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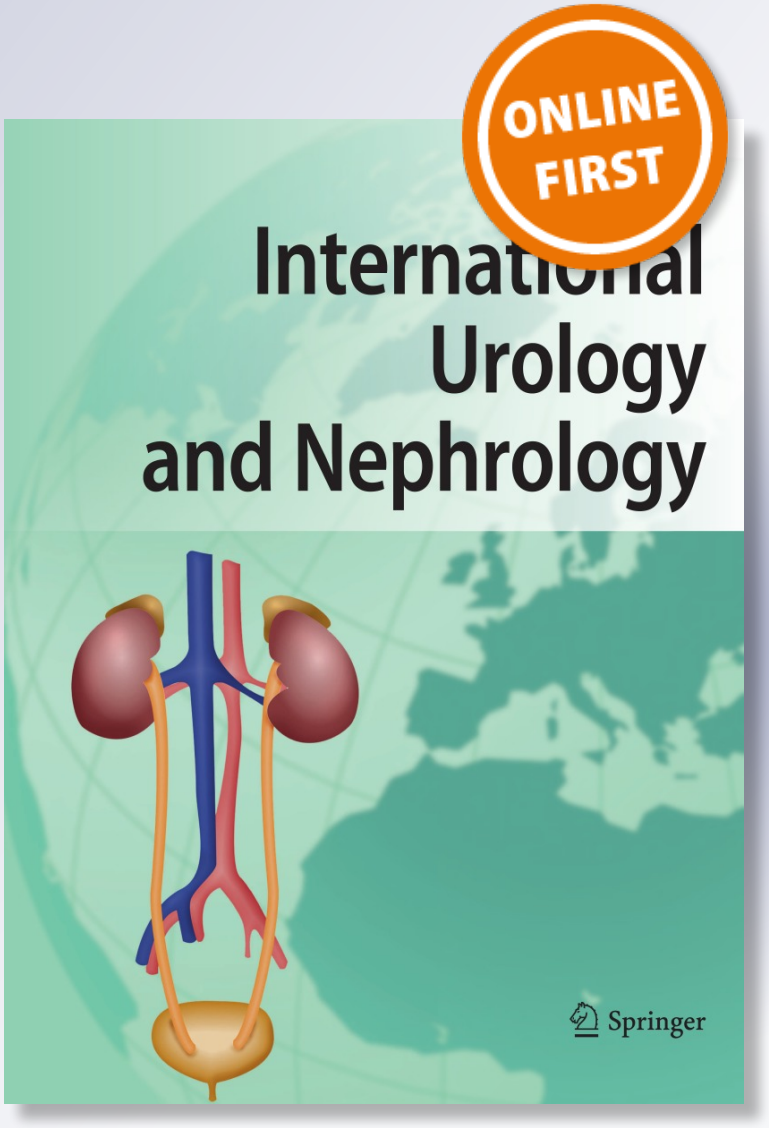

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Protective effects of adiponectin on uncoupling of glomerular VEGF–NO axis in early streptozotocin-induced type 2 diabetic rats

