Original Article
Resolvin D2 prevents inflammation and oxidative stress in the retina of streptozocin-induced diabetic mice

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Abstract: Diabetic retinopathy is the main ocular complication of diabetes mellitus. The aim of this study was to investigate the protective effect and mechanism of resolvin D2 (RvD2) on diabetic retinopathy. Streptozocin-induced C57/BJ diabetic mice were divided into three groups: normal control, diabetes mellitus, and diabetes plus RvD2 treatment. After three months of diabetic model induction, exogenous RvD2 was injected, monthly for three months, into the vitreous cavity of mice in the diabetic treatment group. Retinal vascular leakage, ganglion cell apoptosis, inflammatory factor expression, and oxidative stress factors were detected one month after the last injection. The levels of retinal vascular leakage and ganglion cell apoptosis in diabetic mice treated with RvD2 were significantly lower than those in untreated diabetic mice, as were the retinal levels of inflammatory factors and oxidative stress. In conclusion, RvD2 might be used as a retinal protective factor for diabetes mellitus by reducing inflammation and oxidative stress.

Keywords: Resolvin D2, inflammation, oxidative stress, diabetes, retina

Introduction

Diabetic retinopathy (DR), which seriously threatens visual health, is one of the most important complications of diabetes mellitus. Approximately 60% of patients with diabetes have fundus vascular damage [1-3]. Presently known mechanisms of DR include endoplasmic reticulum stress, oxidative stress, accumulation of advanced glycation end products, and inflammatory stress [1-7]. Although, the detailed mechanism of DR has not been fully elucidated, inflammatory stress is a major cause, and its important role in the occurrence and development of DR has been confirmed by many studies [1, 8]. A high glucose environment can increase the release of inflammatory mediators, such as tumor necrosis factor (TNF)α, interleukin (IL)-1β, and NOD-like receptor family pyrin domain containing-3, in retinal cells. Additional related inflammatory mediators can further activate inflammatory pathways and damage the normal physiologic function of retinal cells [8].

Resolvin D2 (RvD2) is a small molecule derived from omega-3 polyunsaturated fatty acids. In recent years, increasing attention has been paid to the protective role of RvD2 in the development of chronic inflammatory diseases [9, 10]. While previous studies confirmed that RvD2 has physiological effects on inflammatory cell infiltration and apoptosis [9-16], its role in the pathologic process of DR has not been fully elucidated.

In this study, we used a streptozocin-induced diabetic mouse model to induce DR, and treated these mice with RvD2 by intravitreal injection. We compared retinal vascular leakage, retinal inflammatory molecule expression, retinal oxidative stress, and retinal cell apoptosis in treated and untreated diabetic mice, and in controls, to explore the protective effect of RvD2 on DR.
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Table 1. Average blood sugar and body weight in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Age (wk)</th>
<th>Weight (g)</th>
<th>Blood sugar (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>20</td>
<td>24.25±1.31</td>
<td>5.39±1.28</td>
</tr>
<tr>
<td>STZ-group</td>
<td>29</td>
<td>20</td>
<td>18.74±2.03</td>
<td>20.37±2.43</td>
</tr>
<tr>
<td>STZ-RvD2-group</td>
<td>21</td>
<td>20</td>
<td>19.19±1.76</td>
<td>22.41±3.01</td>
</tr>
</tbody>
</table>

STZ mice had significantly higher blood glucose than control mice, while the weight of STZ mice was significantly lower than that of the control animals. *P < 0.01, unpaired t-test relative to corresponding control group.

Methods

Diabetic mouse model

The diabetic model was induced by daily intraperitoneal injection of streptozocin (50 mg/kg) into C57/BJ mice for 5 days. The control group was injected with an equal volume of sodium citrate buffer. Blood glucose levels and body weights of the mice were monitored every month after injection. Blood glucose levels above 15.5 mM indicated diabetes [17]. RvD2 (5 μL of a 1 μg/L solution, Cayman Chemical, Ann Arbor, MI, USA) was injected into the vitreous cavity monthly for three consecutive months. One month after the last RvD2 injection, follow-up tests were performed on diabetic and normal mice of the same age. All mouse experiments were reviewed and approved by the Ethics Committee for Animal Care and Use of Ezhou Hospital, Hubei, China.

