MiR-34b Protects Against Focal Cerebral Ischemia-Reperfusion (I/R) Injury in Rat by Targeting Keap1

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Ischemic stroke is one of the leading causes of death and disability globally and has been regarded as a major public health problem. Understanding the mechanism of ischemia/reperfusion (I/R)-induced oxidative stress injury may provide new treatment for ischemic stroke. Kelch-like ECH-associated protein 1 (Keap1)/ NF-E2-related factor 2 (Nrf2)/ antioxidant response elements (ARE) signaling pathway has been considered to be the major cellular defense against oxidative stress. In the present study, our objective is to evaluate the molecular mechanism of miR-34b/Keap1 in modulating focal cerebral I/R induced oxidative injury. miR-34b was predicted to target the 3′-UTR of the rat Keap1. After focal cerebral I/R, miR-34b expression was downregulated in a time-dependent manner; miR-34b overexpression ameliorated I/R-induced oxidative stress injury in middle cerebral artery occlusion (MCAO) rats by reducing the infarction volume, the neurological severity scores, the levels of nitric oxide (NO) and (3-nitrotyrosine) 3-NT while increasing total (superoxide dismutases) SOD and manganese SOD (MnSOD). Through direct targeting, miR-34b could suppress the protein levels of Keap1 and increase the protein levels of Nrf2 and heme oxygenase (HO-1). Regarding the molecular mechanism, Keap1 overexpression exacerbated, while miR-34b improved H2O2-induced oxidative stress injury; the effect of miR-34b could be partially attenuated by Keap1 overexpression, suggesting that miR-34b modulated oxidative stress injury in vitro and in vivo through targeting Keap1. Taken together, we demonstrate that miR-34b protects against focal cerebral I/R-induced oxidative stress injury in MCAO rats and H2O2-induced oxidative stress injury in rat neuroblast B35 cells through targeting Keap1 and downstream Keap1/Nrf2 signaling pathway. We provided a novel mechanism of focal cerebral I/R injury from the perspective of miRNA regulation.

Key Words: Focal cerebral ischemia-reperfusion (I/R) injury—miR-34b—Keap1—Keap1/Nrf2 signaling—oxidative stress injury

Introduction

Ischemic stroke, one of the leading causes of death and disability globally, is characterized by the sudden loss of blood circulation to an area of the brain, thus leading to a corresponding loss of neurologic function.[1,2] The main cause of brain tissue damage is not ischemia itself but the result of excessive scavenging of free radicals that attack cells, resulting in ischemia/reperfusion (I/R) injury.[3-5] Thus, a better understanding of the cellular and molecular mechanisms underlying the pathological process of oxidative stress injury after focal cerebral I/R may provide new treatment for ischemic stroke.

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The reactive oxygen species (ROS) produced during the process of focal cerebral I/R can directly cause damage to DNAs, lipids, proteins and other macromolecules in brain cells. The Kelch- like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element system is considered to be the major cellular defense against oxidative stress.[6] Under oxidative stressed conditions, suppression of Keap1 activity can provoke activation of Nrf2, a chief transcription regulator, then it can bind to an antioxidant response element found in the promoter region of a battery of genes that encode phase II detoxifying enzymes and/or antioxidant proteins such as heme oxygenase-1 (HO-1), thereby inducing gene expression. These genes or proteins may play an important role in the maintenance of the cellular redox state and preventing oxidative injury by clearing ROS through a series of synergetic mechanisms.[6-8]

MicroRNAs (miRNAs), a family of small noncoding RNAs that bind to the 3′-UTR of downstream target mRNAs to induce transcript degradation and/or inhibit translation,[9] have been reported to play an important role in various pathophysiology processes, including ischemic/reperfusion injury (I/R injury).[10,11] Interestingly, Keap1 has been reported to be a downstream target of several miRNAs in cancers,[12-15] renal mesangial cells[16] and high glucose-induced renal tubular epithelial injury.[17] MiRNA-induced suppression of Keap1 thereby resulted in Nrf2 nuclear translocation and activation of Nrf2-dependent HO-1 gene transcription.[18] Thus, we hypothesized that Keap1-Nrf2 and downstream HO-1 might be regulated by miRNA(s), therefore, participating in the process of focal cerebral I/R.

In the present study, the effect of miR-34b on middle cerebral artery occlusion (MCAO) rat model was evaluated, and the interaction between miR-34b and Keap1/Nrf2 pathway was verified both in vivo and in vitro. Taken together, we provide a novel molecular mechanism of focal cerebral I/R injury from the perspective of miRNA regulation.

Materials and Methods

MCAO/Reperfusion Model Rats

Pathogen-free male Sprague−Dawley rats (weighing 230-250 g) were randomly assigned to 2 groups: sham-operated group and MCAO group (subjected to MCAO and subsequent reperfusion). Rats MCAO models were established according to Longa et al. Briefly, rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g). The carotid artery, internal carotid artery, and external carotid artery were separated. A nylon cord (0.26 mm diameter) was inserted into internal carotid artery from the incision of carotid artery. Resistance implied that the line had reached the beginning of the right middle cerebral artery, thus blocking blood flow of the vessel. The filament was withdrawn after 90 minutes, after which the skin wound was sutured. The sham operation group only received blood vessel separation. All the study was approved by the Laboratory Animal Care and Use Committee of Xinjiang Medical University (Urumqi, China). To ensure the occurrence of ischemia by MCAO, regional cerebral blood flow was monitored using laser Doppler flowmetry (PeriFlux System 5000, Perimed, Stockholm, Sweden). Rectal temperature was controlled at 37°C during and after surgery with a temperature-regulated heating pad. Blood pressure was monitored through a measurement (MP100A-CE, BIOPAC Systems, Inc, CA). All animals were maintained in an air-conditioned room at 25°C ± 1°C after recovering from anesthesia. The infarct volume was determined by 2, 3, 5-triphenyltetrazolium chloride (TTC staining); the neurological severity scores were assessed according to Longa standard.[19] Briefly, a score of 0 indicated no neurologic deficit, a score of 1 (failure to extend left forepaw fully) a mild focal neurologic deficit, a score of 2 (circling to the left) a moderate focal neurologic deficit, and a score of 3 (falling to the left) a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness.

