MicroRNA-429/Cxcl1 Axis Protective Against Oxygen Glucose Deprivation/Reoxygenation-Induced Injury in Brain Microvascular Endothelial Cells

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Abstract

Objective: The objective of the present work was to study the role of Cxcl1 in cerebral ischemia–reperfusion (I/R) injury and to in-depth explore its pathogenesis.

Methods: The expression of Cxcl1 based on the public data was analyzed. Then, we constructed an oxygen glucose deprivation/reoxygenation (OGD/R) model in vitro using mice brain microvascular endothelial cells (BMECs) to simulate cerebral I/R in vivo.

Results: The results of quantitative real-time polymerase chain reaction assay uncovered that Cxcl1 showed higher expression while miR-429 showed lower expression in BMECs damaged by OGD/R, whereas overexpression of Cxcl1 or inhibition of miR-429 expression can strengthen this effect. Hereafter, through dual luciferase reporter assay, we verified that miR-429 directly targets Cxcl1 and negatively regulates Cxcl1 expression. Furthermore, the results also revealed that overexpression of Cxcl1 can reverse the miR-429-mediated effects.

Conclusion: We concluded that miR-429 exerts protective effects against OGD/R-induce injury in vitro through modulation of Cxcl1 and nuclear factor kinase B pathway, hoping provide a new view on the pathogenesis of cerebral I/R injury and a feasible potential therapeutic target.

Keywords
cerebral I/R injury, Cxcl1, miR-429, OGD/R, NF-κB pathway

Introduction

The most sensitive apparatus of ischemia and hypoxia is brain tissue, and cerebral ischemia leads to impaired function of local brain tissue.1 Restoring the blood supply of brain tissue after cerebral ischemia cannot reinstate the physiologic function of brain tissue but aggravate the encephalon injury, which is called cerebral ischemia–reperfusion (I/R) injury.2,3 Cerebral I/R injury is interrelated with the high disability rate and high death rate of cerebrovascular disease.4 It is an extremely complex pathophysiological process, such as endoplasmic reticulum stress, oxidative stress, inflammation, and autophagy, but its specific pathogenesis is not clear and there is still short of efficacious therapy strategies.5 Cerebral microvascular endothelial cells (CMECs) are special structures of brain microvessels, and one of their important functions is to form blood–brain barrier (BBB).6 Further research found that the
initial cause of BBB disruption is CMEC death result from cerebral I/R injury.\(^7,8\) Hence, protecting CMEC from cerebral I/R injury is probably an effective treatment to improve the prognosis of cerebral I/R injury.

Chemokines have the function of promoting nerve recovery.\(^9\) As a member of the CXC subfamily of chemokines, up to now, numerous reports have been published on the application of Cxcl1 in brain diseases. Cxcl1 can be used as a biomarker for diagnosis of Alzheimer disease in the early stage\(^10\) and played a role in promoting the proliferation of neural stem cells in APP/PS1 mice.\(^11\) A study in 2005 by Losy et al discovered that Cxcl1 was involved in the inflammatory response in the early stages of ischemic stroke.\(^12\) Recently, Cxcl1 has been found to be a central gene after 2, 8, and 24 hours of cerebral I/R injury.\(^13\) Additionally, the nuclear factor kinase B (NF-kB) pathway was participated in the regulation of I/R injury\(^14\)-\(^16\) and activation of this pathway can improve the level of Cxcl1 expression.\(^17\) Nevertheless, little work has been done on the application of Cxcl1 as a regulatory gene in cerebral I/R injury.

To date, more than 60% of the genes are considered to be regulated by microRNAs (miRNAs).\(^18\) Bioinformatics predictions revealed that miR-429 is a regulatory miRNA on Cxcl1. MiR-429, as an important member of the miR-200 family, has been reported to be induced by hypoxia-inducible factor 1α, which produced in a hypoxic environment, forming a negative feedback cycle in endothelial cells under hypoxic conditions.\(^19\) Xiao et al have showed that miR-429 can reduce oxygen glucose deprivation/reoxygenation (OGD/R)-induced neuronal damage via upregulating GATA4.\(^5\) However, an miRNA can regulate multiple target genes, and the specific mechanism is still unclear.

In this article, an OGD/R model was established in vitro using mice brain microvascular endothelial cells (BMECs) to simulate cerebral I/R injury. We revealed that miR-429/Cxcl1 axis acts as a potential regulator to against OGD/R-induced injury through NF-kB pathway, which provided a novel and promising targets for cerebral I/R injury.

