

RESEARCH ARTICLE

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Dexmedetomidine attenuates oxygen-glucose deprivation/reperfusion-induced inflammation through the miR-17-5p/TLR4/NF- κ B axis

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Abstract

Background: Dexmedetomidine (DEX) is a selective agonist of α 2-adrenergic receptors with anesthetic activity and neuroprotective benefits. However, its mechanism of action at the molecular level remains poorly defined. In this study, we investigated the protective effects of DEX on oxygen-glucose deprivation/reperfusion (OGD/R)-induced neuronal apoptosis in PC12 cells, and evaluated its underlying mechanism(s) of neuroprotection and anti-inflammation.

Methods: An OGD/R model in PC12 cells was established. PC12 cells were cultured and divided into control, OGD/R, and OGD/R + DEX (1 μ M, 10 μ M, 50 μ M) groups. Cell apoptosis was analyzed by flow cytometry and expression profiles were determined by qRT-PCR, western blot analysis, and enzyme linked immunosorbent assays (ELISA). The interaction between miRNA and its downstream targets was evaluated through luciferase reporter assays.

Results: DEX significantly decreased apoptosis rates and inhibited interleukin 1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), and interleukin 6 (IL-6) release ($P < 0.05$). While expression of the pro-apoptotic proteins Bax and Caspase-3 was down-regulated, expression of Bcl-2 was upregulated in a dose-dependent manner ($P < 0.05$). Interestingly, miR-17-5p expression was down-regulated in the OGD/R group (compared to controls). Toll-like receptor 4 (TLR4), a key regulator of nuclear factor kappa-B (NF- κ B) signaling, was identified as a novel target of miR-17-5p in PC12 cells. miR-17-5p expression was upregulated in the OGD/R + DEX group, suppressing TLR4 expression and reducing the secretion of proinflammatory cytokines.

Conclusion: DEX inhibits OGD/R-induced inflammation and apoptosis in PC12 cells by increasing miR-17-5p expression, downregulating TLR4, and inhibiting NF- κ B signaling.

Keywords: Dexmedetomidine, Oxygen-glucose deprivation/reperfusion, Inflammation, Apoptosis, miR-17-5p, TLR4/NF- κ B signaling

Background

Stroke causes significant disabilities and cognitive impairment in afflicted individuals throughout the world [1]. The basic pathophysiology of cerebral ischemic stroke is complex, involving the interplay of autophagy, apoptosis, oxidative stress, inflammation, and energy attenuation [2, 3]. Recently, evidence has been presented highlighting

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the role of miRNAs in cerebral ischemia-reperfusion injury, identifying miRNAs as potential therapeutic targets [4, 5].

Dexmedetomidine (DEX) is a α_2 -adrenoceptor agonist that exhibits sedative, anxiolytic, and analgesic functions [6]. DEX is known to exert positive effects (in comparison with other sedatives), including mitigation of respiratory depression and hypotension, alleviation of lung and kidney damage, and decreased neuronal apoptosis [7]. DEX also has a long-term neuroprotective influence on cognitive dysfunction and brain injury [8].

The role of microRNAs (miRs), short non-coding RNA molecules, is to bind to mRNAs and inhibit the expression of target genes. The downregulation of miRs in neuronal cells is intricately linked to neurodegenerative disease [9]. Approximately 70% of all known miRs are expressed in the brain (either locally or tissue wide), and these are critical to the functionality of the nervous system [10]. Oxygen-glucose deprivation/reperfusion (OGD/R) miRs are reported to suppress apoptosis of developing hippocampal astrocytes in rodents, thus affording protection against hepatic ischemia/reperfusion injury [11, 12]. An interaction between miR-223-3p and TIAL1 has been demonstrated to contribute to the neuroprotective effects of DEX in hippocampal neuronal cells *in vitro* [13]. Hence, DEX may regulate OGD/R-induced inflammation and apoptosis through miRs. Hao *et al.* (2017) report that miR-17-5p is pro-apoptotic, and that miR-17-5p overexpression induces neuronal death and apoptosis [14]. However, miR-17-5p has also been reported to inhibit neuronal apoptosis and epileptiform discharge in hippocampal neurons following seizures [15].

Thus far, the overall effects of DEX on OGD/R-induced inflammation and apoptosis remain unclear. In the present study, we investigated the hypothesis that DEX-treatment suppresses OGD/R-induced inflammation and apoptosis, and we explored the potential biological mechanisms mediating these protective effects.

Methods

Cell lines and cell culture

PC12 cells obtained from the American Type Culture Collection (ATCC; USA) were cultured in RPMI 1640 containing 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO₂. Cells were passaged after reaching ~80% confluency, and seeded into 96-well plates at a density of 10⁴/mL.