Intracerebroventricular Injection

Three days before MCAO, rats were randomly divided into miRNA and control miRNA mimic groups. The rats were anesthetized with 2% isoflurane in 70% N_2O balance O_2 by facemask and placed in a stereotaxic frame with a rat head holder. Intracerebroventricular injection was performed. The pLVX-miR-34b or pLVX-NC mixture (miRNA or control miRNA 4.2 μL, lipofectamine 2000 1.2 μL, and ddH_2O 0.6 μL) was immediately stereotaxically delivered into the ipsilateral lateral ventricle for more than10 minutes. The bone wound was closed with bone wax.

2, 3, 5-TTC Staining

The rats were sacrificed at 24 hours after reperfusion, and brains were removed quickly. Infarct volumes were measured by TTC staining. Seven 2 mm-thick coronal sections were cut at 3, 5, 7, 9, 11, 13, and 15 mm posterior of the olfactory bulb. Brain slice #3−7 of rats from each group (except for sham group) were used to perform TTC-staining. Slices were incubated in 2% TTC solution for 30 minutes at 37°C, then fixed in 10% formalin. The border zone of infarction was outlined with Image-Pro Plus Analysis Software (Media Cybernetics, MD). Volume calculation with edema correction was performed blindly using the following formula: 100 × (contralateral hemisphere volume-non-infarct ipsilateral hemisphere volume)/contralateral hemisphere volume. The analysis was done by investigators who were blinded to the experimental groups.

Cell Line, Cell Culture, and Cell Transfection

Rat neuroblasts B35 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 100 U/mL

penicillin/streptomycin (Invitrogen). Cells were maintained at 37°C and 5% CO_2.

Rno-miR-34b-3p mimics or Rno-miR-34b-3p inhibitor was transfected into target cells to achieve miR-34b expression (GenePharma, China). Keap1 overexpressing or si-Keap1 vectors were used to achieve Keap1 expression (GenePharma, China). Transfection was conducted with the help of Lipofectamine 2000 (Invitrogen, Waltham, MA). Twenty-four hours later, the cells were treated with H_2O_2 (200 μM) to induce oxidative stress for 24 hours.

Real-time PCR

Total RNA was extracted from the ipsilateral side of brain slice #2 of rats from each group or cells using Trizol (Invitrogen, Carlsbad, CA) according to the protocol supplied with the reagent and then reverse transcribed into cDNA using Superscript III reverse transcriptase kit (Invitrogen). The resulting cDNA was used for polymerase chain reaction (PCR) using SYBR(R) green I Nucleic A (Invitrogen) in triplicates. PCR and data collection were performed on 7500 fast real-time PCR System (Applied Biosystems, Alameda, CA). All quantifications were normalized to an endogenous GAPDH control. The expression of miRNA was detected using the miRNA RT kit (ABI) and TaqMan Universal PCR Master Mix (ABI) according to the manufacturer's instructions. U6 was used as an internal control. The relative quantification value for each target gene was performed using the comparative cycle threshold method.

Immunoblotting

The ipsilateral side of brain slice #2 of rats from each group and cells were homogenized and centrifuged at 12,000 rpm for 30 minutes at 4°C, and then the supernatant was collected. Protein concentration was determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using body surface area as the standard. Protein samples (100 μg per lane) were separated on an 8% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). After blocking in 5% skimmed milk for 2 hours at room temperature, the membranes were incubated overnight at 4°C with the following antibodies: anti-Keap1 (ab119403, Abcam, Cambridge, CA), anti-Nrf2 (ab137550, Abcam), anti-HO-1 (ab13248) and anti-GAPDH (ab8245). Anti-GAPDH antibody was used as an internal loading control. Membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG (1:2000 dilution, Santa Cruz Biotechnology Inc, TX) in blocking solution for 1 hour. The blots were visualized by chemiluminescence (Millipore, Billerica, MA). The relative optical density of protein bands was measured after subtracting the film background.

Luciferase Reporter Assay

The 3′-UTR of the rat Keap1 was synthesized (including a predicted binding site for miR-34b-3p) from Invitrogen. The PCR product was cloned downstream of the Renilla psiCHECK2 vector (Promega, Madison, WI), named wt-Keap1. A mutant luciferase reporter vector (mut-Keap1) was constructed by mutating the putative miR-34b-3p binding site. In 6-well plates, 2.0×10^5 HEK293 cells (ATCC, Manassas, VA) per well were plated and cotransfected with these vectors and miR-34b-3p mimics or miR-34b-3p inhibitor with the help of Lipofectamine 2000 (Invitrogen). Cells were harvested 36 hours after transfection and assayed using Luciferase Assay System (Promega, Madison, WI) according to the protocol.

3-NT Determined by Enzyme-Linked Immunosorbent Assay (ELISA)

The ipsilateral side of brain slice #1 of rats from each group or target cells was used for ELISA. Brain homogenates (10%, w/v) were prepared with cold phosphate buffered saline. 3-NT (Cusabio Biotech Co., Ltd., TX) levels in brain homogenate supernatants were detected by ELISA according to the instructions. 3-NT levels in brain and B35 cells homogenate were normalized to the total protein.

