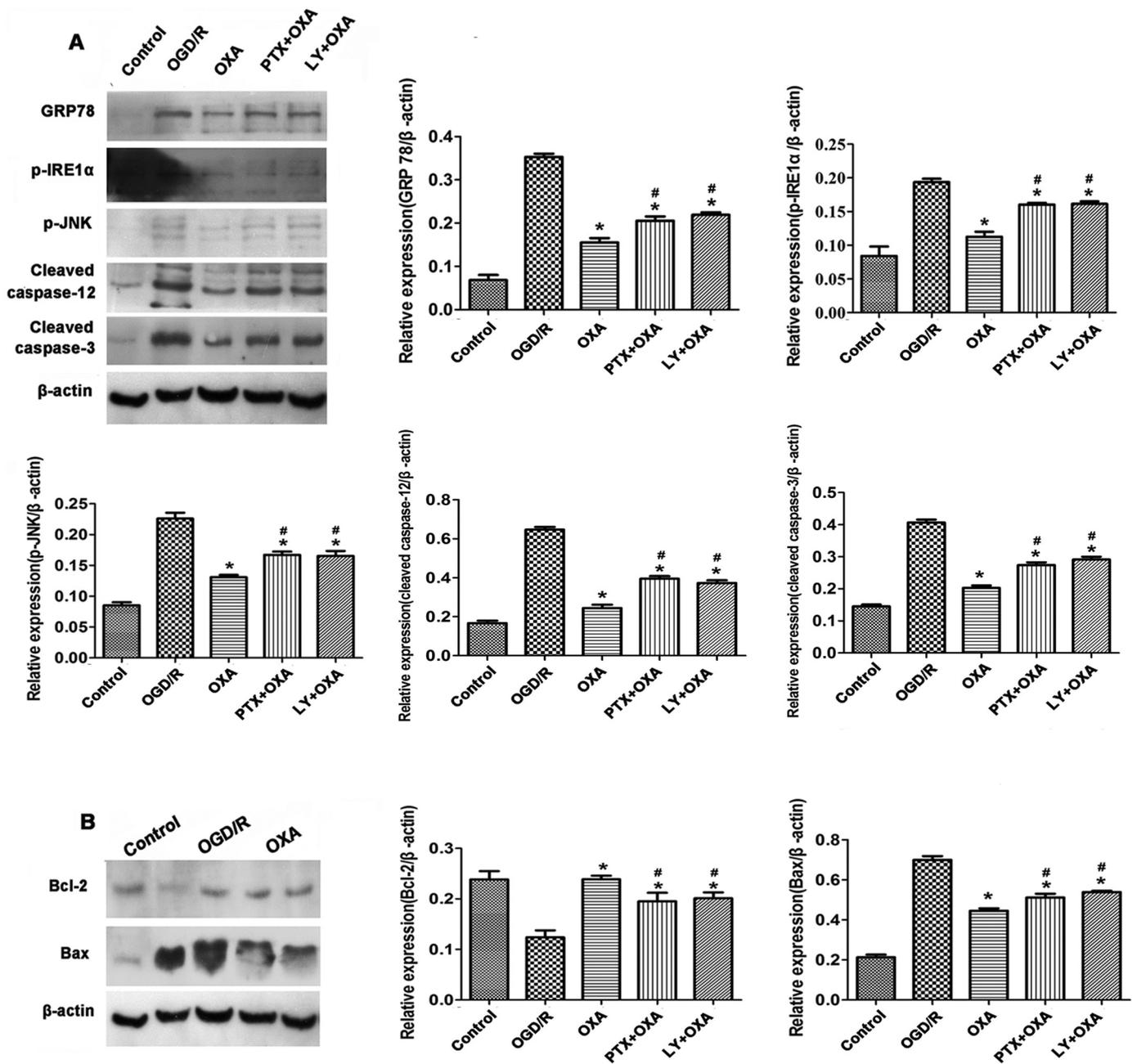
damage, the expression of ERS-related proteins was analyzed by Western blotting after OGD/R. As shown in Fig. 4, compared with the control group, the expression of the ERS-related proteins GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-3, and Cleaved caspase-12 significantly increased after OGD/R treatment ( $p < .05$  and  $p < .01$ ). Compared with the control group, the expression of the apoptosis-related protein Bax increased ( $p < .05$  and  $p < .01$ ), while the expression of the anti-apoptosis factor Bcl-2 decreased ( $p < .05$ ). The results therefore demonstrated that OGD/R does indeed trigger ERS and apoptosis.