Six treatment groups were established: (1) normal control group; (2) model group; (3) solvent group; (4) dexmedetomidine (DEX) low group; (5) DEX medium group; and (6) DEX high group. All treatments were performed in triplicate. In the model DEX groups, cells

were cultured in glucose-free RPMI 1640 containing 30mmol/L Na₂S₂O₄. Cells were treated with DEX (in normal saline) at 1 μmol/L, 10 μmol/L, or 50 μmol/L (an equal volume of saline was added to the control group). The cells in each group were then cultured at 37°C for 4h.

miRNAs, plasmids, and cell transfections

TLR4 overexpression plasmids (pcDNA3.1) and corresponding controls (pcDNA3.1) were generated by GenePharma (Shanghai, China). miR-17-5p mimics, inhibitors, and mimic/inhibitor negative controls (mimic NC and inhibitor NC) were generated by Ribobio (Guangzhou, China). PC12 cells were transfected with LiRNAMax 3000 (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendations. Cells were analyzed 24h post-transfection.

ELISA assays for the determination of interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) levels

Commercially available ELISA kits (R&D Systems, Minneapolis, USA) were used to determine the expression levels of IL-1β (#DLB50), TNF-α (#DTA00D), and IL-6 (#DR600). All ELISA kits were used as per the manufacturer's recommendations.

RNA extraction and qRT-PCR analysis

Total RNA was harvested using commercially available RNA extraction kits (Takara, China) according to the manufacturer's instructions. miR-17-5p was reverse transcribed to cDNA using the MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR amplifications were performed on an ABI7900 Fast Real-Time PCR System using SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific). U6 and GAPDH served as internal controls for miR-17-5p and mRNA expression, respectively. The comparative Ct method was used to calculate relative gene expression levels.

Luciferase reporter assays

The TargetScan V7.2 database was used to assess putative binding sites for miR-17-5p in the TLR4 3'-UTR. Wild-type (WT) fragments of TLR4 3'-UTR were amplified from genomic DNA and subcloned into a pmirGLO reporter vector (Promega, Madison, USA). This construct was termed TLR4 3'-UTR-WT. TLR4 3'-UTR mutations were performed through site-directed mutagenesis kit (Stratagene, San Diego, USA). The construct was termed TLR4 3'-UTR-MUT. For luciferase activity, HEK293 cells were transfected with miRNAs (miR mimic or NC mimic) and luciferase reporter vectors (TLR4 3'-UTR-WT or TLR4 3'-UTR-MUT) using Lipofectamine 3000 reagent

(Invitrogen). Forty-eight hours post-transfection, luciferase activity was assessed using the Dual-Luciferase[®] Reporter (DLR[™]) Assay System (Promega).

Western blot analysis

Cells were lysed in ice-cold radioimmunoprecipitation assay buffer (Roche, Basel, Switzerland) supplemented with protease inhibitors. Protein concentrations were determined using dioctanoic acid assays (Thermo Fisher Scientific). Equal volumes of proteins were separated on 10% sodium lauryl sulfate-polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes and blocked in 1.5% skimmed milk in Tris buffered saline containing Tween 20 (TBST). Membranes were probed with primary antibodies at 4°C overnight and washed in TBST. The membranes were then probed with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 2 h at room temperature. ECL kits (Thermo Fisher Scientific) were used to determine band intensities on the membranes as per the manufacturer's recommendations. The primary antibodies were TLR4, p-p65, p65, p-IκBα, IκBα, Bcl-2, Bax, and caspase-3 (Cell Signaling, Danfoss, Mass.) GAPDH (Santa Cruz, California) was also probed as a loading control.

Apoptosis assays

After 48 h of H₂O₂ treatment, cells were stained with 5 μL Annexin V-FITC and 5 μL PI (BD Biosciences, USA). Apoptosis was then analyzed by flow cytometry (Becton Dickinson, USA). Annexin V-positive cell populations were considered apoptotic.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software. All data were tested for normality and homogeneity of variance, and those that met this criterion were expressed as mean ± standard deviation of the mean (SD). The differences between two groups were evaluated by Student's t-test, while differences between three or more groups were analyzed by ANOVA (with Bonferroni Multiple Comparison tests). Data that did not meet the criterion for normality or homogeneity of variance were transformed into categorical variables and analyzed by Mann-Whitney U tests. The data were graphed using GraphPad Prism Software (version 5, GraphPad Software, Inc., La Jolla, CA). A $P < 0.05$ was used to test for statistical significance.

Results

Dexmedetomidine attenuates OGD/R-induced inflammation and apoptosis in PC12 cells

The overexpression of inflammatory mediators, including IL-6, IL-1β, and TNF-α, is linked to OGD/R. Thus, IL-6, IL-1β, and TNF-α expression levels were higher in the

OGD/R group. Compared to the control, DEX treatment suppressed IL-6, IL-1β, and TNF-α expression levels in a dose-dependent manner ($P < 0.05$; Fig. 1A). Flow cytometry analysis revealed that OGD/R treatment increased the rate of apoptosis in PC12 cells, whilst DEX treatment significantly suppressed OGD/R-induced apoptosis ($P < 0.05$; Fig. 1B). DEX treatment increased the expression of the anti-apoptotic protein Bcl-2, and reduced the expression of the pro-apoptotic proteins Bcl-2-associated X protein (Bax) and Caspase-3 ($P < 0.05$; Fig. 1C). These effects in the OGD/R+DEX group were dose-dependent. Together, these results provide evidence that DEX inhibits apoptosis in OGD/R-treated PC12 cells.

