Original Paper

Inhibition of MiRNA-125b Decreases Cerebral Ischemia/Reperfusion Injury by Targeting CK2 α / NADPH Oxidase Signaling

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Key Words

Ischemia–reperfusion (I/R) • Apoptosis • MiRNAs • MiRNA-125b • CK2 α • NADPH • Oxygen-glucose deprivation and reoxygenation (OGD/R)

Abstract

Background/Aims: Cerebral ischemia-reperfusion (I/R) injury involves multiple independently fatal terminal pathways. CK2 α /NADPH oxidase is an important signaling pathway associated with ischemia-reperfusion injury, and miR-125b can regulate oxidative stress-related injury. In this study, we investigated whether the effect of miR-125b in rat brain I/R injury occurs through its modulation of the CK2 α /NADPH oxidase pathway. **Methods:** Rats were subjected to 2 h of cerebral ischemia followed by 24 h of reperfusion to establish an I/R injury model. Neurological deficit was evaluated using a five-point score. Infarct volume was evaluated with 2, 3, 5-triphenyltetrazolium chloride (TTC) staining, and RT-PCR was used to detect expressions of miR125b and CK2 α . We then examined the association between miR-125b expression and the CK2 α /NADPH oxidative signaling pathway in a PC-12 cell oxygen-glucose deprivation and reoxygenation (OGD/R) injury model. Transfection with miR-125b mimics, an miR-125b inhibitor, and luciferase reporter gene plasmid was accomplished using commercial kits. In these cells, Western blots were used to detect the levels of expression of CK2 α , cleaved caspase-3, NOX2, and NOX4. RT-PCR was used to detect the expressions of CK2 α , miR125b, NOX2, and NOX4. We evaluated Lactate Dehydrogenase (LDH) level, NADPH oxidase activity, and caspase-3 activity using commercial kits. Mitochondrial reactive oxygen species (ROS) were measured by fluorescence microscopy. For both PC-12 cells and rat brains, histological analyses were conducted to observe morphological changes, and apoptosis was measured using a commercial kit. **Results:** I/R rats exhibited an increase in neurological deficit score, infarct volume, and cellular apoptosis, along with miR-125b elevation and CK2 α downregulation. OGD/R treatment increased PC-12 cells' injuries, cellular apoptosis, and ROS levels. These changes were associated with miR-125b elevation, CK2 α downregulation and

activations of NOX2 and NOX4, mimicking our *in vivo* findings. All of these effects were reversed by the inhibition of miR-125b, confirming a strong correlation between miR-125b activity and the CK2 α /NADPH oxidase signaling pathway. **Conclusions:** Based on these observations, we conclude that inhibition of miR-125b protects the rat brain from I/R injury by regulating the CK2 α /NADPH oxidative signaling pathway.

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Introduction

Ischemic stroke is marked by insufficient blood supply to brain tissues and is associated with increasing morbidity and mortality in adults worldwide. Rapid reperfusion represents the standard therapy for cerebral ischemia, depending on the time window after initial insult, but this treatment can exacerbate the inflammation, oxidative stress, and glutamate excitotoxicity induced by the ischemic stroke itself. This process is termed cerebral ischemia/ reperfusion (I/R) injury [1]. Currently, there is no effective clinical treatment for ischemic stroke. Scientists and clinicians are therefore focused on preventing the impaired neurological function induced by neuronal apoptosis following ischemic stroke [2]. It is therefore important to elucidate the molecular mechanisms underlying neuronal apoptosis as we explore effective therapies for ischemic stroke.

miRNAs are small noncoding single-stranded RNA molecules with mature transcripts of 18 to 25 nucleotides long, which are processed from pre-miRNA by the ribonuclease Dicer. miRNAs act as negative regulators of gene expression by binding to the 3'-untranslated region (UTR) of complementary or partially complementary target messenger RNAs (mRNAs), thereby downregulating target mRNAs via degradation or translational inhibition [3]. It has been reported that miRNAs are involved in physiological and pathological processes of pantosomatous organs, including the brain. Numerous studies have suggested a key role for miRNAs in neurodegenerative disorders, neuropsychiatric disorders, and tumors [4], but only a few studies have reported the relationship between miRNAs and cerebral I/R injury. The underlying mechanism of these relationships have not yet been elucidated. Several miRNAs that may relate to cerebral I/R injury have been discovered. One of these, miR-125b, is up-regulated in the peripheral blood of stoke patients and rats [5]. However, whether miR-125b plays an important role in cerebral I/R injury has not been well investigated, and its targets merit further exploration.

