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

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Research article

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Abstract

Background Dexmedetomidine (DEX) is a selective agonist of α2-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 OGD/R-induced neuronal apoptosis in PC12 cells, and evaluated its underlying mechanism(s) of neuroprotection and anti-inflammation.

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

Results Dex significantly decreased the rates of apoptosis rates and inhibited IL-1β, IL-6 and TNF-α release ($p < 0.05$). The expression of the pro-apoptotic proteins Bax and Caspase-3 were down-regulated, whilst Bcl-2 was upregulated in a dose-dependent manner ($p < 0.05$). MiR-17-5p 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-κB) signaling, was identified as a novel target of miR-17-5p in PC12 cells. The expression of miR-17-5p was upregulated in the OGD/R + Dex group which suppressed TLR4 expression and reduced 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-κB signaling.

Background

Stroke causes significant disabilities and cognitive impairment on a global scale [1]. The basic pathophysiology of cerebral ischemic stroke is complex, involving the interplay of autophagy, apoptosis, oxidative stress, inflammation, and energy attenuation [2, 3]. Emerging evidence also highlights the role of miRNAs as targets to treat cerebral ischemia-reperfusion injury [4, 5].

Dexmedetomidine (DEX) is a robust α2-adrenoceptor agonist that exhibits sedative, anxiolytic, and analgesic functions [6]. In comparison to other sedatives, DEX exerts positive effects, including the mitigation of respiratory depression and hypotension, the 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].

MicroRNAs (miRs) are short non-coding RNA molecules that 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 have specific or abundant expression in the brain and are critical to the functionality of the nervous system [10]. Existing studies suggest that OGD/R miRs

suppress apoptosis in the growing hippocampal astrocytes of rodents, offering protection against hepatic ischemia/reperfusion injury [11, 12]. Moreover, the interaction of miR-223-3p/TIAL1 contributes to the neuroprotective effects of DEX in hippocampal neuronal cells *in vitro* [13]. Accordingly, it is speculated that DEX regulates OGD/R-induced inflammation and apoptosis through miRs. miR-17-5p is pro-apoptotic, the overexpression of which induces neuronal death and apoptosis [14]. MiR-17-5p inhibits neuronal apoptosis and epileptiform discharge in hippocampal neurons following seizures [15].

Despite this knowledge, the effects of DEX on OGD/R-induced inflammation and apoptosis remain unclear. In this study, we hypothesized that DEX-treatment suppresses OGD/R-induced inflammation and apoptosis and investigated 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 a RPMI 1640 containing 10% fetal bovine serum at 37 °C, 5% CO_2 in a humidified environment. Cells were passaged when ~ 80% confluent and seeded into 96 well plates at a density of 10^4/ml.

Six treatment groups were established: (1) normal controls; (2) model group; (3) solvent group; (4) dexmedetomidine (Dex) low; (5) Dex medium; and (6) Dex high group. All treatments were performed in triplicate. In model, solvent and dex groups, cells were cultured in glucose-free RPMI 1640 containing 30 mmol/L NaS_2O_4. Cells were treated with Dex at 1, 10, and 50 μmol / L in normal saline. Equal volumes of saline were added to the solvent group. Cells in each group were cultured at 37 °C for 4 h.

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 (mimics NC and inhibitors NC) were generated by Ribobio (Guangzhou, China). PC12 cells were transfected with LiRNAfectamine 3000 (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendations. Cells were analyzed 24 h 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 were used to determine the expression of IL-1β, TNF-α and IL-6 (# DLB50 for IL-1β detection, # DTA00D for TNF-α detection, and # DR600 for IL-6 detection. The R & D system in Minneapolis (USA) was used as per the manufacturers 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). For the detection of TLR4 mRNA, cDNA reverse transcription kits (Thermo Fisher Scientific) were used to convert mRNA to cDNA. Real-time PCR amplifications were performed on a 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 tool (version 7.2) was used to assess the putative binding sites between miR-17-5p and 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 using Lipofectamine 3000 reagent (Invitrogen) with miRNAs (miR mimic or NC mimic) and luciferase reporter vectors (TLR4 3'UTR-WT or TLR4 3'UTR- MUT). Forty-eight hours post-transfection, luciferase activity was assessed using dual luciferase reporter analysis (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. 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 included TLR4, p-p65, p65, p-IκBα, IκBα, Blc-2, Bax, and caspase-3 (Cell Signaling, Danfoss, Mass.) GAPDH (Santa Cruz, California) was probed a loading control.

