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ORIGINAL PAPER

# miR-124-5p/NOX2 Axis Modulates the ROS Production and the Inflammatory Microenvironment to Protect Against the Cerebral I/R Injury

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## Abstract

The reperfusion after an acute ischemic stroke can lead to a secondary injury, which is ischemia–reperfusion (I/R) injury. During ischemia, the reactive oxygen species (ROS) is over-produced, mostly from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX). Besides, miRNAs are also associated with neuronal death in ischemic stroke. MiR-124-5p is selectively expressed within central nervous system (CNS) and is predicted to bind to NOX2 directly. Herein, we successfully set up cerebral I/R injury model in rats through middle cerebral artery occlusion (MCAO) surgery. After 12 h or 24 h of refusion, the superoxide dismutase (SOD) activity was significantly inhibited, accompanied by NOX2 protein increase within MCAO rat infarct area. In vitro, oxygen-glucose deprivation/refusion (OGD/R) stimulation on PC-12 cells significantly increased NOX2 protein levels, ROS production, and the cell apoptosis, while a significant suppression on SOD activity; OGD/R stimulation-induced changes in PC-12 cells described above could be significantly attenuated by NOX2 silence. In vivo, miR-124 overexpression improved, whereas miR-124 inhibition aggravated I/R injury in MCAO rats. miR-124-5p directly bound to the *CYBB* 3′-untranslated region (UTR) to negatively regulate *CYBB* expression and NOX2 protein level. In vitro, miR-124 overexpression improved, while NOX2 overexpression aggravated OGD/R-induced cellular injuries; NOX2 overexpression significantly attenuated the effects of miR-124 overexpression. Besides, miR-124 overexpression significantly repressed NF-κB signaling activation and TNFα and IL-6 production through regulating NOX2. In conclusion, miR-124-5p/NOX2 axis modulates NOX-mediated ROS production, the inflammatory microenvironment, subsequently the apoptosis of neurons, finally affecting the cerebral I/R injury.

**Keywords:** Ischemia–reperfusion (I/R) injury · Reactive oxygen species (ROS) · miR-124-5p · Nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) · NF-κB signaling

## Introduction

Acute ischemic stroke is the second major cause of death worldwide [1–4]. Cerebral blood flow occlusion induces the depletion of energy and nutrients needed for cell metabolism, which results in osmotic gradient imbalance, depolarization of cell membranes and augmented intracellular calcium, thus leading to mitochondrial dysfunction. The reperfusion can be essential for the ischemic brain tissue to regain normal function, but it can also lead to secondary injury, known as ischemia–reperfusion (I/R) injury.

During ischemia, a large number of reactive oxygen species (ROS) can be produced, mostly from the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), which is a family of transmembrane proteins [5–9]. The excessive production of ROS leads to the oxidative stress,

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thus resulting in the activation of the NF-κB signaling and the synthesis of the pro-inflammatory cytokines, including nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6). Subsequently, enhanced neuroinflammation and matrix metalloproteinases-9 (MMP-9) participates in the destruction of extracellular matrix (ECM), as well as the degradation of tight junction proteins, causing blood-brain barrier injury [10, 11]. In theory, the balance between the production and elimination of ROS is maintained through multiple antioxidant defense mechanisms. The most critical of these mechanisms is the superoxide dismutase (SOD) family of enzymes whose activity levels indicate oxidative stress status [12]. Malondialdehyde (MDA) is considered as not only a product of lipid peroxidation but also a marker of oxidative stress to predict lipid peroxidation degree [13]. Activating this system can be essential to protect the brain against I/R injuries.

Of the seven members in the NOX family, NOX2 and NOX4 are considered the main sources of ROS 8–16 h after reperfusion in the stroke model [14]. NOX1 and 2 have been reported to express in neurons [15–17], smooth muscle cells [18, 19], and microglia [15, 16, 19–22]. Savchenko et al. [15] demonstrated that NOX1 and NOX2 expression might alter in microglia and neurons in response to stimulation in vitro; in addition, NOX2 expression is upregulated in microglia in response to activation in cases of multiple sclerosis [20], traumatic brain injury [23], and ischemia [16]. Moreover, NOX2 is increased within the spinal cord after damage [24–26]. Based on these previous findings, developing an in-depth understanding of the role of NOX2 and NOX-mediated ROS production during I/R injury, and searching for promising agents inhibiting NOX2 and ROS levels might provide novel strategies to prevent the patients from cerebral I/R injury.

miRNAs are considered short, non-coding RNAs exerting a regulatory effect on the vast majority of physiological and pathological processes contributing to multiple human diseases, such as stroke [27–29]. Via the specific base pairing to multicellular eukaryotic mRNAs in their 3′-untranslated region (UTR), miRNAs induce target mRNA degradation and/or inhibit their translation so as to suppress the expression of target genes [30], therefore regulating the proliferation, apoptosis, differentiation, invasion, migration, and development of cells [31]. Recently, several miRNAs have been demonstrated to be associated with neuronal death within the ischemic stroke. MiR-124 is almost selectively expressed within central nervous system (CNS), which makes it unique in miRNAs; miR-124 abundance in the CNS can exceed 100 times compared to that within other organs [32]. More importantly, the overexpression of miR-124 remarkably downregulated whereas the silence of miR-124 remarkably upregulated the infarct area of middle cerebral artery occlusion (MCAO) mice in vivo. In vitro, gain-of-function or loss-of-function of miR-124 could respectively lead to the reduction or increase of neuron apoptosis and death caused by oxygen-glucose deprivation/refusion (OGD/R), and the increase or reduction of anti-apoptosis protein (Bcl-2 and Bcl-xl) [33]. Interestingly, according to Targetscan prediction, miR-124 could directly bind to NOX2 (encoded by *CYBB* gene in rats). Thus, we speculate that miR-124 might target NOX2 to regulate NOX2 and NOX-mediated ROS production, finally improving MCAO/R- or OGD/R-induced injury in vivo and in vitro.

Herein, we established a cerebral ischemia–reperfusion model by MCAO operation in rats, evaluated the model, and examined SOD activity, MDA content, and NOX2 protein levels in rats' brain. OGD/R model was then generated in PC-12 cells, and the cellular effects of NOX2 upon SOD activity, MDA content, ROS production, and cell apoptosis were examined. Next, gain or loss miR-124 function was detected in MCAO/R rats, and the putative binding of miR-124-5p to rat CYBB was examined. Afterward, the dynamic effects of miR-124 and NOX2 on SOD activity, MDA content, ROS production, cell apoptosis, as well as NF-κB signaling activation and TNFα and IL-6 production were examined within OGD/R-stimulated PC-12 cells. In summary, we attempted to propose a novel mechanism of miR-124/NOX2 axis modulating the ROS production and the inflammatory microenvironment during cerebral I/R injury, eventually protecting against cerebral I/R injury.

## Materials and Methods

### Establishment of MCAO/Reperfusion Model in Rats

Total 68 SD adult male rats (289 ± 10 g) were obtained from Chengdu Dossy Experimental Animals Co., Ltd. (Chengdu, China) and maintained in a constant 12-h dark/light cycle with standard laboratory chew and food-water available ad libitum. All experimental procedures were performed following the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the Animal Care and Use Ethics Committee of Shunyi District Hospital.

