Original Article

YBX1 Protects against Apoptosis Induced by Oxygen-Glucose Deprivation/Reoxygenation in PC12 Cells via Activation of the AKT/GSK3β Pathway

(YBX1 / AKT/GSK3β pathway / ischaemic stroke / cerebral ischaemia/reperfusion injury)

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Abstract. Reperfusion therapies for ischaemic stroke can induce secondary injury accompanied by neuronal death. The Y-box binding protein 1 (YBX1), an oncoprotein, is critical for regulating tumour cell proliferation and apoptosis. Thus, we wanted to know whether YBX1 could regulate neuronal cell apoptosis caused by cerebral ischaemia/reperfusion (I/R). We established a model of cerebral I/R-induced injury *in vitro* by oxygen-glucose deprivation/reoxygenation (OGD/R) treatment and determined YBX1 expression using Western blot. Next, the effect of YBX1 on the apoptosis and viability of OGD/R-treated PC12 cells was evaluated by flow cytometry, MTT assay, and Western blot. Besides, the release of lactate dehydrogenase (LDH) and the activity of catalase (CAT) and superoxide dismutase (SOD) were detected to evaluate oxidative stress of PC12 cells induced by OGD/R. The regulatory roles of YBX1 in the AKT/GSK3β pathway were examined by Western blot. As a result, OGD/R treatment down-regulated YBX1 expression in PC12 cells. YBX1 over-expression attenuated the growth inhibition and apoptosis of PC12 cells induced by OGD/R. Besides, the increase of LDH release and the decrease of SOD and CAT activities caused by OGD/R were reversed by YBX1 over-expression. Moreover, YBX1 over-expression could activate the AKT/GSK3β pathway in OGD/R-treated PC12 cells. Therefore, YBX1 could protect against OGD/R-induced injury in PC12 cells through activating the AKT/GSK3β signalling pathway, and thus YBX1 has the potential to become a therapeutic target for cerebral I/R-induced injury.

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Abbreviations: CAT – catalase, I/R – ischaemia/reperfusion, LDH – lactate dehydrogenase, NPCs – neural progenitor cells, OGD/R – oxygen-glucose deprivation/reoxygenation, qRT-PCR – quantitative real-time PCR, SOD – superoxide dismutase, YBX1 – Y-box binding protein 1.

Introduction

Stroke is one of the major causes of human mortality and severe disability in the world (Chugh, 2019; Sawale et al., 2021). Ischaemic stroke, also referred to as cerebral infarction, is the most common type of stroke (70–80 % of all cases) (Wang et al., 2017; Uzdensky, 2019). It usually occurs when a blood clot obstructs blood supply to an area of the brain, which leads to neurological deficits (Maida et al., 2020). Treatments focus on rapid reperfusion, including intravenous thrombolysis and mechanical thrombectomy (Rabinstein, 2020; Bae et al., 2021). Although these therapies can reduce disability (Campbell et al., 2019; Barthels and Das, 2020), rapid reperfusion can induce secondary injury, mainly presenting acidosis, oxidative stress, inflammatory injury, intracellular calcium overload, and apoptosis (Huang et al., 2014; Xu et al., 2018). Research on attenuated cerebral ischaemia/reperfusion (I/R)-induced injury has gained a lot of attention (Al-Mufti et al., 2018). Previous studies have demonstrated that the inhibition of neuronal cell apoptosis can protect against I/R-induced neuronal injury (Gong et al., 2017; Wang et al., 2019). Hence, exploring the mechanisms of I/R-induced injury may provide new options for ischaemic stroke treatment.

Y-box binding protein 1 (YBX1), a DNA/RNA-binding protein, is a member of the cold-shock protein superfamily (Budkina et al., 2020). YBX1 is localized in both the nucleus and cytoplasm and plays an important part in regulating DNA damage repair, transcription, and translation (Lyabin et al., 2014). At the cell level, YBX1 is relevant to the cell cycle progression, proliferation, metastasis, and apoptosis (Lyabin et al., 2014;