Apoptosis assays

After 48 hours of H_2O_2 treatment, cells were stained with 5 μL Annexin V-FITC and 5 μL PI (BD Biosciences, USA). Apoptosis was analyzed by flow cytometry (Becton Dickinson, USA). Annexin V-positive cell populations were considered apoptotic.

Statistical analysis

Data are the mean ± standard deviation of the mean (SD). Differences between the two groups were analyzed using a two-tailed Student's t test or one-way analysis of variance with Bonferroni multiple

comparison tests. Statistical analysis was performed using SPSS 18.0 software. $P < 0.05$ was considered statistically significant.

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 are linked to OGD/R. The expression of IL-6, IL-1β and TNF-α were higher in the OGD/R group ($P < 0.05$, Fig. 1A). Compared to the OGD/R + vehicle group, Dex treatment suppressed IL-6, IL-1β and TNF-α expression in a dose-dependent manner ($P < 0.05$, Fig. 1A). Flow cytometry analysis showed that OGD/R treatment increased the apoptotic rates of PC12 cells, whilst Dex treatment significantly suppressed H_2O_2 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 cysteine-containing aspartate Expression of Cysteinyl aspartate specific proteinase-3 (Caspase-3) ($P < 0.05$, Fig. 1C). These effects in the OGD/R + Dex group were dose-dependent. These results suggest 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 from the OGD/R group compared to the control group. Upon comparison to the OGD/R + vehicle group, Dex treatment upregulated miR-17-5p in a dose-dependent manner ($P < 0.05$, Fig. 2A). As TLR4 regulates inflammatory responses through NF-κB signaling, TLR4/NF-κB signaling was further examined. Western blot analysis showed that Dex treatment significantly suppressed the expression of phosphorylated IκBα and phosphorylated p65, but did not affect IκBα and p65. The reduction of TLR4 led to a loss of phosphorylated IκBα and phosphorylated p65 ($P < 0.05$, Fig. 2B). The predicted binding sites between miR-17-5p and the 3'UTR of target genes were analyzed using TargetScan, from which the TLR4 3'UTR (position 4654–4660) was shown to possess complementary binding sites for miR-17-5p ($P < 0.05$, Fig. 2C). After sub-cloning the WT and MUT TLR4 3'UTR into luciferase reporter vectors, luciferase activity was determined in HEK293 cells after co-transfection with miRNAs and luciferase reporter vectors. 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

As TLR4 regulates inflammatory responses via NF-κB signaling, the interaction between miR-17-5p and TLR4/NF-κB were examined. Western blot analysis showed that miR-17-5p overexpression significantly suppressed TLR4, phosphorylated IκBα and phosphorylated p65, but had no effect on IκBα or p65. MiR-17-5p silencing increased the expression of TLR4, phosphorylated IκBα, and phosphorylated p65 ($P < 0.05$, Fig. 3A). As miR-17-5p was down-regulated in the OGD/R group, the effects of miR-17-5p overexpression on the production of pro-inflammatory cytokines in PC12 cells were next determined. Consistently, ELISA analysis showed that the levels of IL-1β, TNF-α and IL-6 in PC12 cells overexpressing miR-17-5p were significantly lower to those transfected with NC mimics. On the other hand, the knockdown of miR-17-5p enhanced the production of pro-inflammatory cytokines in PC12 cells ($P < 0.05$, Fig. 3B). We next examined changes in the apoptotic rates of PC12 cells transfected with miR-17-5p mimics following H_2O_2 stimulation. Flow cytometry analysis revealed that the overexpression of miR-17-5p significantly suppressed H_2O_2 induced apoptosis in PC12 cells, increased Bcl-2 levels, and suppressed Bax and Caspase-3 expression. 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 attenuated 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 in PC12 cells compared to the Dex group ($P < 0.05$, Fig. 4A). TLR4 overexpression and miR-17-5p silencing significantly increased the apoptotic rates of 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 lead to the induction of apoptotic signaling and programmed neuronal cell death [17]. Apoptosis after cerebral ischemia-reperfusion injury is a major form of neuronal death [18]. The inhibition of apoptosis can be used as a potential therapeutic intervention to protect 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* to explore the neuroprotective effects of Dexmedetomidine.

