miR-874 ameliorates retinopathy in diabetic rats by NF-κB signaling pathway

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Abstract

Background. Increased activity of the NF-κB signaling pathway boosts the progression of retinopathy in diabetic rats.

Objectives. Using a bioinformatics website, we identified a site where miR-874 binds to the NF-κB p65. Therefore, we speculated that miR-874 might improve retinopathy in diabetic rats by inhibiting the NF-κB signaling pathway.

Materials and methods. Ten healthy rats were taken as the control group. Sixty streptozotocin (STZ; 60 mg/kg)-induced diabetes model rats were randomly divided into the model group (injection of normal saline), negative control (NC) agomir group (injection of NC mimic), miR-874 agomir group (injection of miR-874 mimic), miR-874 anti-agomir group (injection of miR-874 inhibitor), EVP4593 group (injection of NF-κB signaling pathway antagonist EVP4593), and miR-874 anti-agomir+EVP4593 group (injection of miR-874 inhibitor and EVP4593). All injections were administered into the caudal vein.

Results. miR-874 could target the degradation of p65. Compared with the control group, model rats had reduced miR-874 expression, increased vascular endothelial growth factor (VEGF) and Ang2 protein expression, lowered end-diastolic velocity (EDV) and peak systolic velocity (PSV) of the central retinal artery (CRA) and blood velocity of central retinal vein (CRV) and CRA, heightened plasma viscosity (PV), blood viscosity (BV) and erythrocyte sedimentation rate (ESR) at all shear rates, decreased capillary pericytes (IPCs), increased vascular endothelial cells (VECs), and ascended p65 expression in the retina (all p < 0.05). Thus, it was shown that pathological changes appeared in the retina of diabetic rats. These indices improved in diabetic rats injected with the miR-874 mimic or EVP4593, but deteriorated in those injected with miR-874 inhibitor (all p < 0.05). EVP4593 also could alleviate the aggravation of retinopathy that was caused by miR-874 inhibition in diabetic rats.

Conclusions. miR-874 modulates the NF-κB signaling pathway by targeting the degradation of p65 to further improve the retina of diabetic rats, thus demonstrating the beneficial effect of miR-874 on diabetic retinopathy in rats.

Key words: diabetic retinopathy, NF-κB, Rela, miR-874
Background

Diabetes is a prevalent disease that occurs worldwide. The incidence of diabetes is high in China and there are over 100 million patients diagnosed with this condition. Neuropathy, microangiopathy and macroangiopathy are some of the primary complications of diabetes. However, diabetic retinopathy (DR) is the most common complication and can cause visual disturbances and blindness. Indeed, DR is the leading cause of blindness in the working-age population. It is primarily triggered by the leakage of retinal capillary walls induced by hyperglycemia. However, the pathogenesis of this condition is complicated and not fully understood. Previous studies have suggested that a series of pathophysiological changes in the retina that occur in the presence of persistent hyperglycemia contribute to the etiology of DR.

NF-κB is a nuclear transcription factor that participates in the generation process of multiple cytokines. This protein complex plays a leading role in various biological processes, including immune function and inflammation. The NF-κB protein complex comprises 5 members including RelB, RelA (also known as p65), c-Rel, p100/ p52, and p105/p50. p65-50 is the most widespread and important NF-κB heterodimer. The retina is affected by many metabolic disorders that can induce changes in local gene expression, and the progression of diabetes is associated with retinal capillary cell death and histopathological changes. Diabetic rats show abnormal activation of the NF-κB signaling pathway. This process can augment the generation of reactive oxygen species (ROS), leading to the occurrence of microaneurysms, retinal neovascularization and vitreous hemorrhage, consequently boosting the progression of retinopathy.

Encoded by an endogenous gene, microRNA (miRNA) is a non-coding single-stranded RNA molecule that shows a high degree of conservation, time sequence and tissue specificity. The miRNAs can regulate the protein expression of a specific target gene by interacting with the 3’UTR region of its mRNA through a sequence-specific interaction. Recent studies have revealed that miRNAs participate in the development and progression of DR, and are involved in the multiple pathogenic mechanisms of this disorder. Using a bioinformatics website (www.targetscan.org), we identified a site where miR-874 can bind to NF-κB p65. It has previously been reported that the downregulation of miR-874 expression in rats with myocardial ischemia/reperfusion injury exerts an inhibitory effect on inflammation and injury. However, no study has examined the relationship between miR-874 and DR, and it is unknown whether this miRNA regulates DR, or if NF-κB p65 acts as a downstream regulatory element for miR-874.