Dexmedetomidine inhibits TLR4/ NF-κB signaling through miR-17-5p in PC12 cells

miR-17-5p was significantly down-regulated in PC12 cells in the OGD/R group (compared to the control group). In comparison to the OGD/R+vehicle group, DEX treatment upregulated miR-17-5p in a dose-dependent manner ($P < 0.05$; Fig. 2A). Because TLR4 is known to regulate inflammatory response through NF-κB signaling, TLR4/ NF-κB signaling was further examined. Western blot analysis revealed that DEX treatment significantly decreased the levels of p-IκBα and p-p65 proteins, but not overall IκBα and p65 expression levels. A reduction in TLR4 expression induced a decrease in the levels of p-IκBα and p-p65 ($P < 0.05$; Fig. 2B). The predicted binding sites for miR-17-5p in the 3'-UTR of target genes were analyzed using TargetScan, and the TLR4 3'-UTR (position 4654–4660) was shown to possess complementary binding sites for miR-17-5p ($P < 0.05$; Fig. 2C). The WT and MUT TLR4 3'-UTR regions were subcloned into luciferase reporter vectors, and the resulting luciferase activities in HEK293 cells co-transfected with miRNAs and luciferase reporter vectors were determined. miR-17-5p overexpression repressed the luciferase activity of TLR4 3'-UTR-WT, but had no effect on the activity of TLR4 3'-UTR-MUT in HEK293 cells ($P < 0.05$; Fig. 2D).

miR-17-5p inhibits TLR4/ NF-κB signaling, inflammation, and apoptosis in PC12 cells following OGD/R

Because TLR4 regulates inflammatory responses via NF-κB signaling, the interaction between miR-17-5p and TLR4/ NF-κB was examined. Western blot analysis revealed that miR-17-5p overexpression significantly decreased the levels of TLR4, p-IκBα, and p-p65 proteins, but not overall IκBα and p65 expression levels. In contrast, miR-17-5p silencing increased the levels of TLR4, p-IκBα, and p-p65 proteins ($P < 0.05$; Fig. 3A). As miR-17-5p was down-regulated in the OGD/R group, the effects of miR-17-5p overexpression on the production