Animals were randomly divided into three groups in model establishment step: sham (n = 8), middle cerebral artery (MCAO) + 12 h refusion (n = 10), and MCAO + 24 h refusion (n = 50). Total 40 MCAO/R (24 h refusion) rats were randomly divided into four groups (n = 10 in each group): Agomir-NC (negative control), Agomir-124, Antagomir-NC, and Antagomir-124. Either the Agomir-NC, Agomir-124, Antagomir-NC, or Antagomir-124 (GenePharma, Shanghai, China) was intracerebroventricularly administrated 12 h before the MCAO operation.

Rats in the MCAO/R group were subjected to intraluminal occlusion of the right middle cerebral artery following the methods described previously [34–36]. After 90 min of occlusion, the suture was removed to restore blood flow and allowed reperfusion until 12 or 24 h. Rats in the sham group were subjected to suture insertion into the origin of the internal carotid artery, but not the middle cerebral artery, with the remaining procedures identical to those in the MCAO/R (24 h) injury group. Rat models were selected according to a previously described method [36]. All rats were sacrificed and the entire brain rapidly removed and incubated with triphenyl tetrazolium chloride (TTC) (Beijing Cinontech Co., Ltd.) with the ipsilateral cerebral cortex harvested for ELISA, Immunoblotting, and real-time PCR analyses.

### miR-124 Administration

4.5 µl of 100 µM Agomir-NC, Agomir-124, Antagomir-NC, or Antagomir-124 were incubated with 1.5 µl siRNA-Mate transfection reagent (GenePharma) for 30 min, then stereotaxically delivered into the right lateral ventricle for more than10 min. The bone wound was closed with bone wax.

### Cell Line and Cell Culture

PC-12 (ATCC® CRL-1721™, ATCC, Manassas, VA, USA) cell line was obtained and cultured in RPMI-1640 medium (Catalog No. 30-2001, ATCC) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (Invitrogen, Waltham, MA, USA). Cells were incubated in a humidified incubator with 5% $CO_2$ at 37 °C, with the medium changed every 3 days. After 7 days in culture, the cells were used for subsequent OGD/R model analysis.

### Cell Transfection

PC12 cells ($2\times10^5$) were seeded into a six-wells plate. For NOX2 silence, 50 nmol/l the specific small interfering RNA of NOX2 (si-NOX2) or negative control (si-NC) using 8 µl siRNA-Mate transfection reagent (GenePharma, Co., Ltd., Shanghai, China). For NOX2 overexpression, 1 µg/ml NOX2 overexpression vector (NOX2 OE) or empty vector (NC) were transfected into PC12 cells with 8 µl Lipo2000 (Invitrogen, USA). For miR-124-5p overexpression or suppression, 50 nmol/L of Agomir-124 or Antagomir-124 were transfected into PC12 cells with 8 µl siRNA-Mate transfection reagent (GenePharma), The cells were harvested after 48 h. Western blot analyses or other experiments were performed.

### OGD/R Model in PC-12 Cells

To mimic ischemic-like conditions in vitro, cell cultures were exposed to oxygen-glucose deprivation (OGD) for 4 h followed by culture in 5% $CO_2$ and glucose-containing medium following the methods described previously [37]. PC-12 cells were transfected or non-transfected and transferred into a temperature controlled anaerobic chamber (Forma Scientific, Waltham, MA, USA) with 5% $CO_2$ and 95% $N_2$ (hypoxic condition) at 37 ℃ and cultured in deoxygenated glucose-free RPMI-1640 medium 4 h. After OGD, PC-12 cells were maintained in glucose-containing RPMI-1640 medium supplemented with 10% FBS under normoxia culture conditions for 12 h for reoxygenation. Cells cultured under normal conditions were used as a control. After treatments, the cells were observed under an optical microscope (Olympus, Tokyo, Japan) or collected for further experiments.

### Neurological Function Evaluation

MCAO rats received a neurological function evaluation at 12 h or 24 h after the MCAO operation following a modified 6-point scoring system [38, 39]. A single observer blinded to group assignment performed neurological testing.

### Triphenyl Tetrazolium Chloride (TTC) Staining and Quantitation of Infarct Volume

The whole brain tissue was harvested after 90 min of ischemia followed by 12 or 24 h of reperfusion, ensuring that upon removal, the integrity of the brain was maintained. The brains were rapidly frozen at − 20 °C for 20 min, sliced, and then incubated in 2% TTC solution at 37 °C for 30 min, avoiding light with a foil cover as described before [40], and later photographed using a digital camera. The infarct region appears white, while non-ischemic regions are red. The infarct volume was then calculated from digitized images using the ImageJ software package (NIH, Bethesda, MD, USA) following the methods described previously [41].

### ELISA

The examination of SOD, MDA, TNF-α, and IL-6 was conducted by ELISA assay using rat SOD, MDA, TNF-α, and IL-6 ELISA kits according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The specific binding optical density was assayed immediately at 450 nm with a spectrophotometer (Bio-Rad Laboratories, USA).

### Protein Levels Determination by Immunoblotting

The protein levels of NOX2, p65, p-p65, and IκB were determined using Immunoblotting. Proteins were extracted and the concentrations of proteins were analyzed using the bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, China). After that, the protein samples

were loaded onto an SDS-PAGE minigel for protein separation and transferred onto a PVDF membrane. Afterward, the membrane was probed with the antibodies (dilution 1:1000) listed below: anti-NOX2 (ab80508, Abcam, Cambridge, MA, USA), anti-p-p65 (ab86299, Abcam), anti-p65 (ab16502, Abcam), and anti-IκB (ab32518, Abcam) at 4 °C overnight. Then, the blots were incubated with anti-rabbit or anti-mouse HRP-conjugated secondary antibody (dilution 1:5000, ab205718 and ab205719). Signals visualization was conducted by ECL Substrates (Millipore, MA, USA) using β-actin (ab8226, Abcam) as an endogenous protein for normalization. The gray intensity analysis was performed using ImageJ software (NIH, MD, USA).

### Real-Time PCR

Total RNA was extracted from targeted cells using Trizol reagent (Invitrogen, CA, USA) and the expression of miR-124-5p was determined using PCR-based analyses following the methods described previously [42]. The specific primers were: reverse transcription primer: 5′-GTC GTA TCC AGT GCG TGT CGT GGA GTC GGC AAT TGC ACT GGA TAC GAC ATC AAG-3′; forward: 5′-CGT GTT CAC AGC GGA C-3′; reverse: 5′-CAG TGC GTG TCG TGG A-3′. Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China) were used. RNU6B was employed as endogenous controls for miRNA expression determination (forward:5'-CCT GCT TCG GCA GCA CA-3'; Reverse: 5′-AAC GCT TCA CGA ATT TGC GT-3′). The data were processed using a $2^{-\Delta\Delta CT}$ method.

### Luciferase Reporter Assay

For miR-124-5p binding to rat *CYBB* (gene encoding NOX2 protein in rats) 3′-UTR, the fragment of *CYBB* 3′-UTR was cloned to the downstream of the Renilla psiCHECK2 vector (Promega, Madison, WI, USA) and named wt-*CYBB* 3′-UTR. The mutant type (mut-*CYBB* 3′-UTR) was mutated in the putative miR-124 binding sites. 293T cells were co-transfected with these reporter vectors and agomir-124 or antagomir-124, respectively; the luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was normalized to Firefly luciferase activity for each transfected well.