#### 3.4.2. OXA reduces ERS

Having confirmed that OGD/R causes changes in the expression of ERS-related and apoptosis-related proteins, we investigated the effect of OXA on these changes. Compared with cells treated with OGD/R alone, intervention with OXA significantly decreased the expression of GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-3, and Cleaved caspase-12 ( $p < .05$ ). In addition, the expression of Bax decreased ( $p < .05$ ), and that of Bcl-2 increased ( $p < .05$ ; Fig. 5). Therefore, intervention with OXA can protect from ERS and apoptosis induced by OGD/R.

#### 3.4.3. OXA inhibits ERS via Gi and PI3K signaling

Having confirmed that OGD/R triggers apoptosis induced by ERS, and that OXA can inhibit ERS and apoptosis induced by OGD/R, we then used kinase inhibitors PTX and LY to elucidate signaling pathways involved in OXA activity. Compared with cells treated with OGD/R followed by OXA intervention alone, the results of Western blotting showed that the expression of GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved



**Fig. 6.** Protein expression after adding inhibitors detected by Western blotting. **A.** The expression levels of GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-12, and cleaved caspase-3. **B.** The expression levels of Bcl-2 and Bax. The expression of GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-3, Cleaved caspase-12 and Bax in the PTX + OXA group and the LY + OXA group increased, and the expression of Bcl-2 decreased. Data are expressed as the mean  $\pm$  standard deviation ( $n = 3$ ). \* $p < .05$  compared with the OGD/R group; # $p < .05$  compared with the OXA group.

caspase-3, and Cleaved caspase-12 in the PTX + OXA group and the LY + OXA group increased ( $p < .05$ ), the expression of Bax increased ( $p < .05$ ), and the expression of Bcl-2 decreased ( $p < .05$ ). This indicated that blocking either the Gi or the PI3K pathway influenced the activity of OXA. Meanwhile, compared with the OGD/R group without OXA, the expression of GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-3, and Cleaved caspase-12 in the PTX + OXA and LY + OXA groups decreased ( $p < .05$ ), the expression of Bax decreased ( $p < .05$ ), and the expression of Bcl-2 increased ( $p < .05$ ; Fig. 6). The results indicate that OXA plays a neuroprotective role by blocking the Gi and PI3K pathways.

### 3.5. OXA protects cells from OGD/R damage via Gi and PI3K signaling

To determine the pathways through which OXA attenuates OGD/R-induced cell damage, the CCK-8 assay was used to evaluate viability in OGD/R-treated cells with OXA intervention after PTX or LY treatment. As shown in Fig. 7A, compared with the OGD/R group, the viability of the OXA intervention group was significantly increased by 17.2% ( $p < .05$ ). Meanwhile, compared with the OXA intervention group, cell viability in the PTX + OXA and LY + OXA groups decreased by 5.9% and 5.2%, respectively ( $p < .05$ ), which confirmed that blocking the Gi or the PI3K pathway had a specific effect on the action of OXA. Above all, OXA can act via either the Gi or the PI3K pathway.

To further analyze the protective effect of OXA intervention after PTX and LY treatment, flow cytometry was used to detect apoptosis in