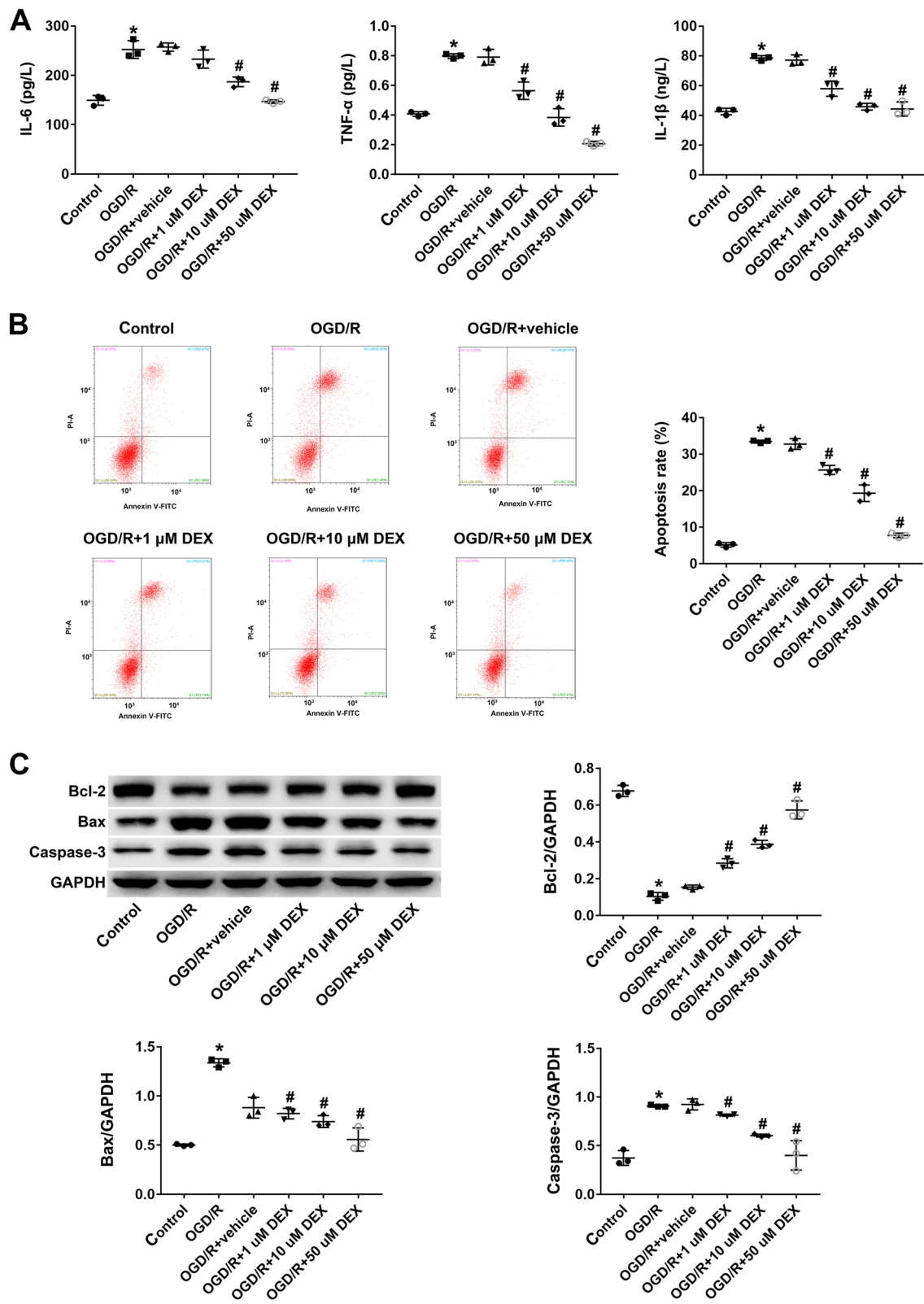