Therefore, in the current study, DR rat models were treated with a genetic intervention to explore the effects of miR-874 on this condition, and to examine the regulatory relationship between miR-874 and the NF-κB signaling pathway. The results deepen our understanding of the pathogenesis of DR and provide a theoretical foundation for establishing miR-874 as a potential drug target for the treatment of this disorder.

Materials and methods

Cell culture

HEK293T cells from the American Type Culture Collection (ATCC, Manassas, USA) were used in the dual-luciferase reporter assay (Promega, Madison, USA). Using routine methods, HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were passaged and cultured in fresh complete medium every 3 days.

Dual-luciferase reporter system assay

A bioinformatics website (www.targetscan.org) was used to identify a potential binding site between miR-874 and Rela (p65), which was then verified using a dual-luciferase reporter system assay. Reporter plasmids containing the target gene Rela (pmirGLO-Rela wild-type (wt)) or a mutated target gene (pmirGLO-Rela mut) were constructed. These 2 reporter plasmids were co-transfected with a negative control (NC) mimic or a miR-874 mimic into HEK293T cells. Twenty-four hours after transfection, the dual-luciferase reporter assay was performed according to the manufacturer’s instructions. Relative luciferase activity was calculated as firefly luciferase activity/renilla luciferase activity.

Establishment of the diabetic rat model

Streptozotocin (STZ) administration was used to induce diabetes in rats. Ninety male Sprague Dawley rats (200–250 g, 8 weeks old, from the Laboratory Animal Center of Chongqing Medical University, Chongqing, China) were fed with standard chew and water, and housed under specific pathogen-free conditions. Ten rats were randomly selected as the control group, and the rest were used to construct the diabetic models. Twenty-four hours after transfection, the dual-luciferase reporter assay was performed according to the manufacturer’s instructions. Relative luciferase activity was calculated as firefly luciferase activity/renilla luciferase activity.

A single intraperitoneal injection of 60 mg/kg of STZ was administered to induce diabetes. One week later, rats with a fasting blood glucose above 250 mg/dL were considered to be diabetic. In total, 71 rats met this criteria. All procedures employed were approved by the Ethics Committee of Xiaogan Hospital Affiliated to Wuhan University of Science and Technology (China) and are in compliance with the Association for Research in Vision and Ophthalmology’s statement for the care and use of laboratory animals in ophthalmology and vision studies.
Nine weeks after grouping, rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg). The retinal hemodynamic and central artery hemorheology indices of the left eyeball of all rats were detected using a color Doppler ultrasound. Following this, blood samples and retinal tissue or the whole eyeballs were collected for the subsequent experiments. All animals were then killed by cervical dislocation and death was confirmed by the absence of respiration.

Treatment

Sixty diabetic rats were randomly selected and divided into 6 groups of 10 rats each. The remaining animals were euthanatized as outlined above. In total, there were 7 groups in this study: control group (healthy rats), model group (diabetic rats injected with normal saline through the caudal vein), negative control (NC) agomir group (diabetic rats injected with the NC mimic through the caudal vein), miR-874 agomir group (diabetic rats injected with the miR-874 mimic through the caudal vein), miR-874 anti-agomir group (diabetic rats injected with a miR-874 inhibitor through the caudal vein), EVP4593 group (diabetic rats injected with EVP4593 through the caudal vein), and miR-874 anti-agomir+EVP4593 group (diabetic rats injected with a miR-874 inhibitor and EVP4593). The details of the groupings are shown in Table 1. EVP4593 is a NF-κB signaling pathway antagonist. The above agents, at a concentration of 4.5 nM, were injected into rats at a dose of 80 mg/kg through the caudal vein, once every 3 days for 4 weeks. Eight weeks later, the rats were fasted for 8 h and blood was then drawn through caudal vein to measure blood glucose with the One Touch II glucometer (LifeScan, Malvern, USA). Rats were also weighed at this point. The experimental design is shown in Fig. 1.

Measurement of retinal hemodynamic and central artery hemorheology indices

Nine weeks after grouping, the left eye of each rat was examined using a color Doppler ultrasound, and hemodynamic indices such as end-diastolic velocity (EDV), peak systolic velocity (PSV) and central retinal vein (CRV) were measured. After the rats were fasted for 20 h, the animals were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg) and anticoagulated blood was drawn from the abdominal aorta. Plasma viscosity (PV), blood viscosity (BV) and erythrocyte sedimentation rate (ESR) at different shear rates were measured with a blood viscometer. Each measurement was performed in triplicate.

