

Original Research Article

Irbesartan protects against type 2 diabetic nephropathy by regulating miR-93/VEGF and its downstream effectors

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Abstract

Purpose: To investigate the protective effect of irbesartan against type 2 diabetic nephropathy (T2DN), and the mechanism involved.

Methods: Wistar rats ($n = 48$) were used in this study. Diabetes mellitus (DM) was established in the rats via injection of streptozotocin (STZ). Thereafter, the DM rats were randomly divided into diabetic nephropathy (DN) group and irbesartan group, with 16 rats in each group. Rats in the control group ($n = 16$) received normal saline in place of irbesartan.

Results: Total cholesterol (TC), triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), as well as protein expression levels of VEGF, FN, collagen IV in DN group were significantly higher in irbesartan group than the corresponding levels in normal control group, while miR-93 protein expression level was significantly lower than that in normal control group ($p < 0.05$). However, the expression level of mir-93 was significantly higher in irbesartan group than in DN group, while levels of TC, TGs, HDL-C, LDL-C and VEGF, as well as protein levels of FN and collagen IV protein were significantly lower than those in DN group ($p < 0.05$). There was no obvious change in the renal tissues of the normal control group. In contrast, in the DN group, glomerular capillary loop hypertrophy, narrow glomerular cavity, thick basement membrane, mesangial matrix, and vacuolar degeneration in renal tubular epithelial cells, were evident. Compared with DN group, the pathological changes in the irbesartan group were significantly mitigated ($p < 0.05$).

Conclusion: Irbesartan protects DM rats against type 2 diabetic nephropathy by regulating miR-93/VEGF and its downstream effector molecules. This provides some ideas for the development of new drugs for the prevention of type 2 diabetic nephropathy.

Keywords: Irbesartan, miR-93, VEGF, Collagen IV, Type 2 diabetic nephropathy

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INTRODUCTION

Diabetic nephropathy (DN) is a microvascular complication of DM which poses a serious threat to human health [1]. Diabetic nephropathy (DN) usually appears in people with more than a 10-year history of DM, and it develops progressively,

eventually leading to end-stage renal failure with poor clinical prognosis [2]. At present, no effective measures are available for the prevention and treatment of DN. Studies have shown that microRNA-93 (miR-93) is abnormally expressed in DN, and it plays an important role in regulating the onset and progression of DN [3].

Vascular endothelial growth factor (VEGF) has been shown to be positively correlated with the severity of type 2 diabetes mellitus (T2DM) [4]. Irbesartan alleviates glomerular perfusion pressure and reduces proteinuria. Furthermore, the drug blocks the activation of renin angiotensin-aldosterone system (RAAS), and it reduces the production and deposition of extracellular matrix (ECM), thereby suppressing fibrosis [5,6]. However, not much is known about the association among irbesartan, miR-93 and VEGF in T2DN.

Therefore, the present study was aimed at investigating the protective effect of irbesartan against type 2 diabetic nephropathy, and the associated mechanism.

EXPERIMENTAL

Animals

Forty-eight (48) male Wistar rats aged 10 weeks were purchased from Nanjing Junke Biological Engineering Co. Ltd. [production license no.: SCXK (Su) 2021-0001. All rats were maintained in SPF animal laboratory at temperature and relative humidity of 22 ± 3 °C and 55 ± 5 %, respectively, in an environment with 12-h light/12-h dark cycle. The rats were allowed *ad libitum* access to feed and water.

Main reagents and equipment

Immunohistochemical kit was bought from Chengdu Zhongneng Biotechnology Co. Ltd. Protein quantitative kit (BCA) was product of Nanjing Novizan Biotechnology Co. Ltd, while SDS-PAGE Gel preparation kit was purchased from Beijing Ita Biotechnology Co. Ltd. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was supplied by Beijing Sizhengbai Biotechnology Co. Ltd. Monoclonal antibodies for Mir-93 and VEGF were purchased from Xibao Biotechnology (Shanghai) Co. Ltd. Hematoxylin and eosin (H & E) staining reagents were bought from Wuhan Jijie Biotechnology Co. Ltd.

