Propofol ameliorates ischemia/reperfusion induced cerebral injury by upregulation of microRNA-206 expression

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Abstract: Aims: The aim of this study is to investigate the role of miR-206 in propofol induced protective effects in cerebral ischemia/reperfusion injury and to explore its possible mechanism. Methods: Hypoxia/reoxygenation (H/R) model of mouse motoneuron-like cell line NSC-43 was established, and the expression miR-206 upon propofol treatment was analyzed by Realtime PCR. Cells were transfected with miR-206 mimic (miR-206) or scramble control (NC) and subjected to H/R, followed by propofol treatment or not. Cell viability and apoptosis was detected by CCK8 assay, flow cytometry and Western blot analysis of apoptosis-related proteins. Target of miR-206 was predicted by bioinformatics analysis and verified by Dual-luciferase reporter gene assay and Western blot. An in vivo model of rat cerebral ischemia/reperfusion injury was used to verify the target of miR-206. Before or after propofol treatment, hippocampi were taken for TUNEL assay and Western analysis of Orthodenticle Homeobox 2 (OTX2) expression. Results: Expression of miR-206 was significantly upregulated after propofol treatment in rat hippocampi. MiR-206 transfection significantly increased cell viability and decreased apoptosis rate, which was consistent with the effect of propofol treatment. Both miR-206 transfection and propofol treatment after H/R inhibited the expression of Bax but upregulated Bcl-2 expression. In addition, 3'untranslated region (3'UTR) of OTX2 has a putative binding site for miR-206. MiR-206 could directly bind to the OTX2 3'UTR and negatively regulate the expression of OTX2 protein. Conclusions: Our results indicate that miR-206 was upregulated after propofol treatment in H/R model to decrease the expression of OTX2 protein, reduce the apoptosis of neural cells and finally suppress cerebral ischemia/reperfusion injury.

Keywords: Propofol, miR-206, hypoxia-reoxygenation, hippocampal neurons

Introduction

Cerebrovascular disease is one of the common clinical diseases with high morbidity and mortality, which greatly endanger human health. Ischemic stroke, with the pathological features of hypoxia-induced brain damage and cerebral ischemia, accounts for 80-85% of the cerebrovascular diseases [1, 2]. Promptly restore blood supply in ischemic area of patients is the major strategy for treatment clinically, but severe brain ischemia-reperfusion injury may occur as a result [3]. Currently, there is no effective clinical treatment against the brain tissue damage caused by hypoxia and ischemia-reperfusion (I/R). In addition, the molecular mechanisms for the development of such damage are not yet clear. Propofol is commonly used in clinical operation as a novel intravenous anesthetic, which is similar to endogenous antioxidants α-tocopherol (VitE) in structure [4]. Previous studies showed that in addition to play the role as anesthetic, propofol can protect organs such as heart and brain during operation [5, 6]. Application of propofol can protect tissues and cells by reducing free radicals in microenvironment, inhibiting the release of excitability neurotransmitter and NOS (nitric oxide synthase) activity, reducing neurological NO content, preventing calcium overload, reducing cerebral blood flow, cerebral metabolic rate and intracranial pressure [7]. However, since the therapeutic effect of propofol on central neurons is challenged by some recent in vitro experiments [8-10], it is still not clear whether propofol has protective effect on...
brain tissue and further investigations are needed.

miRNAs are a class of 18-22 nucleotide (nt), highly conserved, non-coding RNAs that predominantly serve as translational repressor by binding to complementary sequences in the 3' untranslated region (UTR) of their target mRNAs [11]. It is reported that propofol affected the miRNA profile in multiple tissues and cells to regulate cell activity. For example, propofol promoted the apoptosis of ovarian epithelial cells through upregulating let-7i [12], and propofol could inhibit the metastasis of esophageal squamous cell carcinoma by suppressing the expression of miR-143 [13]. It remained to be clarified whether miRNAs are involved during propofol treatment of I/R. Therefore, in this study we attempt to find out whether miR-206 is involved in the cerebral protection of propofol after I/R and to find molecular mechanisms to support the development of propofol as a therapeutic drug.