NO, Superoxide Dismutase (SOD), and Manganese Superoxide Dismutase (MnSOD) Detected by Biochemical Method

The ipsilateral side of brain slice #1 of rats from each group or target cells was used for NO measurement. Brain homogenates (10%, w/v) were prepared with cold phosphate

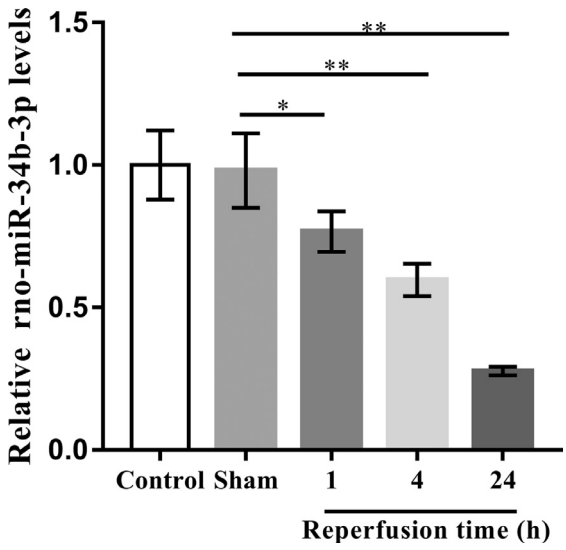

Figure 1. ***The expression of miR-34b in rat focal cerebral ischemia and reperfusion*** *The expression levels of miR-34b in rat in control, Sham-operation and focal cerebral ischemia and reperfusion groups were examined using real-time PCR assays. The data are presented as mean ± SD of 3 independent experiments. *P<0.05, **P<0.01. n = 6.*

buffered saline. NO (Beyotime Institute of Biotechnology, Nantong, China) levels in brain homogenate supernatants were detected by biochemical method. Total SOD activity and MnSOD activity in B35 cells was evaluated by SOD Assay Kit-WST kit (Beyotime Institute of Biotechnology, Nantong, China). NO levels and SOD activity in brain or cell homogenate were normalized to the total protein.

Statistical Analysis

All data were reported as mean ± SD. Statistical analysis was performed using SPSS version 11.0 (SPSS, Chicago, IL). The significance of difference was assessed by one-way analysis of variance followed by Tukey's multiple comparison test or independent sample t-test. For Longa neurological score, the nonparametric statistics were performed. The effects were considered statistically significant with $P < 0.05$.

Results

The Expression of MiR-34b in Rat Focal Cerebral Ischemia and Reperfusion

To evaluate the potential role of miR-34b in I/R injury, the expression of miR-34b in brain samples from untreated rats, sham-operated controls, and rats were subjected to focal cerebral ischemia were examined using real-time PCR. The brain tissues were taken 1, 4, and 24 hours after reperfusion. In comparison with samples from untreated and sham-operated rats, miR-34b expression was significantly downregulated after reperfusion in a time-dependent manner and reached the valley on 24 hours after reperfusion (Fig, 1). The changes