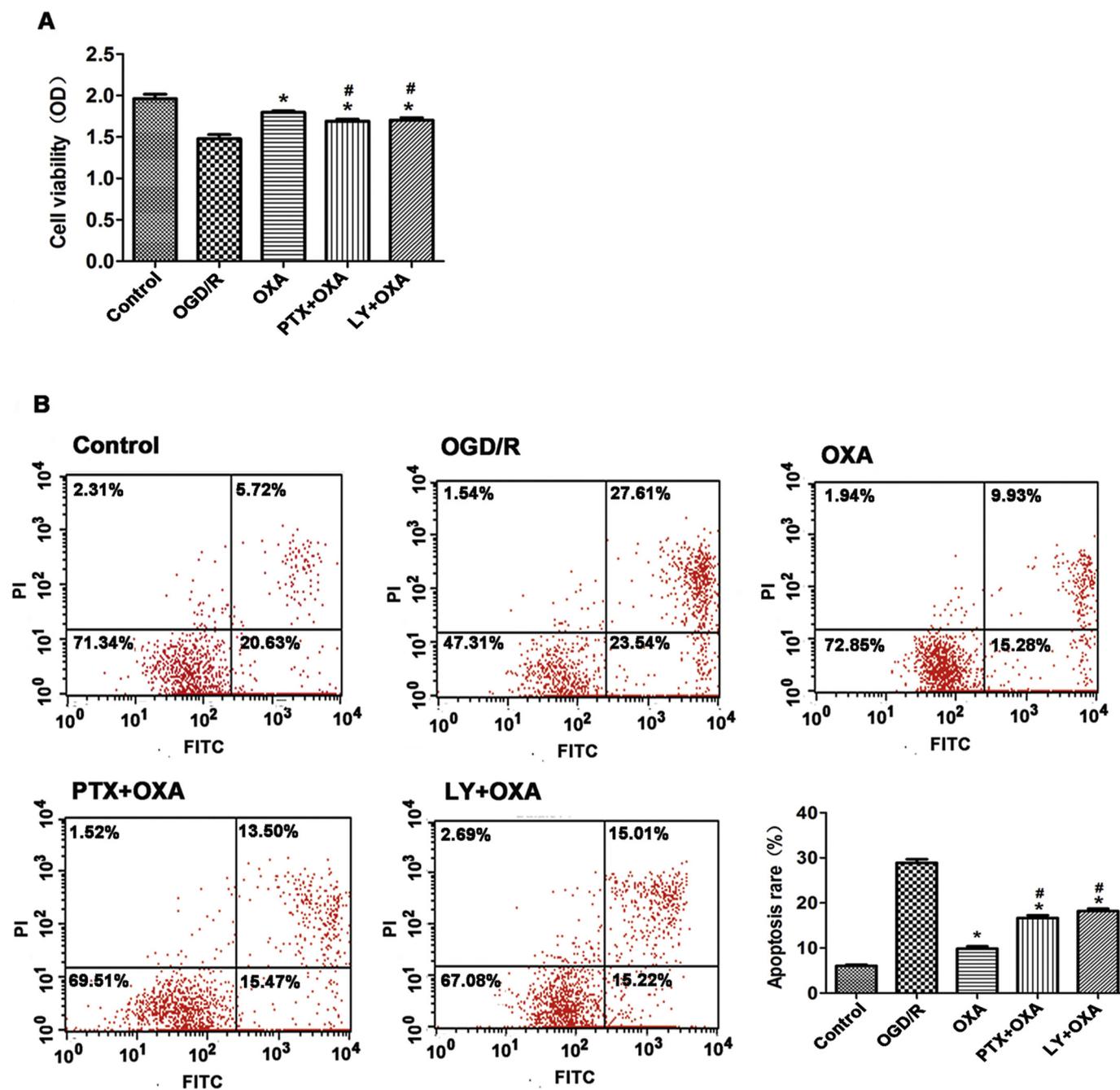
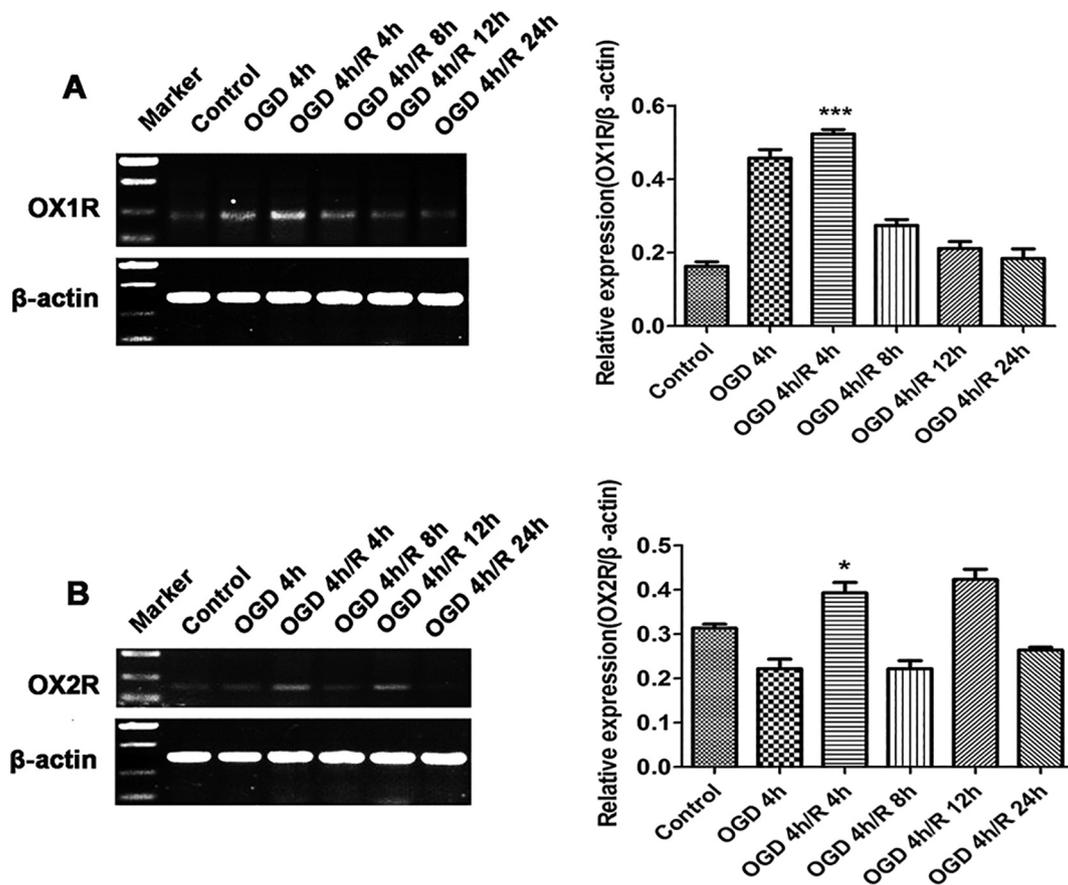


