MicroRNA-135b-5p prevents oxygen-glucose deprivation and reoxygenation-induced neuronal injury through regulation of the GSK-3β/Nrf2/ARE signaling pathway

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Submitted: 27 July 2017
Accepted: 30 August 2017

Arch Med Sci 2018; 14, 4: 735–744
DOI: https://doi.org/10.5114/aoms.2017.71076
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Abstract

Introduction: MicroRNAs (miRNAs) are emerging as critical regulators in the pathological process of cerebral ischemia/reperfusion injury. miRNAs play an important role in regulating neuronal survival. miR-135b-5p has been reported as an important miRNA in regulating cell apoptosis. However, the role of miR-135b-5p in regulating neuronal survival remains poorly understood. Here, we aimed to investigate the role of miR-135b-5p in cerebral ischemia/reperfusion using an in vitro model of oxygen-glucose deprivation and reoxygenation-(OGD/R)-induced neuron injury.

Material and methods: miRNA, mRNA and protein expression was detected by real-time quantitative polymerase chain reaction and Western blot. Cell viability was detected by cell counting kit-8 and lactate dehydrogenase assays. Cell apoptosis was detected by caspase-3 activity assay. Oxidative stress was determined using commercial kits. The target of miR-135b-5p was confirmed by dual-luciferase reporter assay.

Results: We found that miR-135b-5p expression was significantly decreased in hippocampal neurons receiving OGD/R treatment. Overexpression of miR-135b-5p markedly alleviated OGD/R-induced cell injury and oxidative stress, whereas suppression of miR-135b-5p showed the opposite effects. We observed that miR-135b-5p directly targeted the 3’-untranslated region of glycogen synthase kinase-3β (GSK-3β). We found that miR-135b-5p negatively regulates the expression of GSK-3β in hippocampal neurons. Moreover, miR-135b-5p overexpression promotes activation of nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling. However, the restoration of GSK-3β expression significantly reversed the protective effects of miR-135b-5p overexpression.

Conclusions: Overall, our results suggest that miR-135b-5p protects neurons against OGD/R-induced injury through downregulation of GSK-3β and promotion of the Nrf2/ARE signaling pathway-mediated antioxidant responses.

Key words: GSK-3β, ischemia/reperfusion injury, miR-135b-5p, Nrf2.

Introduction

Cerebral ischemia/reperfusion injury is a major cause of death and disability causing a heavy financial burden worldwide [1]. Insufficient blood flow to the brain causes serious brain damage, which is aggravated by blood reperfusion [2]. Ischemia/reperfusion induces calcium overload, inflammation, glutamate excitotoxicity, excessive generation
of reactive oxygen species (ROS), and apoptosis, thus contributing to neuronal damage [3]. Thrombolytic therapy has been applied for the treatment of cerebral ischemia/reperfusion injury in clinics. However, the treatment effect is limited. Therefore, it is important to develop novel therapeutic strategies for the treatment of cerebral ischemia/reperfusion injury.

MicroRNAs (miRNAs) are a subset of small, non-coding RNAs that can negatively regulate gene expression by binding to the 3′-untranslated region (UTR) of mRNA (mRNA), inhibiting translation [4]. Therefore, miRNAs are involved in regulating many biological processes, including cell proliferation, apoptosis, and survival [5]. Increasing evidence has suggested that miRNAs are involved in the pathophysiologic process of cerebral ischemia/reperfusion injury [6]. Various miRNAs are dysregulated during cerebral ischemia/reperfusion injury, and targeting specific miRNAs has been shown to prevent neuronal damage in vitro and in vivo [7, 8]. Therefore, miRNAs are potential and promising targets for treatment of cerebral ischemia/reperfusion injury.

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine protein kinase that plays an important role in many biological activities, including glycogen metabolism, inflammation, embryogenesis, and neuroplasticity [9]. GSK-3β is abundantly expressed in neurons in the central nervous system [10]. Studies have shown that GSK-3β is involved in the regulation of psychiatric and neurodegenerative disorders [11]. A growing body of evidence has shown that GSK-3β plays a critical role in cerebral ischemia/reperfusion injury [12, 13]. Inhibition of GSK-3β attenuates neuronal death and protects neurons against cerebral ischemia/reperfusion injury [14]. Nuclear factor-E2-related factor 2 (Nrf2), a redox-sensitive transcription factor [15], has been suggested as the downstream target of GSK-3β [16]. Nrf2 can bind to the antioxidant response elements (ARE) in the nucleus and activate the expression of antioxidant genes, such as heme oxygenase-1 (HO-1) and NADPH-quinone oxidoreductase 1 (NQO1) [17]. Activation of Nrf2/ARE signaling attenuates the production of ROS and neuron apoptosis, thus having a protective effect during cerebral ischemia/reperfusion injury [18]. GSK-3β impedes the nuclear translocation of Nrf2 and inhibits the antioxidant cell response [19]. Inhibition of GSK-3β prevents the neuronal damage induced by brain ischemia by activation of Nrf2/ARE signaling [20]. Therefore, targeting GSK-3β may lead to the discovery of novel therapeutic strategies for cerebral ischemia/reperfusion injury.