Mordovkina et al., 2020). Numerous reports have shown that aberrantly high expression of YBX1 is associated with a poor outcome and relapse in various cancers (e.g., pancreatic cancer, basal-like breast cancers and brain tumour) (Stratford et al., 2007; Shinkai et al., 2016; Zheng et al., 2016). YBX1 can promote tumour cell proliferation and prevent apoptosis through interacting with the PI3K/Akt/mTOR pathway (Lasham et al., 2013). YBX1 over-expression induces pancreatic cancer cell growth via the GSK3β/cyclin D1/cyclin E1 signalling pathway (Liu et al., 2020). *YBX1* knockdown can up-regulate the levels of Bax and cleaved caspase-3 to induce apoptosis of neuroblastoma cells (Wang H. et al., 2015). Moreover, it is reported that YBX1 regulates the self-renewal of neural progenitor cells (NPCs) and facilitates the differentiation of NPCs to neurons (Evans et al., 2020). Nevertheless, the effect of YBX1 on cerebral I/R-induced injury is rarely reported. Hence, we wanted to know whether YBX1 could regulate neuronal cell apoptosis caused by I/R.

We used the oxygen-glucose deprivation/reoxygenation (OGD/R) method to treat PC12 cells to produce cerebral I/R-induced injury *in vitro* and detected the expression of YBX1. Then, we determined the effect of YBX1 on the viability, apoptosis, and oxidative stress of OGD/R-treated PC12 cells. Furthermore, we investigated the regulatory roles of YBX1 in the AKT/GSK3β pathway, which is closely associated with neuronal cell apoptosis and survival (Fan et al., 2018).

Material and Methods

Cell culture and transfection

We purchased PC12 cells from ACTT (American Type Culture Collection), and cultured PC12 cells in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (foetal bovine serum, Gibco, Rockville, MD), 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma, St. Louis, MO) at 37 °C in a humidified incubator with 95% air/5% CO_2.

BX1 over-expression plasmid (PCMV-AD-plasmid YBX1, Ad-YBX1) and negative control plasmid (PCMV-AD-plasmid control, Ad-NC) were obtained from Genscript (Nanjing, China). These plasmids were transfected into PC12 cells by using Lipofectamine 3000 (Invitrogen, St. Louis, MO), respectively. After 24 h of transfection, the transfected PC12 cells were treated with OGD/R to construct a model of cerebral I/R-induced injury *in vitro.*

Oxygen-glucose deprivation and reperfusion (OGD/R)

To construct a model of cerebral I/R-induced injury *in vitro*, PC12 cells were subjected to OGD/R treatment. There are four groups in this experiment: control, OGD/R, OGD/R+Ad-NC, and OGD/R+Ad-YBX1 groups. The procedures of OGD/R treatment are shown as follows: PC12 cells were separately washed twice with PBS and cultured in DMEM without glucose, followed by maintaining for 2 h in a hypoxia chamber (5 % CO_2 and 95 % N_2). After being treated by oxygen-glucose deprivation (OGD), PC12 cells were removed from the hypoxia chamber, washed with PBS, incubated in complete DMEM for 24 h at 37 °C under normoxic atmosphere (5 % CO_2 and 95 % air) to obtain a model of cerebral I/R-induced injury *in vitro*.

For OGD/R group: PC12 cells were not transfected with any plasmids, but were subjected to the OGD/R treatment. For OGD/R+Ad-NC group: PC12 cells were transfected with Ad-NC plasmid and treated with OGD/R. For OGD/R+Ad-YBX1 group: PC12 cells were transfected with Ad-YBX1 plasmid and treated with OGD/R. For the control group: PC12 cells were cultured under normal conditions, and were subjected to transfection and OGD/R treatment.

Quantitative real-time PCR (qRT-PCR)

Total RNA from PC12 cells was extracted with Trizol reagent (Invitrogen). After quantification by a NanoDrop spectrophotometer (BioTek Epoch, Winooski, VT) and DNase treatment, RNA was used as a template to synthesize cDNA via reverse transcription by using an Invitrogen SuperScript IV kit (Invitrogen). Next, quantitative PCR was performed with a Rotor-Gene SYBR Green PCR Kit (Qiagen, Germantown, MD) using the CFX96 Touch Real-time PCR Detection system (Bio-Rad, Hercules, CA). Primer sequences are shown in Table 1. The relative expression of *YBX1* mRNA was calculated by using the $2^{-\Delta\Delta Ct}$ method, and the expression of *GAPDH* mRNA, an internal control, was applied for normalization.