Fig. 7. Cell viability and apoptosis after treating with Gi and PI3K inhibitors. A. Cell viability after OXA intervention following PTX and LY treatment in the OGD/R model. B. Apoptosis detected by flow cytometry. Compared with the OXA intervention group, cell viability in the PTX + OXA and LY + OXA groups decreased, the apoptosis rate increased. Data are expressed as the mean ± standard deviation (n = 3). \**p* < .05 compared with the OGD/R group; #*p* < .05 compared with the OXA group.

cells damaged by OGD/R after adding PTX or LY. As shown in Fig. 7B, compared with the OGD/R group, the apoptosis rate in the OXA intervention group was reduced by 18.9% (*p* < .05). Compared with the OXA intervention group, the apoptosis rate of cells in the PTX + OXA group increased by 6.7% (*p* < .05), and that of cells in the LY + OXA group increased by 8.3% (*p* < .05), which indicated that the protective effect of OXA was weakened after PTX or LY had blocked the corresponding pathway. In summary, OXA appears to act via the Gi and PI3K pathways, and both pathways are required.

### 3.6. Determination of OX1R and OX2R

We have confirmed that the impact of orexin was mediated by Gi and PI3K activations. Further to demonstrate the specificity of these properties, the use of orexin antagonist as suvorexant (DORA) had been investigated. First, we used RT-PCR to investigate the expression of OX1R and OX2R in SH-SY5Y cells. As shown in Fig. 8A, The expression of OX1R was significantly changed in the OGD/R process of SH-SY5Y cells, and the OGD 4 h/R 4 h group was significantly higher than the Control group (*p* < .001). Moreover, OX1R expression increased first and then decreased. It can be seen from Fig. 8B that the expression of OX2R is also significantly changed, and the OGD 4 h/R 4 h group is



**Fig. 8.** The expression of OX1R and OX2R at different time points of OGD/R in SH-SY5Y cells. A. The expression of OX1R were detected by RT-PCR. B. The expression of OX2R were detected by RT-PCR. The expression of OX1R and OX2R was significantly changed compared with the Control group. Data are expressed as the mean  $\pm$  standard deviation ( $n = 3$ ). \* $p < .05$ , \*\*\* $p < .001$  compared with the Control group.

significantly higher than the Control group ( $p < .05$ ), but it does not follow a certain rule to produce changes.