Protein kinase CK2 (formerly called casein kinase 2) is a serine-threonine kinase ubiquitously expressed in mammalian cells. In contrast to many other protein kinases, CK2 phosphorylates a plethora of different substrates and therefore this kinase participates in numerous different functions such as cell proliferation and survival, cancer, apoptosis, angiogenesis, DNA-damage and repair, the ER-stress response, the regulation of carbohydrate metabolism in the nervous system [6]. CK2 is composed of three different subunits: α , α' , and β . It has been reported that CK2 α exhibits neuroprotective effect in cerebral I/R injury by negatively regulating NADPH oxidase (NOX2 and NOX4)[7].

The present study aimed to investigate the role of miR-125b in cerebral I/R injury *in vitro*, using the ischemic stroke model of oxygen-glucose deprivation and reoxygenation (OGD/R) in PC-12 cells. We demonstrate a direct targeting relationship between miR-125b and CK2 α /NADPH oxidase which suggests a crucial role for miR-125b, and its underlying mechanism, in cerebral I/R. This may provide novel targets for treatment of ischemic stroke.

Materials and Methods

Ethics statement

All animal experiments were performed according to institutional ethical committee guidelines and were approved by the Animal Ethics Committee of the Brain Hospital of Hunan Province. Experiments were conducted in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals." All efforts were made to minimize suffering.

Animals and pharmacological treatment in vivo

A total of 50 male Sprague-Dawley (SD) rats weighing 220–250 g and 25 weeks old were used in the experiments. The animals were housed in polypropylene cages and maintained in a 12-h light/12-h dark cycle, at 50% humidity and $25 \pm 2^\circ\text{C}$. The animals had free access to a standard pellet diet (M/S. Pranav Agro Industries Ltd., Bangalore, India) and water *ad libitum*.

The animals were randomly allocated to three groups ($n = 8$ per group): the control group, the sham group (underwent surgical procedure but without ischemic insult), and the I/R group (subjected to 2h of ischemia followed by 24h of reperfusion). At the end of reperfusion, the neurological deficit score was assessed, and then the plasma was collected for measurement of CK2 α and miR125-b levels. The brain tissue was saved for infarct volume measurement and other measurements (such as the expressions of mRNA or protein).

Assessment of neurological deficit score and infarct volume

A 5-point rating scale of neurological deficit is a well-recognized method for evaluating neurological function in a rat model. Therefore, at the end of reperfusion, an investigator blinded to the experimental groups evaluated the functional consequences of focal cerebral I/R injury according to a five-point neurological deficit score (0=no deficit, 1=fails to extend the left forepaw, 2=decreased grip strength of left forepaw, 3=circles to left after tail is pulled, 4=spontaneous circling).

The infarct volume was evaluated with 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. After assessment of neurological function, the animals were anesthetized and sacrificed. Brains were rapidly removed and were sliced into coronal sections, 2–3 mm in thickness, with the aid of a brain matrix. Sections were incubated with 2% TTC for 10 min at 37°C , followed by immersion in 4% paraformaldehyde overnight, then were scanned into a computer and analyzed with imaging software (Image J, NIH, USA). The absence or presence of infarction was determined by examining TTC stain. The infarct volume (in mm^3) of each section was equal to infarct area (in mm^2) multiplied by the section thickness (2–3 mm). The total infarct volume of each brain was then calculated by summing up the infarct volumes of all sections. To eliminate the effect of edema on the accuracy of the infarct volume assay, the final infarct volume was corrected using the following equation: corrected infarct volume = (total infarct volume)*(left hemisphere volume/right hemisphere volume). Left hemisphere refers to the non-ischemic hemisphere of the brain, while right hemisphere refers to the ischemic contralateral side.