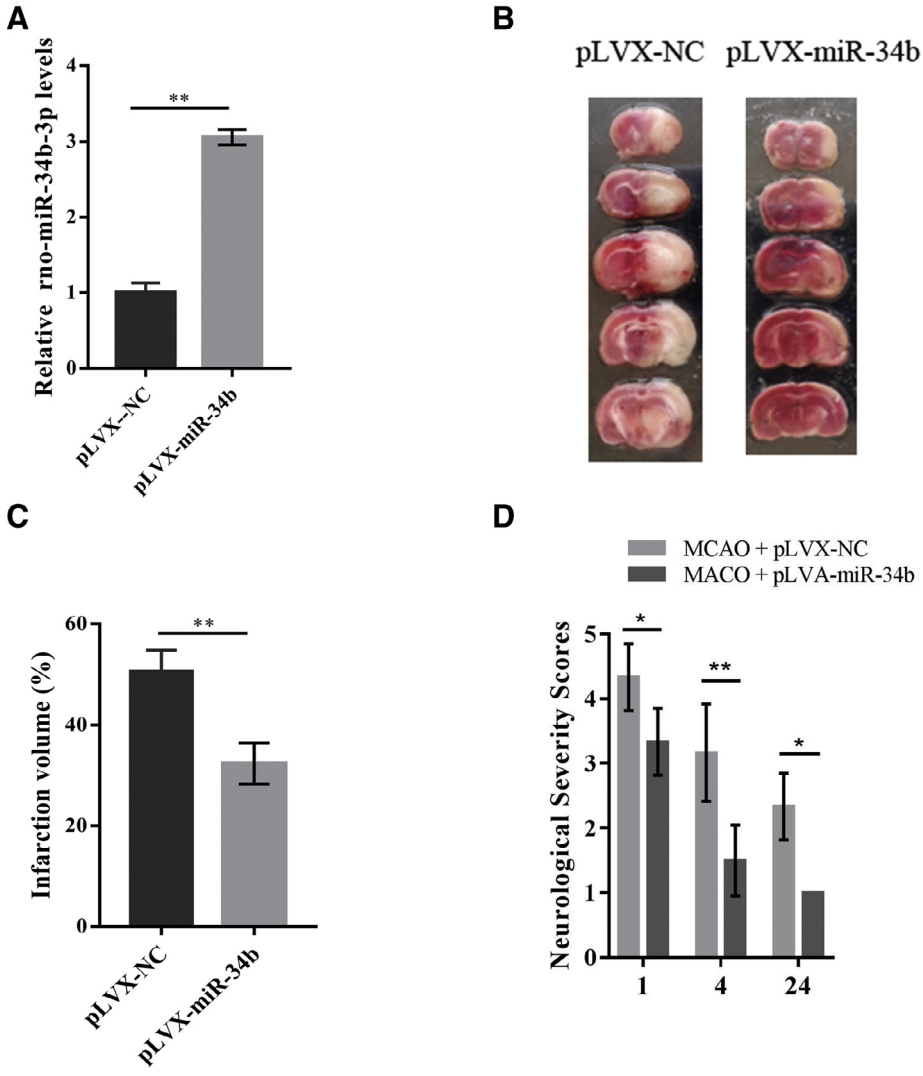

Figure 2. ***The overexpression of miR-34b ameliorates I/R injury*** *(A) The overexpression of miR-34b was achieved by injection of pLVX-miR-34b three days before MCAO/reperfusion, as confirmed using real-time PCR assays. (B-C) After 90 min of focal cerebral ischemia followed by reperfusion, the focal cerebral ischemia volumes in pLVX-NC injection and p-LVX-miR-34b groups were examined using TTC staining. (D) The neurological severity scores of the rats in different groups were assessed according to Longa standard. The data are presented as mean ± SD of 3 independent experiments. *P<0.05, **P<0.01. (Color version of figure is available online.)*

of miR-34b in brain tissues after reperfusion indicate that miR-34b may affect I/R injury.

The Overexpression of MiR-34b Ameliorates I/R Injury

To examine the detailed function of miR-34b in I/R injury, miR-34b overexpression was achieved by intracerebroventricular injection of pLVX-miR-34b, as confirmed using real-time PCR (Fig, 2A). Three days after injection, MCAO rat model was set, the cerebral infarction volume and the rat neurological scores were examined. As shown in Figure 2B, C, the cerebral infarction volume was significantly reduced by miR-34b overexpression. In addition, neurological severity assessment revealed that miR-34b overexpression significantly reduced the neurological severity scores, indicating that miR-34b ameliorated the I/R injury.

MiR-34b Overexpression ROS Injury in Transient MCAO/Reperfusion Rats Model

ROS and reactive nitrogen species (RNS) were known to derive from mitochondria, and manganese superoxide dismutase (MnSOD) is a key antioxidative enzyme located in mitochondria, which protects the energy-generating mitochondria from oxidative damage.[20] To investigate the effect of miR-34b overexpression on transient MCAO-induced brain oxidative stress in vivo, the RNS production including nitricoxide (NO) and oxidation protein products 3-nitrotyrosine (3-NT) levels, as well as protein levels of total SOD and MnSOD were measured in response to miR-34b overexpression. Consistent with cerebral infarction volume alteration, NO and 3-NT levels were observably elevated in the ischemic brain of MCAO rats; miR-34b overexpression significantly reduced NO and 3-NT levels compared with the vector group (Fig 3, A,B). In accordance, total SOD and MnSOD were significantly decreased in the ischemic brain of MCAO rats, while were notably restored by miR-34b overexpression compared with the vector group (Fig 3, C,D). Collectively, miR-34b overexpression increased antioxidative enzyme total SOD and MnSOD, and reduced RNS production after focal cerebral I/R.

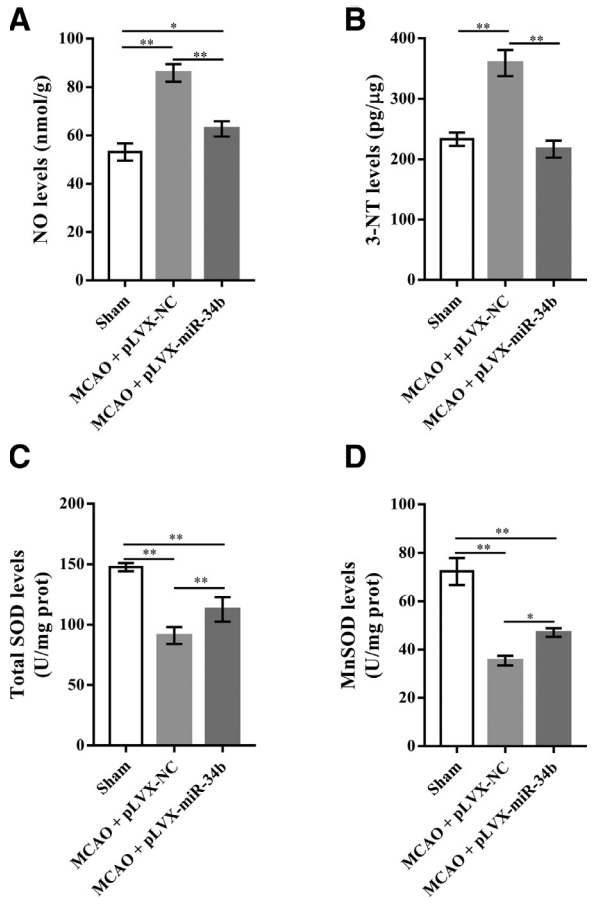

Figure 3. ***miR-34b overexpression reduced oxidative stress injury in transient MCAO/reperfusion rat model*** *The levels of NO, 3-NT, total SOD and MnSOD in p-LVX-NC or p-LVX-miR-34b injection MCAO groups were examined, compared to sham-operation group. The data are presented as mean ± SD of 3 independent experiments. *P <0.05, **P <0.01.*