Ultra-clean workbench was product of Beijing Aerospace Cohen Laboratory Equipment Engineering Technology Co. Ltd. Fluorescence microscope was bought from Shanghai Seeger Biotechnology Co. Ltd. Automatic biochemical analyzer was purchased from Sykonos Biotechnology (Beijing) Co. Ltd. Low temperature, high speed refrigerated centrifuge was purchased (Biogen, USA). Ultraviolet analyzer was product of Wuxi Laifusi Biological Experimental Equipment Co. Ltd. Enzyme

marker was purchased from Shanghai Zoming Machinery trading Co. Ltd.

Ethical matters

This research was approved by the Animal Ethical Committee of Nephrology Ningbo Fenghua District People's Hospital (approval no. 20210983), and performed according to guidelines of "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [7].

Study design and treatments

Sixteen Wistar rats were randomly assigned to normal control group and fed basal diet. The remaining 32 rats were fed high-fat diet. After 6 weeks, DM was induced in the rats via intraperitoneal injection of STZ at a dose of 28 mg/kg, in citric acid medium. Rats in the normal control group were intraperitoneally injected citric acid solution only. Thereafter, DM rats having random blood glucose ≥ 16.7 mmol/L were randomly divided into DN group and irbesartan group, with 16 rats in each group. Rats in irbesartan group were given the drug at a dose of 50 mg/kg via intragastric administration, while rats in normal control group and DN group were given equivalent dose of normal saline through the intragastric route. The three groups were fed continuously for 22 weeks.

Evaluation of parameters and indices

Serum TC, LDL-C, HDL-C and TG of rats

At 22 weeks, blood was collected from the posterior orbital venous plexus of each rat in each group, and centrifuged. Serum TC (total cholesterol), Low density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C) and Triacylglycerol (TG) of rats in each group were determined using automatic biochemical analyzer.

Thereafter, all rats were sacrificed via decapitation. The bilateral kidneys were excised and fixed in 4 % paraformaldehyde. After paraffin embedding, the tissues were sliced into 2- μ m sections in a microtome, and the slices were subjected to H & E staining using standard histological procedures.

The protein expression levels of miR-93 and VEGF

Western blot assay was used to determine the protein expression levels of miR-93 and VEGF in kidney tissues of rats in each group. Total protein

was extracted using RIPA buffer, and the protein concentration of the lysate was measured using BCA protein quantitative kit. Then, equal amounts of protein were resolved on 10 % SDS-PAGE and transferred to PVDF membrane which was blocked by incubation with 5 % skimmed milk powder for 2 h.

Thereafter, the membrane was incubated overnight at 4 °C with primary antibodies, followed by rinsing with TBST. Then, the membrane was incubated with horse radish peroxidase-linked secondary antibody at room temperature for 2 h, after which ECL reagent was added to develop the protein bands. The protein bands were analyzed using Image J, and the gray values were calculated.

Levels of FN and collagen IV

The levels of FN and collagen IV in kidney tissues of rats in each group were determined using immunohistochemistry. Kidney tissue was subjected to de-greasing and hydration treatment, and then placed in a pressure cooker for medium antigen repair, following treatment with 3 % H₂O₂, followed by PBS-washing (3 times, each for 5 min).

After adding primary antibody and secondary antibodies, the tissues were rinsed thrice with PBS, 5 min each, followed by addition of working solution of DAB color reagent, counterstaining with hematoxylin, and differentiation with hydrochloric acid and alcohol. Thereafter, the tablets were dehydrated, cleared and sealed. Finally, the results were analyzed using Image J analysis system.

Statistical analysis

All data were analyzed using SPSS 19.0. Levels of TC, TGs, HDL-C and LDL-C are expressed as mean \pm standard deviation (SD). Comparison among multiple groups was done with one-way analysis of variance (ANOVA), while SNK-Q test was used for comparison between two groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Levels of TC, TG, HDL-C and LDL-C

The levels of TC, TG, HDL-C and LDL-C were significantly higher in DN group than in control group ($p < 0.05$). However, the levels of TC, TG, HDL-C and LDL-C in the irbesartan group were

significantly lower than those in DN group ($p < 0.05$; Table 1).