### Flow Cytometry

For intracellular ROS level measurement, cells were incubated with 10 μmol/l DCFH-DA (Sigma, USA) in the dark for 20 min at 37 °C prior to harvest, and then washed with PBS. The fluorescence intensity of the cells was determined by flow cytometry (BD, CA,USA). For apoptosis assay, cells were harvested and incubated with 5 μl annexinV-FITC solution and 5 μl propidium iodide solution (Keygene, Nanjing, China) in the dark for 10 min at room temperature and determined by flow cytometry (BD, CA,USA).

### Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End-Labeling (TUNEL) Assay

TUNEL assay was performed to detect OGD/R-mediated apoptosis in PC-12 cells using TUNEL analysis kit (Roche, Basel, Switzerland). The cell nucleus was presented as blue color, and the positive nucleus was indicated as red color. The number of positive nuclei was determined by manually counting (magnification × 400) the positively labeled nuclei in five randomly selected fields under a microscope. Apoptosis rate was considered as the percentage of TUNEL-positive cells from the total cell number. The positive control and negative control for TUNEL assay were shown in Fig.S1.

### Statistical Analysis

Results from at least three independent experiments are expressed as mean ± standard deviation (S.D.). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or by Student's *t* test. A $P$ value of $< 0.05$ indicates statistical significance.

## Results

### NOX2 is Up-Regulated in the Cerebral Ischemia–Reperfusion Model (I/R) in Rats

Firstly, I/R model was established within adult SD rats and examined. After minutes of ischemia and then 12 or 24 h of reperfusion, the infarct (white region) and non-ischemic (red region) regions in sham, MCAO + 12 h refusion, and MCAO + 24 h refusion groups were analyzed by TTC staining followed by the quantitative analyses. Figure 1a, b showed that MCAO operation remarkably increased the infarct volume after 12 and 24 h of refusion. Consistently, the neurological function scores of MCAO rats were significantly increased after 12 and 24 h of refusion (Fig. 1c), indicating the existence of ischemic injury.

NOX [43] and NOX-mediated reactive oxygen species (ROS) could react with lipids, proteins, and DNA during ischemia, thus activating apoptosis pathways, and eventually exacerbating cerebral ischemic damage [44, 45]. Thus, we also monitored the SOD activity, MDA content, and NOX2 protein levels in three groups. Consistent with previous studies, SOD activity was significantly impaired in MCAO rats after 12 and 24 h of refusion (Fig. 1d); in the

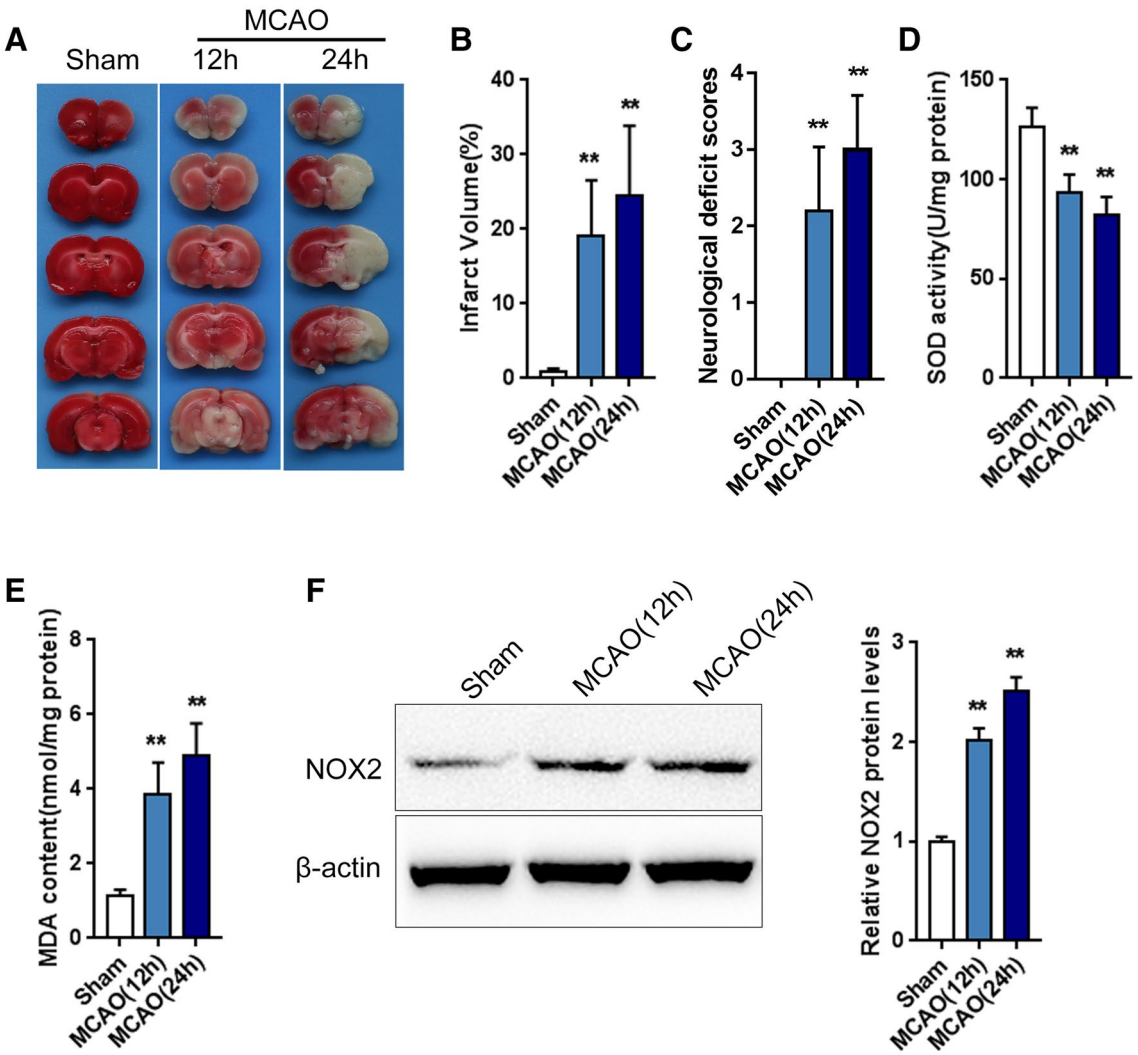


**Fig. 1** NOX2 is up-regulated in the ischemia reperfusion model (I/R) in rats. **a** The infarct (white region) and non-ischemic (red region) regions in sham, MCAO + 12 h refusion, and MCAO + 24 h refusion groups revealed by TTC staining (n = 5). **b** The quantitative analysis on infarct volumes in three groups by using the ImageJ software package. n = 5. **c** MCAO rats underwent neurological function evaluation at 12 h or 24 h after MCAO surgical procedure following a modified 6-point scoring system n = 10. **d, e** SOD activity and MDA content in three groups determined by ELISA. n = 5. **f** NOX2 protein levels in three groups determined by Immunoblotting. n = 5. All results were represented as means ± SD from three independent experiments. **P < 0.01.

meantime, MDA content and NOX2 protein levels were significantly increased in MCAO rats after 12 and 24 h of refusion (Fig. 1e, f), indicating the increase of NOX and NOX-mediated ROS.