miR-135b-5p has been reported as an anti-apoptotic miRNA [21, 22]. A recent study has shown that miR-135b-5p has a neuroprotective effect in Alzheimer’s disease [23]. However, little is known about the role of miR-135b-5p in regulating neuronal survival during cerebral ischemia/reperfusion injury. Here, we aimed to investigate the functional significance of miR-135b-5p in regulating neuron survival using an OGD/R cellular model. We found that miR-135b-5p expression was significantly decreased in hippocampal neurons receiving OGD/R treatment. Overexpression of miR-135b-5p markedly alleviated OGD/R-induced injury. GSK-3β was identified as a target gene of miR-135b-5p. Moreover, overexpression of miR-135b-5p inhibited GSK-3β and promoted activation of the Nrf2/ARE signaling pathway. The restoration of GSK-3β expression significantly reversed the protective effects of miR-135b-5p overexpression. Taken together, our study suggests that miR-135b-5p protects neurons against OGD/R-induced injury through downregulation of GSK-3β and promotion of the Nrf2/ARE signaling pathway-mediated antioxidant responses.

Material and methods

Cell culture

The mouse hippocampal neuronal HT22 cell line was purchased from JENNIO Biological Technology (Guangzhou, China). HT22 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 4.5 g/l glucose and 1% penicillin/streptomycin mix (Sigma, St. Louis, MO, USA). Cells were cultured in a humidified incubator with 5% CO₂ at 37°C.

Induction of oxygen-glucose deprivation and reoxygenation (OGD/R) injury

Cells were cultured in glucose-free Hank’s Balanced Salt Solution and incubated in a hypoxia incubation chamber (Thermo Fisher Scientific, Shanghai, China) containing 1% O₂, 5% CO₂, 94% N₂ at 37°C. After culture for 6 h, the medium was replaced with growth medium containing 4.5 g/l glucose and cultured under normal conditions for 24 h reoxygenation. Cells without OGD/R treatment were used as a control.

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA was reverse-transcribed into cDNA using the miScript Reverse Transcription Kit (Qiagen, Dusseldorf, Germany) or M-MLV Reverse Transcriptase (TaKaRa, Dalian, China). PCR reactions were performed using SYBR Green.

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ROS measurement

Reactive oxide species levels were measured using a dichlorofluorescein diacetate (DCFH-DA) assay. Briefly, cells were incubated with 50 µM DCFH-DA (Sigma) for 30 min at 37°C. Then, cells were washed, and the fluorescence intensity was measured by a fluorescence spectrophotometer (Bio-Tek) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Superoxide dismutase (SOD) and malondialdehyde (MDA) measurement

The levels of SOD and MDA were evaluated using commercial kits (Nanjing Jiancheng Biotechnology Institute, Nanjing, China) in accordance with the manufacturer’s protocols.

Dual-luciferase reporter assay

The fragments of wild-type or mutant GSK-3β 3′-UTR containing the predicted binding site of miR-135b-5p was inserted into the pmirGLO vector (Promega, Madison, WI, USA). HT22 cells were cotransfected with a pmirGLO vector containing wild-type or mutant GSK-3β 3′-UTR and miR-135b-5p mimics or inhibitor using Lipofectamine 2000 (Invitrogen). After culturing for 48 h, cells were lysed, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). For measurement of ARE activity, HT22 cells were cotransfected with ARE reporter vector (Promega), phRL-TK Renilla luciferase vector (Promega) and miR-135b-5p mimics or inhibitor. After culturing for 48 h, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Western blot analysis

Equivalent amounts (40 µg) of proteins from each sample were fractionated by 10% sodium dodecyl sulfate polyacrylamide gels. The separated proteins were then electroblotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membrane was incubated with anti-GSK-3β (Cell Signaling Technology, Danvers, MA, USA), anti-Nrf2, and anti-β-actin (Abcam, Cambridge, UK) overnight at 4°C. Afterwards, the membrane was probed with horseradish peroxidase-conjugated secondary antibodies (1 : 5000; Sigma) for 1 h at room temperature. Protein bands were visualized with the chemiluminescent reagents (Millipore). Band density was analyzed by Image-Pro Plus 6.0 software. β-actin served as the loading control for...
normalization of GSK-3β and Nrf2 protein expression.