Table 1. The sequences of primers

Gene	Sequence (5'-3')
YBX1 forward	GACCGACCAGACTCTCATCC
YBX1 reverse	TCACCAACCTCCATGTTCCT
GAPDH forward	GGTGGTCTCCTCTGACTTCAACA
GAPDH reverse	GTTGCTGTAGCCAAATTCGTTGC

Western blot

PC12 cells were lysed by using ice-cold RIPA lysis buffer (Beyotime). After centrifugation at 4 °C for 20 min at 16,099 *g*, cell supernatant was aspirated and placed in a tube on ice. The quantification of proteins in the supernatant was carried out by the BCA method. Equal amounts of protein were loaded into 10% SDS-PAGE gels (Invitrogen), followed by transferring from the gel to PVDF membranes. The above membranes, after being blocked using 5% skim milk in TBST (Bio-Rad), were washed with TBST and incubated with dilutions of primary antibodies overnight at 4 °C. The membranes, after being rinsed with TBST (three times, 5 min each), were further incubated with the dilution of HRP-

conjugated secondary antibodies (Cat. No. ab205718, Goat Anti-Rabbit IgG H&L, dilution: 1/2000, ABCAM) for 1 h at 37 °C and detected by an enhanced ECL (chemiluminescence) system (Thermo Fisher Scientific, Waltham, MA). The quantification of the protein intensity was analysed by ImageJ software. β-Actin, an internal control, was used in this assay. Primary antibodies in this study included YBX1 (Cat. No. ab76149, dilution: 1/1000, ABCAM), BDNF (brain-derived neurotrophic factor, ab108319, 1/1000), Bax (ab32503, 1/1000), Bcl-2 (ab182858, 1/2000), cleaved caspase-3 (ab2302, 1/500), cleaved PARP (ab32561, 1/1000), p-AKT (Cat no: #4060, dilution: 1/2000, Cell Signaling Technology, Danvers, MA), AKT (#9272, 1/1000), p-GSK3β (ab75814, 1/10000), GSK3β (ab32391, 1/5000) and β-actin (ab8227, 1/1000) antibodies.

MTT

The transfected PC12 cells were added into 96-well plates (cell density: 5×10^3 cells/well) and cultured in complete DMEM for 24 h. Next, the cells were processed by the OGD/R method and MTT reagent was added (5 mg/ml, 25 µl) for 4 h of incubation at 37 °C. To dissolve formazan crystals, dimethyl sulphoxide (DMSO) (100 µl) was added into each well and mixed thoroughly. Subsequently, the sample absorbance was assessed at 490 nm by a Bio-Rad's absorbance microplate reader.

Flow cytometry

PC12 cells, after being treated by OGD/R, were collected and resuspended in 1× Binding Buffer (cell density: 1×10^6 cells per ml). Next, cell suspension (100 µl) was placed in each tube, stained with propidium iodide (PI) (5 µl) and annexin V-FITC (5 µl) for 15 min in the dark at room temperature, and subsequently 400 µl of 1× Binding Buffer was added. Next, flow cytometry was utilized to assess PC12 cell apoptosis.

Lactate dehydrogenase (LDH) leakage assay

LDH release from cells after membrane damage is a key indicator of cell membrane integrity. After being treated by OGD/R for 24 h, the extracellular medium of PC12 cells was collected and centrifuged to get the supernatant. LDH levels in the supernatant were measured by using a cytotoxicity detection kit (Takara Bio, Kusatsu, Shiga, Japan) based on the manufacturer's instructions. Finally, a microplate reader (Bio-Rad) was applied to examine the sample absorbance at 490 nm.

Catalase (CAT) and superoxide dismutase (SOD) enzyme activity assay

SOD and CAT, antioxidant enzymes, play important roles in the antioxidant defence mechanism of cells. PC12 cells, after being treated by OGD/R, were collected, washed with PBS, and then resuspended in PBS. The cell suspension was sonicated in an ice-water bath for 25 s, and subsequently centrifuged at 4 °C at 1,200 *g* for 15 min, followed by harvesting the supernatant for further determination. The corresponding commercially available kits (Beyotime, Haimen, Jiangsu, China) were used to detect SOD and CAT activities.

Statistical analysis

In this study, all experiments were repeated at least three times, and the experimental data are expressed as the mean ± SD (standard deviation). The data analysis was carried out by GraphPad Prism software (Version 7.0). Statistical comparisons among groups were performed by the Student's *t*-test or one-way ANOVA with Sidak's multiple comparison test. The difference among groups was considered statistically significant when the P value < 0.05.