Keap1 is a Direct Downstream Target of MiR-34b

Activation of the Keap1-Nrf2 pathway allows survival and adaptation under various conditions of stress, including oxidative stress, and has protective effects in many animal models.[21] As shown in Figure 4 A,B, Keap1 expression was decreased in ischemic brain of MCAO rats. MiR-34b further reduced the Keap1 expression. In contrast, Nrf2 and HO-1 expression was increased in ischemic brain of MCAO rats MiR-34b further promoted the Nrf2 and HO-1 expression. These results indicated that Keap1-Nrf2 pathway was protectively activated in MCAO rats. Interestingly, Keap1 has been predicted as a direct downstream target of miR-34b by online tools. Thus, the binding and regulation between miR-34b and Keap1 were verified. Rat neuroblasts B35 cells were transfected with miR-34b mimics or miR-34b inhibitor to achieve miR-34b expression, as confirmed using real-time PCR (Fig, 4C). The protein levels of Keap1, and its downstream Nrf2 and HO-1 in response to miR-34b overexpression were examined. The ectopic miR-34b expression dramatically reduced the protein levels of Keap1 while increased the protein levels of Nrf2 and HO-1 (Fig 4, D, E). Regarding the molecular mechanism, luciferase reporter gene assays were performed to verify the predicted interaction between miR-34b and Keap1. Two different types of reporter vectors were constructed: a wt-Keap1 3′-UTR vector containing wild-type miR-34b binding site and a mut-Keap1 3′-UTR vector containing mutated miR-34b binding site (Fig 4F). These vectors were cotransfected into HEK293 cells with miR-34b mimics or miR-34b inhibitor; the luciferase activity was examined. As shown in Figure 4E, the luciferase activity of wt-Keap1 3′-UTR was significantly suppressed by miR-34b mimics while enhanced by miR-34b inhibitor; after mutating the putative miR-34b binding site, the alterations of the luciferase activity were eliminated

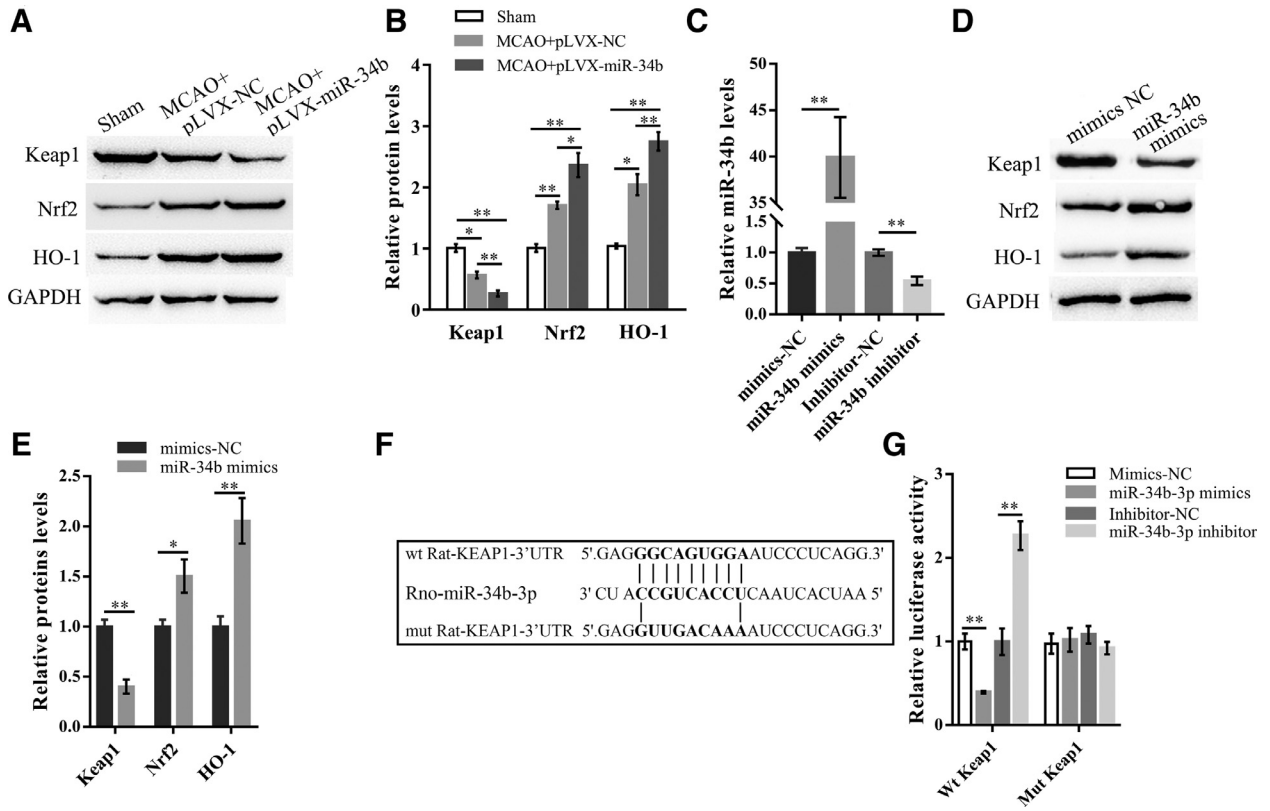

Figure 4. Keap1 is a direct downstream target of miR-34b (A-B) The protein levels of Keap1, Nrf2 and HO-1 in sham, p-LVX-NC or p-LVX-miR-34b injection MCAO groups were examined using Immunoblotting. (C) The expression of miR-34b in B35 cells was achieved by transfection of miR-34b mimics or miR-34b inhibitor, as confirmed using real-time PCR. (D-E) B35 cells were transfected with miR-34b mimics or miR-34b inhibitor; the protein levels of Keap1, Nrf2 and HO-1 were examined using Immunoblotting. (F) The 3′-UTR of the rat Keap1 was predicted to possess miR-34b binding site; two different types of Keap1 3′-UTR luciferase reporter vectors were constructed: wt-Keap1 3′-UTR containing the wild-type miR-34b binding site and mut-Keap1 3′-UTR containing mutated miR-34b binding site. (G) These vectors were co-transfected into HEK293 cells with miR-34b mimics or miR-34b inhibitor; the luciferase activity was examined. The data are presented as mean ± SD of 3 independent experiments. *P <0.05, **P <0.01.