Statistical analysis

Data were expressed as mean ± standard deviation. Student’s t-test and one-way analysis of variance (ANOVA) with Bonferroni post hoc test were used to determine the significance levels of differences using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences with p-values less than 0.05 were considered statistically significant.

Results

MiR-135b-5p is downregulated in hippocampal neurons receiving OGD/R treatment

To investigate the potential role of miR-135b-5p in cerebral ischemia/reperfusion injury, we examined the expression pattern of miR-135b-5p in hippocampal neurons receiving OGD/R treatment by RT-qPCR. We found that miR-135b-5p was significantly downregulated in hippocampal neurons receiving OGD/R treatment compared to the control group (Figure 1 A), implying an important role of miR-135b-5p in hippocampal neurons receiving OGD/R treatment.

Overexpression of MiR-135b-5p attenuates OGD/R-induced injury

To investigate the precise biological function of miR-135b-5p in hippocampal neurons receiving OGD/R treatment, we detected the effect of miR-135b-5p overexpression on OGD/R-induced injury. Transfection of miR-135b-5p mimics significantly upregulated the expression of miR-135b-5p in cells receiving OGD/R treatment (Figure 1 B). CCK-8 assay showed that miR-135b-5p overexpression significantly improved the viability of hippocampal neurons receiving OGD/R treatment (Figure 2 A). The LDH assay showed that miR-135b-5p overexpression markedly attenuated OGD/R-induced cell death (Figure 2 B). Furthermore, OGD/R-induced cell apoptosis was also significantly reduced by miR-135b-5p overexpression (Figure 2 C). By contrast, suppression of miR-135b-5p significantly aggravated OGD/R-induced injury in hippocampal neurons (Figures 2 A–C). These results suggest that miR-135b-5p attenuates OGD/R-induced injury.

Overexpression of MiR-135b-5p inhibits oxidative stress induced by OGD/R treatment

To further investigate the biological function of miR-135b-5p in hippocampal neurons receiving OGD/R treatment, we detected the effect of miR-135b-5p overexpression on ROS production induced by OGD/R treatment. We found that overexpression of miR-135b-5p significantly reduced the ROS production induced by OGD/R treatment (Figure 3 A). Moreover, overexpression of miR-135b-5p significantly promoted the expression of SOD (Figure 3 B) while decreasing the expression of MDA (Figure 3 C). In contrast, suppression of miR-135b-5p showed the opposite effects (Figures 3 A–C). These results suggest that miR-135b-5p inhibits OGD/R oxidative stress.

GSK-3β is a target gene of MIR-135b-5p

To investigate the molecular mechanism by which miR-135b-5p regulates OGD/R-induced injury, we predicted the downstream target genes of miR-135b-5p by bioinformatics analysis. Interestingly, we found that GSK-3β, an important gene involved in cerebral ischemia and reperfusion [12, 13], was a potential target gene of miR-135b-5p (Figures 4 A). To investigate whether miR-135b-5p

Figure 1. Expression of miR-135b-5p in hippocampal neurons receiving OGD/R treatment. A – RT-qPCR analysis of miR-135b-5p expression in HT22 cells with OGD/R treatment. Cells cultured in normal condition were used as a control. B – RT-qPCR analysis of miR-135b-5p expression in HT22 cells transfected with miR-135b-5p mimics or inhibitor for 24 h followed by OGD/R treatment

*p < 0.05 vs. control and *p < 0.05 vs. OGD/R + NC.
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directly targeted the 3′-UTR of GSK-3β, we performed dual-luciferase reporter assays. We found that overexpression of miR-135b-5p significantly decreased the luciferase activity of a reporter vector containing the wild-type GSK-3β 3′-UTR, whereas suppression of miR-135b-5p promoted luciferase activity (Figure 4 B). However, miR-135b-5p showed no significant effect on the luciferase activity of the reporter vector containing the mutant GSK-3β 3′-UTR (Figure 4 B). To confirm

Figure 2. Overexpression of miR-135b-5p attenuates OGD/R-induced injury. HT22 cells were transfected with miR-135b-5p mimics or inhibitor for 24 h and then subjected to OGD/R treatment. A – Cell viability was measured by CCK-8 assay. B – Cell death was evaluated by LDH assay. C – Cell apoptosis was detected by caspase-3 activity assay

\*p < 0.05 vs. control and \#p < 0.05 vs. OGD/R + NC.