## Cellular Effects of NOX2 on OGD/R Model Within PC-12 Cells

After confirming increases in NOX2 and NOX-mediated ROS during ischemic injury, next, we further investigated the cellular effects of NOX2 on OGD/R model established within PC-12 cells. We transfection si-NC or si-NOX2 to conduct NOX2 silence within OGD/R-stimulated PC-12 cells, and performed Immunoblotting to verify the transfection efficiency (Fig. 2a). PC-12 cells cultured in normal condition were taken as a control. NOX2 protein levels could be significantly induced by OGD/R stimulation while decreased by si-NOX2 transfection (Fig. 2a). Consistently, SOD activity was significantly inhibited by OGD/R stimulation while rescued by NOX2 silence (Fig. 2b). In the meantime, the content of MDA and the production of ROS could be significantly enhanced within OGD/R-stimulated PC-12 cells while suppressed by NOX2 silence (Fig. 2c, d). As a result of ROS increase, the apoptosis of PC-12 cells was significantly enhanced upon OGD/R stimulation while suppressed by NOX2 silence

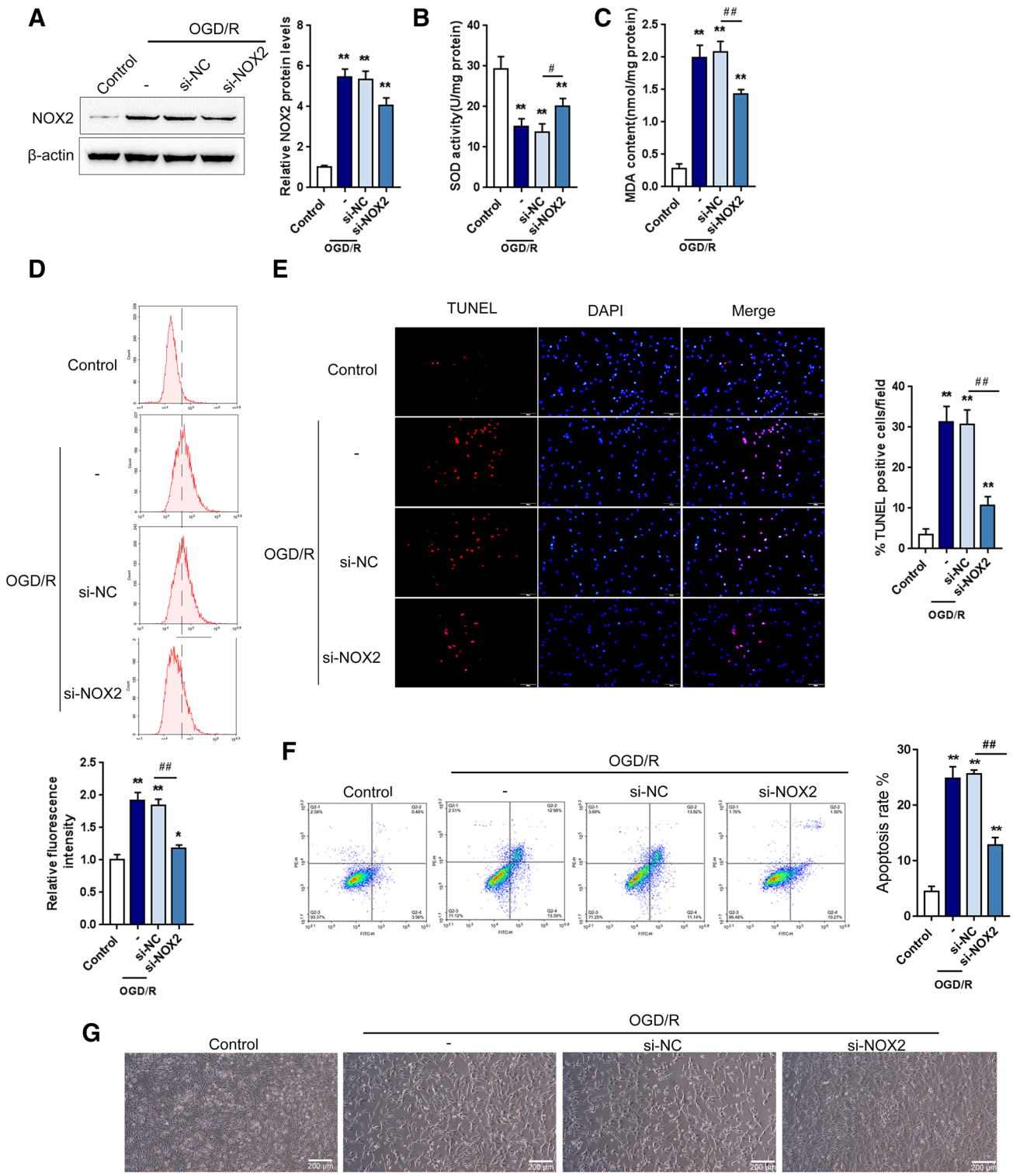


**Fig. 2** Cellular effects of NOX2 on OGD/R model in PC-12 cells. PC-12 cells were not transfected or transfected with si-NC (negative control) or si-NOX2, subjected to OGD/R stimulation, and then examined for **a** NOX2 protein levels by Immunoblotting; n = 4. **b**, **c** SOD activity and MDA content in three groups by ELISA; n = 4. **d** ROS production by Flow cytometry; n = 4. **e**, **f** cell apoptosis by TUNEL staining and flow cytometry n = 4. (scale bar: 50 μm). **g** The morphology change of PC12 cells was observed by optical microscope (scale bar: 200 μm) n = 3. All results were represented as means ± SD from three independent experiments. *P < 0.05, **P < 0.01, compared to control group; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, compared to OGD/R + si-NC group.

(Fig. 2e, f). Consistently, the morphology change of PC12 cells shown that OGD/R stimulation reduced the cell numbers while NOX2 silence partly reversed the reduction effect of OGD/R stimulation to some extent (Fig. 2g). These data indicate that NOX2 and NOX-mediated ROS could be significantly induced upon OGD/R stimulation, which further results in enhanced PC-12 cell apoptosis and cellular damage; NOX2 silence remarkably reverses OGD/R stimulation-induced damages within PC-12 cells.

## Protective Effects of miR-124 on MCAO Rats

Since miR-124 can not only improve I/R-induced cerebral damage and dysfunction via modulating Ku70 [46], but also be predicted to target NOX2-encoding gene, *CYBB*; here, we hypothesize that miR-124 might also exert its protective effect on MCAO rats via regulating NOX2. To validate this hypothesis, we first examined miR-124 expression in rats received sham or MCAO operation and then 24 h-refusion. Figure 3a showed that miR-124 expression