Pathological and morphological changes in renal tissues

There were no obvious changes in kidney tissue of rats in the normal control group. In contrast, the DN group manifested loop hypertrophy of glomerular capillaries, narrowing of glomerular lumen, thickening of basement membrane, increased mesangial matrix, and vacuolar degeneration of renal tubular epithelial cells. The pathological changes in the irbesartan group were significantly reduced, when compared with DN group. These results are shown in Figure 2.

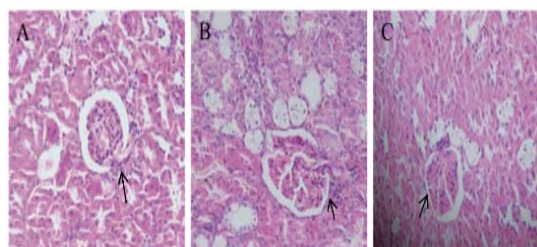


Figure 2: Pathological morphological changes in renal tissues of rats in each group (H & E staining, x200). A: normal control group; B: DN group; C: Irbesartan group

Protein expression levels of miR-93 and VEGF

The expression level of miR-93 protein in DN group was significantly lower than that in normal control group, but the expression level of VEGF protein in DN group was significantly higher than that in normal control group ($p < 0.05$). The expression level of miR-93 protein in irbesartan group was significantly higher than that in DN group, but the expression level of VEGF protein was significantly lower than that in DN group ($p < 0.05$). These results are presented in Table 1 and Figure 2.

Protein expression levels of FN and Collagen IV

The protein expression levels of FN and collagen IV in DN group were significantly higher than those in normal control group ($p < 0.05$). However, the protein expression levels of FN and collagen IV in irbesartan group were significantly lower than those in DN group ($p < 0.05$). These results are shown in Table 3 and Figure 3.

Table 1: Levels of TC, TG, HDL-C and LDL-C in each group (n = 16)

Group	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control	1.75±0.32	0.93±0.31	0.87±0.23	0.66±0.09
DN	8.48±0.91*	1.82±0.96*	1.90±0.84*	8.36±3.27*
Irbesartan	1.88±0.27#	0.90±0.41#	0.89±1.03#	0.90±0.28#
F	708.55	11.06	9.15	82.940
P-value	<0.001	<0.001	0.001	<0.001

*P < 0.05, compared with normal control group; #p < 0.05, compared with DN group. Values are expressed as mean ±SD

Table 2: Protein expression levels of miR-93 and VEGF in each group (n = 16)

Group	miR-93	VEGF
Control	1.00±0.01	0.19±0.02
DN	0.45±0.02*	0.43±0.03*
Irbesartan	0.63±0.02#	0.31±0.01#
F	4193.780	493.710
P-value	<0.001	<0.001

Note: Values are presented as mean ± SD. *P < 0.05; compared with normal control; #p < 0.05, compared with DN group

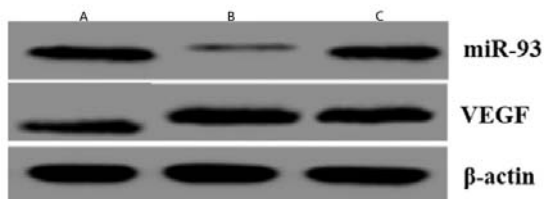


Figure 2: Protein expression levels of Mir-93 and VEGF in each group. A: normal control group; B: DN group; C: Irbesartan group

Table 3: Protein expression levels of FN and collagen IV in each group (n = 16)

Group	FN	Collagen IV
Control	0.28±0.02	0.32±0.02
DN	0.34±0.02	0.53±0.02
Irbesartan	0.31±0.02	0.35±0.01
F	36.000	688.000
P-value	<0.001	<0.001

Values are presented as mean ± SD. *P < 0.05; compared with normal control; #p < 0.05, compared with DN group