Results

YBX1 expression is down-regulated in OGD/R-treated PC cells

We used the OGD/R method to treat PC12 cells to construct the model of cerebral I/R-induced injury *in vitro* and next carried out the detection of YBX1 levels. The mRNA level of *YBX1* was down-regulated in OGD/R-treated PC12 cells when compared with untreated PC12 cells (control, Fig. 1A). YBX1 protein expression was also decreased after exposure of PC12 cells to OGD/R (Fig. 1B). Hence, the YBX1 level was down-regulated in PC12 cells after the OGD/R treatment.

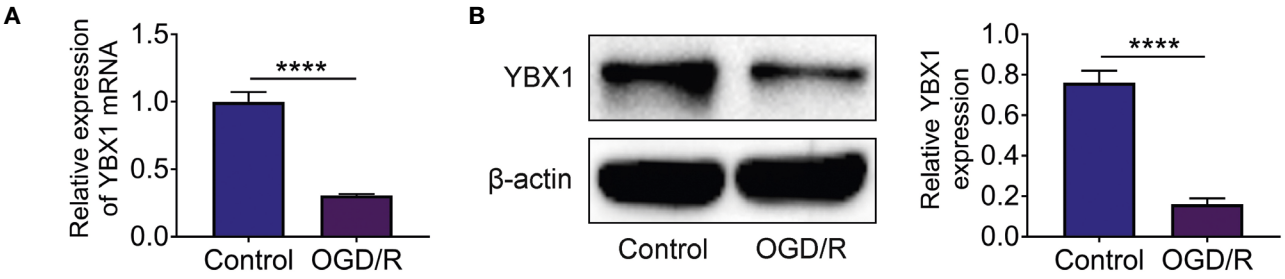

Fig. 1. YBX1 expression is down-regulated in OGD/R-treated PC cells. (**A**) After OGD/R treatment, the mRNA expression of *YBX1* in PC12 cells was measured using qRT-PCR. (**B**) The protein expression of YBX1 in PC12 cells was determined by Western blot. ****P < 0.0001.

YBX1 attenuates OGD/R-induced growth inhibition of PC12 cells

To detect the effect of YBX1 on the growth of PC12 cells, we transfected YBX1 plasmid (Ad-YBX1) and negative control plasmid (Ad-NC) into PC12 cells, treated the transfected cells with OGD/R, measured the YBX1 expression, and assessed the cell viability by MTT assay. Compared with the untreated PC12 cells, OGD/R treatment decreased the mRNA expression of *YBX1* in PC12 cells (Fig. 2 A). After OGD/R treatment, *YBX1* mRNA expression in PC12 cells showed no significant difference between Ad-NC transfection and non-transfection groups (Fig. 2 A). Nevertheless, PC12 cells transfected with Ad-YBX1 showed higher expression of *YBX1* mRNA than the cells transfected with or without Ad-NC, indicating high transfection efficiency of YBX1 (Fig. 2A). Meanwhile, the YBX1 protein expression had the same changing tendency (Fig. 2B). Additionally, BDNF, a neurotrophin, is known to promote the survival of nerve cells (Phillips, 2017). Fig. 2B suggests that BDNF expression was decreased after exposure of PC12 cells to OGD/R, and yet its protein levels in OGD/R-treated PC12 cells were increased by the introduction of Ad-YBX1. Furthermore, the analysis of MTT assay suggested that OGD/R treatment considerably suppressed PC12 cell viability in comparison to the untreated PC12 cells, while YBX1 over-expression attenuated OGD/R-induced inhibition of cell viability (Fig. 2C). Collectively, YBX1 attenuated the growth inhibition of PC12 cells caused by OGD/R.

YBX1 suppresses OGD/R-induced apoptosis of PC12 cells

To evaluate the effect of YBX1 on the apoptosis of PC12 cells, flow cytometry was performed, and its results revealed that OGD/R treatment induced apoptosis of PC12 cells compared to the control, whereas the cellular apoptosis was suppressed by the introduction of Ad-YBX1 (Fig. 3A). Additionally, expression of Bax, cleaved caspase-3 and cleaved PARP, also known as pro-apoptotic proteins, was up-regulated, but the level of an anti-apoptotic protein (Bcl-2) was down-regulated in PC12 cells after the OGD/R treatment (Fig. 3B). Nevertheless, YBX1 over-expression decreased the expression of Bax, cleaved caspase-3 and cleaved PARP but increased Bcl-2 expression in OGD/R-treated PC12 cells (Fig. 3B). Therefore, YBX1 suppressed PC12 cell apoptosis induced by OGD/R.