**Materials and Methods**

**Data Collection**

The samples of I/R injury in wild-type mice were downloaded from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov GEO; access number: GSE23160). Four different cases of I/R-2 hours (I/R-2h), I/R-8 hours (I/R-8h), I/R-24 hours (I/R-24h), and Sham were selected for different gene analysis. The miRNA gene prediction sites miRTarBase (http://miRTarbase.mbc.edu.tw/php/index.php) and TargetScan (http://www.targetscan.org) were used to predict the upstream regulatory miRNA of Cxcl1.

**Cell Culture and OGD/R Model Construction**

The mice BMECs were cultured in deoxygenated glucose-free Hanks' Balanced Salt Solution. To simulate I/R, we created the OGD/R cell model. The BMECs were transferred to an anaerobic chamber (5% CO\(_2\), 95% N\(_2\)) and incubated in sugar-free Dulbecco modified Eagle medium at 37°C for 4 hours, followed by replacing with normal glucose level medium and incubated the BMECs for 24 hours at room temperature for reoxygenation (95% air, 5% CO\(_2\)). Simultaneously, the control cells were cultured under normal conditions.

**Cell Transfection**

The miR-429 mimic/inhibitor and corresponding negative control (NC) were obtained from Shanghai GenePharma Co, Ltd. (Shanghai, China), and the si-Cxcl1, si-con, and pcDNA3.1-Cxcl1 were designed and synthesized by Shanghai GenePharma Co, Ltd (Shanghai, China). The cells were seeded in a 6-well plate 1 day before transfection. The transfections were carried out with Lipofectamine 2000 (Invitrogen, USA) when the cell fusion achieved 80%, based on the manufacturer’s instructions. After that, the cells were cultured at 37°C with 5% CO\(_2\) for 6 hours, followed by replacing the medium into a complete medium. After 48 hours of transfection, follow-up experiments were performed.

**Quantitative Real-Time Polymerase Chain Reaction Assay**

TRIZol reagent (Invitrogen, Carlsbad, California) was used to extract RNA, and complementary DNA was synthesized by reverse transcriptase using miScript RT Kit (Qiagen, Düsseldorf, Germany) for miRNA or Prime Script RT Kit (RR036A; Takara Biotechnology Co., Ltd, Dalian, China) for messenger RNA (mRNA). Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) assay was performed to test the expression level of miR-429 and Cxcl1 using RT qPCR kit (Kapa Biosystems, Wilmington, Massachusetts), according to the manufacturer’s instructions. The 2\(^{- \Delta \Delta Ct} \) method was used to calculate the expression of miRNA and mRNA, while U6 and GAPDH were used as internal controls. The primer sequences were:

- **miR-429:** F: 5’-ATACTGTCTGGTAATGCCCG-3’
- **R:** 5’-GAACATGTCGGCTATCTC-3’
- **U6:** F: 5’-CTCGCTTCGGCAGCATTACTC -3’
- **R:** 5’-ACGCTTCAGAATTTTGGTCTC-3’
- **CXCL1:** F: 5’-TCCAGGCTTTGAAGTGTTGCC-3’
- **R:** 5’-AACCAAGGGAGCTCAGAGTCAGC-3’
- **GAPDH:** F: 5’-TGTGCCATGGTGATCTGA-3’
- **R:** 5’-CCTGCTTCACCACCTTCTTGAG-3’.

**Western Blot**

Cells were lysed with lysis buffer containing protein inhibitors to extract the total protein, then BCA kit was used to test the protein concentration. Hereafter, 20 μg of protein samples were detached by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The polyvinylidene fluoride membrane was transferred with the proteins and then sealed with 5% skimmed milk powder for 1 hour. After that, the blotted membrane was
incubated with the primary antibody at 4°C overnight, followed by washing it 3 × 5 minutes with TBST. Then the membrane was incubated with the second antibody for 1 hour at room temperature. An ECL detection kit (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) was used to develop the protein signaling after washing the membrane. The protein level was evaluated by using GAPDH as an internal control.

**Cell Counting Kit-8 Assay**

The transfected cells were prepared into a cell suspension and then seeded the cell suspension in a 96-well plate with the density of 1 × 10^3/well and then performed OGD/R treatment. Subsequently, cells were conventional cultured in an incubator with CO₂ for 24 hours. Using a microplate reader to detect optical density values with 450 nm excitation light. Before detection, 10 μL of CCK8 reagent (Dojindo, Kumamoto, Japan) was added into each well, and incubated the plate for 1.5 hours in a 37°C incubator.