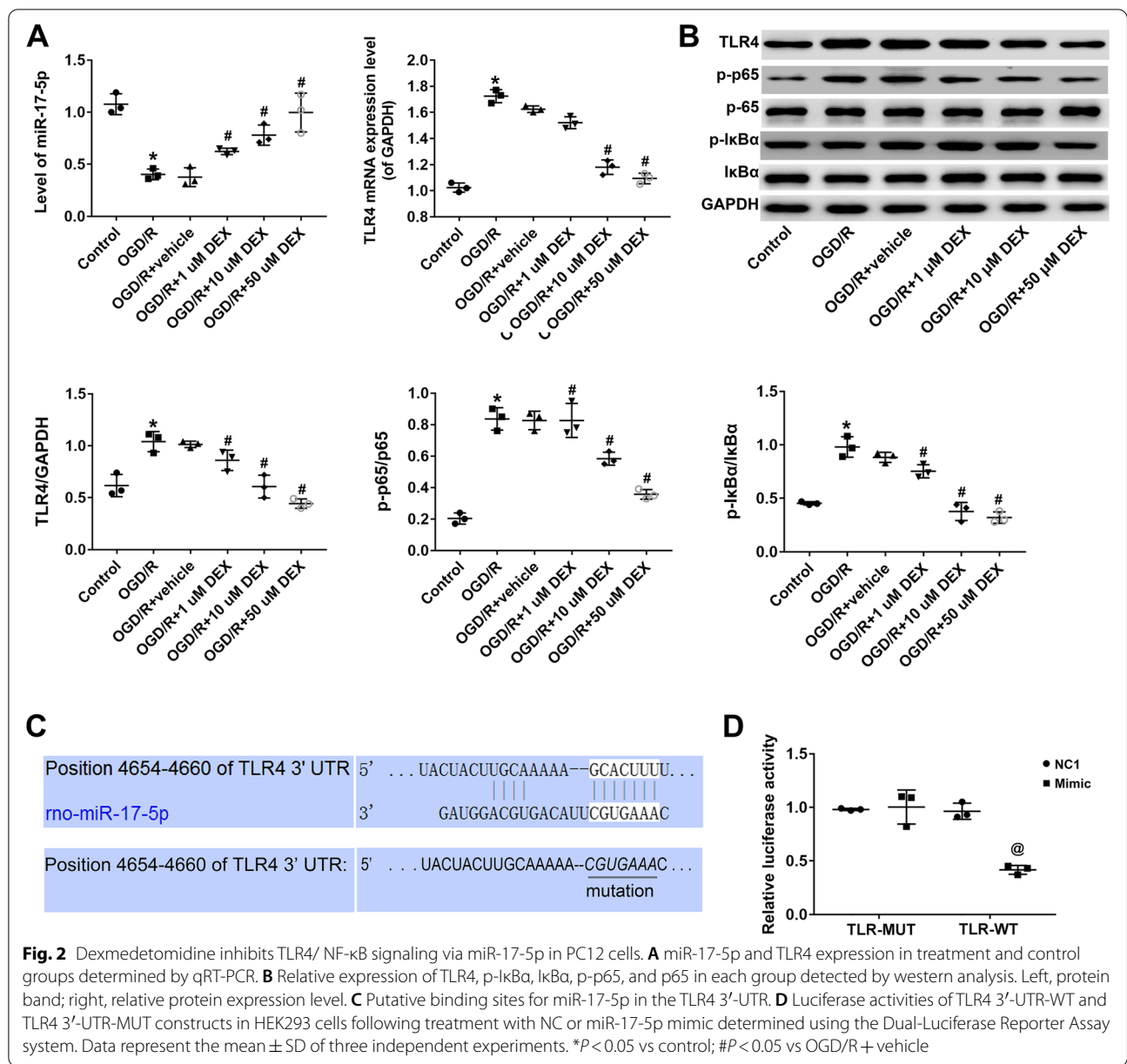


