Downregulation of miR-93 elevates Nrf2 expression and alleviates reactive oxygen species and cell apoptosis in diabetic retinopathy

Yan Yin¹, Xiquan Zhao², Zhanyun Yang³, Xiangrong Min¹

¹Department of Ophthalmology, Jining NO.1 People’s Hospital, Jining 272000, Shandong, China; ²Department of Ophthalmology, Shanxi Ophthalmic Medical Center, Xi’an 710004, Shaanxi, China; ³Department of Anesthesiology, Jining NO.1 People’s Hospital, Jining 272000, Shandong, China

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Abstract: Diabetic retinopathy (DR) is a severe complication of diabetes and is related with reactive oxygen species (ROS). Nrf2-Keap1-ARE pathways play an important role in DR. Previous studies have shown abnormally elevated levels of microRNA-93 (miR-93) in DR. Bioinformatics studies have suggested complementary binding sites between miR-93 and 3’-UTR of Nrf2 mRNA. The current study, therefore, established a type 2 DR rat model, in which expression of miR-93 and Nrf2/ARE signal pathway proteins were examined to investigate the roles of miR-93 in DR. Serum levels of fibrinogen (FBG), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), and high density lipoprotein cholesterol (LDL-C) were quantified. ROS content, caspase-3 activity, cell apoptosis, and expression of miR-93, Nrf2, heme oxygenase-1 (HO-1), and γ-glutamylcysteine synthetase (γ-GCS) were compared in retinal tissues. The content of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) in aqueous humor was measured. DR rats were treated with antagomir-93 for measurement of those parameters. DR rats had significantly elevated FBG, TC, TG, and LDL-C, while HDL-C levels were decreased. DR rats had higher ROS, caspase-3 activity, and cell apoptosis level in retinal tissues, plus elevated MDA content and lower SOD or GSH-Px in aqueous humor. Antagomir-93 treatment inhibited miR-93 expression, elevated Nrf2, HO-1, and γ-GCS, decreased retinal cell apoptosis or ROS, lowered aqueous MDA, and raised SOD and GSH-Px. miR-93 upregulation is an adverse factor for oxidative stress in DR rat retinas. Inhibition of miR-93 can alleviate retinal apoptosis or ROS via enhancing Nrf2 expression.

Keywords: Diabetic retinopathy, Keap1-Nrf2-ARE, cell apoptosis, ROS, miR-93

Introduction

Diabetic retinopathy (DR) is a prominent symptom in diabetes-related microvascular degeneration. As an idiopathic fundus disease, DR is a commonly occurring severe complication of diabetic patients [1, 2]. Under DR conditions, the body will initiate endogenous mechanisms to antagonize oxidative stress (OS), to alleviate the injury on cells and tissues by OS or product of reactive oxygen species (ROS) [3, 4]. Nuclear factor-E2 related factor 2 (Nrf2)/antioxidant response element (ARE) is the most important endogenous anti-OS signal pathway ever found. Under OS conditions induced by diabetes, activation and nuclear translocation of Nrf2 can alleviate OS injury and cell apoptosis inside the body, protecting the pathology of tissue and cells [5-7]. Multiple studies have shown that activation of Nrf2 plays crucial roles in suppressing OS response and in alleviating DR related retinal injuries [8, 9].

MicroRNA (miR) is one group of small non-coding RN with about 22~25 nucleotides length in eukaryotes. It can bind with 3’-untranslated region (3’-UTR) of target gene mRNA via complete or incomplete complementary manners. It can regulate more than 30% of human gene expression via degrading or inhibiting gene mRNA translation. Increasing evidence has shown that miRs play important roles, regulating body OS response under diabetic conditions [10, 11]. Studies have shown that miR-93 expression levels were abnormally elevated in peripheral serum [12] and vitreous body [13],
suggesting that miR-93 upregulation might participate in DR pathogenesis. This study established a type 2 DR rat model. Using normal rats as the control, expression of miR-93 and Nrf2/ARE pathway related protein was examined in rat retinal tissues, aiming to investigate whether miR-93 plays a role in mediating Nrf2-Keap1-ARE pathway and DR.