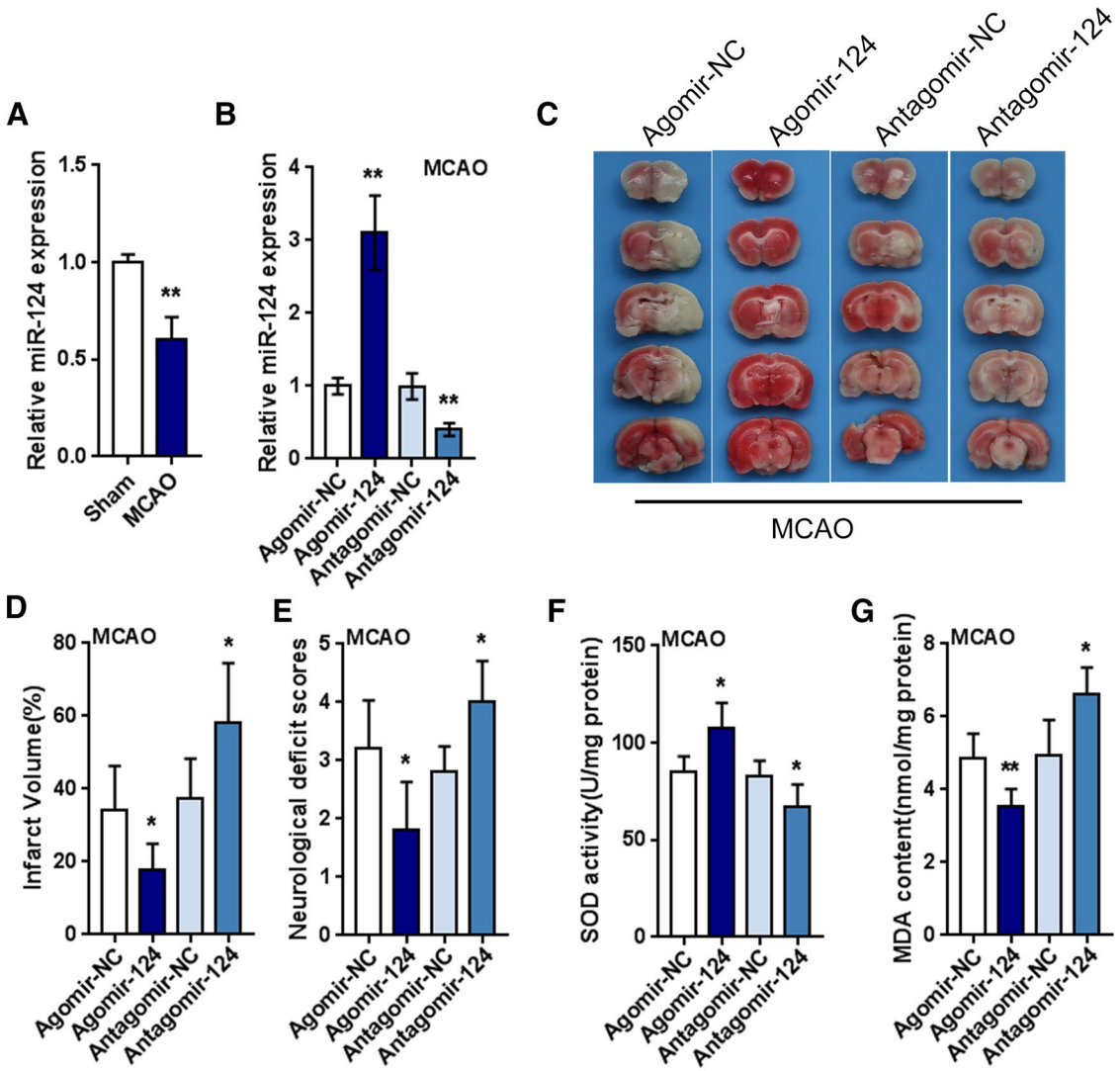


**Fig. 3** Protective effects of miR-124 on MCAO rats. **a** Rats were subjected to sham or MCAO operation followed by refusion for 24 h and examined for miR-124 expression by real-time PCR. n = 4. **b** miR-124 overexpression or miR-124 inhibition was conducted in MCAO rats via administrating the Agomir-NC (negative control), Agomir-124, Antagomir-NC (negative control), or Antagomir-124 (50 nmol/kg) by intracerebroventricularly injection at 12 h before MCAO operation, as confirmed by real-time PCR. (n = 5). **c** The infarct (white region) and non-ischemic (red region) regions in miR-124-overexpressed or miR-124-inhibited MCAO rats' brains revealed by TTC staining. n = 5. **d** The quantitative analysis on infarct volumes in three groups by using the ImageJ software package. n = 5. **e** MCAO rats in four groups underwent neurological function evaluation at 24 h after MCAO surgical procedure following a modified 6-point scoring system. n = 10. **f, g** SOD activity and MDA content in four groups determined by ELISA. n = 5. All results were represented as means ± SD from three independent experiments. *P < 0.05, **P < 0.01.

could be remarkably suppressed within MCAO rats, indicating the effect of miR-124 during ischemic injury. Next, we conducted miR-124 overexpression/inhibition within MCAO rats via administrating the Agomir-NC (negative control), Agomir-124, Antagomir-NC (negative control), or Antagomir-124 with intracerebroventricularly injection, and performed real-time PCR to verify the efficiency (Fig. 3b). Figure 3c, d showed that MCAO/R-induced increases in the infarct volumes were dramatically decreased via the overexpression of miR-124 while further increased via the inhibition of miR-124. Consistently, neurological function scores of MCAO/R rats were dramatically decreased via the overexpression of miR-124 while increased via the inhibition of miR-124 (Fig. 3e). Meanwhile, miR-124 overexpression significantly promoted SOD activity and inhibited MDA content; miR-124 inhibition inhibited SOD activity and promoted MDA content (Fig. 3f, g). In summary, miR-124 overexpression can protect MCAO/R rats in a ROS-related manner.

## miR-124 Targets NOX2 to Inhibit Its Expression in PC-12 Cells

Next, we validated the putative binding of miR-124-5p to *CYBB* 3′-UTR. We examined miR-124 expression after OGD/R stimulation on PC-12 cells; As shown in Fig. 4a, the expression of miR-124 is remarkably decreased within OGD/R PC-12 cells. Next, we transfected Agomir-NC (negative control), Agomir-124, Antagomir-NC (negative control), or Antagomir-124 to conduct miR-124 overexpression/inhibition in OGD/R model within PC-12 cells, and performed real-time PCR to verify the transfection efficiency (Fig. 4b). Within OGD/R PC-12 cells, the protein levels of NOX2 could be significantly downregulated via the overexpression of miR-124 while upregulated via the inhibition of miR-124 (Fig. 4c).

To validate the predicted binding, we constructed two types of *CYBB* 3′-UTR luciferase reporter vectors, wild-type and mutant-type (wt-*CYBB* 3′-UTR and mut-*CYBB* 3′-UTR), which contain wild or mutated miR-124 binding