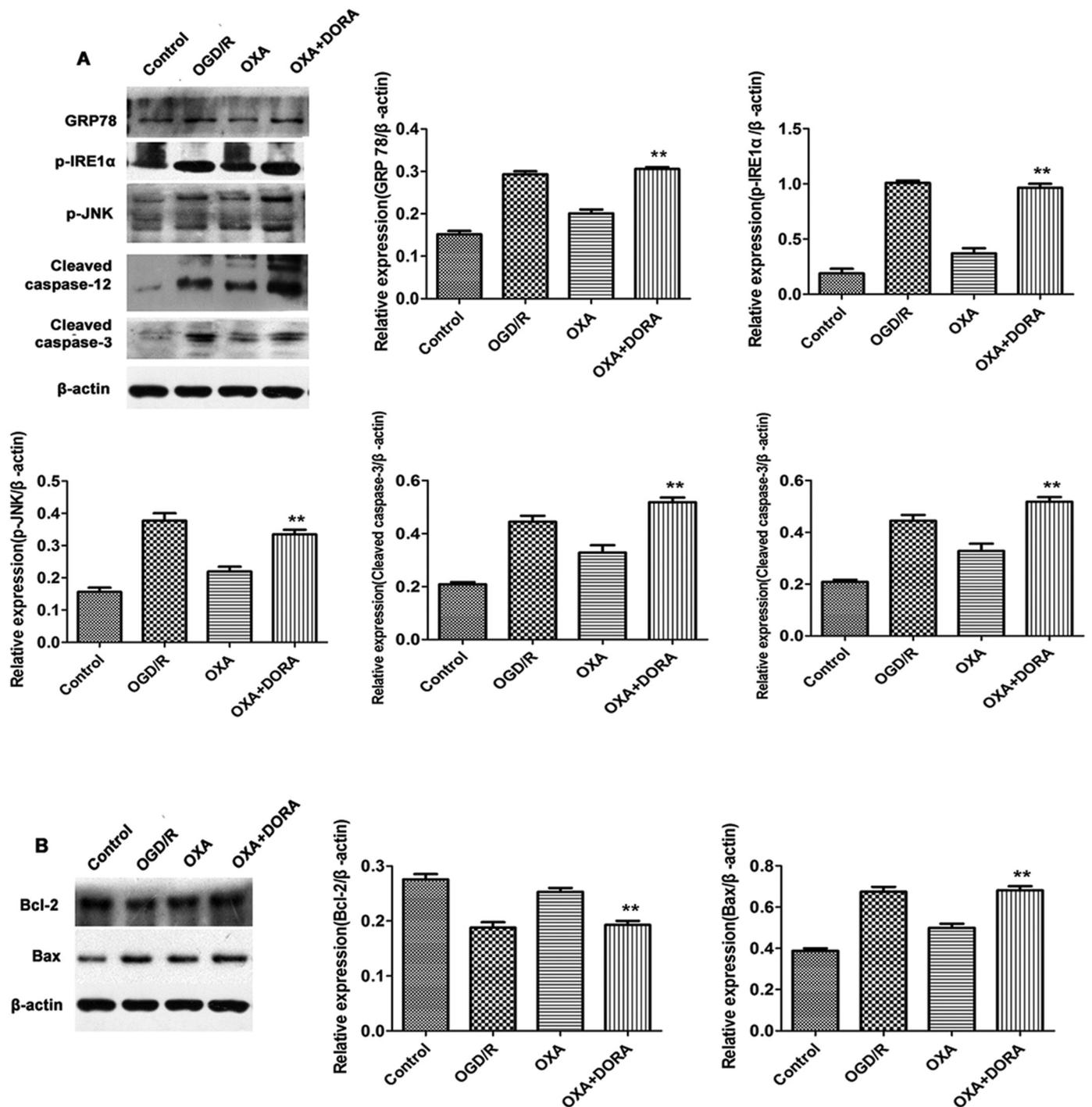
On this basis, DORA and OXA co-treatment groups were added, and Western blotting was used to detect the changes of corresponding protein expression after DORA intervention. As shown in Fig. 9, the expression of GRP78, p-IRE1 $\alpha$ , p-JNK, Cleaved caspase-3, and Cleaved caspase-12 in the OXA + DORA group increased ( $p < .01$ ), the expression of Bax increased ( $p < .01$ ), and the expression of Bcl-2 decreased ( $p < .01$ ), compared with the OXA group. It is indicated that the protective effect of OXA cannot be exerted after the blocking of OX1R and OX2R, which is consistent with the traditional view that OXA needs to bind to corresponding receptors to exert its effect, as shown in Fig. 10.