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**Table 1. Grouping methods of rats**

<table>
<thead>
<tr>
<th>Group</th>
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<th>Treatment</th>
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<tr>
<td>Control</td>
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<td>normal saline</td>
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<tr>
<td>Model</td>
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<td>streptozotocin</td>
</tr>
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<td>Negative control agomir</td>
<td>diabetic</td>
<td>streptozotocin + negative control mimic</td>
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<td>diabetic</td>
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<td>miR-874 anti-agomir</td>
<td>diabetic</td>
<td>streptozotocin + miR-874 inhibitor</td>
</tr>
<tr>
<td>EVP4593</td>
<td>diabetic</td>
<td>streptozotocin + EVP4593 (NF-κB signaling pathway antagonist)</td>
</tr>
<tr>
<td>miR-874 anti-agomir + EVP4593</td>
<td>diabetic</td>
<td>streptozotocin + miR-874 inhibitor + EVP4593</td>
</tr>
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**Fig. 1.** Experimental design flow diagram

qRT-PCR – real-time fluorescence quantitative polymerase chain reaction.
Measurement of the number of retinal vascular endothelial cells and pericytes

The eyeballs of all rats were fixed and retinal vascular digest preparations were performed. The numbers of retinal vascular endothelial cells (VECs) and capillary pericytes (IPCs) were counted using a microscope. All measurements were performed in triplicate.

Separation of retinal tissue

The eyeballs were extirpated under aseptic conditions and the bulbar conjunctiva was removed. The cornea was separated at 1 mm from the posterior of corneoscleral limbus, followed by evisceration of the crystalline lens and removal of the vitreous body under a stereomicroscope. The retina was isolated along with the under part of the retina, and the optic nerve was cut off. The retina was dissociated and cut into pieces.

Quantitative real-time fluorescence polymerase chain reaction

Total RNA in the retinal tissue was extracted using the Trizol method (Invitrogen, Calsbad, USA). After purity determination, the RNA was reverse transcribed into cDNA according to the manufacturer's instructions (TaqMan MicroRNA Assays Reverse Transcription primer, 4427975; Applied Biosystems, Waltham, USA), with reaction conditions of 37°C for 30 min and 85°C for 5 s. Primers were synthesized by the Wuhan Branch of Sangon Biotech Co. Ltd. (Shanghai, China) and the sequences are listed in Table 2. The reaction conditions for quantitative real-time polymerase chain reaction (qRT-PCR) were pre-denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extending at 72°C for 34 s. The reagents used for the qRT-PCR were 10 µL of SYBR Premix Ex Taq™ II, 0.8 µL of PCR forward primer (10 µM), 0.8 µL of PCR reverse primer (10 µM), 0.4 µL of ROX Reference Dye II, 2.0 µL of cDNA templates, and 6.0 µL of sterilized distilled water. GAPDH was the internal reference for CD40, RORyt, Foxp3, interleukin (IL)-17, IL-10, tumor necrosis factor (TNF)-α, IL-23 and IL-8. The reactions were performed with an ABI7500 quantitative PCR amplifier (7500; Applied Biosystems). 2−ΔΔCt showed the relative expression of the target gene. Each measurement was performed in triplicate.

Western blotting

RIPA buffer (Beyotime Biotechnology Co. Ltd., Shanghai, China) was mixed with protease inhibitors and phenylmethylsulfonyl fluoride (PMSE) to lyse cells on ice for 30 min. Protein concentration was measured using a BCA protein assay kit (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China). Protein was separated using SDS-PAGE for 2 h and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was sealed with 5% milk for 2 h and incubated at 4°C with various primary antibodies, including rabbit anti-human angiopoietin2 (Ang2; 1 : 2500, ab155106; Abcam, Cambridge, USA), p65 (1 : 2500, ab32536; Abcam), vascular endothelial growth factor (VEGF; 1 : 2500, ab1316; Abcam) and GAPDH (1 : 2500, ab9485; Abcam). After the membrane was washed with Tris-buffered saline with Tween (TBST) 3 times, horseradish peroxidase (HRP)-labeled IgG (1 : 10,000, ab6721; Abcam) was added and incubated at room temperature for 1 h. The membrane was then washed with TBST 3 times. Color development was carried out by electrogenerated chemiluminescence solutions. The relative expression of each protein was calculated using the gray value of the target protein band divided by the gray value of the GAPDH band. Each measurement was performed in triplicate.