Figure 3. Overexpression of miR-135b-5p inhibits OGD/R-induced oxidative stress. HT22 cells were transfected with miR-135b-5p mimics or inhibitor for 24 h and then subjected to OGD/R treatment. A – ROS levels were measured by DCFH-DA assay. Levels of SOD (B) and MDA (C) were measured using commercial kits

\*p < 0.05 vs. control and \#p < 0.05 vs. OGD/R + NC.
that GSK-3β is a direct target gene of miR-135b-5p, we detected the effect of miR-135b-5p on GSK-3β expression by RT-qPCR and Western blot analysis. We found that overexpression of miR-135b-5p significantly decreased the mRNA and protein expression of GSK-3β while suppression of miR-135b-5p promoted GSK-3β expression (Figures 5 A and B). Overall, these results suggest that GSK-3β is a target gene of miR-135b-5p.

Overexpression of Mir-135b-5p promotes activation of the Nrf2/ARE signaling pathway

To further investigate the molecular basis of miR-135b-5p in regulating OGD/R-induced injury, we detected the effect of miR-135b-5p on the Nrf2/ARE signaling pathway, the downstream pathway of GSK-3β [19, 20]. We found that overexpression of miR-135b-5p significantly promoted the expression of Nrf2 in cells with OGD/R treatment (Figure 6 A). Overexpression of miR-135b-5p also significantly increased ARE activity (Figure 6 B). Furthermore, the expression of Nrf2/ARE downstream target genes, including HO-1 (Figures 6 C) and NQO1 (Figure 6 D), was also markedly upregulated by miR-135b-5p overexpression. These results suggest that miR-135b-5p overexpression increases activation of the Nrf2/ARE signaling pathway.

Restoration of GSK-3β expression reverses the protective effect of Mir-135b-5p overexpression

To confirm that GSK-3β is a functional target gene of miR-135b-5p, we performed rescue experiments by restoration of GSK-3β expression in cells transfected with miR-135b-5p mimics. We found that transfection of the pcDNA3.1/GSK-3β vector significantly restored the expression of GSK-3β in cells transfected with miR-135b-5p mimics (Figure 7 A). Moreover, restoration of GSK-3β expression significantly blocked the effect of miR-135b-5p overexpression on activation of the Nrf2/ARE signaling pathway (Figures 7 B–D). As expected, the protective effect of miR-135b-5p overexpression against OGD/R-induced injury was significantly abrogated by GSK-3β overexpression (Figures 8 A–C). These results suggest that GSK-3β is a functional target gene of miR-135b-5p in regulating OGD/R-induced injury.

Discussion

Cerebral ischemia/reperfusion injury is a complex and severe pathological process that involves
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inflammation, protein synthesis inhibition, and impaired mitochondrial function and energy metabolism, which cause neuron loss and brain damage [24]. Therefore, the concept of single-drug intervention cannot be effective [24]. Further experimental research is needed to gain a better understanding of the molecular pathogenesis of cerebral ischemia/reperfusion injury and to explore novel and effective therapeutic strategies. Here, we have revealed an important role for miR-135b-5p in regulating OGD/R-induced injury in neurons. We found that miR-135b-5p is significantly decreased in response to OGD/R treatment. Overexpression of miR-135b-5p significantly attenuates OGD/R-induced injury in neurons. The underlying mechanism for this might involve the Nrf2/ARE defense pathway through the inhibition of GSK-3β. Overall, our study indicates that the miR-135b-5p/GSK-3β/Nrf2/ARE signaling pathway may play an important role in regulating neuronal survival under OGD/R-induced injury.

Studies have demonstrated that miR-135b-5p is involved in regulating inflammation and cell differentiation [25, 26]. Various studies have shown that miR-135b-5p promotes tumorigenesis, chemoresistance, and radioresistance by inhibiting cell apoptosis [21, 27, 28]. Fan et al. reported that miR-135b-5p protects osteoblastic cells from dexamethasone-induced apoptosis [29]. Overexpression of miR-135b-5p has a neuroprotective effect on Alzheimer’s disease by targeting the β-site APP-cleaving enzyme 1 [23]. miR-135b-5p is decreased in the hippocampus of a rat model of temporal lobe epilepsy and may be involved in the regulation of hippocampal neuron apoptosis [30]. In mouse hippocampal neurons, miR-135b-5p is deregulated under oxidative stress [31]. However, little is known about the role of miR-135b-5p in cerebral ischemia/reperfusion injury. Herein, we investigated the potential role of miR-135b-5p in regulating neuronal survival using OGD/R-induced injury, an in vitro model of cerebral ischemia/reperfusion injury. We found that miR-135b-5p was decreased in hippocampal neurons receiving OGD/R treatment. The overexpression of miR-135b-5p markedly attenuated OGD/R-induced injury, implying a neuroprotective role for miR-135b-5p in OGD/R-induced injury.