**Apoptosis Assay**

The cells were stained and quantified with Annexin V-FITC/Propidium iodide (PI) kit (4 A Biotech, Beijing, China) according to the manufacturer’s instructions. The transfected cells were incubated in 200 μL binding buffer containing 10 μL of Annexin V-FITC and 5 μL of PI in the dark at room temperature for 1 hour. Then apoptotic analysis was performed by flow cytometry. Finally, Flowjo software was used to analyze the flow results.

**Dual-Luciferase Reporter Assay**

In order to further confirm whether miR-429 is an upstream regulatory miRNA of Cxcl1, dual-luciferase reporter assay was carried out. First, wild and mutant Cxcl1-3'-untranslated region (UTR) containing the binding site of miR-429 were cloned into pmirGLO luciferase vector to construct Cxcl1-wild-type (WT) and Cxcl1-mutant (MUT) vectors. Then HEK 239T cells were cotransfected with miR-429 mimic or miR-429 mimic NC and Cxcl1-WT or Cxcl1-MUT using lipofectamine 2000, according to the standard protocol. After 48 hours of transfection, the luciferase activity was detected using the Dual-Luciferase TM system (Promega) based on the standard protocol.

**Statistical Analysis**

In this article, the data were analyzed by statistical analysis software SPSS22.0 (SPSS, Inc, Chicago, Illinios). Student t test (2 groups) or 1-way analysis of variance analysis with posttest of Dunnett (3 groups and above) was used for comparison between groups. Statistical results were considered to be significantly different when P < .05.

**Results**

**Cxcl1 and MiR-429 Were Differential Expressed in Cerebral I/R Injury**

First, the data including I/R-2h (n = 4), I/R-8h (n = 4), I/R-24h (n = 4), and Sham (n = 4) samples were analyzed by GEO Online Analysis Tool GEO2R. It can be seen in Figure 1A that the Cxcl1 expression in I/R-2h group, I/R-8h group, and I/R-24h group was markedly increased compared with the sham group, P < .01, which was constantly enhanced with the prolongation of I/R time. B, Cxcl1 was highly expressed in OGD/R treated BMECs. **P < .01. C, MiR-429 expression was low in OGD/R-treated BMECs. **P < .01. BMECs indicates microvascular endothelial cells; I/R, ischemia–reperfusion; OGD/R, oxygen glucose deprivation/reoxygenation.
The Transfection Efficiency of pcDNA3.1-Cxcl1/si-Cxcl1 and MiR-429 Mimic/Inhibitor in BMECs Suffered From OGD/R

To verify the function of Cxcl1 and miR-429 in BMECs, we divided the cells into Cxcl1 overexpression groups, Cxcl1 knockdown group, and miR-429 mimic/inhibitor group. At the same time, the control group in which cells treated with transfection reagent was set up. As can be seen from Figure 2A, the expression of Cxcl1 in cells transfected with pcDNA3.1-Cxcl1 was markedly increased than that in cells transfected with vector and control ($P < .01$). In the meantime, the results in Figure 2B indicated that the expression of Cxcl1 in cells transfected with si-Cxcl1 was significantly decreased than that in cells transfected with si-con and control ($P < .01$). Then, we analyzed the data in the miR-429 mimic/inhibitor group. As described in Figure 2C, the miR-429 expression in cells transfected with miR-429 mimic was obviously higher than that in control group ($P < .01$), and the miR-429 expression in cells transfected with miR-429 inhibitor was clearly lower than that in the control group ($P < .01$). These results indicated that miR-429 and Cxcl1 were significantly decreased or increased in BMECs after transfection, laying the foundation for subsequent experiments.

Knockdown of Cxcl1 Enhances the Viability While Inhibition of MiR-429 Expression Reduced the Viability of OGD/R-Damaged BMECs

So as to examine the influence of Cxcl1 and miR-429 on the viability of BMECs, CCK-8 assay was carried out. We can see from Figure 3A and B that the cell viability was markedly declined in OGD/R-treated BMECs than in sham group ($P < .01$), and this influence was markedly strengthened by
overexpression of Cxcl1 and reversed by knocking down of Cxcl1 \( (P < .01) \). Furthermore, we found that transfection of miR-429-mimic can promote the viability of OGD/R-treated BMECs than that of OGD/R group, whereas inhibition of miR-429 showed an opposite effects (Figure 3A, \( P < .01 \)). These findings demonstrated that Cxcl1 and miR-429 can regulate the cell viability of OGD/R-treated BMECs.

