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The role of microRNA-146a in regulating the expression of IRAK1 in cerebral ischemia-reperfusion injury

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The role of microRNA-146a in regulating the expression of IRAK1 in cerebral ischemia-reperfusion injury

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Abstract

MicroRNA-146a (miR-146a) is reportedly implicated in the pathogenesis of ischemia/reperfusion (I/R) injury. Its role in the cerebral I/R injury is however unclear and requires further investigation. In this study, cerebral I/R injury was established in mice via middle cerebral artery occlusion (MCAO), and the expression of miR-146a was detected in the brain tissue via quantitative real-time PCR. We found that the expression of miR-146a was up-regulated. Furthermore, the endogenous miR-146a was antagonized by its specific inhibitor. The results indicated that the inhibition of miR-146a deteriorated I/R-induced neurobehavioral impairment, exaggerated the infarct size and exacerbated blood brain barrier leakage. Cerebral I/R injury-induced generation of inflammatory cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6, was further promoted by miR-146a inhibitor. The expression of interleukin-1 receptor associated kinase 1 (IRAK1), a target of miR-146a, was up-regulated upon miR-146a inhibition. In addition, the nuclear factor κ B (NF- κ B) signaling pathway was over-activated when miR-146a was antagonized as manifested by the increased levels of phospho-NF- κ B inhibitor α and nuclear p65. In summary, our findings demonstrate that the elevation of miR-146a may be one of the compensatory responses post the cerebral I/R injury and suggest miR-146a as a potential therapeutic target for cerebral I/R injury.

Key words: miR-146a, cerebral I/R injury, inflammatory response, IRAK1, NF- κ B

Introduction

Cerebral ischemia, one of the cerebrovascular diseases, is related to the failure of circulation supply to the local brain. The number of patients with cerebral ischemia is increasing worldwide every year (Amadatsu et al. 2016). Restoring blood flow to the brain is the key to the treatment of cerebral ischemia, but the ischemia/reperfusion (I/R) injury that further aggravates tissue and organ damage may occur (Xu et al. 2017). Thus, there is an urgent need to explore the molecular mechanisms involved in cerebral I/R injury.

MicroRNAs are a family of small non-coding RNAs that regulate various physiological and pathological processes via silencing their target mRNAs (Watier & Sanchez 2017). Growing evidence has demonstrated an involvement of miR-146a in I/R injury of multiple organs. It has been reported that miR-146a can protect against intestinal, renal and myocardial I/R injuries by inhibiting the acute inflammatory response (Chassin et al. 2012; Dai et al. 2016; Wang et al. 2013). Interestingly, a previous study indicated that the expression of miR-146a was elevated in the brain tissues of experimental stroke Wistar rats (Liu et al. 2017). IRAK1 is a member of interleukin-1 receptor associated kinase (IRAK) family, which can mediate the activation of nuclear factor κ B (NF- κ B) and trigger a series of inflammatory responses (Song et al. 2016). Moreover, increasing evidence has reported IRAK1 as a target of miR-146a (Gao et al. 2015; Li et al. 2013). Nonetheless, whether miR-146a participates in I/R-induced cerebral injury and whether it regulates IRAK1 expression during this process remain unclear.

In our present study, the cerebral I/R injury model was established in mice. We investigated the role of miR-146a in cerebral I/R injury with an emphasis on NF- κ B signaling pathway.

Materials and methods

Animals and establishment of middle cerebral artery occlusion (MCAO) model

Male C57BL/6J mice (22-25 g) were obtained from Beijing Vital River Laboratory Animal Co., Ltd. (Beijing, China). Temporary focal cerebral I/R injury was induced in mice by MCAO surgery according to a previous study with some modifications (Wang et al. 2016). Briefly, the mice were anesthetized by injection of 10% chloral hydrate (3.5 ml/kg, i.p.). After exposure of the right common carotid artery (CCA), internal carotid artery (ICA), and right external carotid artery (ECA), the CCA and proximal ECA were nipped. Then a nylon suture (0.23 mm in diameter) was inserted into the ICA through the ECA stump, and gently advanced until feeling resistance to occlude the middle cerebral artery. After 60-min MCAO, the mice were received reperfusion by cautiously removing the suture. Sham-operated mice were underwent the same procedure just without the suture insertion. All surgical procedures were performed in sterile conditions. The animals were cared for in accordance with the principles and guidelines for the Care and Use of Laboratory Animals from the Canadian Council on Animal Care. All experimental procedures performed in studies involving animals were in accordance with the Institutional Animal Care and Use Committee guidelines of Shandong University.