Experiments in Cell cultures

The PC-12 cell line was purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences of Shanghai. Cells were cultured in DMEM medium supplemented with 10% FBS and penicillin/streptomycin in 95% air and 5% CO_2 for 2 days, and then were washed with PBS and cultured in serum free DMEM for 24h before the experiments.

Oxygen-Glucose Deprivation and Reperfusion (OGD/R)

To mimic ischemic-like conditions *in vitro*, cell cultures were exposed to oxygen-glucose deprivation (OGD) for 4 hours and then were returned to 95% air, 5% CO_2 , and glucose containing medium for different recovery times as previously described [8]. First, PC-12 cells were transferred into a temperature controlled (37°C) anaerobic chamber (Forma Scientific) containing a gas mixture composed of 5% CO_2 and 95% N_2 . The culture medium was replaced with deoxygenated glucose-free Hanks' Balanced Salt Solution (Invitrogen) and cells were maintained in the hypoxic chamber for 4 hours. After OGD, PC-12 cells were maintained in DMEM supplemented with 10% FBS under normoxic culture conditions for 12 hours.

Histopathological examination and Nissl staining

A total of 5 rats from each group were used for H&E and Nissl staining. After being anesthetized with choral hydrate (300 mg/kg), the rats were perfused with 0.9% NaCl and the brain tissues were removed and fixed with 4% formaldehyde. The brain tissues were dehydrated with xylene and were then embedded in paraffin. Hippocampal sections were observed under a microscope (Olympus, Tokyo, Japan) by a pathologist blinded to the groups under study.

Determination of apoptosis with Hoechst 33258 staining

To detect apoptosis, Hoechst 33258 staining was performed as previously described [9]. After OGD/R, PC-12 cells were placed in a fixing solution for 5 min. They were then washed three times in PBS, incubated

in Hoechst 33258 solution (0.5 ml) for 5 min, and again washed three times in PBS. For each treatment group, the number of cells undergoing apoptosis per unit area was calculated using fluorescence microscopy by randomly selecting 5 fields and counting the number of apoptotic cells.

Western Blot Analysis

PC-12 cells from each group were lysed on ice with a tissue or cell protein extraction reagent containing a cocktail of 0.1 mM dithiothreitol and proteinase inhibitor. The protein concentration was determined using a BCA kit (Pierce Corporation, Rockford, USA). Equal amounts of protein fractions were separated with 12% SDS-PAGE and then transferred onto nitrocellulose membranes (Millipore Corporation, USA) in Tris-glycine buffer at 100V for 55 min. The membranes were blocked with 5% (w/v) nonfat milk powder in Tris-buffer with 0.05% (v/v) Tween-20 (TBST) at room temperature for 2 h. After incubation overnight at 4°C with appropriate primary antibodies, the membrane was washed three times with TBST, incubated with secondary antibodies for 2 h at room temperature, and then washed again three times with TBST. Protein blots were developed using an enhanced chemiluminescence solution. The protein expression levels were visualized with Image Lab Software (Bio-Rad, USA).

Measurement of mRNA expression

Real-time PCR was used to analyze the miRNA and mRNA levels. Total RNA was extracted by using TRIzol reagent (TakaRa, Dalian, China); the concentration and purity of RNA were determined spectrophotometrically. 200ng of RNA from each sample was used for the reverse transcription reaction conducted according to instructions for a commercial transcription kit (DRR037A; TaKaRa, Dalian, China). Real-time PCR was used to quantitatively determine the miR-125b and CK2α mRNA expression levels using SYBR Premix Ex Taq (TaKaRa Dalian, China) and ABI 7300. Data analysis was performed with ABI software using the comparative Ct method.

Bio-informatics analysis

The target gene of a given miRNA and the target miRNA of a given gene were predicted by the online systems TargetScan and miBase. We focused on the gene CK2α, which is downregulated, and miR-125b, which is upregulated in our PC-12 model. Based on this analysis, CK2α was predicted as the target of miR-125b.

miR-125b mimics and inhibitor transfection

Experiments involving transfection of miR-125b mimics or an miR-125b inhibitor were conducted according to the instructions of the manufacturer. Before transfection, a mix including transfection agent lip2000 and either miR-125b mimics, or an miR-125b inhibitor, were prepared. The mix was then added into a 24 well plate containing PC-12 cells, to make a final concentration of 100 nM. Transfection proceeded for 6 h, after which the cells were collected for mRNA or protein analysis.