Methods and materials

Equipment and reagents

Healthy adult male SD rats (6 weeks old, body weight 220–240 g) were purchased from Vital River (Beijing, China). DMEM medium, fetal bovine serum (FBS), type I collagenase, and trypsin were purchased from Gibco (US). Rabbit anti-Nrf2, Keap1, HO-1, and γ-GCS antibody were purchased from Abcam (US). Rabbit anti-β-actin and HRP conjugated goat anti-rabbit IgG (H+L) secondary antibody were purchased from Sangon (China). Streptozotocin (STZ)-citrate buffer and DCFH-DA probe were purchased from Sigma (US). Mondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and Annexin V-FITC/PI cell apoptosis test kit, as well as the caspase 3 activity kit, were purchased from Beyotime (China). RNA extraction kit, PrimeScript™ RT reagent Kit, and SYBR Green were purchased from Takara (Dalian, China). Antagomir-93 and antagomir-NC were designed and synthesized by RioBio (Guangzhou, China). Dual-Glo™ Luciferase Assay System kit and pGL3 plasmid were purchased from Promega (US). Model 7180 fully automatic biochemical analyzer was purchased from Toshiba (Japan). Gallios flow cytometry apparatus was purchased from Beckman Coulter (US). UV-1100 spectrophotometry was purchased from Meipuda Instrument (Shanghai, China). Model CFX96 fluorescent quantitative PCR cycler was purchased from Bio-Rad (US).

Rats were used for all experiments and all procedures were approved by the Animal Ethics Committee of the First People’s Hospital of Shandong (Jining, Shandong, China).

Generation of DR model rats

Model rats received 2 months of a high-fat and high-glucose diet. After 12 hours of fasting, 1% STZ-citrate buffer was injected by intraperitoneal route (50 mg/kg dosage). After 1-week of continuous treatment, fasting plasma glucose (FPG) was measured from tail vein blood samples. Rats with FPG>16.7 mmol/L were identified as type 2 diabetes mellitus (DM). These rats continuously received a high-fat and high-glucose diet until the end of experiment. Control group rats received normal diet feeding, receiving citrate buffer injections only, followed by normal diet feeding.

Grouping and treatment of spontaneous hypertensive rats (SHR) rats

DR model rats were randomly assigned into two groups. Antagomir-93 group rats received injections of 10 μg/kg antagomir-93 into subretinal space 5 days before sampling. Antagomir-NC group rats received injections of an equal volume of antagomir-NC into subretinal space 5 days before sampling.

Sample collection

Twelve weeks after model preparation, blood samples were collected from the two groups of rats. Next, 10% hydrate chloral was injected into peritoneal cavity for anesthesia. Aqueous humor was collected at coronal edge. Content of MDA, SOD, and GSH-Px was examined by test kits. Rat eyeballs were collected to remove retinal tissues and RNA was extracted using the RNAiso kit. Total proteins were extracted by RIPA lysis buffer and caspase-3 activity was measured by the test kit. Cell apoptosis and ROS content were measured by flow cytometry. Cardiac and peripheral blood samples were collected from measuring fibrinogen (FBG), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), and high density lipoprotein cholesterol (LDL-C) levels.

Cell apoptosis in retinal tissues

Rat retinas were separated and digested by 0.1% type I collagenase and 0.125% trypsin to obtain single cell suspension. After PBS rinsing, 0.5 mL binding buffer was used to re-suspend cells, which were mixed with 5 μL Annexin V-FITC buffer for 15 minutes of dark incubation at room temperature. Next, 5 μL PI dye was added for 5 minutes of staining. Cell apoptosis was then measured by flow cytometry.

ROS content assay

Rat retinal tissues were separated and incubated in the dark at 37°C with DCFH-DA staining buffer (0.1%). After rinsing in PBS, cells
were resuspended in 400 μL PBS and ROS content was measured by flow cytometry.

**Caspase-3 activity assay**

Following manufacturer instructions for the test kit, pNA standard samples with gradient concentrations were prepared. Absorbance values at 405 nm were measured to plot standard curve based on concentrations. Next, 100 μL lysis buffer was added into each 5 mg retinal tissue. After preparing homogenate, tissue lysate was processed at 4°C for 10 minutes, then centrifugated at 10,000 g for 10 minutes at 4°C. The supernatant was saved to quantify caspase 3 activity. The relative enzymatic activity unit was calculated as A_{405} of experimental group/A_{405} of control group.