#### 4. Discussion

ERS is implicated in I/R and ischemia-hypoxia injury, and the damaged cells generate stress protection measures through ERS itself. However, severe or persistent ERS activates apoptosis signaling pathways, induces cell apoptosis, and aggravates cerebral I/R injury. Studies have confirmed that ERS participates in I/R and OGD/R injury, suggesting that ERS plays an important role in cerebral I/R injury [12–14]. Inhibition of ERS after I/R injury could be used as a key therapeutic approach for neuroprotection. In the present study, we designed an in vitro model of cerebral I/R injury and found that expression of a key ERS protein GRP78 was increased after OGD/R, indicating that OGD/R could induce ERS. The phosphorylation level of proteins downstream GRP78, including PERK, IRE1 $\alpha$ , and ATF6 was simultaneously investigated, but only p-IRE1 $\alpha$  was increased, implying that OGD/R induces the IRE1 $\alpha$  signaling pathway. JNK and caspase-12 downstream of

IRE1 $\alpha$  were also induced by OGD/R. Some studies have confirmed that caspase-12, one of the caspase family of proteases, is located in the ER and is activated by ERS [15], which in turn activates cytoplasmic caspase-3 [16], culminating in ERS-mediated apoptosis. In the present study, we also found caspase-3 to be induced by OGD/R. Based on our results, we consider that ERS-mediated apoptosis induced by OGD/R is mainly carried out through GRP78/IRE1 $\alpha$ /JNK and GRP78/IRE1 $\alpha$ /caspase-12/caspase-3 pathways, findings that help to elucidate the mechanism of ERS-mediated apoptosis (Fig. 8).

OXA is a neuropeptide secreted by the hypothalamus and has many physiological functions. Previous studies have revealed that OXA can promote the survival of cultured neurons derived from rat cerebral cortex [17], providing new insights for OXA as a potential neuroprotective factor. Other studies have shown that the regulation of post-ischemic glucose intolerance by OXA suppresses cerebral ischemic neuronal damage, providing evidence of the neuroprotective effect of OXA [18,19]. Studies on OXA and I/R injury are now gathering pace. OXA has a protective effect on I/R-induced gastric mucosa injury by reducing neutrophil activation and lipid peroxidation [20]. In in vivo experiments, OXA has been confirmed to change the pathological mechanism of cerebral ischemia and have a neuroprotective effect [21]. Consistent with these reports, we also found in the present study that OXA was neuroprotective in the OGD/R model by inhibiting apoptosis and promoting viability. The mechanism via which OXA alleviates cerebral I/R injury, however, is currently unclear. It is well known that ERS is involved in cerebral I/R injury. Therefore, we hypothesized that in the OGD/R in vitro model of I/R injury the neuroprotective effect of OXA occurs through its reduction of ERS-mediated apoptosis. Consistent with this hypothesis, we found that OXA did indeed produce an anti-apoptosis effect by reducing the expression of members of the ERS-