Enzyme-linked immunosorbent assay (ELISA)

The level of RvD2 was determined by an ELISA. Retinal tissues were collected from mouse eyes and ultrasonicated in radioimmunoprecipitation assay lysis buffer. After high-speed centrifugation (1000 rpm), the supernatant was subjected to an ELISA in accordance with the provider’s recommendation (EMD-Millipore, Bedford, MA, USA). The protein concentration was quantified by a bicinchoninic acid protein assay kit (Thermo Fisher, Waltham, MA USA).

Retinal vascular leakage

Retinal vascular leakage was detected using Evans Blue (20 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) as described previously [18-20]. In brief, intravenous perfusion with 0.3 mL of Evans Blue was conducted in mice via the superior vena cava. Five minutes after intravitral circulation, the eyes were enucleated and placed in paraformaldehyde (40 g/L) for 40 minutes. Then, retinas were dissected and flat-mounted onto glass slides. Retinal vessels were visualized under an Olympus BX60 fluorescence microscope (Olympus America, Center Valley, PA, USA), and retinal vascular leakage was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Retinal cell apoptosis

Retinal cell apoptosis was measured using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Roche Applied Science, Penzberg, Germany). Retinal paraffin sections were dewaxed, dripped with protease K working fluid (10-20 g/mL), and incubated at 37°C for 20 minutes. The samples were then treated with 80 μL of 3,3’diaminobenzidine solution, re-stained with hematoxylin, dehydrated, and then sealed with neutral gum. Sections were observed with a microscope (Nikon E100 Biomicroscope, Japan) and images were acquired.

Real-time polymerase chain reaction (RT-PCR)

Vascular endothelial growth factor (VEGF) and inflammatory marker mRNA (NF-κB, TNF-α, and monocyte chemoattractant protein (MCP)-1) levels in the retina were determined by RT-PCR. In brief, mouse retinas were dissected rapidly, total RNA was extracted using an RNA Extraction Kit (Takara Bio, Kusatsu, Japan), and cDNA was generated using the RevertAid First Strand cDNA Synthesis Kit (Takara) following the manufacturer’s instructions. Gene-specific primer sequences and annealing temperatures are shown in Table 1.

Western blot analyses

The expression of inflammatory- and vascular-related proteins in the retina was assessed by western blot analyses. Total protein was extracted from retinal tissue using twelve alkyl sulfonates. Proteins were separated using polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and incu-
Table 2. Primer sequences for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>5’-GAGCGGAGCAGGCAGAGTG-3’</td>
<td>5’-TTCAATGGCCGCGTTCCGA-3’</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5’-CTGCAATTGGCTATAATGGC-3’</td>
<td>5’-ACAAGTTCACTGAGATTACG-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’-CGACCCACGGATTGCTCT-3’</td>
<td>5’-CGGACTCGAGAAGCTAAAG-3’</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5’-CTGACCAACTGCAACTG-3’</td>
<td>5’-ACTGGAATACTGGTCTCTATC-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-CTAGGCGAACCCTGTAGGA-3’</td>
<td>5’-ACCAGAGGCATACAGGACA-3’</td>
</tr>
</tbody>
</table>

Detection of retinal oxidative stress

Malondialdehyde (MDA) levels were determined in fresh retinal tissues following the instructions of the MDA kit (S0131, Beyotime Biotechnology, Shanghai, China). In brief, retinal tissue was lysed using cell lysis solution (P0013, Beyotime Biotechnology) and the supernatant was centrifuged for 10 minutes at 12,000 g. A bicinchoninic acid protein assay kit (P0009, Beyotime Biotechnology) was used to determine protein concentrations. Pyrolysis solution (0.1 mL) was used as the blank control. 0.1 mL of standard MDA was used to construct the standard curve, 0.1 mL of sample was used for concentration determinations, and 0.2 mL of the thioarbituric acid working fluid was added. The mixture was then mixed and incubated at 100°C for 15 minutes. The reaction was cooled to room temperature (20°C) in a water bath and centrifuged at 1000 × g at room temperature for 10 minutes. Absorbance was measured at 532 nm. The MDA content in each sample was calculated and expressed as µmol/mg protein or µmol/mg tissue.

Glutathione (GSH) levels were determined in fresh retinal tissues homogenized in 0.1 M phosphate buffer (pH 8.0) containing 5 mM EDTA. After centrifugation, a quantity of the resulting supernatant was removed for storage at -80°C for subsequent protein determination by the bicinchoninic acid assay. GSH determinations were carried out on the freshly prepared tissue extract following the instructions of the GSH kit (S0053, Beyotime Biotechnology). Absorbance was measured at 410 nm. The GSH content in the sample was expressed as µmol/mg protein or µmol/mg tissue.