**Lipid oxidation and anti-oxidative index**

Following manual instructions, the content of MDA, SOD, and GSH-Px was measured by ultraviolet spectrometry, evaluating oxidative stress levels and anti-oxidative stress.

**Luciferase reporter gene assay**

HEK293 cells were lysed by TRIzol Reagent to extract mRNA. Using it as the template, 3'-UTR of Nrf2 gene containing targeted binding site or mutant form was amplified to extract DNA by electrophoresis. Products were digested by restriction enzymes and were ligated into pGL3 plasmid to transform DH5α competent cells. Primary screening was performed for positive selection of those bacterial with plasmid insertion. Sequencing was performed to select plasmids with correct insertion, named as pGL3-Nrf2-wt and pGL3-Nrf2-mut.

Lipo2000 was used to co-transfect 200 ng pGL3-Nrf2-wt or pGL3-Nrf2-mut, plus 50 nmol miR-93 mimic or miR-93 inhibitor or miR-NC, and 50 ng pRL-TK into HEK293 cells. After 48 hours of incubation, the Dual-Glo™ Luciferase Assay System kit was used to measure relative luciferase activity.

**qRT-PCR for gene expression assay**

RNAiso Plus was used to extract RNA. It was used to produce cDNA by reverse transcription using the PrimerScript™ RT reagent kit. Using cDNA as the template, PCR amplification was used under TaqDNA polymerase. In a 10 μL reaction system, one added 5.0 μL 2×SYBR Gr-}

**Western blot for protein expression**

Retinal tissues were lysed by RIPA lysis buffer to extract proteins. A total of 40 μg protein samples were loaded onto SDS-PAGE (12% separating gel and 4% condensing gel) for separation under 45 V for 2.5 hours. Proteins were transferred to PVDF membranes under 250 mA for 1.5 hours. Membranes were blocked in 5% defatted milk powder at room temperature for 60 minutes. Primary antibody (Nrf2 at 1:1000, HO-1 at 1:2000, γ-GCS at 1:2000 and β-actin at 1:10000) was added for 4°C overnight incubation. Unbounded primary antibody was washed away and HRP conjugated goat anti-rabbit IgG (H+L) secondary antibody was added for 60 minutes at room temperature. ECL approach was used to develop the membrane, followed by exposure and imaging for data processing.

**Statistical analysis**

SPSS 18.0 was used for data analysis. Measurement data are presented as mean ± standard deviation (SD). Comparisons between the two groups of measurement data were performed by t-test or Mann-Whitney U-test. Comparisons among multiple groups of measurement data were performed first by one-way analysis of variance (ANOVA), followed by Bonferroni’s post-hoc comparison. Statistical significance is defined as P<0.05.

**Results**

**Targeted regulation between miR-93 and Nrf2**

Online prediction by micorRNA.org showed the existence of complementary binding sites be-
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between miR-93 and 3'-UTR of Nrf2 mRNA (Figure 1A). Dual luciferase reporter gene assays showed that the transfection of miR-93 mimic or miR-93 inhibitor significantly decreased or increased the relative luciferase activity in HEK293 cells transfected with pGL3-Nrf2-wt. No significant changes of relative luciferase activity were found in HEK293 cells transfected with pGL3-Nrf2-mut (Figure 1B), suggesting targeted regulation between miR-93 and Nrf2 mRNA.

Metabolic disorder of blood glucose and lipids in model rats

Of the 20 rats used for generating DM models, 12 of them were successful (60%). Test results showed that, compared to the control group, DM model rats showed significantly elevated levels of FBG (Table 1). Assays for blood lipids showed that, compared to control rats, DM model rats had remarkably higher levels of TC, TG, and LDL-C, while HDL-C content was significantly lower than control rats (Table 1).

Significantly enhanced retinal cell apoptosis and OS in DR model group

Of the 12 DM rats, 5 showed DR occurrence. Spectrometry assays showed that, compared to control rats, DR rats had significantly elevated caspase-3 activity in retinal tissues (Figure 2A). Flow cytometry results showed that, compared to control rats, DR rats had significantly elevated cell apoptosis in retinal tissues (Figure 2B). DCFH-DA staining showed significantly elevated intensity (Figure 2C). UV spectrometry showed remarkably elevated MDA in aqueous humor of DR rats, compared to control rats (Figure 2D), while the activity of SOD (Figure 2E) and GSH-Px (Figure 2F) was significantly depressed.