Dex is a highly selective and specific α2 adrenoceptor agonist that exhibits a broad spectrum of effects, including sedation, hypnosis, anesthesia and analgesia [19]. Dex is 1600-fold more selective for α2 over α1, and inhibits apoptosis, exerting neuroprotective effects in the developing brain [20]. The mechanism(s) mediating these effects are however poorly understood.

MiRNAs participate in a range of essential biological processes including neuronal apoptosis during ischemic stroke and nervous system dysfunction [21]. Here, we explored the underlying biological mechanisms of DEX in OGD/R-induced neurotoxicity and assessed the involvement of miR-17-5p and potential molecular factors.

TLR4 belongs to the Toll-like receptor family. These represent innate pattern recognition receptors that mediate the host response to pathogen infection [22]. TLR4 activation promotes the production of inflammatory cytokines, such as IL-1β, TNF-α, and IL-6 [23]. Aberrant IL-1β and IL-6 responses induced by TLR4 were observed in patient.

We found that miR-17-5p was downregulated in the OGD/R group and mediated OGD/R-induced inflammation and apoptosis. Dex treatment increased miR-17-5p expression in a dose-dependent manner in PC12 cells, the overexpression of which led to the dampening of inflammatory responses due to NF-κB inhibition. The downregulation of miR-17-5p produced the opposite phenotype. To explore the detailed mechanisms underlying these effects, the StarBase v2.0 database was employed with miR-17-5p identified as a potential miRNA. Further results showed that miR-17-5p negatively correlated with TLR4, with luciferase gene reporter assays showing that miR-17-5p binds to TLR4. However, the functions of miR-17-5p were not defined. To further investigate the role of miR-17-5p in OGD/R-induced inflammation and apoptosis in PC12 cells, miR-17-5p mimics and inhibitors were respectively transfected into each group. We found that miR-17-5p inhibits OGD/R-induced inflammation and apoptosis. We identified NF-κB signaling as a potential mediator of miR-17-5p and found that miR-17-5p inhibits TLR4/NF-κB signaling. Taken together, we postulate that Dex upregulates miR-17-5p which inhibits NF-κB, thereby reducing OGD/R-induced inflammation and apoptosis. Moreover, we found that the inhibition of OGD/R-induced inflammation and apoptosis were suppressed following TLR4 overexpression and miR-17-5p silencing.

Previous studies have shown that Dexmedetomidine attenuates oxygen-glucose deprivation/reperfusion-induced inflammation and apoptosis in PC12 cells through its effects on the miR-17-5p/TLR4/NF-κB axis. These data suggest that Dex represents a novel intervention strategy for cerebral ischemia-reperfusion injury. These findings now require verification in human stroke patients.

Some limitations should be noted. Firstly, the optimal concentration of Dex *in vivo* was not investigated. As such, multiple doses of Dex require investigation in further *in vivo* experiments. Secondly, we found that Dex up-regulates the expression of miR-17-5p in PC12 cells. Further studies are now required to confirm these effects *in vivo*.

Abbreviations

DEX: Dexmedetomidine; ELISA: enzyme linked immunosorbent assays; miRs: MicroRNAs; Dex: dexmedetomidine; mimics NC: mimic negative controls; inhibitors NC: inhibitor negative controls; IL-1β: 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: Cysteinyl aspartate specific proteinase-3

Declarations

Ethics approval and consent to participate

All protocols 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.

Availability of data and materials

The datasets used and/or analysed during the current study are available

from the corresponding author on reasonable request.

Competing interests

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

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Authors' contributions

LS: Supervision, Methodology, Investigation, Datas Curation, Writing – Original Draft, Writing – Review & Editing, Visualisation. MW: Conceptualization, Resources, Dats Curation, Writing – Review & Editing, Project Administration. All authors read and approved the final manuscript.

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None to declare.