Statistical analysis

Data analysis was performed using SPSS v. 11.5 software (SPSS Inc., Chicago, USA) and the data are expressed as mean ± standard deviation (SD). Comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by post hoc least significant difference (LSD) tests. An α value of p < 0.05 was considered statistically significant.

Results

miR-874 targets regulating the expression of p65

The bioinformatics website predicted that there was a binding site between miR-874 and Rela (p65; Fig. 2A), which was verified using the dual-luciferase reporter system assay. The results showed that there were no significant differences in luciferase activity between the pmirGLO-Rela mut+NC mimic and the pmirGLO-Rela mut+miR-874 mimic. However, the pmirGLO-Rela wt+miR-874 mimic group had a significantly reduced luciferase activity compared to the pmirGLO-Rela mut+miR-874 mimic group (Fig. 2B), indicating a targeted regulation of miR-874 on p65.
**p65 expression in the retina of diabetic rats is affected by miR-874**

miR-874 expression in the retina was measured using qRT-PCR and Rela (p65) protein expression was measured with western blot (Fig. 3). Compared with the control group, retinal miR-874 expression was significantly decreased and p65 expression significantly increased in the model group. The miR-874 agomir group and EVP4593 group had increased miR-874 expression and inhibited p65 expression, while the miR-874 anti-agomir group showed opposite effects. EVP4593 reversed the promoting effect of miR-874 anti-agomir on p65 expression. These results suggest that miR-874 inhibits the NF-κB signaling pathway by suppressing p65 in the retina of diabetic rats.

**Retinal injury associated with STZ-induced diabetes in rats and the ameliorative effects of miR-874**

After STZ treatment, blood glucose levels were significantly higher in the model group than in the control group (4 mmol/L). miR-874 agomir and EVP4593 treatment slowed the soaring increase of blood glucose in model rats, but miR-874 anti-agomir exacerbated this effect. EVP4593 neutralized the effect of miR-874 anti-agomir on blood glucose levels in diabetic rats (Fig. 4A).

Rats in the control group exhibited typical weight gain. Compared with the control group, rats in the model group weighed significantly less. miR-874 agomir and EVP4593 treatment increased the weight of the rats, and the miR-874
anti-agomir treatment resulted in lower weight. EVP4593 reduced the weight loss caused by the miR-874 anti-agomir in diabetic rats (Fig. 4B).

Abnormal expression of Ang2 and VEGF could cause retinal VEC damage and a pro-inflammatory response. Thus, the protein expression levels of VEGF and Ang2 were measured using western blotting (Fig. 4C,D). The expression of VEGF and Ang2 was significantly increased in the retinas of diabetic rats ($p < 0.05$), indicating that pathological changes occurred in the retina. The increases in VEGF and Ang2 protein expression were inhibited in the miR-874 agomir and EVP4593 groups, and promoted in the miR-874 anti-agomir group. EVP4593 neutralized the promoting effect of miR-874 anti-agomir on VEGF and Ang2 protein expression.

**Retinopathy relief in diabetic rats administered miR-874**

Hemodynamic indices in diabetic rats were measured to detect retinal blood perfusion and blood supply (Fig. 5). Compared with the control group, EDV, PSV and CRV values in the model group were decreased by varying degrees, indicating insufficient retinal blood perfusion and
blood supply in diabetic rats. Diabetic rats with *miR*-874 agomir and EVP4593 treatment had increased EDV, PSV and CRV values, while the *miR*-874 anti-agomir group showed opposite effects. EVP4593 neutralized the inhibitory effect of *miR*-874 anti-agomir on the EDV, PSV and CRV values (all p < 0.05). Hence, it was shown that *miR*-874 had an influence on the hemodynamic indices of rats with DR, and that *miR*-874 could alleviate the retinopathy of diabetic rats.

Hemorheology indices in diabetic rats were also measured to further verify the alleviation of *miR*-874 on DR (Fig. 6). Compared with the control group, BV, PV and ESR values were increased in the model group. Diabetic rats with *miR*-874 agomir and EVP4593 treatment showed decreased BV, PV and ESR values, while the *miR*-874 anti-agomir group exhibited opposite effects. EVP4593 neutralized the promoting effect of *miR*-874 anti-agomir on BV, PV and ESR values at all shear rates (all p < 0.05). Thus, it was demonstrated that *miR*-874 relieved retinopathy in diabetic rats.