Material and methods

Cerebral infraction and the reperfusion rat model

A total of 60 adult male SD rats weighing 230-250 g were used. They were allowed standard chow pellets and drinking water ad libitum. The experimental protocol was ethically approved by the Animal Care Committee of Xi'an Ninth Hospital. Rats were randomly divided into four groups (15 animals each). Rats of the sham-operated control (sham) and ischemia/reperfusion (I/R) groups were intravenously (i.v.) administered saline solution. Rats of the lipid microsphere +I/R group (LM+I/R) were i.v. administered 1 ml/kg lipid microsphere, and propofol +I/R group were received propofol preparation (50 mg/kg) dissolved in lipid microsphere 24 hr before I/R. All rats except those of the sham control group were exposed to 90 min of cerebral ischemia followed by 24 hr of reperfusion, and another 24 hr of reperfusion was performed after the rats were fully awake. After I/R or sham operation, all rats were sacrificed by decapitation. The brains were carefully dissected out on ice for following experiments.

Cerebral I/R was performed as described previously [14]. Briefly, rats were anesthetized at the time of the operation, the right and left common carotid arteries were exposed by a midline ventral incision in the neck. The bilateral carotid artery was separated from the adjacent tissues and vagus nerve. Ischemia was induced by bilateral clamping of the common carotid arteries for 90 min. Following cerebral ischemia, the arteries were declamped to restore circulation. The skin was sutured for reperfusion. Rats of the sham control group were exposed to the same procedure except for carotid occlusion.

Construction of H/R model in NSC-43

Mouse motoneuron-like NSC-43 cells were cultured in DMEM (Gibco, Thermo Fisher Scientific Inc, Vilnius, Lithuania, USA) medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C, 5% CO₂. Cells were seeded into 24-well plates at 1 x 10⁵ cells/well. For hypoxia, the culture media was replaced by synthetic ischaemia solution and the NSC-43 s were then placed in hypoxic conditions with 5% CO₂ and 95% N₂ at 37°C for 24 h. After hypoxia, the medium was washed off, and the NSC-43 s were returned to reperfusion solution and cultured with 5% CO₂ and 95% O₂ for 6 h. At the same time, prepared propofol was added to the medium at the concentration of 150 μM.

RNA extraction and real-time PCR assay

The total RNA from each treatment was extracted using TRizol® isolation reagent (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's construction. Following gel electrophoresis verification of RNA integrity and quantification using UV spectrophotometer, 0.5 ug of total RNA was reverse transcribed into cDNA using a miRNA cDNA Kit (TAKARA, Dalian, China) with specific primers. The expression of small nuclear U6 was used as internal control. Quantitative Real-time PCR was performed using KAPA SYBR FAST qPCR Kits (Kapa biosystems, Boston, Massachusetts, USA). Forward primer used for miR-206 amplification was 5'-AATGTAAGGAAGTGTG-3', and the reverse primer is provided by kit. Primers used for amplification of U6 are as follows, forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCAGAATTTGCGT-3'. The relative expression levels were evaluated using the 2^(-ΔΔCt) method.
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MiRNA transfection of NSC-43

NSC-43 cells were seeded at 24-well plate and incubated to a contingency of 70%-90% prior to transfection with 31.25 uM of miR-206 mimics or negative control (NC) (Ribobio, Guangzhou, China) using Lipofectamine 2000 (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer’s instructions. Cells were collected at 48 h post-transfection for further experiments or subjected to H/R treatment.

CCK-8 assay

After hypoxia culture, NSC-43 cells were returned to reperfusion solution and cultured with 5% CO₂ and 95% O₂ for 6 h. After washing twice with PBS, cells were suspended in fresh DMEM containing 10% of Cell Counting Kit-8 (Biyuntian, Beijing, China) and incubated for 1 h at 37°C. The absorbance values at 450 nm in each well were measured with ELx800 (BioTek, Winooski, Vermont, USA).