8-Hydroxydeoxyguanosine (8-OHdG) levels were determined using a competitive ELISA BioAssay Kit (385070, US Biological, Salem, MA, USA). The procedure was performed according to the manufacturer’s protocol. Standard 8-OHdG was assayed over a concen-
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The enzyme activity of manganese superoxide dismutase (MnSOD) was measured in 5-10 μg of retinal protein using a kit (S0103, Beyotime Biotechnology). Sample preparation and protein quantitation were similar to those described above. According to the protein concentration, the sample was diluted with the SOD detection buffer provided by this kit. The standard curve was generated using a quality-controlled SOD standard. MnSOD activity was determined by performing the assay in the presence of potassium cyanide to inhibit Cu-ZnSOD, thus measuring only residual MnSOD activity. Absorbance was measured at 450 nm. MnSOD activity in the original sample was calculated and expressed as μmol/mg protein or μmol/mg tissue.

Figure 2. Retinal vascular leakage in resolvin D2 (RvD2)- and streptozocin (STZ)-treated mice. Evans Blue retinal flat-mounts of (A) STZ, and (B) STZ + RvD2 mice. (C) Quantitative analysis of the relative area of leakage in retinas from each group. Retinal vascular leakage was lower in RvD2 than STZ mice (n = 4, P < 0.05).

Figure 3. DNA damage and apoptosis in resolvin D2 (RvD2)- and streptozocin (STZ)-treated mouse retinas. TUNEL staining of (A) STZ, and (B) STZ + RvD2 retinal cells (TUNEL-positive cells have brown-yellow particles in the nucleolus). (C) Quantitative analysis of TUNEL-positive cells in the GCL. RvD2 reduces DNA damage and apoptosis in retinas from diabetic mice and prevents diabetes-induced cell death. Data are expressed as mean ± SD (n = 4, P < 0.05). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

Statistical analyses

Normally distributed data from assays performed in triplicate were analyzed statistically using the independent two-sample t-test or one-way analysis of variance with
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Figure 4. The levels of apoptosis related protein expression. A. Western blot analysis of apoptosis related protein (Bcl-2, Caspase-3 and P53) in the retina; B. Quantitative analysis of relative protein level in the retina; RvD2 reduced the level of Caspase-3 and P53 protein, but increased that of Bcl-2. (n = 4, P < 0.05).

SPSS 15.0 software (IBM, Chicago, IL, USA). Data are presented as mean ± standard deviation. Differences between groups were considered significant at P < 0.05.

Results

Average blood sugar and body weights in mice

Body weights of streptozocin-induced type 1 diabetic mice were significantly lower than those of the normal control group (P < 0.01). Additionally, the blood sugar level of streptozocin-induced type 1 diabetic mice was significantly higher than that of the normal control group (P < 0.01) (Table 2).

RvD2 levels in retinas and serum of normal and diabetic mice

The levels of RvD2 in the retina and serum of streptozocin-induced diabetic mice did not significantly differ from those measured in the control group after one month (n = 4, P > 0.05), but decreased significantly in the third month (n = 4, P < 0.05) (Figure 1).

Exogenous RvD2 reduces retinal vascular leakage in diabetic mice

RvD2 treatment significantly reduced retinal vascular leakage in diabetic mice compared with that observed in normal mouse group (Figure 2, n = 4, P < 0.05).

RvD2 reduces the level of retinal ganglion cell apoptosis in diabetic mice

To observe the level of retinal ganglion cell apoptosis in DR, the retina was TUNEL stained. In addition, we measured the levels of Bcl-2, Caspase, and P53, which are anti- and pro-apoptotic proteins, respectively, by western blot. The number of apoptotic retinal ganglion cells in the RvD2 group was significantly lower than that in the non-intervention group (Figure 3, n = 4, P < 0.05). The level of Bcl-2 protein in the RvD2 group was significantly higher than in the diabetes group, while the level of Caspase-3 and P53 were lower (Figure 4, n = 4, P < 0.05).

RvD2 reduces the expression of VEGF and inflammatory factors in diabetic mice

RT-PCR and western blot results showed that the mRNA and protein levels of VEGF, NF-κB, IL-6, IL-8, TNF-α, and MCP-1 were significantly reduced in retinal tissue of the RvD2 intervention group compared to the streptozocin alone group (Figure 5, P < 0.05).