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Figures

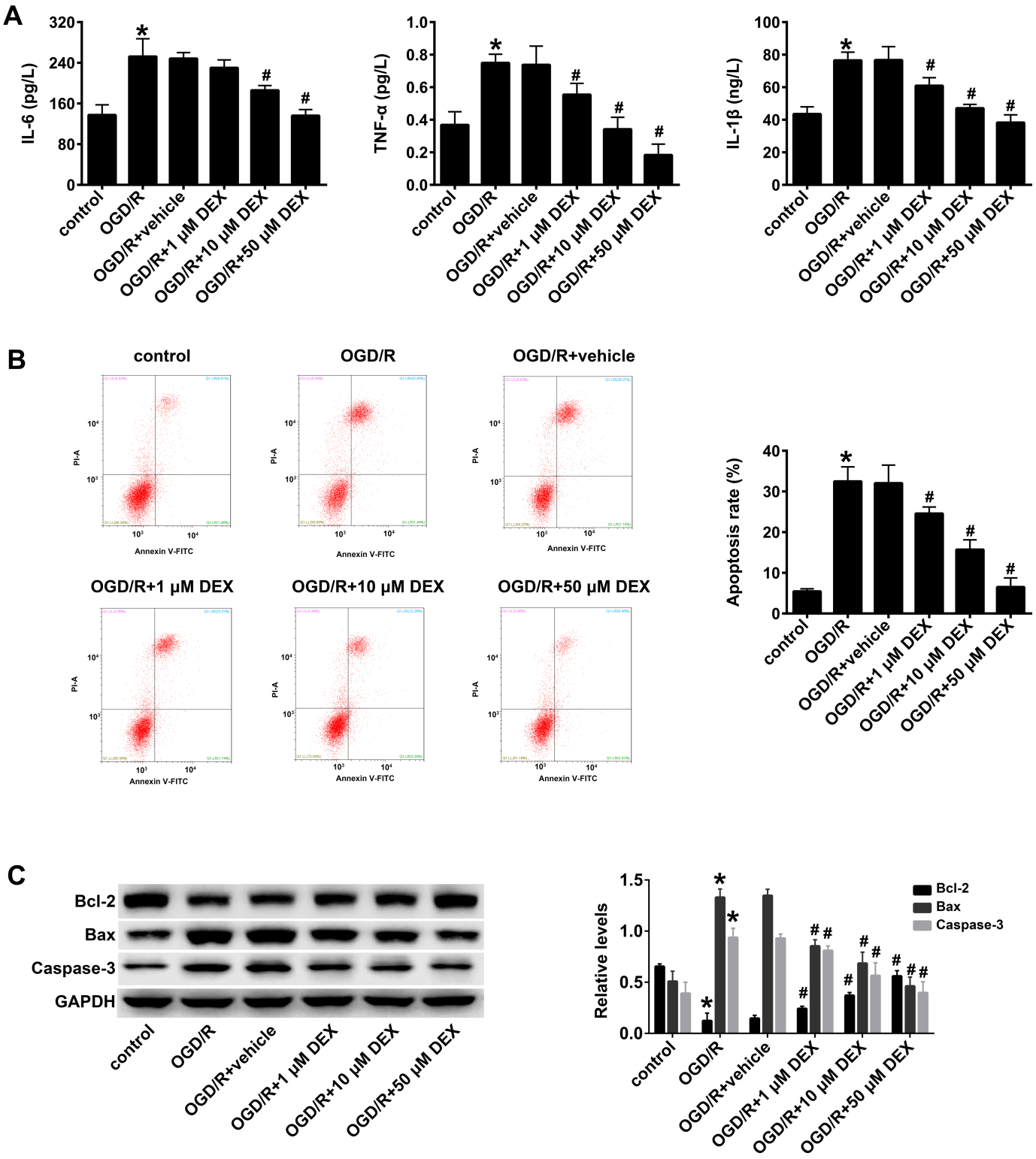

Figure 1

Dexmedetomidine attenuates OGD/R-induced inflammation and apoptosis in PC12 cells. (A) IL-1β, TNF-α and IL-6 production in PC12 cells determined by ELISA. (B) Apoptosis rates detected by flow cytometry in H2O2 treated PC12 cells treated with the indicated interventions. (C) Expression of Bcl-2, Bax, and

caspase 3 detected by Western blotting. Data represent the mean ± SD of three independent experiments. *P<0.05 vs control; #P<0.05 vs OGD/R+vehicle.