Down-regulation of miR-146a in mice

The miR-146a inhibitor and inhibitor negative control (NC) were purchased from Genepharma Co., Ltd (Shanghai, China). The sequences were as follows: miR-146a inhibitor: AACCCAUGGAAUUCAGUUCUCA; NC: CAGUACUUUUGUGUAGUACAA. Briefly, the mice were anesthetized and fixed in a stereotaxic apparatus. Twenty-four hours before MCAO operation, the miR-146a inhibitor or NC (0.5 nmol in 1 μ l *in vivo* transfection reagent (Entranster™-in vivo, Engreen, China) was injected into the right lateral ventricle (bregma: 2.0 mm posterior, 1.5 mm dorsoventral, 1.8 mm lateral) at a rate of 0.2 μ l/min with a microsyringe.

Neurobehavioral evaluation

Neurobehavioral evaluation was performed at 24 h after reperfusion by an observer blinded to the experiment, according to previous methods reported by Longa *et al.* (Longa et al. 1989). The neurobehavioral findings were assessed by a five-point scale: a score of 0 showed no neurological impairment; a score of 1 showed mild degree of neurological impairment and indicated failure to extend the opposite forepaw fully; a score of 2 showed moderate degree of neurological impairment and exhibited circling to the opposite side; a score of 3 showed severe degree of neurological impairment and indicated falling to the opposite side; a score of 4 showed failure to walk spontaneously and had decreased level of consciousness.

2, 3, 5-triphenyltetrazolium chloride (TTC) staining

At 24 h after reperfusion, the infarct volume was measured by TTC (Solarbio, China) staining. Briefly, the brain tissues were removed and sectioned into five coronal slices (1 mm thick). Then the slices were immersed in 1% TTC at 37°C for 15

min. Images were taken by a digital camera and the infarct area and whole brain area were measured by Image-Pro Plus Analysis Software (Media Cybernetics, USA). The infarct volume was calculated by the following formula: Infarct volume % = $(\sum \text{Infarct area} \times \text{thickness}) / (\sum \text{whole brain area} \times \text{thickness}) \times 100\%$.

Brain water content

After reperfusion for 24 h, brain water content was detected to evaluate the extent of brain edema. Briefly, the brains were removed and the lesion hemispheres were weighted to obtain the wet weight data. Then the hemispheres were placed in a desiccating oven at 100°C for 24 h and weighted to obtain their dry weight. The percentage of brain water content was calculated by the following formula: Brain water content % = $[(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100\%$.

Evans blue (EB) staining

EB staining assay was performed to assess BBB leakage at 24 h after reperfusion. In short, 0.1 ml 2% EB was intravenously injected via tail vein and allowed to circulate for 60 min. Then the mice were anesthetized, the adequate heart perfusion with saline was performed. Subsequently, the mice were euthanized and their brains were removed and homogenized in PBS. After centrifuged at 15000 g for 30 min, the supernatant was collected and incubated with an equal volume of 50% trichloroacetic acid at 4°C overnight, and then centrifuged. The results were measured at 615 nm by a microplate reader (BioTek, USA) and quantitatively calculated via a standard curve. The EB content is expressed as the amount of EB per brain tissue weight ($\mu\text{g/g}$).

Quantitative real-time PCR

Total RNAs were extracted by a RNeasy total RNA fast isolation kit (Qiagen, China). Then the total RNAs were reversely transcribed to cDNAs by a Super M-MLV Reverse Transcriptase (Qiagen). RNA expression was detected by quantitative real-time PCR in Real-Time Quantitative Thermal Block (BIONEER, Korea) using the SYBR-Green reagent (Solarbio, China). For the miR-146a expression, U6 was used as an internal control. β -actin was used as an internal reference gene for IRAK1. The primer sequence information was listed in Table 1.