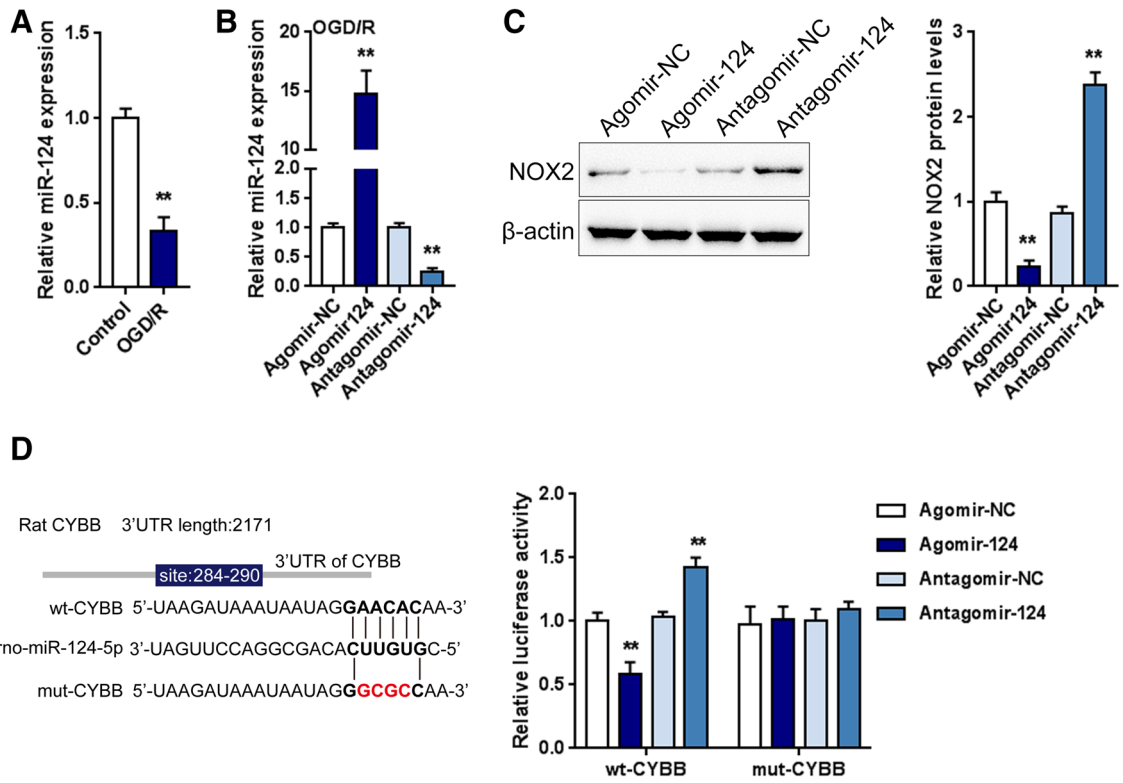


**Fig. 4** miR-124 targets NOX2 to inhibit its expression in PC-12 cells. **a** PC-12 cells were subjected to OGD/R stimulation and examined for miR-124 expression. n = 6. **b** miR-124 overexpression or miR-124 inhibition was conducted in OGD/R model in PC-12 cells by transfection of Agomir-NC (negative control), Agomir-124, Antagomir-NC (negative control), or Antagomir-124, as confirmed by real-time PCR. n = 4. **c** NOX2 protein levels were determined in miR-124-overexpressed or miR-124-inhibited OGD/R model in PC-12 cells by Immunoblotting. n = 4. **d** A schematic diagram showing the predicted miR-124 binding site in rat CYBB 3′-UTR. Two types of CYBB 3′-UTR luciferase reporter vectors, wild- and mutant-type, were constructed as described in the Materials and methods section and co-transfected in PC-12 cells with Agomir-NC, Agomir-124, Antagomir-NC, or Antagomir-124; the luciferase activity was determined. n = 4. All results were represented as means ± SD from three independent experiments. **P < 0.01.

site (Fig. 4d). We co-transfected these luciferase reporter vectors in PC-12 cells with Agomir-NC, Agomir-124, Antagomir-NC, or Antagomir-124 and validated the luciferase activity. The overexpression of miR-124 dramatically suppressed, while the inhibition of miR-124 enhanced the luciferase activity of wt-*CYBB* 3′-UTR; mutating the putative miR-124 binding site could abolish the alterations in luciferase activity (Fig. 4d). In summary, miR-124 could target *CYBB* 3′-UTR to reduce NOX2 protein level in PC-12 cells.

### miR-124 Inhibits NOX2 to Suppress ROS Production in OGD/R-Stimulated PC-12 Cells

To confirm whether miR-124 exerts its protective effects on ischemic injury via NOX2 and NOX-mediated ROS, we determined the dynamic effects of miR-124 and NOX2 upon the ROS production within OGD/R PC-12 cells. These cells were co-transfected with Agomir-124 and NOX2-overexpressing vector (NOX2 OE) and examined for NOX2 protein levels; within OGD/R-stimulated PC-12 cells, the protein levels of NOX2 could be significantly suppressed via the overexpression of miR-124 while enhanced via NOX2 OE transfection. NOX2 overexpression significantly attenuated the effects of miR-124 overexpression (Fig. 5a). SOD activity could be enhanced via the overexpression of miR-124 whereas inhibited via the overexpression of NOX2; MDA content and ROS production were reduced via the overexpression of miR-124 whereas increased via the overexpression of NOX2; NOX2 overexpression could dramatically attenuate the effects of miR-124 overexpression (Fig. 5b–d), indicating that miR-124 suppresses ROS production via downregulating NOX2. Consistently, OGD/R PC-12 cell apoptosis was significantly suppressed via the overexpression of miR-124 whereas enhanced via the overexpression of NOX2; NOX2 overexpression could remarkably attenuate the effects of miR-124 overexpression (Fig. 5e, g). The optical microscope results further confirmed that overexpression of miR-124 reduced cell numbers while NOX2 overexpression partly blocked the reduction effect under OGD/R stimulation. These results suggested that miR-124 exerts its protective effects on PC-12 cell upon OGD/R stimulation via inhibiting NOX2 and NOX-mediated ROS.

### miR-124/NOX2 Axis Modulates the Inflammatory Microenvironment in OGD/R PC-12 Cells

The excessive production of ROS leads to oxidative stress, thus activating the nuclear factor kappa-beta (NF-κB) signaling pathway to synthesize pro-inflammatory cytokines, including the tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) that enhance neuroinflammation [10, 11]. To further investigate the molecular mechanism, we co-transfected OGD/R-stimulated or non-stimulated PC-12 cells with Agomir-124 and NOX2-overexpressing vector and examined p-p65, p65, and IκB protein levels. Consistent with previous studies, OGD/R stimulation dramatically enhanced p-p65/p65 ratio while suppressed IκB expression (Fig. 6a). OGD/R stimulation-induced increases in p-p65/p65 ratio and suppression on IκB protein could be significantly attenuated via the overexpression of miR-124 whereas enhanced via the overexpression of NOX2; NOX2 overexpression could remarkably attenuate the effects of miR-124 overexpression (Fig. 6a). Consistently, the production of TNFα and IL-6 was induced by OGD/R stimulation (Fig. 6b, c). OGD/R stimulation-induced TNFα and IL-6 production could be suppressed via the overexpression of miR-124 whereas enhanced via the overexpression of NOX2; NOX2 overexpression could dramatically attenuate the effects of miR-124 overexpression (Fig. 6b–c). In summary, miR-124 might improve inflammatory microenvironment within OGD/R-stimulated PC-12 cells via inhibiting NOX2 and NOX-mediated ROS.

## Discussion

Herein, we successfully set up a cerebral I/R injury model in rats through MCAO surgery and evaluated the model. After 12 h or 24 h of refusion, the SOD activity was significantly inhibited, while the MDA content and NOX2 protein levels could be dramatically upregulated within MCAO rat infarct area. OGD/R stimulation on PC-12 cells significantly increased NOX2 protein levels, MDA content, ROS production, and the cell apoptosis, while a significant suppression on SOD activity; OGD/R stimulation-induced changes in PC-12 cells described above could be significantly attenuated by NOX2 silence. In vivo, the overexpression of miR-124 improved, whereas the inhibition of miR-124 aggravated I/R injury in MCAO rats. In PC-12 cells, miR-124 directly bound to the *CYBB* 3′-UTR to negatively regulate *CYBB* expression and NOX2 protein level. In vitro, miR-124 overexpression also improved, while NOX2 overexpression aggravated OGD/R-induced cellular injuries; NOX2 overexpression significantly attenuated the effects of miR-124 overexpression. In addition, miR-124 overexpression significantly repressed NF-κB signaling activation and TNFα and IL-6 production, which was promoted by NOX2 overexpression; similarly, the effects of miR-124 overexpression were significantly reversed by NOX2 overexpression.