Statistical analysis

SPSS software (Version 11, SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data were expressed as mean ± SEM. Differences in measured values among the multiple groups were evaluated using analysis of variance with Bonferroni's multiple comparisons correction. The chi-squared test was used to compare the frequency distribution between groups. Differences were considered significant when $P < 0.05$.

Results

Cerebral I/R contributes to severe brain tissue injury

To investigate the pathological mechanism of ischemic stroke, a rat cerebral I/R model was established and induced brain damage was evaluated. As shown in Fig. 1A, compared to sham rats, 2h of cerebral ischemia plus 24h of reperfusion caused a significant increase in the neurological deficit score. The results of TTC staining and analysis of infarction volume indicated that the ischemic hemispheres of the brain suffered from infarction (Fig. 1B-C). Nissl staining revealed that Nissl bodies were subjected to severe damage, suggesting that cell

biosynthesis was hindered (Fig. 1D). Concordant with the results of Nissl staining, edema and cell shrinkage in brain tissues of the I/R group were observed with H&E staining (Fig. 1E). These results indicate that I/R induced neurological deficit and neuronal cell death.

miR-125b and CK2 α are involved in cerebral I/R injury

Several studies have demonstrated that miR-125b and CK2 α are involved in I/R injury, but their interactions have not been clearly elucidated. To explore this question, levels of expression of miR-125b and CK2 α were examined in brain tissues of rats with I/R injury. As shown in Fig. 2A, compared to the sham group, the miR-125b levels were elevated in brain tissues of rats with I/R injury, while expression of CK2 α decreased. This significant down-regulation of CK2 α mRNA and protein, with up-regulation of miR-125b, suggests that miR-125b and CK2 α play important roles in cerebral I/R injury. To investigate the relationships between miR-125b and CK2 α , we performed a bioinformatics analysis as described above. The results revealed that CK2 α is a potential target gene of miR-125b, and that miR-125b likely interacts with CK2 α by binding with its 3'UTR (Fig. 2D).

miR-125b directly suppresses the expression of CK2 α

To further describe the interaction between miR-125b and CK2 α , we transfected PC-12 cells with miR-125b mimics. As shown in Fig. 3A, expression of miR-125b increased significantly in PC-12 cells transfected with miR-125b mimics, and this overexpression of miR-125b significantly suppressed mRNA and protein expression of CK2 α (Fig. 3B-C). To confirm the hypothesis that miR-125b directly inhibits expression of CK2 α , a luciferase reporter gene plasmid was added, as shown in Fig. 3D. The effect of miR-125b on the relative luciferase activity in PC-12 cells transfected with wild or mutated CK2 α -3'UTR was observed. Overexpression of miR-125b significantly decreased the relative luciferase activity

Fig. 1. Cerebral ischemia/reperfusion contributes to severe brain tissue injury in rats. A. Neurological score of each group (n=8); B. TTC staining; C. Infarct volume of each group (n=8); D. Nissl staining; E. H&E staining. All values were expressed as means $\pm$ SEM. I/R: ischemia/reperfusion injury; *P<0.005 vs control.

Fig. 2. miR-125b and CK2 α are involved in cerebral ischemia/reperfusion injury. A. The expression of miR-125b in brain tissue; B. CK2 α mRNA expression level of each group in brain tissue; C. CK2 α mRNA expression level of each group in brain tissue; D. Schematic diagram for interaction of miR-26 and CK2 α . All values were expressed as means $\pm$ SEM. *P<0.05 vs control.

in cells transfected with CK2 α -3'UTR-WT plasmid, but those transfected with the CK2 α -3'UTR-MU plasmid did not show a similar effect (Fig. 3 E). These results indicate that miR-125b directly suppresses the expression of CK2 α by binding to its 3'UTR.