Fig. 1 Dexamethasone attenuates OGD/R-induced inflammation and apoptosis in PC12 cells. **A** ELISA-based detection of IL-1β, TNF-α, and IL-6 production in PC12 cells. **B** Apoptosis rates detected by flow cytometry in H₂O₂-treated PC12 cells. **C** Expression of Bcl-2, Bax, and caspase 3 detected by western analysis. Data represent the mean ± SD of three independent experiments. **P* < 0.05 vs control; #*P* < 0.05 vs OGD/R + vehicle

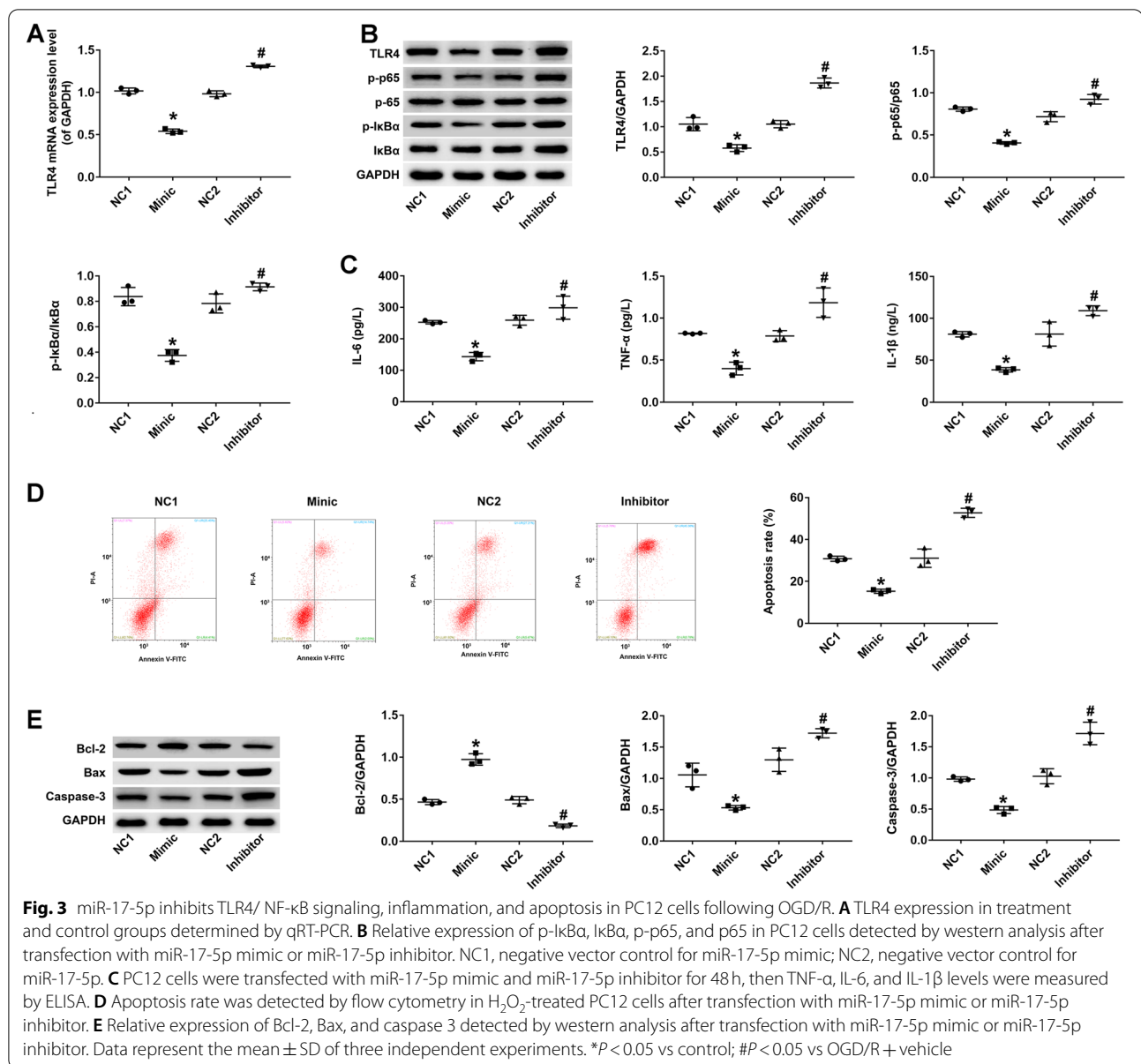


of pro-inflammatory cytokines in PC12 cells were also determined. ELISA analysis demonstrated that the levels of IL-1β, TNF-α, and IL-6 proteins in PC12 cells overexpressing miR-17-5p were significantly decreased (compared with those transfected with NC mimic). In contrast, knockdown of miR-17-5p enhanced the production of pro-inflammatory cytokines in PC12 cells (*P* < 0.05; Fig. 3B). Next, we examined the rate of apoptosis in PC12 cells transfected with miR-17-5p mimic after H₂O₂ stimulation. Flow cytometry analysis revealed that overexpression of miR-17-5p significantly suppressed H₂O₂-induced apoptosis in PC12 cells, increased Bcl-2

levels, and suppressed Bax and Caspase-3 expression. As expected, miR-17-5p silencing produced the opposite phenotype (*P* < 0.05; Fig. 3C, D).

TLR4 overexpression and miR-17-5p silencing inhibit DEX-induced anti-inflammatory and anti-apoptotic phenotypes in PC12 cells following OGD/R

Rescue experiments were performed to determine if the combined overexpression of TLR4 and miR-17-5p silencing could attenuate the effects of DEX treatment on OGD/R-induced inflammation and apoptosis in PC12 cells. TLR4 overexpression and miR-17-5p knockdown



markedly increased the production of IL-1β, TNF-α, and IL-6 proteins in PC12 cells compared to the DEX group (*P* < 0.05; Fig. 4A). Furthermore, TLR4 overexpression and miR-17-5p silencing significantly increased the rate of apoptosis in PC12 cells, decreased the expression of Bcl-2, and increased Bax and Caspase-3 expression (*P* < 0.05; Fig. 4B, C).

Discussion

The mechanism(s) of cerebral ischemia-reperfusion injury are complex, involving excitatory amino acid toxicity, oxidative stress, and inflammatory responses [16]. These factors are interconnected and ultimately induce

apoptotic signaling and programmed neuronal cell death [17]. Apoptosis after cerebral ischemia-reperfusion injury is a major form of neuronal death [18]. Inhibition of apoptosis can be used as a potential therapeutic intervention for cerebral ischemia-reperfusion injury. In this study, an OGD/R cell model of PC12 cells was established to simulate cerebral ischemia-reperfusion injury *in vitro*, and to explore the neuroprotective effects of DEX. Li *et al.* (2016) report that DEX is 1600-fold more selective for α2 over α1, inhibiting apoptosis and exerting neuroprotective effects in the developing brain [19]. DEX preconditioning is reported to protect the heart from apoptosis following ischemic/ reperfusion injury in a diabetic rat