Western blotting assay

Twenty-four hours after the reperfusion, the peri-infarct brain tissues were collected and homogenized in RIPA buffer (Beyotime, China) and then denatured. The nuclear or cytoplasmic protein was extracted by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) according to the manufacture's instructions. The concentrations of total, cytosolic and nuclear proteins were determined by a BCA Protein Assay Kit (Beyotime). Subsequently, protein samples were separated on SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, USA). Then the membranes were blocked with 5% skimmed milk for 1 h at room temperature and incubated with primary antibodies against IRAK1 (1:400, BOSTER, China), phospho-NF- κ B inhibitor α (p-I κ B α) (1:500, Bioss, China), I κ B α (1:500, Bioss, China), NF- κ B (1:500, Bioss, China), β -actin (1:500, Bioss, China), and Histone H3 (1:500, Bioss, China) at 4°C overnight, respectively. The secondary antibodies were added to the membranes at 37°C for 45 min. The enhanced chemiluminescence (ECL, Beyotime, China) was used to visualize the bands.

Densitometry quantification of the bands was carried out by Gel-Pro-Analyzer 4 software (Media Cybernetics, USA).

ELISA assay

At 24 h after reperfusion, the levels of pro-inflammatory cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6, in brain tissues were determined by commercial ELISA detection kits (Boster, China), according to the manufacture's instructions.

Statistical analysis

The experimental data were presented as mean $\pm$ standard deviation (SD). The statistical analyses of data among multiple groups were carried out by one way ANOVA followed by Bonferroni's Multiple Comparison Test using GraphPad Prism 5 software. A *p* value less than 0.05 was considered statistical significant.

Results

Effect of cerebral I/R injury on miR-146a expression in the brain

First, at various time points after reperfusion, the expression of miR-146a in the brain tissue of mice received sham operation or 60-min MCAO was detected. As shown in Fig. 1, there was no difference in miR-146a expression between the pre-operation and sham groups ($P>0.05$). The miR-146a expression was significantly increased from 24 h to 72 h post reperfusion ($P<0.001$ for all).

Down-regulation of miR-146a exacerbated the cerebral I/R injury

To further evaluate the role of miR-146a on cerebral I/R injury in mice, the expression of miR-146a was down-regulated by intracerebral injection of miR-146a

inhibitor at 24 h before MCAO (Fig. 2(A)) ($P<0.001$). Then the neurological deficit scores were assessed at 24 h after reperfusion and the results were shown in Fig. 2(B). Sham-operated mice displayed no neurological deficits. Mice underwent MCAO surgery exhibited mild to moderate degree of neurological impairment ($P<0.001$ for all), and inhibition of miR-146a further exacerbated such neurological impairment ($P<0.01$). The infarct volume was determined by TTC staining. As shown in Fig. 2(C)&(D), inhibition of miR-146a further exaggerated I/R-induced infarction ($P<0.01$). Moreover, as indicated in Fig. 2(E), I/R led to obvious brain edema in mice ($P<0.01$). Although miR-146a inhibitor caused an upward trend of water content in the brain of MCAO mice as compared with NC inhibitor, there was no statistical difference ($P>0.05$). As shown in Fig. 2(F), BBB leakage was detected by EB staining. Inhibition of miR-146a significantly deteriorated the EB leakage induced by cerebral I/R ($P<0.05$).

Down-regulation of miR-146a promoted I/R-induced inflammation in the brain

We further examined the role of miR-146a in cerebral I/R injury-related inflammatory response. As shown in Fig. 3(A-C), the levels of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 were significantly increased in the brain tissue of mice subjected to cerebral I/R surgery (for TNF- α , $P<0.01$; for IL-1 β , $P<0.05$; for IL-6, $P<0.05$), which could be further up-regulated by miR-146a inhibition (for TNF- α , $P<0.05$; for IL-1 β , $P<0.01$; for IL-6, $P<0.001$).

Antagonism of miR-146a promoted cerebral I/R injury-induced activation of NF- κ B pathway

As the activation of NF- κ B pathway may promote inflammatory response via inducing the expressions of various pro-inflammatory cytokines, the role of miR-146a in regulating NF- κ B pathway in cerebral I/R injury was assessed. As shown in Fig. 4 (A-D), I/R injury enhanced the phosphorylation of I κ B α ($P<0.05$) and accelerated the nuclear accumulation of NF- κ B p65 ($P<0.001$). Down-regulation of miR-146a could further promote the above changes (for p-I κ B α , $P<0.001$; for nuclear NF- κ B, $P<0.001$).

Knockdown of miR-146a enhanced I/R-induced IRAK1 expression in mouse brain

IRAK1 has been proved to be a target of miR-146a, so we evaluated the effect of miR-146a on IRAK1 expression in mice with cerebral I/R injury. As shown in Fig. 5(A), miR-146a inhibitor further augmented cerebral I/R-induced up-regulation of IRAK1 mRNA ($P<0.01$). Moreover, as shown in Fig. 5 (B), knockdown of miR-146a also up-regulated the protein level of IRAK1 in the brain tissue ($P<0.001$).