Ischemia induces complex metabolic issues which might result in non-reversible damage and death of neuronal cells within the ischemia nuclear. The cerebral blood flow recovery (reperfusion) leads to an increase in blood oxygen concentration and ROS production. In turn, the occurrence of oxidative stress following ischemic reperfusion is the main

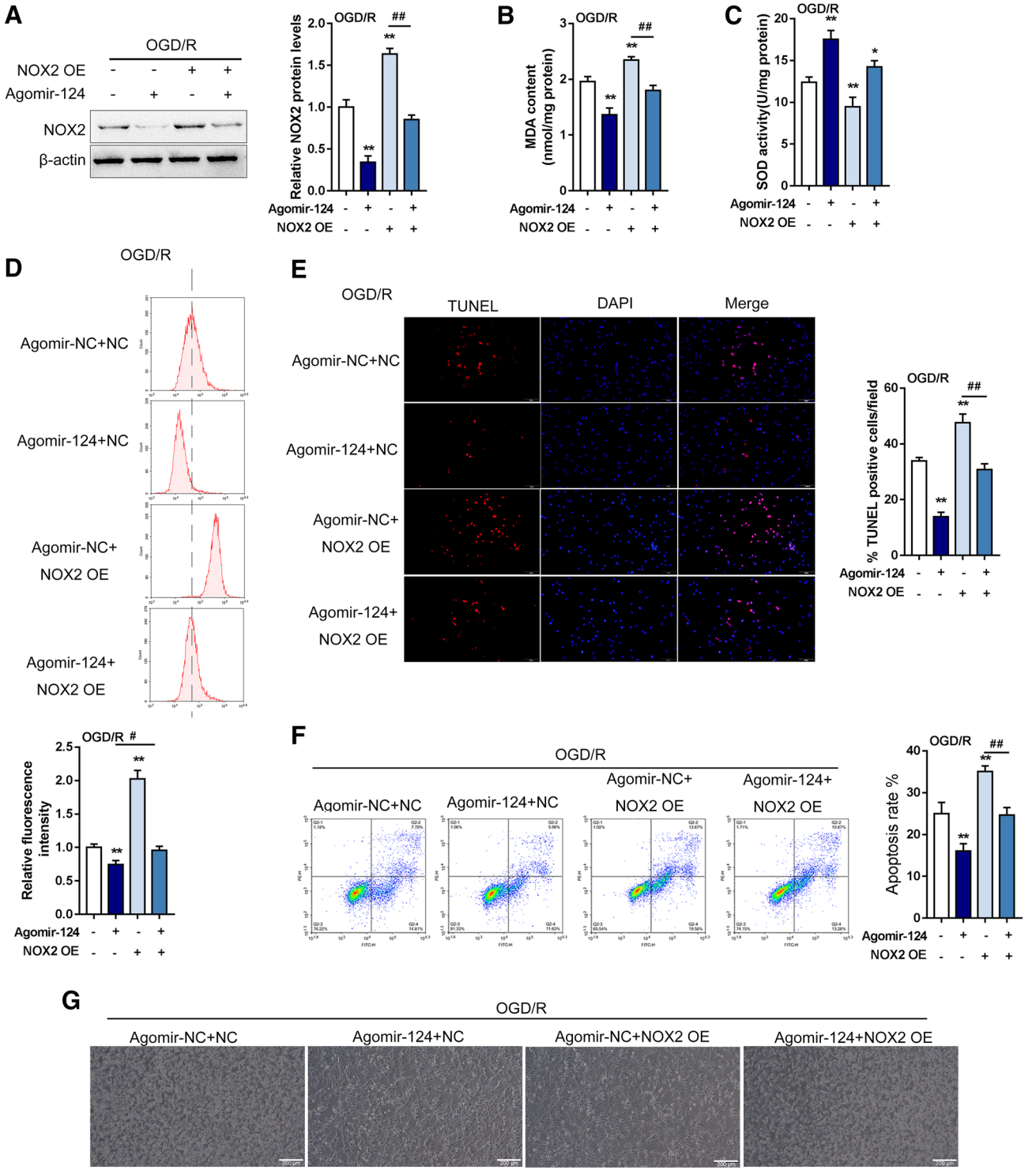


**Fig. 5** miR-124 inhibits NOX2 to suppress ROS production in OGD/R PC-12 cells PC-12 cells were co-transfected with Agomir-124 and NOX2-overexpressing vector and subjected to OGD/R operation and examined for **a** NOX2 protein levels by Immunoblotting; **b**, **c** SOD activity and MDA content in three groups by ELISA; **d** ROS production by Flow cytometry; **e**, **f**) cell apoptosis by TUNEL staining and flow cytometry n = 3. (scale bar:50 μm). **g** The morphology change of PC12 cells was observed by optical microscope (scale bar: 200 μm) n = 3. All results were represented as means ± SD from three independent experiments. *P < 0.05, **P < 0.01, compared to control group; #P < 0.05, ##P < 0.01, compared to NOX2 + Agomir-NC group.