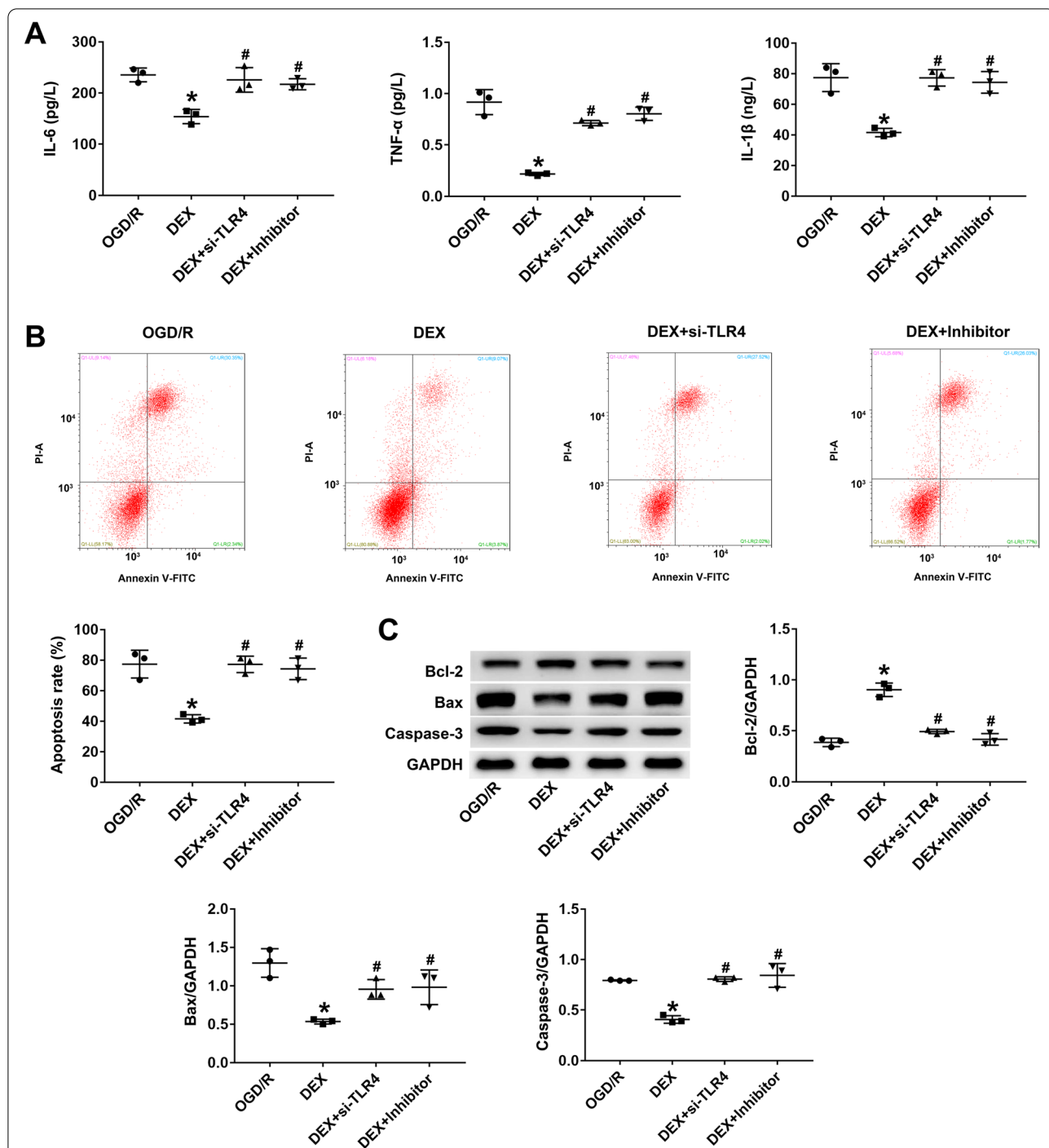


Fig. 4 TLR4 overexpression and miR-17-5p silencing inhibit the anti-inflammatory and anti-apoptotic effects of DEX in PC12 cells following OGD/R. **A** PC12 cells were transfected with pcDNA3.1-TLR4 and miR-17-5p inhibitor, and TNF-α, IL-6, and IL-1β levels were measured by ELISA. **B** Apoptosis rate was detected by flow cytometry in H₂O₂-treated PC12 cells after transfection with pcDNA3.1-TLR4 and miR-17-5p inhibitor. **C** Relative expression of Bcl-2, Bax, and caspase 3 in PC12 cells transfected with pcDNA3.1-TLR4 and miR-17-5p inhibitor detected by western blot. Data are the mean ± SD of three independent experiments. **P* < 0.05 vs OGD/R; #*P* < 0.05 vs DEX

model (both *in vivo* and *in vitro*) by activating PI3K/ Akt signaling [20]. Furthermore, DEX preconditioning protects the heart from apoptosis in ischemic/ reperfusion injury in diabetic rats by activating PI3K/ Akt signaling *in vivo* and *in vitro* [21]. However, the mechanisms by which DEX regulates these effects remain to be elucidated.

miRNAs participate in a range of essential biological processes, including neuronal apoptosis during ischemic stroke and nervous system dysfunction [22]. Here, we explored the underlying biological mechanism of DEX attenuation of OGD/R-induced neurotoxicity, and we investigated the involvement of miR-17-5p and potential molecular factors. miR-17-5p is significantly upregulated in the early stage of cerebral ischemia-reperfusion injury (within 4 h). Moreover, onset of miR-17-5p upregulation is earlier than the observed changes in urea nitrogen level and neutrophil gelatinase-related lipocalin (NGAL) concentration [23]. miR-17-5p is known to be induced by p53 and to protect from renal ischemia-reperfusion injury by targeting death receptor 6 [24]. In addition, miR-17-5p is down-regulated by the Act A/ Smads signaling loop, thus enhancing the neuroprotective effect after ischemic injury [25]. Although these effects of miR-17-5p may modulate the therapeutic efficacy of DEX on OGD/R-induced neurotoxicity, no reports to this effect have been published thus far.

TLR4 belongs to the Toll-like receptor family. These innate pattern recognition receptors mediate the host response to pathogen infection [26]. TLR4 activation promotes the production of inflammatory cytokines, such as IL-1 β , TNF- α , and IL-6 [27]. Aberrant IL-1 β and IL-6 responses induced by TLR4 have been observed in patients. TLR4 mRNA is reported to have a binding site for miR-17-5p [28]. Thus, miR-17-5p may regulate inflammation induced by oxygen and glucose deprivation/ reperfusion via TLR4/ NF- κ B [29].