Anexin V-FITC staining and flow cytometry analysis

NSC-43 cells were harvested after trypsin digestion, washed with PBS twice and stained by Annexin V-FITC Apoptosis Detection Kit I (BD bioscience, San Jose, CA, USA) according to the manufacturer’s instruction. Cells were immediately analyzed by flow cytometry. Cells undergoing apoptosis were Annexin V positive and PI negative, and cells that were necrosis were Annexin V negative and PI positive. Cells observed to be Annexin V and PI positive were in end stage apoptosis or already dead.

Western blot analysis

Cells were collected and re-suspended in RIPA lysis buffer with 1% PMSF to extract the total protein. Each sample was centrifuged at 12,000 × g for 10 min at 4°C for collecting the supernatant. Protein assay kit (Biyuntian, Beijing, China) using bovine serum albumin (BSA) as the standard was used to measure the total protein. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and expression of BAX, Bcl-2, OTX2 and GAPDH was detected with the following antibodies: rabbit anti-rat OTX2 (ab11413; 1:1000), rabbit anti-rat monoclonal anti-GAPDH (ab181602; 1:5000), Goat anti-rabbit (ab181603; 1:5000) or goat anti-mouse (ab 1018; 1:2000) antibodies conjugated to HRP were used as secondary antibodies. All antibodies were purchased from Abcam (Cambridge, MA, USA). Signal detection was performed using chemiluminescence reaction (ECL) (Beyotime Institute of Biotechnology, Beijing, China).

Dual-luciferase reporter gene assay

One of the miR-206 binding sites on the 3’UTR of Otx2 gene with lowest P value was chosen. Luciferase reporter plasmids were generated by insertion of wild type or mutant Otx2 3’UTR fragment containing the miR-206 binding site into the multiple cloning site (Spe-1 and Hind III) downstream of the luciferase reporter gene in the pMIR-REPORT™ Luciferase (Thermo Fisher Scientific, Vilnius, Lithuania). HEK293T cells were seeded at a concentration of 5 × 10⁴ cells per well in 24-well plates and transfected with 1 ug constructed luciferase reporters and 10 ng pMIR-REPORT™ β-gal Control Plasmid as an internal control for transfection efficiency. And, 100 nmol miRNA mimics or negative control RNA were also transfected. Luminescence was measured at 24 h after transfection using the Dual-Luciferase® Reporter Assay System (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer’s instructions. Measurements of luminescence were performed on the luminometer (Glomax 20/20, Promega, Madison, WI, USA).

Immunocytochemistry

Tissues were fixed in 10% formaldehyde and embedded in paraffin. Paraffin sections were cut at 4 um and sections were dewaxed and rehydrated in graded alcohols. Then sections were incubated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase activity. Antigen retrieval was achieved by microwave heating. After blocking, sections were incubated with rabbit anti-rat OTX2 (ab11413; 1:200) at 37°C in the dark for 1 h. After washing with PBS, biotinylated secondary antibodies were added and incubated in dark for 30 minutes. Then sections were developed with DAB chromogenic reagent. Finally, sections were counterstained with haematoxylin.
transparency, sections were mounted with neutral gum.

Cells with yellow or brown granules in the cytoplasm or membrane of hippocampal cells were positive cells under light microscopy. Five fields at high-magnification were randomly taken and positive cells were counted. Positive rate was the ratio of the number of positive cells to the number of total cells. Cells without positive staining or with a positive rate less than 1% were scored as 1. Cells with a positive rate more than 1% and less than 25% were scored as 2. Cells with a positive rate between 25% and 50% were scored as 3. Cells with a positive rate over 50% were scored as 4. According to the strength of staining, cells with no staining were scored as 0; cells with yellow staining were scored as 1; cells with medium brown staining were scored as 2; cells with dark brown staining were scored as 3. The product of positive rate score and staining strength score was accepted as the final score. Score 0-1 was negative, 2-3 was weakly positive, 4-6 was medium positive, and >6 was strong positive.