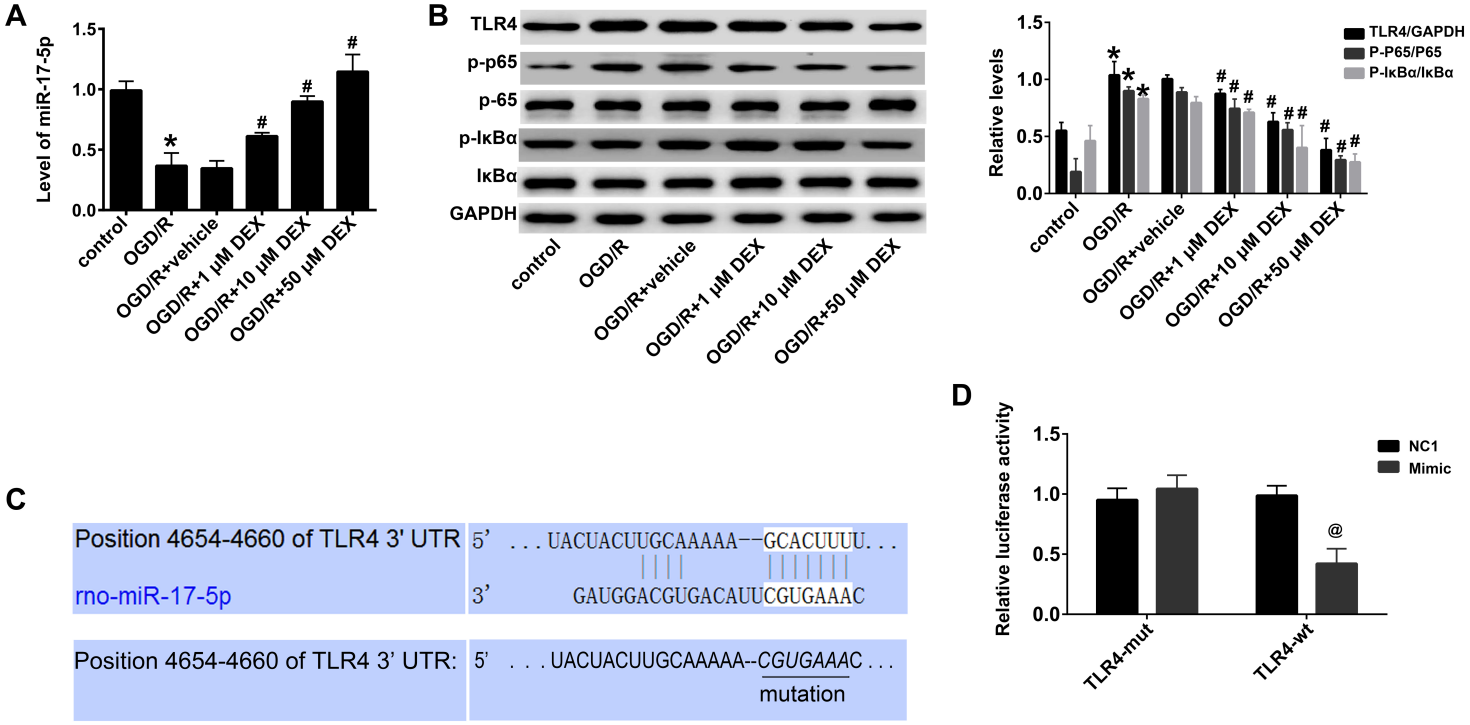

Figure 2

Dexmedetomidine inhibits TLR4/NF-κB signaling through miR-17-5p in PC12 cells. (A) miR-17-5p expression in treatment and control groups determined by qRT-PCR. (B) Relative expression of TLR4, phosphorylated IκBα, IκBα, phosphorylated p65 and p65 in each group (C) Putative binding sites between miR-17-5p and TLR4 3'UTR. (D) Luciferase activity of TLR4 3'UTR-WT and TLR4 3'UTR-MUT in HEK293 cells following treatment with NC or miR-17-5p mimics determined using the Dual-Luciferase Reporter Assay system. Data represent the mean ± SD of three independent experiments. *P<0.05 vs control; #P<0.05 vs OGD/R+vehicle.