In our present study, we report that miR-17-5p was downregulated in the OGD/R group, and that miR-17-5p mediated OGD/R-induced inflammation and apoptosis. DEX treatment increased miR-17-5p expression in a dose-dependent manner in PC12 cells. Moreover, miR-17-5p overexpression suppressed the inflammatory response by inhibiting NF- κ B. Conversely, miR-17-5p downregulation produced the opposite phenotype. To explore the mechanisms underlying these effects, the TargetScan V7.2 database was employed to identify miR-17-5p regulated genes. miR-17-5p was predicted to bind to TLR4 mRNA. Our results reveal that miR-17-5p levels were negatively correlated with TLR4 levels, and that miR-17-5p binding to the 3'-UTR of TLR4 suppressed expression in a luciferase gene reporter assay. However, the role of miR-17-5p remained undefined.

To further investigate the role of miR-17-5p in OGD/R-induced inflammation and apoptosis in PC12 cells, miR-17-5p mimic and miR-17-5p inhibitor were separately transfected into each group. miR-17-5p mimic was observed to inhibit OGD/R-induced inflammation and apoptosis. Moreover, we identified NF- κ B signaling as a potential mediator of miR-17-5p inhibition, and we subsequently demonstrated that miR-17-5p mimic could inhibit TLR4/ NF- κ B signaling. For many drugs such as soy isoflavones and genistein, phosphorylation of I κ B α and P65 is necessary for their neuroprotective effects [30]. From a consideration of these results, we propose that DEX upregulates miR-17-5p, and that miR-17-5p inhibits NF- κ B subsequently reducing OGD/R-induced inflammation and apoptosis. In addition, we observed that inhibition of OGD/R-induced inflammation and apoptosis was suppressed following TLR4 overexpression or miR-17-5p silencing, which suggests that DEX attenuates oxygen-glucose deprivation/ reperfusion-induced inflammation and apoptosis in PC12 cells through an effect on the miR-17-5p/ TLR4/ NF- κ B axis. Together, our data demonstrate the potential of DEX as a novel intervention strategy for cerebral ischemia-reperfusion injury. These findings require further verification in human stroke patients.

Our study had several limitations worth noting. First, the optimal concentration of DEX *in vivo* was not investigated. Therefore, DEX dosage should be further investigated in *in vivo* experiments. Second, DEX has only been observed thus far to up-regulate the expression of miR-17-5p in PC12 cells. Further studies are required to confirm these effects *in vivo*. On the side, in this study, DEX mainly exerts its protective effects through its anti-inflammatory activity, which is an ancillary activity compared to its main property (hypnotic drug). Would glucocorticoids, the reference anti-inflammatory drugs, provide the same protection while avoiding undesired central effects? Several studies in recent years have reported on this, Wang et al. [31] found knockout in studies glucocorticoid-regulated kinase 1 (SGK1) knockdown upregulated beclin-1 and LC-3 expression mediated by Intracarotid cold saline infusion (ICSI), This suggests that ICSI has a neuroprotective effect on ischemic stroke after reperfusion through up-regulation of SGK1 and inhibition of autophagy. The role of glucocorticoid signaling was also reported in the study of Perović et al. [32], They found Food restriction (FR) applied prior to TBI significantly changes p-GR levels, and it's transcriptional activity during the recovery period after TBI. Moreover, as a pretreatment, FR modulates other protective factors in response to TBI, such as 11 β -HSD1, NF- κ B (p65) and HSP70 that act in parallel with GR in it's

anti-inflammatory and neuroprotective effects in the rat model of brain injury. That's what we're going to do next.
Conclusion.

DEX inhibits OGD/R-induced inflammation and apoptosis in PC12 cells by increasing miR-17-5p expression, downregulating TLR4, and inhibiting NF- κ B signaling. These results preliminarily explain the neuroprotective mechanism of DEX in ischemic stroke, and provide a direction for further searching for therapeutic targets for ischemic stroke.

Abbreviations

DEX: Dexmedetomidine; ELISA: Enzyme Linked Immunosorbent Assays; miRs: microRNAs; mimic NC: Mimic Negative Controls; inhibitor NC: Inhibitor Negative Controls; IL-1 β : Interleukin 1 beta; TNF- α : Tumor Necrosis Factor-alpha; IL-6: Interleukin 6; WT: Wild-type; TBST: Tris Buffered Saline containing Tween 20; SD: Standard Deviation of the mean; Bax: Bcl-2 associated X protein; Caspase-3: CysteinyI aspartate specific proteinase-3.

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None to declare.

Authors' contributions

LS: Supervision; Methodology; Investigation; Data Curation; Writing – Original Draft; Writing – Review & Editing; Visualization. MW: Conceptualization; Resources; Data Curation; Writing – Review & Editing; Project Administration. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.
 The authors declare that there was no conflict of interest in this paper.

Declarations

Ethics approval and consent to participate

All protocols used during our study followed the requirements of the Animal Experiment Center of the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences.

Consent for publication

Not applicable.

Competing interests

No potential conflicts of interest relevant to this article are reported.

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