TUNEL staining

Brains of rats were dissected and fixed in 10% formaldehyde and embedded in paraffin. Paraffin sections were cut at 4 μm and sections were dewaxed and rehydrated in graded alcohols. Subsequently, the sections were incubated for 20 min at 20-37°C in proteinase K (without DNase). Then sections were incubated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase activity, followed by PBS washing for 3 times. TUNEL staining was performed according to the instructions of the in situ cell death detection kit-POD (Roche, Basel, Switzerland). The incorporated fluorescein-dUTP was detected with 50 ul of biotinylated antibody for 60 min at 37°C, followed by PBS or HBSS washing once. The reaction was stopped by 2 × SSC for 10 min at room temperature. After PBS washing, 50 ul Streptavidin-HRP were added to slides and the labeled antibody was visualized DAB chromogenic reagent. The frequency of TUNEL-positive cells that with yellow or brown staining were evaluated under a 400-fold microscope magnification. Five fields at high-magnification were randomly taken and positive cells were counted. The average of positive cells was calculated.

Statistical analysis

Data were expressed as mean ± S.D. Statistical significance was determined with paired t-tests using SPSS 16.0 (SPSS Statistics/IBM Corp, Chicago, IL, USA). P-values < 0.05 were considered statistically significant.

Results

MiR-206 is upregulated after propofol treatment

We first evaluated the expression of miR-206 in rat ischemia/reperfusion model with or without propofol treatment by Real-time PCR. MiR-206
expression was significantly downregulated in NSC-34 cells subjected to H/R compared with untreated cells (P < 0.05), and its expression was significantly upregulated after propofol treatment (Figure 1) (P < 0.05). These results indicate that miR-206 may play a role in the protection of neural cell injury induced by H/R.

Overexpression of miR-206 inhibits apoptosis after exposure to H/R

We next investigate the role of miR-206 in regulating cell apoptosis after exposure to H/R. NSC-34 cells were stained with Annexin V-FITC and propidium iodide, and then analyzed by flow cytometry. There was an obvious reduction in the number of apoptotic cells when propofol was administrated compared with the untreated H/R group (P < 0.05) (Figure 3A). A similar reduction in the number of apoptotic cells was observed when cells were transfected with miR-206 compared with that transfected with NC (Figure 3B). To confirm this finding, apoptosis-related proteins were analyzed by Western blot after exposure to H/R. Apoptotic protein Bax was significantly downregulated while anti-apoptotic protein Bcl-2 was upregulated upon propofol treatment compared with untreated cells (Figure 4A), and miR-206 transfection resulted in the same expression tendency of these proteins compared with cells transfected with NC (Figure 4B). Overall, overexpression of miR-206 induced apoptosis of NSC-34 cells, which may contribute to the protection effect of propofol.

OTX2 is a target of miR-206

To figure out the molecular mechanisms in which the miR-206 are involved, its target...
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Figure 4. Western blot analysis of apoptosis-related proteins. A. NSC-34 cells administrated with H/R were treated with propropofol (H/R+ propofol) or left untreated (H/R). Cell lysates were prepared and analyzed by Western blot for BAX, Bcl-2 and GAPDH protein. B. NSC-34 cells were transfected with miR-206 mimic or NC, and underwent H/R. Cell lysates were prepared and analyzed by Western blot for BAX, Bcl-2 and GAPDH protein. *P < 0.05, t test.

Figure 5. Otx2 is a direct target of miR-206. A. The wildtype Otx2 3’UTR luciferase reporter construct (Otx2) or mutant Otx2 3’UTR luciferase reporter construct (Otx2_mutant) was co-transfected with miR-206 mimic or NC, and luciferase activities were assayed 24 h post transfection. *P < 0.05 compared with NC, t test. B. NSC-34 cells were transfected with miR-206 mimic or scramble (NC). Cell lysates were prepared and analyzed by Western blot for Otx2 and GAPDH protein. Representative Western blot results were shown. *P < 0.05, t test.