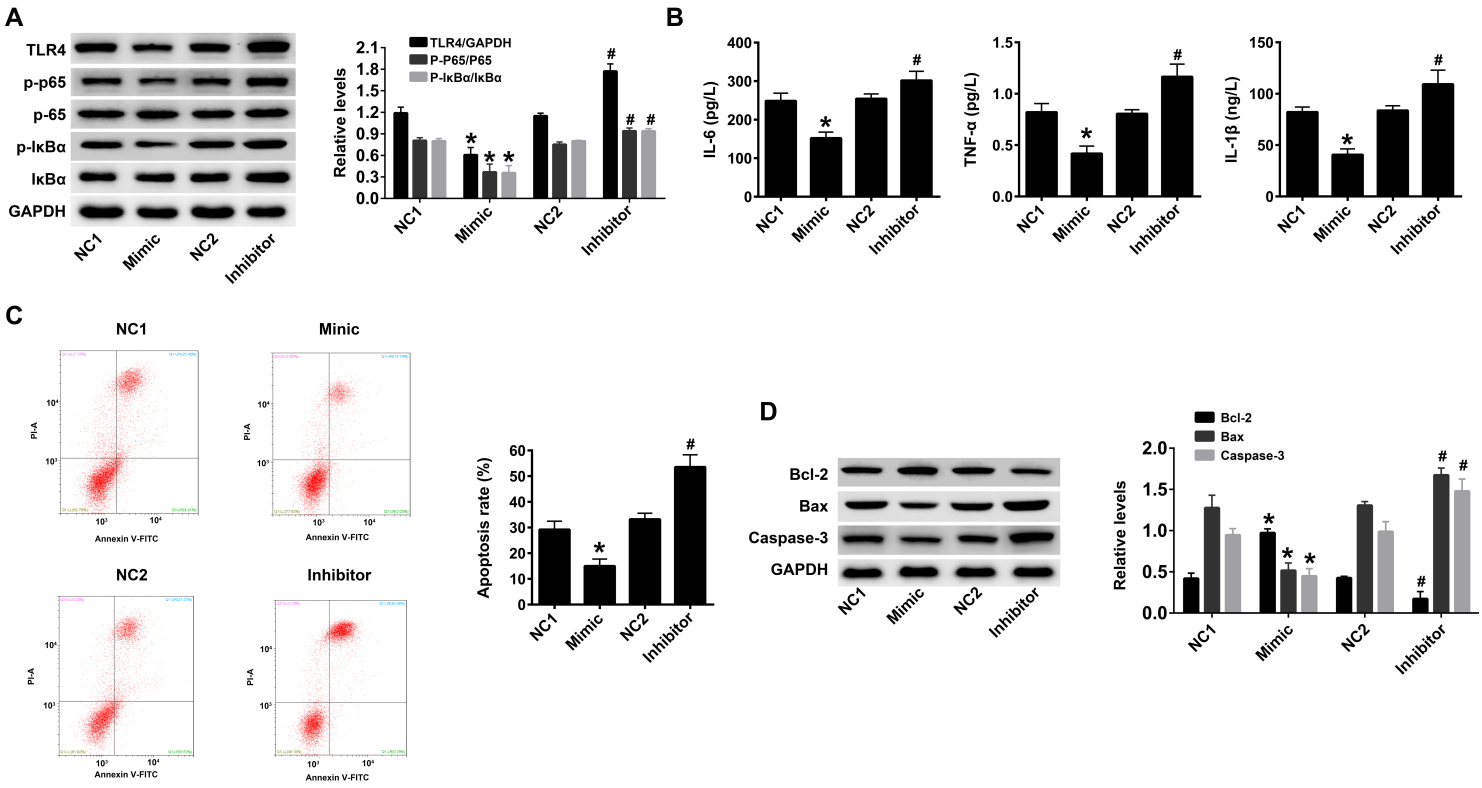

Figure 3

MiR-17-5p inhibits TLR4/NF-κB signaling, inflammation and apoptosis in PC12 cells following OGD/R. (A) Relative expression of phosphorylated IκBα, IκBα, phosphorylated p65 and p65 in PC12 cells after transfection with miR-17-5p mimics and inhibitors determined by western blot assays. (B) PC12 cells were transfected with miR-17-5p mimics and inhibitors for 48 h and TNF-α, IL-6 and IL-1β levels were measured by ELISA. (C) Apoptosis was detected by flow cytometry in H2O2 treated PC12 cells after transfection with miR-17-5p mimics and inhibitors. (D) Protein expression of Bcl-2, Bax and caspase 3 detected by western blotting after transfection with miR-17-5p mimics and inhibitors. Data represent the mean ± SD of three independent experiments. *P<0.05 vs control; #P<0.05 vs OGD/R+vehicle.