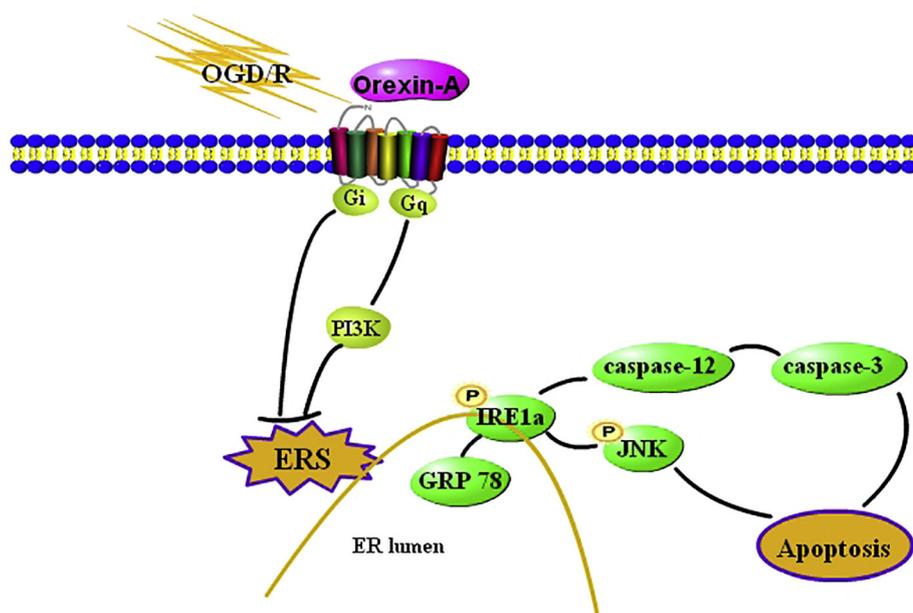
**Fig. 9.** Effect of DORA on the expression of ERS-related proteins detected by Western blotting. A. The expression levels of GRP78, p-IRE1α, p-JNK, Cleaved caspase-12, and cleaved caspase-3. B. The expression levels of Bcl-2 and Bax. The expression of GRP78, p-IRE1α, p-JNK, Cleaved caspase-3, Cleaved caspase-12 and Bax in the OXA + DORA group increased, and the expression of Bcl-2 decreased, compared with the OXA group. Data are expressed as the mean ± standard deviation (n = 3). \*\**p* < .01 compared with the OXA group.

related signaling pathways GRP78/IRE1α/JNK and GRP78/IRE1α/caspase-3/caspase-12, which provides new mechanistic evidence of the neuroprotective effect of OXA.

Studies have shown that the Gi pathway [22,23] and Gq pathway [24–26] are involved in the action of OXA, and other signaling pathways such as cAMP, MAPK-ERK1/2, PI3K/Akt, and JNK are also involved [25]. Further studies have confirmed that OXA plays a protective role in diseases and pathophysiological processes through the PI3K/AKT pathway [27–29]. However, there is little research on how OXA protects against cerebral I/R injury. Since we showed in our initial

experiments that OXA can protect against OGD/R-induced damage by inhibiting ERS-induced apoptosis, we hypothesized that OXA activates the Gi or the PI3K signaling pathway. Treatment with OXA after intervention with the Gi inhibitor PTX and the PI3K inhibitor LY in the in vitro OGD/R model revealed that the protective effect of OXA could be weakened after blocking each of the two pathways. Considering the complexity of physiological functions of cells and organisms, it is entirely possible. Whether other pathways are also involved now needs to be explored.

In conclusion, OGD/R triggered ERS, while OXA intervention



**Fig. 10.** Scheme of the mechanism of action of OXA. Treatment with OXA in the OGD/R model attenuates apoptosis induced by ERS through the Gi and PI3K pathways. OGD/R leads to severe damage of ER function, and unfolded protein accumulates in the ER and activates IRE1 $\alpha$ , an ERS sensor protein, to induce the ER response. IRE1 $\alpha$  is triggered by dissociation of GRP78 from the ER luminal domains. When ER function is seriously damaged, activation of the JNK and caspase-12 pathways induces apoptosis. Moreover, through the Gi and PI3K pathways, OXA attenuates apoptosis induced by ERS.

reduced the cellular damage induced by ERS. The use of inhibitors verified that the pathways involved in the neuroprotective effect of OXA in the OGD/R model were the Gi and the PI3K pathways. These findings provide a basis for further study of the role and mechanism of action of OXA, and for exploring a new potential therapeutic approach in the clinical treatment of I/R injury.

#### Author's contribution

C.-M.W. and J.C. conceived and designed the experiments; T.-T.K. and K.-X. Q. conducted the experiments and wrote the manuscript; M.-H. L. and C.-Q.Y. performed the data analysis; Y.-Y.P. contributed to the cell culture; B.-H.C. and B.B. revised the manuscript.

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#### Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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