Expression of OTX2 is downregulated upon propofol treatment

As the above results were obtained from in vitro experiments, we next sort to investigate the protection effect of propofol and the involvement of OTX2 protein in vivo. Rat cerebral ischemia/reperfusion (I/R) model was used, and cell apoptosis in hippocampus was detected by TUNEL staining. As shown in Figure 6A and 6C, a visible elevation in the number of TUNEL-positive cells was observed in hippocampus of I/R group as compared to sham control. Pre-administration of propofol to cerebral I/R-exposed rats caused a reduction of TUNEL-positive cell number as compared to I/R and lipid microsphere (+I/R (LM+I/R) groups. The number of OTX2 protein expressed cells

genes were predicted by TargetScan (www.targetscan.org/), and we found that the 3’UTR of Otx2 contains potential binding sites for miR-206. As it is reported that OTX2 participates in cell apoptosis, miR-206 may inhibit NSC-34 cell apoptosis through targeting OTX2. To validate whether miR-206 could directly target OTX2, the 3’UTR fragment of Otx2 containing the miR-206 binding site was cloned into the pMIR-
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was upregulated in I/R group compared to sham control, and decreased upon pre-administration of propofol when compared to I/R and LM+I/R groups (Figure 6B). These results indicated that OTX2 protein may be involved in propofol-induced protection.

Discussion

Ischemia-reperfusion (I/R) injury that caused by ischemia and perfusion is a common pathophysiological process during operations, which has been paid more and more attention. MiR-206 is a recently identified miRNA that play important roles in the differentiation of myocytes as well as tumor metastasis and apoptosis. Dey et al. reported that miR-206 promoted myogenesis and accelerated myogenic differentiation by inhibiting Pax7 expression [15]. Studies of Fu et al. demonstrated that miR-206 could repress tumor proliferation and invasion in breast cancer by targeting Cx43 [16]. In addition, miR-206 could inhibit MET protein expression, cell migration and invasion of the low-metastatic lung cancer cell by regulating the HGF-Met signal pathway [17].

In this study, we investigated the role of miR-206 in propofol induced protective effects in cerebral I/R injury and to explore its possible mechanism. In in vitro experiments using NSC-34 cells, we found that miR-206 was significantly downregulated in the H/R model, but its expression was significantly upregulated after propofol treatment. Upon propofol treatment, increase of cell viability and inhibition of apoptosis was also observed, demonstrating the protective effect of propofol in neural cells. Furthermore, overexpression of miR-206 mimicked the effect of propofol treatment in cell viability and neural cell apoptosis, indicating the involvement of miR-206 in propofol-induced protection. By bioinformatics searching we found that Otx2 is a potential target of miR-206. The transcription factor OTX2, a member of the OTX family that is characterized by the presence of a conserved 60-amino acid residue homeodomain, binds to the promoter sequence of corresponding DNA sequence to promote gene transcription [18]. In pre-streak embryos, Otx2 is expressed in the entire murine epiblast, but during gastrulation, its expression becomes progressively restricted to the rostral part of the embryo that corresponds to the presumptive fore- and mid-brain [19]. Actually, OTX2 is important for brain morphogenesis and some sensory organ development [20]. Here we confirmed the direct binding of miR-206 to the 3'UTR of Otx2 mRNA, and proved that OTX2 protein was significantly downregulated upon miR-206 overexpression.

We also constructed a rat model of cerebral I/R injury to study the function of propofol and OTX2 in vivo. Propofol treatment ameliorated...
the damage in hippocampus caused by I/R and in turn, upregulated OTX2 protein expression, demonstrating the protective effect of propofol and the involvement of OTX2 protein.

In summary, propofol ameliorates cerebral damage after I/R, and one possible mechanism is the upregulation of OTX2 expression mediated by downregulation of miR-206. Therefore, miR-206 could be applied as a therapeutic target for the treatment of cerebral I/R.

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Disclosure of conflict of interest

None.

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