YBX1 inhibits OGD/R-induced oxidative stress in PC12 cells

To study the effect of YBX1 on oxidative stress induced by OGD/R, we investigated the LDH release and activity of antioxidant enzymes (SOD and CAT). In comparison to the control, the release of LDH, a key indicator of cell membrane integrity, was higher in PC12 cells after 24-h exposure to OGD/R, demonstrating the PC12 cell injury caused by OGD/R (Fig. 4A). However, the LDH release in PC12 cells treated with OGD/R was down-regulated by YBX1 over-expression (Fig. 4A).

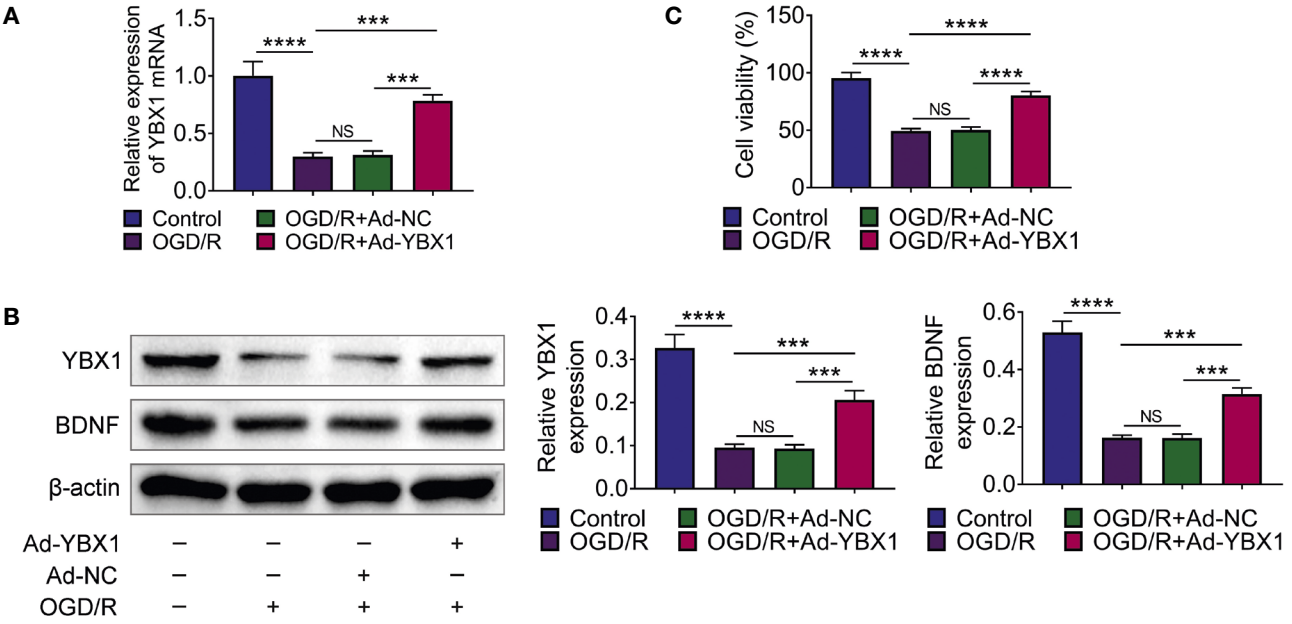

Fig. 2. YBX1 attenuates OGD/R-induced growth inhibition in PC12 cells. PC12 cells were transfected with YBX1 plasmid (Ad-YBX1) or negative control plasmid (Ad-NC) and then treated with OGD/R. (**A**) the mRNA expression of *YBX1* was detected by qRT-PCR; (**B**) the protein levels of YBX1 and BDNF were evaluated by Western blot; (**C**) the viability of PC12 cells was assessed by MTT assay. $^{***}P < 0.001$, $^{****}P < 0.0001$.