GSK-3β has been suggested as an important regulator in cerebral ischemia/reperfusion injury [12, 13]. Inhibition of GSK-3β attenuates neuronal death and protects neurons against cerebral ischemia/reperfusion injury [14, 32, 33]. GSK-3β promotes cerebral ischemia/reperfusion injury
Figure 7. Restoration of GSK-3β expression reverses the effect of miR-135b-5p overexpression on Nrf2/ARE signaling. HT22 cells were cotransfected with pcDNA3.1/GSK-3β vector and miR-135b-5p mimics for 24 h and then subjected to OGD/R treatment. Expression of GSK-3β (A) and Nrf2 (B) was detected by Western blot. C – ARE activity was detected by dual-luciferase reporter assays. D – The mRNA expression of HO-1 was detected by RT-qPCR

\*p < 0.05.

Figure 8. Restoration of GSK-3β expression reverses the protective effect of miR-135b-5p overexpression. HT22 cells were cotransfected with pcDNA3.1/GSK-3β vector and miR-135b-5p mimics for 24 h and then subjected to OGD/R treatment. A – Cell viability was measured by CCK-8 assay. B – Cell apoptosis was detected by caspase-3 activity assay. C – ROS levels were measured by DCFH-DA assay

\*p < 0.05.
through downregulation of the Nrf2/ARE signaling pathway [12, 13]. Therefore, inhibition of GSK-3β can promote activation of the Nrf2/ARE signaling pathway and alleviate neuronal damage [20]. Various neuroprotective drugs inhibit neuron apoptosis by targeting the GSK-3β/Nrf2/ARE signaling pathway [34–37], suggesting that the GSK-3β/Nrf2/ARE signaling pathway plays an important role in regulating neuron apoptosis. In this study, we found that GSK-3β was a potential target gene of miR-135b-5p. We observed that overexpression of miR-135b-5p inhibits the expression of GSK-3β in neurons and protects neurons against OGD/R-induced injury by promoting activation of the Nrf2/ARE signaling pathway. Activation of the Nrf2/ARE signaling pathway can inhibit the inflammation and oxidative stress induced by ischemia and reperfusion [38, 39]. Research has shown that downregulation of the inflammatory response is beneficial to alleviate ischemia/reperfusion injury [40]. Our results suggested that miR-135b-5p may serve as an inhibitor of GSK-3β which can thus activate the Nrf2/ARE signaling pathway, representing a novel molecular target for protecting neurons against OGD/R-induced injury.

A growing body of evidence has demonstrated that GSK-3β is regulated by various miRNAs, including miR-769 [41], miR-709 [42], and miR-26a [43]. In this study, we found that GSK-3β is also a target gene of miR-135b-5p. In line with our findings, several studies have shown that miR-135b-5p targets and regulates GSK-3β expression. Xiao et al. reported that miR-135b-5p promotes the radiosensitivity of glioblastoma multiforme cells by targeting GSK-3β [27]. miR-135b-5p mediates podocyte injury through targeting GSK-3β [44]. Our study further confirmed the functional relationship between miR-135b-5p and GSK-3β.

Overall, our results show that overexpression of miR-135b-5p attenuates OGD/R-induced injury hippocampal neurons by downregulation of GSK-3β and upregulation of the Nrf2/ARE signaling pathway-mediated antioxidant responses (Figure 9). These results suggest a neuroprotective role for miR-135b-5p in OGD/R-induced injury. However, the precise role of miR-135b-5p in the modulation of neuronal survival by the GSK-3β/Nrf2/ARE signaling pathway in vivo requires further study using animal models.

**Acknowledgments**

This study was supported by the People’s Liberation Army General Logistics Department Special Innovation Project (No. 16CXZ022), the International Science and Technology Cooperation Program of China (No. 2013DFA32610) and the National Natural Science Foundation of China (No. 81271450).

**Conflict of interest**

The authors declare no conflict of interest.

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**Figure 9.** miR-135b-5p protects hippocampal neurons against OGD/R-induced injury by downregulation of GSK-3β and upregulation of Nrf2/ARE signaling pathway-mediated antioxidant responses.
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