The numbers of retinal capillary VECs and IPCs were also measured (Fig. 7). Compared with the control group, the numbers of IPCs were significantly reduced, and numbers of VECs significantly increased, in the retinal capillaries of the diabetic group. Diabetic rats treated with *miR*-874 agomir and EVP4593 had increased numbers of IPCs and decreased VEC proliferation in the retinal capillaries, while the *miR*-874 anti-agomir group showed opposite effects. EVP4593 partly reversed the IPC number decrease and the VEC proliferation increase caused by *miR*-874 anti-agomir (all p < 0.05). Thus, *miR*-874 relieved the retinopathy of diabetic rats.
Discussion

The main treatment methods for DR are laser photocoagulation, hyperbaric oxygen and drug therapy. However, these methods may lead to severe adverse side effects, including decreases in visual acuity and contrast sensitivity, and visual field damage. Therefore, safer therapeutic strategies need to be developed for DR patients.

Previous work has shown that NF-κB is activated in the retina of diabetics, and that the activated NF-κB increases capillary cell apoptosis. These changes are observed prior to development of the histopathological features characteristic of DR. Once NF-κB is activated early in the pathogenesis of DR, its effects could only be inhibited partially by early reconstruction. Moreover, if the hyperglycemia induced by diabetes was not well controlled for 7 months, it could be irreversible. In this study, there were heightened EDV, PSV of central retinal artery (CRA) and blood velocity of CRV, lowered PV, BV and ESR at all shear rates, an increased number of retinal capillary pericytes, decreased endothelial cell proliferation, and a significantly reduced p65 expression in the retina in the EVP4593 group as compared to the model group. These results indicate that inhibition of the NF-κB signaling pathway significantly improves the condition of DR in rats, which is consistent with the literature that we reviewed.
Recently, miRNA has been identified as a biomarker for the diagnosis of various diseases, including DR. Moreover, there is growing evidence that miRNAs play a key role in regulating NF-κB activation and its downstream functions. However, the effects of miRNA on NF-κB activation or inhibition in DR have not been fully revealed. miR-874 plays a role in various diseases, including several types of cancers, such as colorectal, gastric and non-small cell lung cancers.29 miR-874 is significantly downregulated in the sertoli cells of diabetic rats and miR-874 overexpression relieves renal injury in these animals.30 However, there are no studies on the relationship between miR-874 and DR.

In this study, we used bioinformatics prediction to identify a targeted site where miR-874 binds to p65, which is the most important protein in the NF-κB signaling pathway. Moreover, a dual-luciferase reporter system assay confirmed that p65 is a target gene of miR-874. A DR rat model was successfully established to explore the relationship between miR-874 and NF-κB signaling pathway. The results showed that, in DR rats, a miR-874 mimic has similar therapeutic effects to the NF-κB signaling pathway antagonist EVP4593. In addition, DR was aggravated after treatment with a miR-874 inhibitor in diabetic rats. EVP4593 also counteracted the aggravation of retinopathy that was caused by the miR-874 inhibitor. These results indicate that miR-874 upregulation inhibits the activity of the NF-κB signaling pathway, alleviating retinopathy in diabetic rats.

In addition, we found that blood glucose levels in the miR-874 agomir and EVP4593 groups were the lowest for the diabetic rat groups. In this study, diabetes in rats was first established and then treated. Moreover, compared with the miR-874 agomir and EVP4593 groups, the NF-κB signaling pathway in other groups of diabetic rats was not inhibited or only partially inhibited, and correspondingly their retinopathy was more severe. Therefore, the amelioration effect of miR-874 on diabetic retinopathy was due not to low blood glucose levels, but to the fact that miR-874 inhibited the abnormally high activity of the NF-κB signaling pathway (the NF-κB impairment) in the retinas of diabetic rats. As signaling pathways in the body are complicated, it remains to be elucidated whether the decrease in blood glucose is related to inhibition of the NF-κB signaling pathway.

Limitations

While the effects of miR-874 on retinopathy in diabetic rats have been confirmed, the specific molecular mechanism between miR-874 and NF-κB is not clear. Moreover, further experiments will be necessary to determine whether overexpressed miR-874 mimic can be applied in clinical treatment.

Conclusions

miR-874 can inhibit p65, an important protein in the NF-κB signaling pathway, in the retina of diabetic rats, and further inhibit NF-κB signaling, leading to an alleviation of retinopathy. It may be a new drug target in the treatment of DR.

References