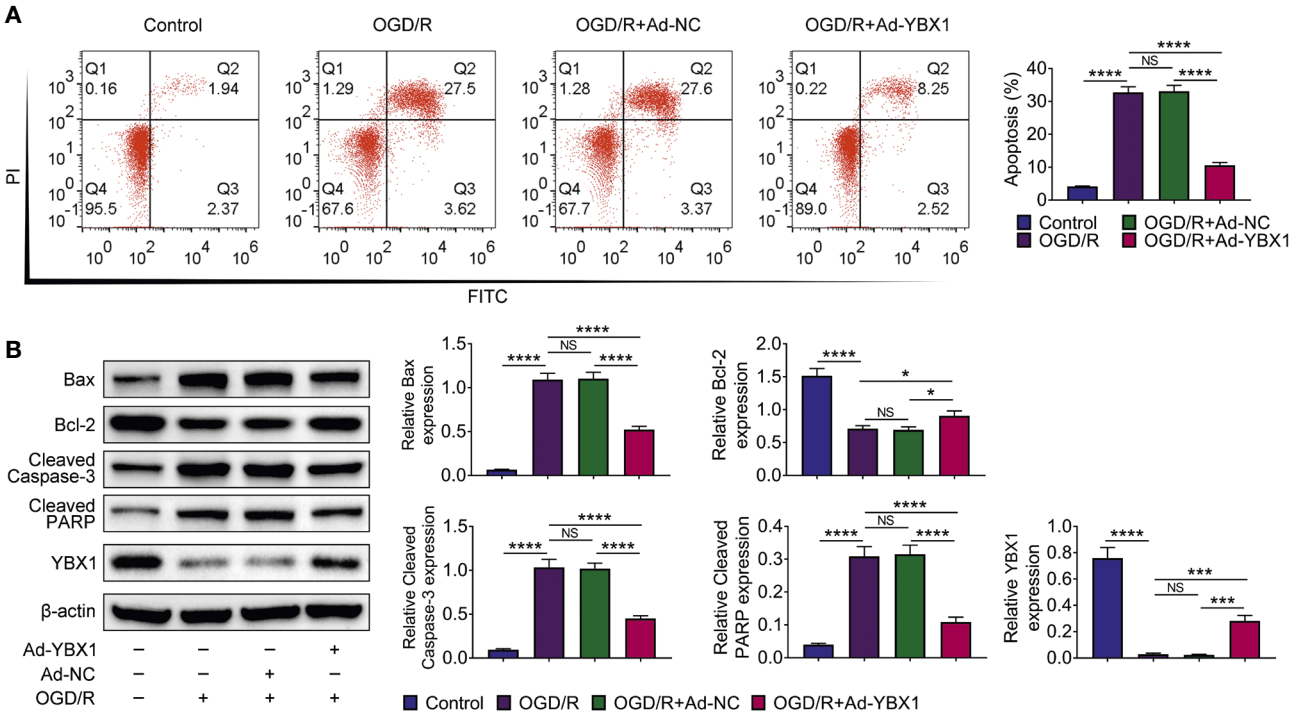

Fig. 3. YBX1 suppresses OGD/R-induced apoptosis in PC12 cells. PC12 cells were transfected with YBX1 plasmid (Ad-YBX1) or negative control plasmid (Ad-NC) and then treated with OGD/R. (**A**) The apoptosis of PC12 cells was examined by flow cytometry; (**B**) the levels of YBX1, pro-apoptotic proteins (Bax, cleaved caspase-3 and cleaved PARP) and an anti-apoptotic protein (Bcl-2) were determined by Western blot. $^{*}P < 0.05$, $^{****}P < 0.0001$.