Elevated expression of miR-93 and Nrf2 in DR rat retinal tissues

qRT-PCR results showed that, compared to control rats, DR rat retinal tissues had significantly elevated miR-93, Nrf2 mRNA expression (Figure 3A). Western blot results showed that, compared to control rats, DR rats had significantly elevated Nrf2 protein expression in retinal tissues, accompanied with decreased expression of anti-oxidase HO-1 and γ-GCS proteins (Figure 3B).

Injections of antagomir-93 into retina elevate Nrf2 expression and suppress OS or apoptosis

qRT-PCR results showed that, compared to rats receiving antagomir-NC injections, the antagomir-93 group showed significantly suppressed miR-93 expression in rat retinal tissues, while Nrf2 mRNA expression was remarkably elevated (Figure 4A). Western blot results showed that, compared to antagomir-NC rats, antagomir-93 rat retinal tissues had significantly elevated expression of Nrf2, HO-1, and γ-GCS proteins (Figure 4B). Spectrometry results showed that, compared to the antagomir-NC group, antagomir-93 rats showed significantly depressed caspase-3 activity (Figure 4C). Flow cytometry results showed that, compared to antagomir-NC rats, antagomir-93 rat retinal tis-
Figure 2. Potentiated cell apoptosis and OS in DR retinal tissues. A. Spectrometry for caspase-3 activity in retinal tissues. B. Flow cytometry for cell apoptosis in rat retinal tissues. C. ROS content in rat retinal tissues by flow cytometry. D. UV spectrometry for MDA content in aqueous humor. E. UV spectrometry for SOD enzymatic activity in humor aqueous; F. GSH-Px enzymatic activity in humor aqueous by UV spectrometry. *, P<0.05 compared to control group.

Nrf2/ARE is the most important endogenous anti-OS signaling pathway ever found. Under OS conditions, Nrf2 can be translocated into the nucleus for binding with ARE. This is to initiate the transcription and expression of downstream phase II detoxification enzymes and anti-oxidase and anti-apoptotic genes, enhancing cell resistance against OS or electrophilic macromolecules, thereby alleviating body ROS and cell apoptosis, protecting tissues and cell pathology [5-7]. Nrf2 is an OS-sensitive gene transcription regulator containing seven structural domains from Neh1 to Neh7. Of these, Neh2 is the structural domain for Nrf2 protein to bind and couple with Keap1, playing crucial roles in mediating Nrf2 functional activity [14-16]. When body OS levels are within physiological range, Nrf2 couples with Keap1 to bind with cytoplasmic actin for anchoring into cytoplasm.
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It is under minimal transcriptional activity with continuous degradation and low expression. When OS is significant, structural changes of Keap dissociate Nrf2 and Keap1 for nuclear translocation to bind with ARE and upregulation of SOD, glutathione transferase (GST), HO-1, γ-GCS, and nicotinamide adenine dinucleotide (phosphate) (NADH or NADPH). These are anti-OS proteasome components, alleviating OS levels and rescuing injury [17-20]. Therefore, Nrf2 plays crucial roles in anti-OS. Multiple studies have shown the critical role of Nrf2 activation in depressing OS response and alleviating DR related retinal injury [8, 9]. Previous studies have found possible relationship between miR-93 upregulation and DR pathogenesis [12, 13]. The current study investigated if miR-93 plays a role in modulating Nrf2-Keap1-ARE pathways and DR.