Discussion

In this study, we first demonstrated that miR-146a expression was up-regulated with perfusion time in mouse brain and that its down-regulation aggravated cerebral I/R injury-induced neurological impairment, infarction and BBB leakage in mice. These pathological alterations were accompanied with the up-regulation of IRAK1 and the over-activation of NF- κ B signaling pathway.

BBB disruption promotes inflammatory response via activating the pro-inflammatory NF- κ B signaling pathway, resulting in severe cerebral vascular

permeability and eventual brain edema (Mao et al. 2015; Xu et al. 2016). Brain edema further induces neuronal cell death, deteriorates cerebral infarction, and causes neurological deficits. Therefore, restoration of BBB is a focus for the clinical treatment of cerebral I/R injury. Our data here showed that the suppression of miR-146a further worsened I/R-induced BBB leakage in mice, indicating that miR-146a played a role in maintaining the integrity of BBB and that its elevation post the I/R injury may be one of the compensatory responses.

The inflammatory response participates in the pathological course of cerebral I/R injury which has been confirmed to lead to stroke (Siniscalchi et al. 2014). The inflammatory cells tend to aggregate in the damaged areas post cerebral I/R injury and release more inflammatory cytokines, which further aggravates the brain lesion (DeGraba 1998; Stoll et al. 2002). TNF- α is a critical pro-inflammatory cytokine that is mostly produced by microglial cells and astroglia cells post the localized cerebral lesion (Suzuki et al. 2004). The level of TNF- α was positively correlated with the severity of focal cerebral ischemic injury (Feuerstein et al. 1998; Zhai et al. 1997), and its inhibition could significantly reduce the cerebral infarct in ischemic mice (Yang et al. 1998). IL-1 β and IL-6 can be synthesized and secreted by the central nervous system cells upon being stimulated. Increasing evidence has demonstrated an over-generation of IL-1 β and IL-6 in the serum and brain post cerebral I/R injury (Bai et al. 2017; Fann et al. 2013). Like the beneficial effects induced by TNF- α interference, blockade of IL-1 β by recombinant IL-1 receptor also has been found to attenuate ischemic brain injury in rats (Pradillo et al. 2012). These results indicate that

the elevation of these pro-inflammatory cytokines contributes to I/R-induced brain lesion. We found that miR-146a inhibitor further augmented I/R-induced over-generation of TNF- α , IL-1 β and IL-6. These results suggest that the miR-146a inhibitor deteriorates the cerebral I/R injury at least due to its inflammation-promoting effects.

The activation NF- κ B signaling can be characterized by the nuclear translocation of p65. This signaling pathway is well-known to regulate the inflammatory response during cerebral I/R injury (Chen et al. 2016; Xu et al. 2016). In un-stimulated cells, the I κ Bs family inhibitors sequester the NF- κ B p50-p65 heterodimer in the cytoplasm in an inactive form (Ghosh et al. 1998). When activated by stimulus, the I κ B kinases are activated to phosphorylate the I κ B α , and finally promote the I κ B α degradation via ubiquitination. Then p65 is freed and translocates into the cell nucleus, and regulates the expression of its target genes, such as TNF- α , IL-1 β and IL-6. Blockade of NF- κ B signaling transduction could alleviate brain tissue injury after reperfusion (Lille et al. 2001). Beside the further elevation of pro-inflammatory cytokines induced by miR-146a inhibition, the over-activation of NF- κ B pathway was also observed (enhanced phosphorylation of I κ B α and nuclear accumulation of p65).

IRAK1 is a kinase that partially mediates the activation of NF- κ B signaling pathway, (Landais et al. 2015; Song et al. 2016), and it is a predicted target of miR-146a. Therefore, its expression in mouse brain was further detected. A study from Loubaki et al. suggested that up-regulating the expression of miR-146a reduced the IRAK1 level, and restrained NF- κ B activation in LPS-activated monocytes

(Loubaki et al. 2017). In the early phase of hyperglycemic condition, miR-146a is up-regulated to decrease the NF- κ B activity through inhibition of IRAK1 in human umbilical vein endothelial cells (Kamali et al. 2016). As expected, our results also showed that the mRNA and protein expression levels of IRAK1 in I/R brain tissue were further up-regulated by inhibition of miR-146a.