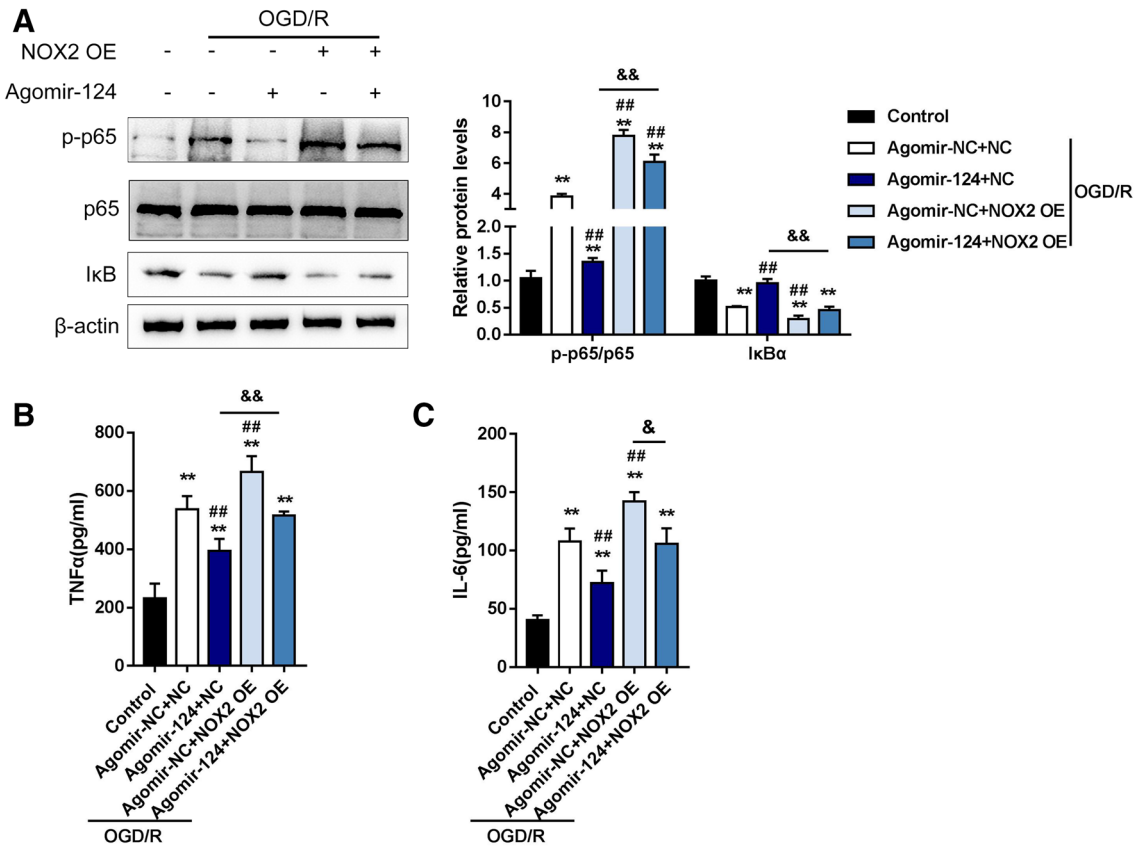


**Fig. 6** miR-124/NOX2 axis modulates the inflammatory microenvironment in OGD/R PC-12 cells. PC-12 cells were co-transfected with Agomir-124 and NOX2-overexpressing vector and subjected to OGD/R operation or non-operation and examined for **a** the protein levels of p-p65, p65 and IκB by Immunoblotting; n = 4. **b**, **c** the production of TNFα and IL-6 by ELISA. n = 4. All results were represented as means ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to control group; $^{\#\#}P < 0.01$, compared to Agomir-NC + NC group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, compared to Agomir-NC + NOX2 group.

reason for aggravating the process of I/R injury [47]. The overproduction of ROS may subsequently lead to the peroxidation of lipid, protein, and nucleic acids [48], further decrease Bcl-2/Bax ratio and finally result in cell apoptosis [49]. SOD is an endogenous oxygen free radical scavenger and its level could reflect the ability of eliminating free radicals [50]. On the contrary, MDA is a stable end product of lipid peroxidations and could be determined to evaluate the degree of cell damage [51]. In the present study, we generated a cerebral I/R model in adult rats by MCAO operation in which we observed not only the upregulation of infarct areas but also significantly decreased SOD activity and increased MDA content, indicating the existence of ROS-mediated oxidative stress during the cerebral I/R injury.

NOX represents an important ROS source which can mediate oxidative stress in the process of heart, brain, lung and renal I/R injury [52, 53]. Previous studies have shown that the increased expression and activation of Nox induced the massive production of ROS in cerebral I/R injury [16]. Nox activation and ROS production may lead to activation of microglia and production of inflammatory mediators, such as IL-1β and TNF-α in a mice model with focal cerebral I/R injury [15]. Among the members of Nox family, NOX2 may play greater roles than other members in cerebral I/R injury [22]. NOX2 increases from 24 to 72 h after reperfusion in endothelial cells and microglia of the penumbra in a mice model of MCAO [54]. Consistent with these previous studies, NOX2 protein levels were significantly increased after 12 h and 24 h of refusion in MCAO rats, suggesting the specific role of NOX2 in cerebral I/R injury.

It has been previously reported that NOX2 inhibition might not only impair ROS production and lipid peroxidation, but also reduce infarction area [14]. Pretreatment with NOX2-specific inhibitor gp91ds-tat or potent ROS scavenger melatonin could reduce the infarct sizes in brain tissues of rats and effectively suppress I/R-induced increase in ROS levels, neuronal apoptosis and degeneration [55, 56]. However, these previous studies mainly addressed the in vivo effects of NOX2 on cerebral I/R injury. To confirm the cellular effects and mechanism of NOX2, we set up an OGD/R

model within PC-12 cells and conducted NOX2 silence in OGD/R-stimulated PC-12 cells. It has been predicted that NOX2 silence significantly promoted the activity of SOD while suppressed MDA content and the production of ROS within PC-12 cells suffered to OGD/R injury. As previously mentioned, SOD family exerts ROS scavenging capacity [12] and MDA is regarded as a marker of oxidative stress to predict lipid peroxidation degree [13], here, NOX2 silence indeed attenuates ROS production. More importantly, the apoptosis of nerve cells represents an essential part of the pathophysiology of brain stroke [57]; here, NOX2 silence significantly inhibited OGD/R-induced cell apoptosis and cell number reduction within PC-12 cells, indicating that inhibiting NOX2 might be a potent strategy for improving cerebral I/R injury.

There is currently evidence that miRNAs exert a critical effect on modulating neuronal death upon ROS-mediated oxidative stress during cerebral ischemic stroke [58]. NOX family members have also been reported to be targets of miRNAs [59–61]. miR-124 is regarded as a critical miRNA in CNS which can protect MCAO mice in vivo and OGD/R neurons in vitro [33]. Herein, we observed significantly downregulated miR-124 expression in both MCAO rats and OGD/R-stimulated PC-12 cells. Regarding the functions, early miR-124 injection in MCAO mice has been reported to result in a significantly increased neuronal survival [62]. In vivo, miR-124 overexpression significantly decreased the infarct area of MCAO mice. In vitro, the gain of miR-124 function resulted in reduced neuron apoptosis and death induced by OGD, and increased anti-apoptosis protein, Bcl-2 and Bcl-xl, respectively [33]. Consistent with the previous studies, miR-124 overexpression significantly decreased MCAO-induced upregulation in infarct area and ROS production in vivo and miR-124 overexpression attenuated OGD/R-induced ROS production and cell apoptosis in vitro, indicating the protective role of miR-124 against I/R injury. As for the molecular mechanism, via direct binding to rat *CYBB* in its 3′-UTR, miR-124 can suppress its expression and decrease NOX2 protein level. More importantly, NOX2 overexpression exerted opposing effects on OGD/R-treated PC-12 cells by enhancing OGD/R-induced ROS production and cell apoptosis in vitro, and significantly reversed the effects of miR-124 overexpression. In summary, miR-124 exerts its protective effects against MCAO- and OGD/R-induced injuries via suppressing NOX2 and NOX-mediated ROS production.

To provide further evidence, we also examined the dynamic effects of miR-124 and NOX2 upon NF-κB signaling pathway activation and TNFα and IL-6 production. Accordingly, oxidative stress caused by ROS overproduction induced NF-κB signaling pathway activation, resulting in pro-inflammatory cytokine release, including IL-1β, IL-6, and TNFα [63, 64], thus leading to blood-brain barrier disruption and angioedema. Herein, miR-124 overexpression inhibited the phosphorylation of p65 while increased the protein levels of IκB, as well as reduced the production of both TNFα and IL-6, indicating that miR-124 overexpression improved the inflammatory microenvironment caused by OGD/R stimulation in PC-12 cells. Conversely, NOX2 overexpression enhanced OGD/R-induced activation of NF-κB signaling and cytokines release, while significantly reversed the effects of miR-124 overexpression.

In conclusion, miR-124/NOX2 axis modulates NOX-mediated ROS production, the inflammatory microenvironment, subsequently the apoptosis of neurons, finally affecting the cerebral I/R injury. Our findings provide a rationale for further studies on the potential therapeutic benefits of miRNA/mRNA axis in ischemic stroke treatment.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

**Ethical Approval** All experimental procedures were performed following the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85 – 23, revised 1996) and approved by the Animal Care and Use Ethics Committee of Shunyi District Hospital.

**Informed Consent** Consent for publication was obtained from the participants.

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