Gene and protein expression assays showed that, compared to controlled rats, DR rat retinal tissues had significantly elevated miR-93 and Nrf2 expression, while anti-oxidase HO-1 and γ-GCS were depressed. Results showed that, under DR conditions, the body can initiate anti-OS mechanisms via potentiating expression and function of Nrf2, although the actual anti-oxidative stress potency of the body remains relatively low, with prominently elevated ROS content. Liu et al. found that, compared to the control group, diabetic rats showed significantly elevated Nrf2 expression, ROS, and cell apoptosis in retinal tissues [23]. Deliyanti et al. showed elevated Nrf2 expression plus OS levels in diabetic rat retinas [8], in accord with present results. It is worth noticing that this study also observed significantly elevated miR-93 expression in DR rat retinal tissues. Due to the existence of targeted inhibition between miR-93 and Nrf2, it was further proposed that miR-93 upregulation is an inhibitor for Nrf2 expression, compromising initiation of anti-OS mechanisms and depression of ROS levels. Therefore, this study further investigated regulatory roles of miR-93 in DR. Injections of antagomir-93 into rat retinal tissues can decrease miR-93 expression and further accelerate Nrf2 expression, as well as elevate expression of anti-oxidase, such as HO-1, and γ-GCS decrease of ROS, MDA content, or cell apoptosis in DR rat retinas. Results showed that miR-93 upregulation is a negative factor for anti-OS in DR rats. On the other hand, inhibition of miR-93 more effectively upregulates Nrf2 expression and decreases OS levels or cell apoptosis rates in DR rat retinas. Zou et al. also showed significantly elevated ROS content in retinal cells, plus higher cell apoptosis, under high-glucose stress [21]. Sun et al. found that, under diabetic conditions, rat retinal tissues had significantly improved cell apoptosis and oxidative stress levels, suggesting the close correlation between OS and apoptosis in DR pathogenesis [22]. In this study, DR rats showed remarkably elevated retinal cell apoptosis and ROS content, supporting the results from Cao et al. [21] and Sun et al. [22].

Dual luciferase reporter gene activity results showed that, compared to transfected with pGL3-Nrf2-wt, confirming targeted regulation between miR-93 and Nrf2 mRNA. Blood glucose and lipid assays showed significantly elevated blood glucose in model rats, plus abnormal blood lipid levels, suggesting successful generation of the type 2 DM model. Assays on DR rats showed significantly elevated cell apoptosis and ROS content in DR retinal tissues, as well as abnormally higher MDA content in humor aqueous, while the enzymatic activity of SOD and γ-GCS was remarkably decreased. Results suggest prominent OS in DR retinal tissues plus suppressed anti-oxidative potency. Cao et al. also showed remarkably elevated ROS content in retinal cells, plus
Figure 4. Antagomir-93 injections into retina elevate Nrf2 expression and suppress OS or cell apoptosis. A. qRT-PCR for miR-29a and Nrf2 mRNA expression in retinal tissues. B. Western blot for retinal protein expression. C. Spectrometry for caspase-3 activity in retinal tissues. D. Flow cytometry for cell apoptosis in rat retinal tissues. E. Flow cytometry for ROS content in rat retina. F. UV spectrometry for MDA content in humor aqueous. G. UV spectrometry for SOD enzymatic activity in humor aqueous. H. UV spectrometry for GSH-Px enzymatic activity in humor aqueous. *, $P<0.05$ compared to antagomir-NC group.
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cohort, and miR-93 expression levels were closely correlated with disease progression [12]. Hirota et al. showed abnormally elevated miR-93 expression in vitreous body of DR patients [13]. These studies have shown that miR-93 upregulation might be a negative factor for DR, consistent with present observations. In a study about Nrf2 in protecting DR, Liu et al. showed that Fenofibrate treatment further enhanced Nrf2 expression in DR rat retinas, elevating expression of target genes, including quinine oxidoreductase-1 (NQO-1) and HO-1 for suppression of ROS production [23]. Deliyanti et al. showed that dh404 treatment further enhanced Nrf2 expression in DR rat retinas, elevating NQO-1 and HO-1 expression to decrease OS levels [8]. In contrast with Liu et al. [23] or Deliyanti et al. [8], the current study used interference miR-93 to upregulate Nrf2 expression, further alleviating apoptosis and OS injury of DR rat retinal tissues. These observations have not been mentioned so far, indicating the novelty of the current study. However, only an animal model was examined. Thus, it is still not clear whether miR-93 and Nrf2 expression is altered during human DR pathogenesis. Future studies should collect human DR retinal samples. Expression of miR-93 and Nrf2 should be examined to reveal whether miR-93 participates in human DR pathogenesis. This was a limitation of the current study.

Conclusion

miR-92 upregulation is an adverse factor for suppression of Nrf2 expression and OS injuries in DR rat retinal tissues. Inhibition of miR-93 can decrease cell apoptosis or ROS levels of DR rat retinas via enhancing Nrf2 expression and body anti-OS levels.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiangrong Min,
Department of Ophthalmology, Jining NO.1 People’s Hospital, Jining 272000, Shandong, China. Tel: +86-0537-6056666; +86-0537-6056666; E-mail: yunwota@163.com

References


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