(Fig 4G), indicating that miR-34b may directly bind to the predicted binding site in Keap1 3′-UTR, thus negatively regulating its protein level.

The Function of Keap1 on H_2O_2-Induced Oxidative Stress Injury in B35 Cells

After confirming the binding between miR-34b and Keap1, the molecular functions of Keap1 knockdown or overexpression in H_2O_2-induced oxidative stress injury in B35 cells were evaluated. Keap1 expression was achieved by transfection of Keap1 overexpressing or si-Keap1 vectors, as confirmed using Immunoblotting assays (Fig 5, A, B). Upon 200 μM H_2O_2 stimulation, NO and 3-NT levels were increased while total SOD and MnSOD were reduced, indicating the existence of H_2O_2-induced oxidative stress (Fig 5, C-F). Keap1 overexpression exacerbated H_2O_2-induced oxidative injury by further increasing NO and 3-NT levels and reducing total SOD and MnSOD, while Keap1 knockdown ameliorated oxidative injury by reducing NO and 3-NT levels and increasing total SOD and MnSOD (Fig 5, C-F). The above findings indicate that Keap1 knockdown may ameliorate H_2O_2-induced oxidative injury.

MiR-34b Modulates H_2O_2-Induced Oxidative Injury in B35 Cells Through Keap1

Since miR-34b negatively regulated Keap1 expression through direct binding; next, the dynamic effect of miR-34b and Keap1 on H_2O_2-induced oxidative injury in B35 cells were assessed. H_2O_2-induced NO and 3-NT increasing was reduced while H_2O_2-induced suppression of total SOD and MnSOD was attenuated by miR-34b overexpression; on the contrary to miR-34b overexpression, Keap1 overexpression exerted an opposing effect on the above indexes; more importantly, the effect of miR-34b overexpression could be partially attenuated by Keap1 overexpression (Fig 6, A-D), indicating that miR-34b modulates H_2O_2-induced oxidative injury through Keap1.

Discussion

In the present study, we demonstrate that miR-34b expression is continuously downregulated after focal cerebral I/R in MCAO rats; miR-34b overexpression can ameliorate focal cerebral I/R injury by reducing infarction volume, neurological severity scores, NO and 3-NT levels and increasing total SOD and MnSOD levels. Moreover,