**MiR-429 is an Upstream MiRNA of Cxcl1 and Directly Targets Cxcl1**

First, through bioinformatics analysis, we predicted that miR-429 was the upstream target gene of Cxcl1, and Cxcl1 has the binding sites for miR-429 in the 3' UTR region (Figure 4A). To further confirm whether miR-429 directly targets Cxcl1, dual luciferase reporter assay was carried out. WT-Cxcl1 or MUT-Cxcl1 and miR-429 mimic or miR-429 mimic NC were cotransfected into HEK 239T cells to determine the luciferase activity. As shown in Figure 4B, luciferase activity in vectors containing WT-Cxcl1 showed a significantly downswing when transfected with miR-429 mimic \( (P < .01) \), and this influence was hindered in mutations. This result illustrated that miR-429 directly targets Cxcl1.

To further examine the regulation of miR-429 on Cxcl1, we performed qRT-PCR and Western blot experiments. Figure 4C-E indicate that the mRNA and protein expression levels of Cxcl1 were markedly escalated in BMECs after OGD/R treatment than that in sham group \( (P < .01) \), while this effect was reversed after upregulation of miR-429 expression \( (P < .01) \). On the contrary, inhibition of miR-429 can markedly increase the mRNA and protein expression levels of Cxcl1 in OGD/R-treated BMECs than that in OGD/R group. These results illustrated that miR-429 can negatively regulate Cxcl1 levels, further confirming that miR-429 was the upstream regulatory miRNA of Cxcl1.

**Effect of MiR-429/Cxcl1 Axis on Cell Biological Function**

Moreover, we set up a rescue experiment to confirm whether miR-429 affects the biological behavior of OGD/R-treated BMECs by regulating Cxcl1. First, miR-429 mimic and miR-429 mimic plus pcDNA3.1-CXCL1 were transected into OGD/R-treated BMECs, respectively, and the control group was treated with transfect reagent. After that, CCK8 assay was performed to detect cell viability, and flow cytometry was carried out to detect cell apoptosis. The results of flow cytometry assay illustrated that after overexpression of miR-429 in
OGD/R-treated BMECs, the number of apoptotic cells was markedly declined compared with the control group (Figure 5A and B, \( P < .01 \)), whereas the number of apoptosis was significantly increased in OGD/R-treated BMECs cotransfected with miR-429 mimic and pcDNA3.1-Cxcl1 compared with the miR-429 mimic group (\( P < .01 \)). Simultaneously, we can see the result of CCK-8 from Figure 5E that upregulation of miR-429 expression can markedly induce the cell viability in OGD/R-treated BMECs than that in control (\( P < .01 \)), while overexpression of Cxcl1 reversed this effect (\( P < .01 \)). Furthermore, Western blot assay was performed to detect the effect of miR-429/Cxcl1 axis on apoptotic proteins. It can be seen from Figure 5C and D that overexpressed miR-429 can markedly decreased the expressions of proapoptosis protein Bax, while this decrease was hindered when the OGD/R-treated BMECs cotransfected with pcDNA3.1-Cxcl1 and miR-429 mimic (\( P < .01 \)). BCL-2, as an antiapoptosis protein, showed the opposite results. Taken together, these results provided that miR-429 can exert its biological function in OGD/R-treated BMECs by regulating Cxcl1.

NF-\( \kappa \)B Pathway Mediates the Regulation of MiR-429/Cxcl1 on OGD/R-Induced Injury in BMECs

To in-depth study the protective mechanism of miR-429/Cxcl1 axis on OGD-R-induced injury, we carried out Western blot experiment to detect the protein expression of pathway-related factors and proinflammatory factors. From Figure 6A and B, we can see that the protein level of phosphorylation NF-\( \kappa \)B, tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)), and interleukin (IL) 1\( \beta \) in miR-429 mimic group showed a significantly decrease than those in control group in OGD/R-treated BMECs (\( P < .01 \)), while upregulation of Cxcl1 hindered the decrease caused by overexpression of miR-429 (\( P < .01 \)). Contrarily, the protein level of total NF-\( \kappa \)B was almost not altered. Overall, these results illustrated that miR-429/Cxcl1 axis can protect OGD/R-induced injury through NF-\( \kappa \)B pathway, hoping to provide a new scheme to protect cerebral I/R injury in clinical therapeutics.

Discussion

In this article, we conclude that Cxcl1 as a novel gene participated in regulating OGD/R-induced injury in vitro, which was highly expressed in OGD/R-treated BMECs, and knocking down of Cxcl1 enhances the cell viability. Then, we identified that miR-429 directly targets Cxcl1, and miR-429 was lowly expressed in BMECs after OGD/R treatment. Further data indicated that miR-429/Cxcl1 axis can protect BMECs against OGD/R-induced injury, while this protection was achieved by the mediation of the NF-\( \kappa \)B pathway.