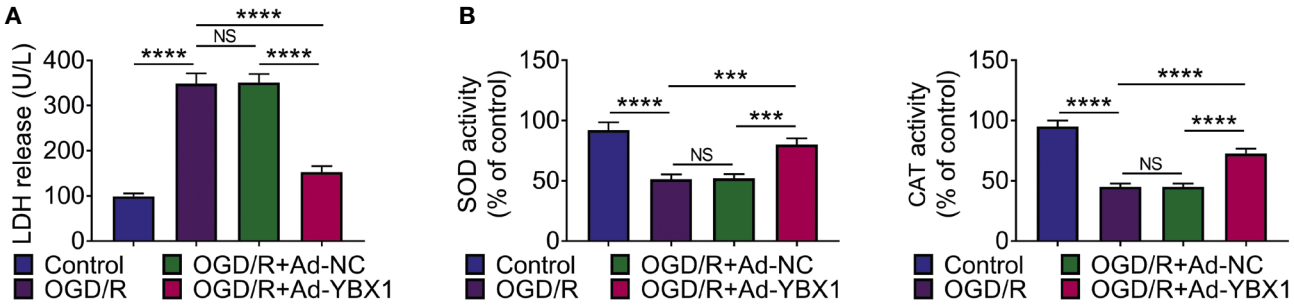

Fig. 4. YBX1 inhibits OGD/R-induced oxidative stress in PC12 cells. PC12 cells were transfected with YBX1 plasmid (Ad-YBX1) or negative control plasmid (Ad-NC) and then treated with OGD/R. (**A**) The release of LDH and (**B**) the activity of SOD and CAT were respectively measured by corresponding commercially available kits. $^{***}P < 0.001$, $^{****}P < 0.0001$.

Furthermore, the activity of SOD and CAT was suppressed by OGD/R treatment in comparison to the control, while their activity in OGD/R-treated PC12 cells showed an obvious elevation after the introduction of YBX1 over-expression plasmid (Fig. 4B). Hence, YBX1 could inhibit oxidative stress of PC12 cells induced by OGD/R.

YBX1 activates the AKT/GSK3β pathway in OGD/R-treated PC12 cells

To investigate the molecular mechanism of YBX1 activity in OGD/R-treated PC12 cells, the regulatory role of YBX1 in the AKT/GSK3β pathway was assessed. After OGD/R treatment, the protein levels of p-AKT and p-GSK3β were remarkably reduced in PC12 cells compared to the control, indicating deactivation of the AKT/GSK3β pathway caused by OGD/R (Fig. 5). Nevertheless, YBX1 over-expression caused an enhance-

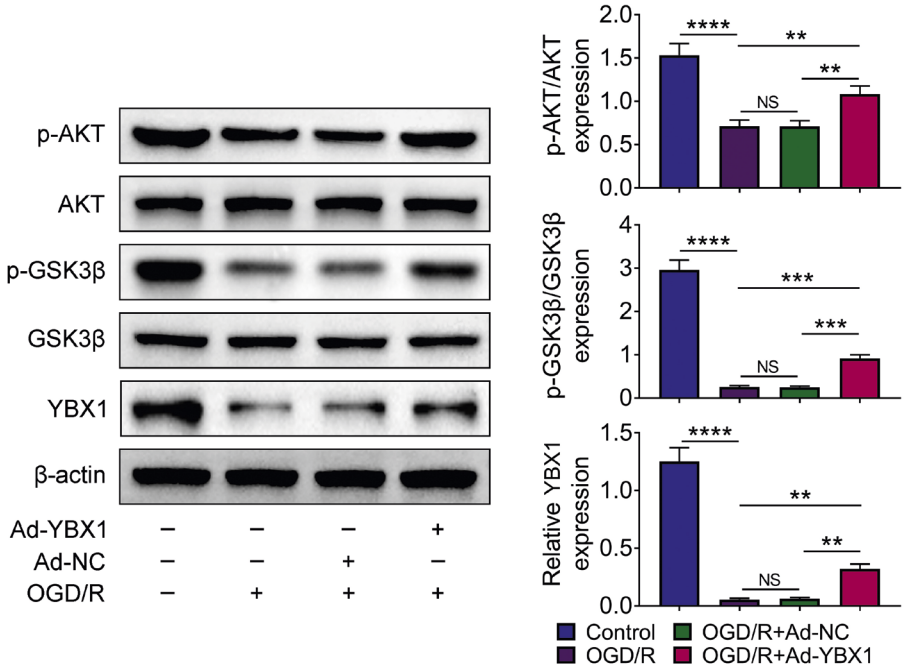

Fig. 5. YBX1 activates the AKT/GSK3β pathway in OGD/R-treated PC12 cells. After transfection of PC12 cells with YBX1 plasmid (Ad-YBX1) or negative control plasmid (Ad-NC) and then treatment with OGD/R, the expression levels of key proteins (AKT, p-AKT, GSK3β, and p-GSK3β) of the AKT/GSK3β pathway and YBX1 were determined by Western blot. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

ment in p-AKT and p-GSK3β expression in OGD/R-treated PC12 cells (Fig. 5). Thus, YBX1 activated the AKT/GSK3β pathway in PC12 cells treated with OGD/R.