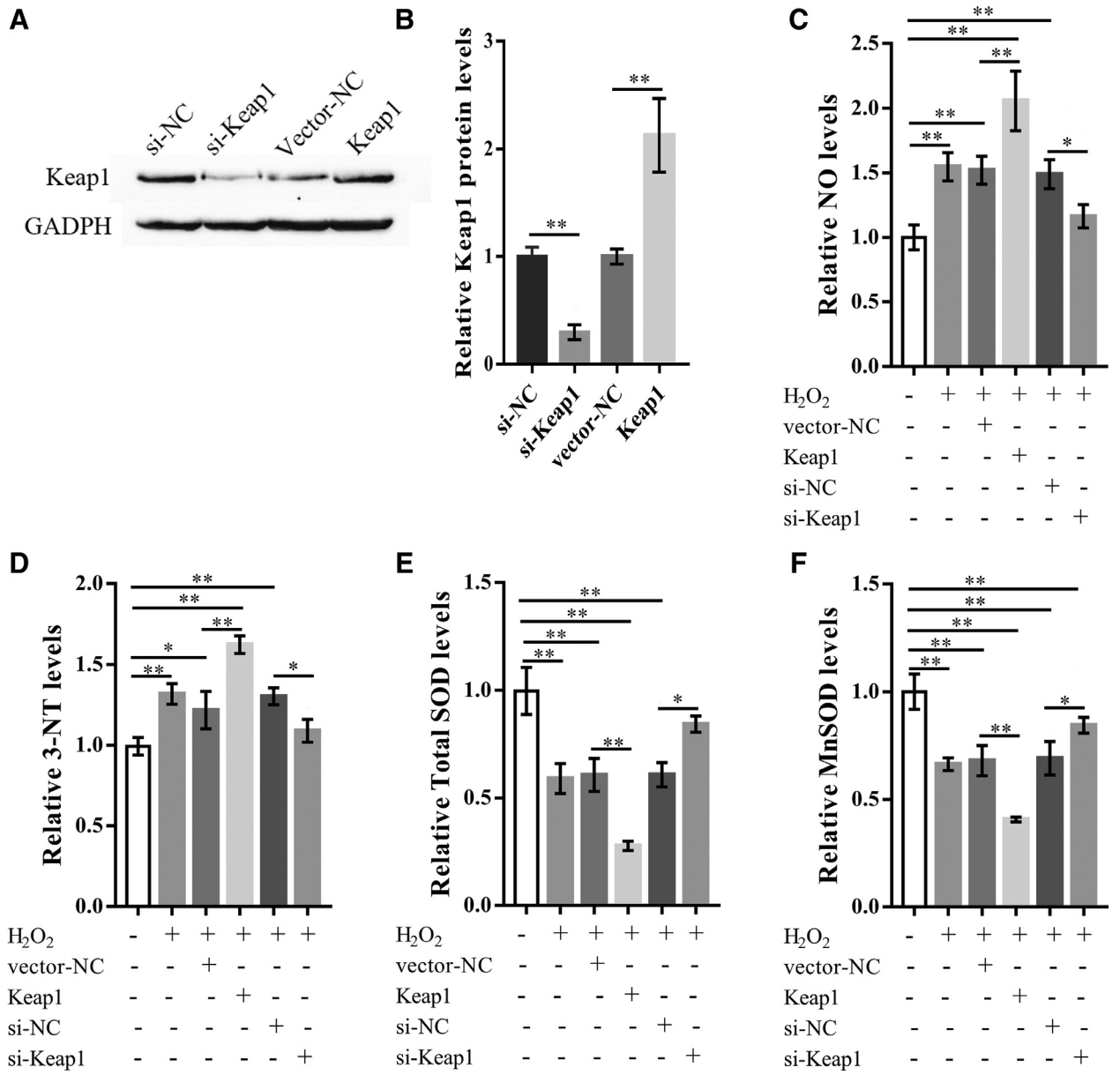

Figure 5. ***The function of Keap1 on H_2O_2-induced oxidative stress injury in B35 cells*** *(A-B) Keap1 overexpression of knockdown was achieved by transfection of Keap1 overexpressing or si-Keap1 vectors, as confirmed using Immunoblotting. (C-F) B35 cells were transfected with Keap1 or si-Keap1 vectors; the levels of NO, 3-NT, total SOD and MnSOD were examined in the presence or absence of 200 μM H_2O_2 stimulation. The data are presented as mean ± SD of 3 independent experiments. *P <0.05, **P <0.01.*

Keap1 is a direct downstream target of miR-34b; Keap1 overexpression exacerbates H_2O_2-induced oxidative injury in B35 cells; by binding to the 3′-UTR of Keap1, miR-34b ameliorates H_2O_2-induced oxidative injury in B35 cells, and its effect can be partially attenuated by Keap1 overexpression.

The role of miR-34b in cell response to oxidative stress has been previously reported. By using in silico analysis, Consales et al[22] revealed that miR-34b/c control reactive oxygen species production and affect mitochondrial oxidative stress triggered by extremely low-frequency magnetic fields (ELF-MFs), likely by modulating mitochondria-related miR-34 targets in neuronal cells. In addition, miR-34b/c downregulation has been regarded as an early event in Parkinson's disease, and that miR-34b/c deregulation tags mitochondrial integrity and oxidative stress pathways, which are major hallmarks of Parkinson's disease.[23] In the present study, miR-34b was predicted to target Keap1, a chemical sensor responsible for Nrf2 activation.[21] Since Keap1/Nrf2 pathway has been regarded as a primary cellular defense against the cytotoxic effects of oxidative stress,[24] miR-34b might modulate oxidative stress injury during the process of focal cerebral I/R in MCAO rats through Keap1.

Indeed, the expression of miR-34b was significantly downregulated 1, 4, and 24 hours after focal cerebral I/R

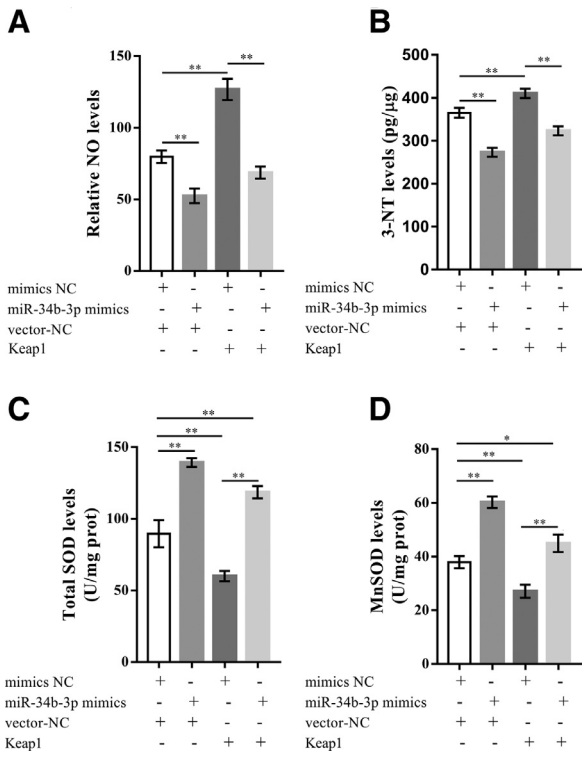

Figure 6. ***miR-34b modulates H_2O_2-induced oxidative injury in B35 cells through Keap1*** *B35 cells were co-transfected with miR-34b mimics and Keap1 vector under 200 μM H_2O_2 stimulation; the levels of NO (A), 3-NT (B), total SOD (C) and MnSOD (D) were examined. The data are presented as mean ± SD of 3 independent experiments. *P<0.05, **P<0.01.*

in a time-dependent manner, further suggesting that miR-34b might be involved in focal cerebral I/R-induced oxidative stress injury. Consistent with its expression after focal cerebral I/R, miR-34b overexpression ameliorated focal cerebral I/R injury by reducing the infarction volume, neurological severity scores, and the levels of NO and 3-NT which were regarded as markers of oxidative stress injury,[25] while increasing the levels of total SOD and MnSOD, the key antioxidative enzyme located in mitochondria which protects the energy-generating mitochondria from oxidative damage.[20]

Regarding the molecular mechanism, it was confirmed that miR-34b directly bound to the 3′-UTR of Keap1 to negatively regulate the protein levels of Keap1, as well as key components of Keap1/Nrf2 pathway, Nrf2 and HO-1.[21] In MCAO rat, miR-34b overexpression further activated the Keap1/Nrf2 pathway to ameliorated focal cerebral I/R injury. In H_2O_2-stimulated B35 cells, Keap1 overexpression enhanced, while Keap1 knockdown ameliorated H_2O_2-induced oxidative stress injury by affecting the levels of NO, 3-NT, total SOD and MnSOD, which was consistent with previous studies.[26-28] Furthermore, H_2O_2-induced oxidative stress injury was ameliorated by miR-34b overexpression while exacerbated by Keap1 overexpression; the effect of miR-34b overexpression was significantly attenuated by Keap1 overexpression, indicating that miR-34b modulated H_2O_2-induced oxidative stress injury in B35 cells through Keap1.