So far, there have been studies highlighting the role of Cxcl1 in I/R injury. Zheng et al based on bioinformatics analysis indicated that Cxcl1 might be participated in the reinfusion
period of liver I/R injury.\textsuperscript{20} In addition, alveolar type II epithelial cells participate in lung I/R injury by producing strong chemokines such as CXCL1.\textsuperscript{21} For cerebral I/R injury, Losy et al found that CXCL1 was escalated in the cerebrospinal fluid of patients with stroke at 2, 8, and 24 hours after reperfusion.\textsuperscript{12} In our study, the data obtained from bioinformatics analysis appear to be similar to the reported earlier by Losy et al that the expression level of Cxcl1 in cerebral I/R group was obviously enhanced than that in the sham group, and its level was constantly enhanced with the prolongation of I/R time. Further experimental results indicated that Cxcl1 was highly expressed in OGD/R-treated BMECs, and knocking down of Cxcl1 enhances the viability of BMECs damaged by OGD/R. These results suggested that Cxcl1 may contribute to protecting cells against the OGD/R-induced injury.

Previous research has shown that most of mRNAs function in diseases through the regulation of miRNAs.\textsuperscript{18} In the present study, we predicted upstream miRNAs that might regulate Cxcl1, and the results indicated that miR-429 was an upstream molecule of Cxcl1. Moreover, this prediction was further confirmed by the dual luciferase assay. MiR-429 has been reported to function in tumor suppressor genes in multiple cancers, such as nasopharyngeal carcinoma,\textsuperscript{22} pancreatic cancer,\textsuperscript{23} thyroid cancer,\textsuperscript{24} nephroblastoma,\textsuperscript{25} and so on. Recently, there have also been many reports showed that miR-429 was contributed to the development of various brain diseases. MALAT-1 can accelerate apoptosis of hippocampus neurons through negative regulation of miR-429.\textsuperscript{26} In addition, upregulation of miR-429 expression can significantly increase the permeability of blood–tumor barrier, so that chemotherapeutic drugs can better act on brain tumors.\textsuperscript{27} Lee et al found that miR-429 plays a neuron-protective role by targeting prolife hydroxyls 2.\textsuperscript{28} Our data indicated that miR-429 was downregulated in OGD/R-treated BMECs and overexpression of miR-429 can increase the cell viability while downregulation of miR-429 can decrease the cell viability. Furthermore, the rescue experiment data revealed that overexpression of Cxcl1 can reverse these effects. These findings suggested that miR-429/Cxcl1 axis can against the injury caused by OGD/R in BMECs.

Nuclear factor kinase B can participate in many biological functions of cells,\textsuperscript{14} and it is activated in a variety of cerebrovascular diseases.\textsuperscript{29} Nuclear factor kinase B has been proved to be a key factor in the regulation of inflammation,\textsuperscript{30} which activation can promote the production of extensive inflammatory factors, such as IL-1 and IL-6, thus aggravating the cerebral I/R injury.\textsuperscript{29} Similarly, a recent study has indicated that baicalein plays a neuron-protective role in mice with cerebral I/R injury by inhibiting NF-κB signal transduction to reduce neuron-inflammation and apoptosis.\textsuperscript{31} Kim et al have demonstrated that Dexmedetomidine protects neurons from cerebral I/R injury through the anti-inflammatory effect of inactivation of toll-like receptor 4/NF-κB pathway.\textsuperscript{32} This study demonstrated that miR-429 can reduce the protein expression of pathway-related factor phosphorylation NF-κB and proinflammatory factors TNF-α and IL-1β, whereas upregulation of Cxcl1 expression can block this effect. These results indicated that the protective effect of the miR-429/Cxcl1 axis on OGD/R-induced injury was achieved through NF-κB pathways.

**Conclusion**

To sum up, on the basis of bioinformatics and experimental data analysis, our results provide substantial evidence for the initial hypothesis that miR-429/ Cxcl1 axis in part acts as a potential regulator to against OGD/R-induced injury through NF-κB pathway in BMECs. It supplied a new opinion for the mechanism of cerebral I/R injury and its clinical treatment. However, the findings of this study are restricted to OGD/R models in vitro, the effects of miR-429/Cxcl1 axis on cerebral I/R injury in vivo need further study.
Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

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