Inhibition of miR-125b attenuates the oxidative stress induced by OGD/R in PC-12 cells

To investigate the role of miR-125b in ischemic stroke, we introduced an miR-125b inhibitor into our OGD/R model PC-12 cells as described above. As shown in Fig. 4, OGD/R significantly increased the expression of miR-125b, while CK2 α mRNA and protein expression were down-regulated. However, these changes were reversed after introduction of an miR-125b inhibitor. This suggests that miR-125b is responsible for the decrease in CK2 α mRNA and protein expression after OGD/R in PC-12 cells (Fig. 4A-C). We also studied the effects of an miR-125b inhibitor on oxidative stress in PC-12 cells during OGD/R. As expected, OGD/R in PC-12 cells led to significant up-regulation of NOX2 and NOX4 mRNA and protein expression, with concomitant increases in NOX enzyme activity and ROS levels (Fig. 4D-I). However, these effects were reversed by an miR-125b inhibitor (Fig. 4D-I). This suggests that an miR-125b inhibitor might decrease oxidative stress after OGD/R in PC-12 cells, and the underlying mechanism might involve the regulation of expression of CK2 α .

Inhibition of miR-125b attenuates the apoptosis induced by OGD/R in PC-12 cells

Considering the important role of miR-125b in OGD/R-induced oxidative stress, we examined the effect of miR-125b on cell death in PC-12 cells. As shown in Fig. 5, OGD/R significantly increased the levels of LDH, caspase-3 protein expression and activity, and apoptosis in PC-12 cells. Transfection of an miR-125b inhibitor into PC-12 cells significantly reversed these OGD/R-induced effects. These results indicate that inhibition of miR-125b is associated with a decrease in cellular apoptosis after OGD/R in PC-12 cells.

Discussion

miRNAs may regulate as many as 60% of all human mRNAs, including mRNAs involved in practically all cellular and molecular functions. miRNAs are emerging as key regulators of neuronal development and function [3]. Accordingly, miRNAs have been extensively

Fig. 3. miR-125b directly suppresses the expression of CK2 α . A. MiR-125b expression level; B. CK2 α mRNA expression level; C. CK2 α protein level; D. Schematic diagram for the luciferase reporter gene plasmid; E. Relative luciferase activity. All values were expressed as means $\pm$ SEM. *P<0.05 vs control.

Fig. 4. Inhibition of miR-125b attenuates the oxidative stress induced by OGD/R in PC-12 cells. A. miR-125b expression level; B. CK2 α mRNA expression level; C. CK2 α protein level; D. NOX2 mRNA expression level; E. NOX2 protein expression level; F. NOX4 mRNA expression level; G. NOX4 protein expression level; H. Total NOX enzyme activity; I. ROS level. All values were expressed as means $\pm$ SEM. +NC miRNA: OGD/R+negative control miRNA; +miR-125b inhibitor: OGD/R+miR-125b inhibitor; OGD/R: oxygen-glucose deprivation/reperfusion. * $P < 0.05$ vs control; ** $P < 0.01$ vs control; *** $P < 0.001$ vs control; # $P < 0.05$ vs OGD/R; ## $P < 0.01$ vs OGD/R; ### $P < 0.001$ vs OGD/R.

investigated as biomarkers or target molecules for the nervous system [10]. For example, the level of circulating miR-125b has been confirmed as a potential biomarker of Alzheimer's Disease [11]. In addition, large amounts of data have indicated that miR-125b is up-regulated in the peripheral blood of stroke patients and rats [5]. In the present study, we established a model of cerebral I/R in rats, which was confirmed by increased neurological dysfunction, brain tissue injury, and neuronal cell death, and found that the expression of miR-125b was up-regulated in the brains of I/R rats. These

Fig. 5. Inhibition of miR-125b attenuates the apoptosis induced by H/R in PC-12 cells. A. LDH release; B. Caspase-3 protein expression level; C. Caspase-3 enzyme activity; D. Representative images of Hoechst staining. All values were expressed as means $\pm$ SEM. +NC miRNA: OGD/R+negative control miRNA; +miR-125b inhibitor: OGD/R+miR-125b inhibitor. * $P < 0.05$ vs control; # $P < 0.05$ vs H/R.

results suggest miR-125b may be involved in cerebral I/R injury. In order to confirm this hypothesis, we further investigated the role of miR-125b in apoptosis of PC-12 cells in an OGD/R model.