RvD2 reduces oxidative stress in the retinas of diabetic mice

MDA, GSH, 8-OHdG, and MnSOD are the main markers of oxidative stress. Levels of these markers in the retinas of each group were compared and differed significantly (Figure 6). Specifically, MDA and 8-OHdG contents in reti-
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![Figure 5.](image)

**Figure 5.** Vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB, and monocyte chemoattractant protein (MCP)-1 expression in retinas. (A) Representative western blot. Quantitative analysis of (B) VEGF, (C) NF-κB, (D) TNF-α, and (E) MCP-1 protein expression in the retina. Quantitative analysis of mRNA levels of (F) MCP-1, (G) VEGF, (H) NF-κB, and (I) TNF-α. Resolvin D2 (RvD2) suppresses the expression of VEGF, TNF-α, NF-κB, and MCP-1 mRNAs in retinas from streptozocin (STZ) diabetic mice (P < 0.05, n = 4).
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Figure 6. Levels of oxidative stress markers in retinas. (A) Manganese superoxide dismutase (MnSOD) activity is lower, and (B) the malondialdehyde (MDA) content is significantly higher, in retinas of streptozocin (STZ)-treated mice than those in the normal control group (n = 5, P < 0.05 and P < 0.01, respectively). These retinal levels are significantly improved in resolvin D2 (RvD2)-treated mice compared to retinas of the diabetic model group (n = 5, P < 0.05). (C) 8-Hydroxydeoxyguanosine (8-OHdG) levels increase and (D) glutathione (GSH) levels decrease in retinas of diabetic model mice (n = 5, P < 0.05, P < 0.01, respectively). Contents of 8-OHdG and GSH in the retinas of RvD2-treated mice are significantly improved compared to the diabetic model group (n = 5, P < 0.05).

Discussion

DR is the main ocular complication of diabetes mellitus. Inflammation, oxidative stress, and endoplasmic reticulum stress are the main pathophysiologic factors of DR [1-8]. Because current DR treatments are suboptimal, it is very important to find an effective target.

RvD2 is a new member of the resolvin family. RvD2 is an effective leukocyte regulator and has a regulatory effect on inflammation [9-16]. However, the role of RvD2 in the occurrence and development of DR has not been elucidated. In this study, we provide evidence for the role of RvD2 in diabetic retinopathy through the introduction of exogenous RvD2 to the vitreous cavity of diabetic mice. We focused on the effects of RvD2 on the retina of diabetic mice, as well as on inflammatory pathways, oxidative stress, and other related factors. We specifically examined its effects on the expression of IL-6, IL-8, MCP-1, TNF-α, IL-12, NF-κB, and VEGF.

An early pathologic feature of DR is increased retinal vascular permeability [1, 2, 19]. Our
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results confirmed that RvD2 can significantly reduce vascular permeability. Ganglion cell apoptosis is another main characteristic of DR [1, 2, 21]. Intravescial injection of RvD2 into diabetic mice significantly reduced the level of retinal apoptosis and pro-apoptotic protein expression. Hyperglycemia can damage vascular endothelial cells and aggravate retinal damage. Anti-VEGF injection has become a first-line method in the clinical treatment of DR [22, 23]. Inflammatory factors such as NF-κB, TNF-α, IL-6, IL-8, and MCP-1 play important roles in the inflammatory pathway, immune response, cell proliferation, and apoptosis, and participate in the pathologic process of DR [1, 2, 4]. Our results show that RvD2 significantly reduced the levels of these inflammatory factors, and reduced the expression of VEGF.

Oxidative stress also plays a key role in DR. The level of reactive oxygen species increases in high glucose environments, which leads to abnormal cell metabolism, aggravates retinal ischemia and hypoxia, and promotes DR progression [7, 21, 24]. Inhibition of reactive oxygen species has become an important strategy in DR research [7, 21, 24, 25]. Our results suggest that RvD2 can significantly reduce oxidative stress in retinal cells.

In summary, as an inflammatory regulator, RvD2 plays an important role in DR. RvD2 can mitigate retinal damage by reducing inflammation and oxidative stress, and may be a target for protection against DR in patients with diabetes mellitus.

Acknowledgements

Jinpeng-Chen and Zheng Zhang designed and conducted the experiments, and prepared the manuscript. Huiyong-Xu and Lin Liao conducted the experiments and analyzed the data. Zheng Zhang is the guarantor of this work, prepared and reviewed the manuscript, and takes responsibility for the integrity of the data and the accuracy of the data analysis. The work was supported by the Natural Science Foundation of Ezhou Central Hospital of Hubei (no. EZ2016KY005).

Disclosure of conflict of interest

None.

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References

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