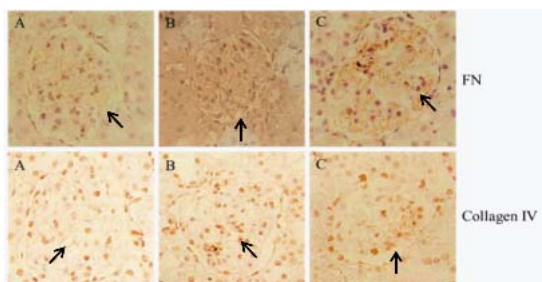


Figure 3: Protein expression levels of FN and Collagen IV in each group, as determined using immunohistochemistry (×100)

DISCUSSION

The onset of DN is closely related to metabolic abnormalities, genetics and hemodynamics [8]. However, the specific mechanism involved in the process has not been fully understood. Therefore, studies on the pathogenesis of DN will aid the development of new treatment methods for DN. Irbesartan is an angiotensin receptor blocker which inhibits the proliferation of glomerular mesangial cells, suppresses oxidative stress response and maintains the integrity of podocytes, thereby exerting renal protection [9]. In addition, irbesartan inhibits angiotensin II and angiotensin receptor 1 resistance [10].

Dyslipidemia occurs in most DN patients. It may lead to podocyte apoptosis, enhanced glomerular macrophage infiltration and abnormal extracellular matrix generation in diabetic environment, thereby inducing DN [11]. Activation of peroxisome proliferating receptor δ (PPAR δ) induces glucose metabolism and enhances fatty acid β -oxidation [12]. Angiotensin receptor blockers reduce levels of TC and TG in type 2 diabetes, and suppress metabolic syndrome in rats by activating PPAR δ [13]. In this study, analysis of rat blood lipid levels showed that TC, TG, HDL-C and LDL-C in the DN group were significantly higher than those in DN group, while the levels of TC, TG, HDL-C and LDL-C in irbesartan group were significantly lower than those in DN group. These results suggest that irbesartan effectively reduced the level of blood lipids in DN rats due to activation of PPAR δ .

It has been reported that miRNA is involved in the onset and progression of DN. Indeed, miR-93 is involved in tumor formation, invasion, migration and angio-proliferative diseases [14]. For example, abnormal expression of miR-93 has been closely linked to the occurrence of DN, and its expression in the serum of DN patients is significantly reduced, when compared with the normal population [15]. It has been demonstrated that up-regulation of the expression of Mir-93 in adult mice effectively mitigated the biochemical

indicators and histological characteristics of DN in mice [16].

It was also suggested that the drug-like repair of this gene may have important therapeutic implication for DN, and that it may be crucial in nuclear small body weight structure through mitogen and stress-induced protein kinase-2 and histones under high glucose conditions. At physiological level, VEGF effectively maintains the functional structure of the kidney. However, increased expression of VEGF is induced by DN [17]. This leads to proteinuria, glomerular hypertrophy and mesangial dilatation, all of which can be effectively alleviated if the expression of VEGF is down-regulated. Studies have shown that Mir-93 blocks the expressions of downstream related proteins FN and collagen IV by negatively controlling the expression of VEGF [18].

In the present study, it was found that the protein expression levels of VEGF, FN and collagen IV in DN group were significantly higher than those in the normal control group, while the protein expression level of miR-93 was significantly lower than that in the normal control group. The protein expression level of miR-93 in irbesartan group was significantly higher than that in DN group, while the protein expression levels of VEGF and collagen IV in irbesartan group were significantly lower than the corresponding levels in DN group. Irbesartan reduced the DN-induced renal pathological changes, when compared with the DN group. These results suggest that miR-93 may be involved in the pathogenesis of DN, and that irbesartan exerted a protective effect on the kidney by affecting the expression of VEGF regulated by miR-93 and downstream collagen fibers.

CONCLUSION

Irbesartan protects against type 2 diabetic nephropathy in rats by regulating the expressions of miR-93/VEGF and its downstream effectors. The findings provide an insight for the development of new drugs for the prevention of type 2 diabetic nephropathy.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article and all liabilities

pertaining to claims relating to the content of this article will be borne by the authors. Fu Zhao designed the study, supervised the data collection, and analyzed the data. Fu Zhao interpreted the data and prepared the manuscript for publication. Fu Zhao, Yifei Chen and Fuhai Yu supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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