Taken together, we demonstrate that miR-34b ameliorated focal cerebral I/R-induced oxidative stress injury in MCAO rats and H_2O_2-induced oxidative stress injury in B35 cells through targeting Keap1 and downstream Keap1/Nrf2 signaling pathway. We provided a novel mechanism of focal cerebral I/R injury from the perspective of miRNA regulation.

Reference

1. Goldstein LB. Medical therapy for acute ischemic stroke. Curr Atheroscler Rep 2001;3:299-306.
2. Zhang Q, et al. [Intra-arterial thrombolysis in patients with acute ischemic stroke of the cervical internal carotid artery occlusive disease], Zhongguo Yi Xue Ke Xue Yuan Xue Bao. Acta Academiae Medicinae Sinicae 2002;24:646-649.
3. Singhal AB, et al. Effects of normobaric hyperoxia in a rat model of focal cerebral ischemia-reperfusion. J Cereb Blood Flow Metab 2002;22:861-868.
4. Gursoy-Ozdemir Y, Can A, Dalkara T. Reperfusion-induced oxidative/nitrative injury to neurovascular unit after focal cerebral ischemia. Stroke 2004;35:1449-1453.
5. Awooda HA, et al. Oxidative/nitrosative stress in rats subjected to focal cerebral ischemia/reperfusion. Int J Health Sci (Qassim) 2015;9:17-24.
6. Kobayashi M, Yamamoto M. Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. Adv Enzyme Regul 2006;46:113-140.
7. Jaiswal AK. Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic Biol Med 2004;36:1199-1207.
8. Holtzclaw WD, Dinkova-Kostova AT, Talalay P. Protection against electrophile and oxidative stress by induction of phase 2 genes: the quest for the elusive sensor that responds to inducers. Adv Enzyme Regul 2004;44:335-367.
9. Ruike Y, et al. Global correlation analysis for micro-RNA and mRNA expression profiles in human cell lines. J Hum Genet 2008;53:515-523.
10. Zhai F, et al. Expression profiles of microRNAs after focal cerebral ischemia/reperfusion injury in rats. Neural Regen Res 2012;7:917-923.
11. Min XL, et al. MicroRNAs: a novel promising therapeutic target for cerebral ischemia/reperfusion injury? Neural Regen Res 2015;10:1799-1808.
12. Eades G, et al. miR-200a regulates Nrf2 activation by targeting Keap1 mRNA in breast cancer cells. J Biol Chem 2011;286:40725-40733.
13. Murray-Stewart T, et al. Histone deacetylase inhibition overcomes drug resistance through a miRNA-dependent mechanism. Mol Cancer Ther 2013;12:2088-2099.
14. van Jaarsveld MT, et al. MiR-141 regulates KEAP1 and modulates cisplatin sensitivity in ovarian cancer cells. Oncogene 2013;32:4284-4293.
15. Kabaria S, et al. MicroRNA-7 activates Nrf2 pathway by targeting Keap1 expression. Free Radic Biol Med 2015;89:548-556.
16. Wei J, et al. Aldose reductase regulates miR-200a-3p/141-3p to coordinate Keap1-Nrf2, Tgfbeta1/2, and Zeb1/2 signaling in renal mesangial cells and the renal cortex of diabetic mice. Free Radic Biol Med 2014;67:91-102.

17. Zhou L, et al. High glucose induces renal tubular epithelial injury via Sirt1/NF-kappaB/microR-29/Keap1 signal pathway. J Transl Med 2015;13:352.
18. Shi L, et al. MiR-141 activates Nrf2-dependent antioxidant pathway via down-regulating the expression of keap1 conferring the resistance of hepatocellular carcinoma cells to 5-fluorouracil. Cell Physiol Biochem 2015;35:2333-2348.
19. Longa EZ, et al. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989;20:84-91.
20. Wani WY, et al. Protective efficacy of mitochondrial targeted antioxidant MitoQ against dichlorvos induced oxidative stress and cell death in rat brain. Neuropharmacology 2011;61:1193-1201.
21. Baird L, Dinkova-Kostova AT. The cytoprotective role of the Keap1-Nrf2 pathway. Arch Toxicol 2011;85:241-272.
22. Consales C, et al. Fifty-Hertz magnetic field affects the epigenetic modulation of the miR-34b/c in neuronal cells. Mol Neurobiol 2018;55:5698-5714.
23. Minones-Moyano E, et al. MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. Hum Mol Genet 2011;20:3067-3078.
24. Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem 2009;284:13291-13295.
25. Visavadiya NP, et al. Cellular and subcellular oxidative stress parameters following severe spinal cord injury. Redox Biol 2016;8:59-67.
26. Mann GE, et al. Targeting the redox sensitive Nrf2-Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones. Curr Opin Pharmacol 2009;9:139-145.
27. Hu LY, et al. Expression of Nrf2-Keap1-ARE signal pathway in traumatic lung injury and functional study. Eur Rev Med Pharmacol Sci 2018;22:1402-1408.
28. Vriend J, Reiter RJ. The Keap1-Nrf2-antioxidant response element pathway: a review of its regulation by melatonin and the proteasome. Mol Cell Endocrinol 2015;401:213-220.