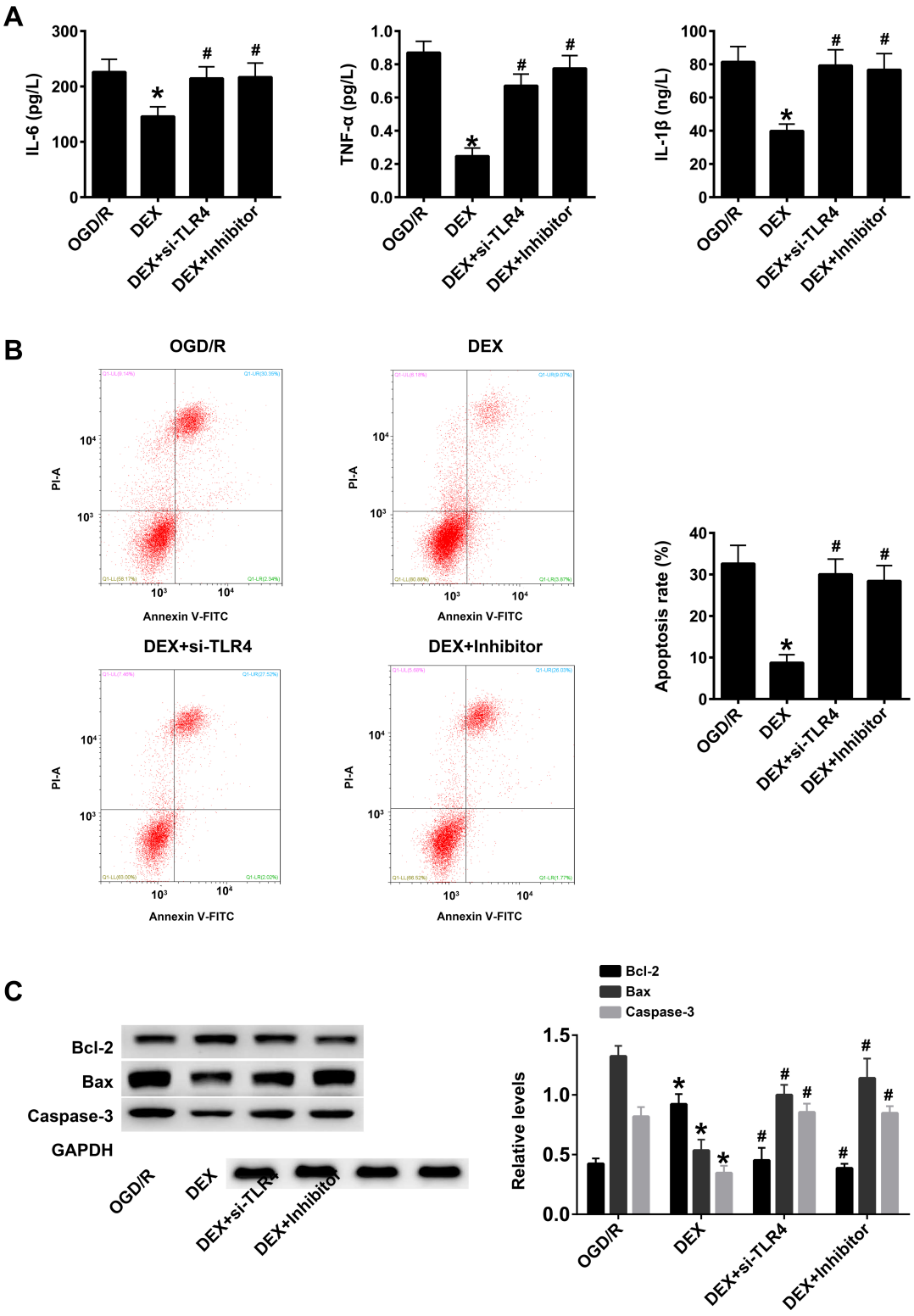

Figure 4

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 inhibitors and TNF-α, IL-6 and IL-1β levels were measured by ELISA. (B) Apoptosis was detected by flow cytometry in H2O2 treated PC12 cells after transfection with pcDNA3.1-TLR4 and miR-17-5p inhibitors. (C) Bcl-2, Bax and caspase 3 expression detected by western blot in PC12 cells transfected with

pcDNA3.1-TLR4 and miR-17-5p inhibitors. Data are the mean ± SD of three independent experiments. *P<0.05 vs OGD/R; #P<0.05 vs DEX.