Conclusion

Taken together, the present study suggests that inhibition of miR-146a aggravates the cerebral I/R injury in mice by triggering inflammatory response via promoting the IRAK1/NF- κ B signaling pathway activation. Our results provide evidence that miR-146a may be a potential therapeutic target for cerebral I/R injury.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 Oligonucleotide primer sets for real-time PCR

Name	Sequence(5'-3')	Length
IRAK1 F	TCCACCAAGCAGTCAAGCC	19
IRAK1 R	AAACCACCCTCTCCAATCC	19
β-actin F	CTGTGCCCATCTACGAGGGCTAT	23

β -actin R	TTTGATGTCACGCACGATTTC	22
miR-146a F	CCGCTGAGAACTGAATTCC	22
miR-146a R	GTGCAGGGTCCGAGGTATTC	20
U6 F	CTCGCTTCGGCAGCACA	17
U6 R	AACGCTTCACGAATTTGCGT	20

Draft

Figure legends

Figure 1. Effect of cerebral I/R injury on miR-146a expression in the brain tissue of mice. The expression of miR-146a in the brain tissue of mice that from pre-operation,

sham and MCAO groups following different durations of reperfusion (6 h, 24 h, 72 h) was determined by quantitative real-time PCR. The results were obtained from at least three independent experiments. Data were presented as means $\pm$ SD (n=6). ***P < 0.001, versus the sham group.

Figure 2. Effect of miR-146a on cerebral I/R injury in mice. (A) The expression of miR-146a was inhibited by the injection of miR-146a inhibitor into the brain at 24 h before MCAO. The expression of miR-146a in various groups was detected by quantitative real-time PCR. (B) The neurobehavioral scores of mice were evaluated at 24 h after the reperfusion. (C) TTC staining was performed to detect the cerebral infarction at 24 h after the reperfusion. (D) The infarct volume of each group was quantitatively analyzed. (E) The brain water content in various groups was detected at 24 h after the reperfusion. (F) EB staining was used to assess the BBB leakage and the EB content of each group was shown. The results were obtained from at least three independent experiments. Data were presented as means $\pm$ SD (n=6). *P < 0.05, **P < 0.01, ***P < 0.001, versus the sham group. #P < 0.05, ## P < 0.01, ### P < 0.001 versus the MCAO+NC group.

Figure 3. Effect of miR-146a on cerebral I/R injury-induced inflammatory response. The levels of TNF- α (A), IL-1 β (B), and IL-6 (C) in the brain tissue were determined by ELISA assay at 24 h after the reperfusion. The results were obtained from at least three independent experiments. Data were presented as means $\pm$ SD (n=6). *P < 0.05, **P < 0.01, ***P < 0.001, versus the sham group. #P < 0.05, ## P < 0.01, ### P < 0.001 versus the MCAO+NC group.

Figure 4. Effect of miR-146a on cerebral I/R injury-induced the activation of NF- κ B pathway. (A)&(B) The protein levels of p-I κ B α and I κ B α in the brain tissue were assessed by western blot assay at 24 h after the reperfusion. The gray-scale value of the bands was quantitatively analyzed. The protein level of NF- κ B in cytoplasm (C) and nucleus (D) in the brain tissue was determined by western blot assay at 24 h after the reperfusion. The results were obtained from at least three independent experiments. Data were presented as means $\pm$ SD (n=6). *P < 0.05, **P < 0.01, ***P < 0.001, versus the sham group. ## P < 0.01, ### P < 0.001 versus the MCAO+NC group.

Figure 5. Effect of miR-146a on mRNA and protein expressions of IRAK1 in MCAO mice. (A) The mRNA expression of IRAK1 in the brain tissue was assessed by quantitative real-time PCR at 24 h after the reperfusion. (B) The IRAK1 protein level was evaluated by western blot assay at 24 h after the reperfusion. The results were obtained from at least three independent experiments. Data were presented as means $\pm$ SD (n=6). *P < 0.05, **P < 0.01, ***P < 0.001, versus the sham group. ## P < 0.01, ### P < 0.001 versus the MCAO+NC group.

Figure 1

173x96mm (300 x 300 DPI)

Figure 2

209x196mm (300 x 300 DPI)

Figure 3

209x117mm (300 x 300 DPI)

Figure 4

220x213mm (300 x 300 DPI)

Figure 5

209x99mm (300 x 300 DPI)