Neuronal cell apoptosis is a critical part of cerebral stroke pathophysiology [12], and current evidence has suggested a pivotal role of miRNAs in regulating neuronal death during cerebral ischemic stroke [13]. For instance, miR-134 regulates I/R injury-induced neuronal cell death by regulating CREB signaling [14]. Similarly, the level of expression of miR-323 was increased after OGD/R treatment in rat hippocampal neurons, and inhibition of miR-323 suppressed apoptosis induced by OGD/R [15]. miR-99a decreases neuronal damage following focal cerebral I/R injury in mice [16]. Upregulation of miR-9 inhibits neuronal apoptosis in ischemic stroke [17]. In the present study, the expression of miR-125b was up-regulated in OGD/R treated PC-12 cells, which is consistent with our *in vivo* results. Furthermore, inhibition of miR-125b decreased OGD/R-induced apoptosis and LDH release. These results suggest that miR-125b plays a key role in OGD/R-induced neuronal cell apoptosis, and is involved in cerebral I/R injury.

To further investigate the role of miR-125b in OGD/R-induced neuronal cell apoptosis, we hypothesized that miR-125b might target a downstream gene to regulate this process. Bioinformatics analysis was used to predict the target gene of miR-125b; this analysis suggested that miR-125b potentially targeted the 3'-UTR of CK2 α . The dual-luciferase reporter assay confirmed this result: up-regulation of miR-125b suppressed the expression of CK2 α . These results suggest that CK2 α is the direct downstream target of miR-125b.

CK2 α , a crucial subunit of CK2, has been reported to act as a neuroprotectant in mice after cerebral ischemia. Several lines of study have demonstrated that the protein level of CK2 α is significantly reduced after transient focal cerebral ischemia, and deactivation of CK2 in the mouse brain enhances neuronal cell death [7]. As shown in previous studies, we found that the expression of CK2 α was down-regulated in the brains of I/R rats. OGD/R induced a decrease of CK2 α expression in PC-12 cells, while transfection with an miR-125b inhibitor reversed the down-regulation of CK2 α induced by OGD/R. These findings imply that CK2 α is an intermediary for the regulatory effect of miR-125b in OGD/R-induced neuronal cell apoptosis.

CK2 has been reported to act as a negative regulator of NADPH oxidase (NOX) activity in cerebral I/R injury [7]. It is well known that the complex NADPH oxidase is a major contributor to production of detrimental oxygen-derived free radicals during the ischemic period. The family of NOX contains seven members, NOX1 to NOX5, dual oxidase (Duox)-1, and Duox-2. Among these isoforms, NOX2 and NOX4 play a major role in mediation of OGD/R-induced neuronal cell apoptosis in cerebral I/R injury [18]. On the basis of the above findings, we investigated the effect of miR-125b on NADPH oxidase activity and ROS, and found that inhibition of miR-125b reduced the OGD/R-induced up-regulation of NOX2 and NOX4 expression. NADPH oxidase activity and ROS production were also decreased with introduction of an miR-125b inhibitor. These results suggest that miR125b negatively regulates CK2 α , and affects NADPH oxidase activity, in OGD/R-induced neuronal cell apoptosis. These findings may be extrapolated to cerebral I/R injury.

Conclusion

In summary, the present study investigated the role of miR-125b in cerebral I/R injury. We found that miR-125b is involved in regulating OGD/R-induced neuronal cell apoptosis through direct suppression of CK2 α expression, and subsequent activation of NADPH oxidase. These findings provide novel insights into the molecular basis of ischemic stroke, which may provide clues for therapeutic targets. However, our study focused on the role of miR-125b/CK2 α /NADPH oxidase *in vitro*. Future studies on the effect of miR-125b on cerebral I/R *in vivo* will provide further evidence for possible pharmacological treatments for ischemic stroke.

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Disclosure Statement

The authors declare no Disclosure Statement.

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