Discussion

In this study, YBX1 expression levels were down-regulated in PC12 cells after OGD/R treatment. Additionally, YBX1 was proved to suppress OGD/R-induced growth inhibition, apoptosis, as well as oxidative stress in PC12 cells. Furthermore, YBX1 activated the AKT/GSK3β pathway in OGD/R-treated PC12 cells.

Reperfusion therapies for ischaemic stroke effectively salvage the brain and reduce physical disability (Khoshnam et al., 2017). However, cerebral I/R disrupts the tight junctions of cerebral microvascular endothelial cells to induce secondary injury accompanied by neuronal death such as apoptosis and necrosis (Huang et al., 2014; Xu et al., 2018). Many studies suggest that apoptosis is intensively involved in cerebral I/R-induced injury (Gong et al., 2017; Wang et al., 2019). YBX1, an oncoprotein, is critical for regulating the proliferation and apoptosis of tumour cells (Lasham et al., 2013). It also promotes the differentiation of NPCs to neurons (Evans et al., 2020).

Therefore, we wanted to know whether YBX1 could regulate the apoptosis of neuronal cells caused by I/R. We first constructed a model of cerebral I/R-induced injury *in vitro* by using OGD/R treatment, then determined YBX1 expression and assessed the effect of YBX1 on neuronal cells. Experimental results demonstrated that YBX1 expression levels in PC12 cells were down-regulated after OGD/R treatment. In addition, the growth inhibition of PC12 cells caused by OGD/R treatment was attenuated by YBX1 over-expression. Besides, over-expression of YBX1 inhibited apoptosis of PC12 cells induced by OGD/R. Furthermore, the increase of LDH release and the decrease of SOD and CAT activities caused by OGD/R treatment were reversed by YBX1 over-expression, indicating that YBX1 suppressed oxidative stress in PC12 cells caused by OGD/R. Therefore, YBX1 could protect against OGD/R-induced injury in PC12 cells.

The regulatory role of YBX1 in the AKT/GSK3β pathway was investigated to study the molecular mechanism of YBX1 activity in OGD/R-treated PC12 cells. AKT, a Ser/Thr kinase, is regarded as an essential signalling node and its activation can phosphorylate diverse downstream molecules such as GSK3β to mediate numerous cellular processes (Majewska and Szeliga, 2017). The AKT/GSK3β pathway has been proved to modulate neuronal cell apoptosis and survival (Zhang et al., 2014; Qian et al., 2015). Many studies suggest that this pathway activation can alleviate cerebral I/R-induced injury (Chen et al., 2014; Yu et al., 2021). For instance, Xu et al. (2019) have indicated that AKT/GSK3β activation induced by FCPR03 protects neuronal cells against cell death and oxidative stress to alleviate cerebral I/R injury. Zhang et al. (2017) have shown that activation of the AKT/GSK3β signalling

caused by parthenolide increases neuronal cell viability, inhibits apoptosis, and prevents oxidative stress to attenuate OGD/R-evoked neuronal injury.

Furthermore, YBX1 interacts with the PI3K/Akt/mTOR pathway to regulate tumour cell proliferation and apoptosis (Lasham et al., 2013). For example, an aberrantly high level of YBX1 has a positive correlation with the invasion and metastasis of colorectal cancer lymph node, and inhibition of YBX1 can deactivate PI3K/AKT signalling to reduce colorectal cancer cell motility and proliferation (Kim et al., 2020). Additionally, YBX1 can induce GSK3β expression to promote growth of pancreatic cancer cells (Liu et al., 2020). However, interaction of YBX1 with the AKT/GSK3β pathway has not been reported. Therefore, our study performed related experiments and found that the decrease of p-AKT and p-GSK3β caused by OGD/R was reversed by YBX1 over-expression. Hence, we speculated that YBX1 could activate the AKT/GSK3β pathway to attenuate OGD/R-induced injury.

In conclusion, YBX1 could protect against OGD/R-induced injury in PC12 cells through activating the AKT/GSK3β signalling pathway. These findings contribute to understanding the molecular mechanism of cerebral I/R-induced injury and provide potential therapeutic biomarkers. The present work is a preliminary study and our next research option is to further investigate the more detailed interaction mechanism among YBX1, AKT and GSK3β in OGD/R-induced neuronal injury.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Contribution of authors

T. T. and X. L. designed the study, supervised the data collection; F. H. analysed the data, interpreted the data; T. T., Y. W., X. W. and L. M. prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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