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Abstract
Purpose To determine whether adiponectin could reduce microalbuminuria and provide renal protective effects by improving endothelial dysfunction and uncoupling of the glomerular vascular endothelial growth factor (VEGF)–nitric oxide (NO) axis in streptozotocin-induced type 2 diabetic rats.
Methods Wistar rats were randomly divided into normal control group, diabetic nephropathy (DN) group induced by high-fat feeding and streptozotocin, diabetic rats injected with adenovirus-expressed adiponectin (AD-AdipoQ), and diabetic rats injected with AD-IRES-EGFP as control. Blood and urine samples were collected. Endothelium-dependent vasodilatation (EDV) of the aorta was measured. Renal tissues were collected for CD34 immunohistochemistry. Glomerular NO and VEGF levels were measured by the Griess reaction and Western blot testing, respectively.
Results Injections of AD-AdipoQ significantly increased serum adiponectin levels and reduced the urinary albumin-to-creatinine ratio in diabetic rats ($P < 0.05$). The levels of plasma glucose, serum insulin, high-sensitivity C-reactive protein, and malondialdehyde were significantly reduced in diabetic rats after injections of AD-AdipoQ ($P < 0.05$). Severe EDV impairment was observed in the DN group, which was improved by AD-AdipoQ. CD34 expression in the glomeruli was also higher in diabetic rats, indicating increased proliferation of glomerular endothelial cells. However, AD-AdipoQ improved the increased proliferation of endothelial cells in the glomeruli. Diabetic rats showed increased glomerular VEGF levels and reduced NO levels. This uncoupling of the VEGF–NO axis was partially improved by AD-AdipoQ.
Conclusion Adiponectin reduces the degree of microalbuminuria and has renal protective effects by improving endothelial dysfunction and uncoupling of the glomerular VEGF–NO axis in early diabetic nephropathy.

Keywords Adiponectin · Microalbuminuria · Vascular endothelial growth factor · Nitric oxide · Diabetic nephropathy

Introduction

Diabetic nephropathy is one of the most severe, long-term, microvascular complications of diabetes mellitus and is the dominant cause of end-stage kidney disease [1]. It is characterized by increasing levels of albuminuria, glomerular sclerosis, and the eventual decline of the glomerular filtration rate, often over a period of 10–20 years. Early diabetic nephropathy is defined as persistent microalbuminuria with an albumin excretion of 30–300 mg per 24 h, or an albumin-to-creatinine ratio (ACR) of 30–300 mg/g [1]. Microalbuminuria, an early marker of renal damage, is also an independent risk factor for ischemic cardiovascular disease [2, 3]. This may be caused by generalized endothelial dysfunction, which is usually seen in diabetic nephropathy [2]. The relationship between vascular endothelial growth factor (VEGF) and nitric oxide (NO), known as the VEGF–NO axis, plays an important role in maintaining glomerular endothelial cell function [4,

N. Hou · N. Huang · J. Zhao · X. Liu · X. Sun (✉)
Department of Endocrinology, Affiliated Hospital of Weifang Medical University, No. 2428, Yuhe Road, Weifang 261031, China
e-mail: sxdfriend@sina.com

F. Han
Department of Pathology, Affiliated Hospital of Weifang Medical University, Weifang, China

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5]. In diabetes, high glucose levels cause endothelial dysfunction by reducing NO, which could result in increased expression of VEGF and an uncoupling of glomerular VEGF from NO, thus leading to increased vascular permeability and albumin leakage. This uncoupling of the VEGF–NO axis is considered to be an important mechanism of early diabetic nephropathy [4, 6].

Adiponectin is a 30-kDa protein secreted by adipocytes and is an insulin-sensitizing, anti-inflammatory, and vascular protective cytokine. Adiponectin level is an important predictive factor for cardiovascular mortality in patients with renal dysfunction and plays a protective role in improving renal disease [7]. Adiponectin improves NO bioavailability and endothelial dysfunction [8]; however, the chronic effects of adiponectin on the glomerular VEGF–NO axis in early type 2 diabetic nephropathy remain to be fully elucidated. We hypothesized that adiponectin could have renal protective effects by improving endothelial dysfunction and improving the uncoupling of the glomerular VEGF–NO axis in early diabetic nephropathy. In this study, we examined the effects of adiponectin on impaired endothelial function and urinary albumin excretion in streptozotocin-induced type 2 diabetic rats. We also investigated the possible mechanisms of action of adiponectin on the VEGF–NO axis in glomerular endothelial cells.

Materials and methods

Materials

Streptozotocin (STZ), norepinephrine (NE), acetylcholine (Ach), and sodium nitroprusside (SNP) were purchased from Sigma (St. Louis, MO, USA). The insulin radioimmunoassay kit was purchased from Linco Research, Inc. (St. Louis, MO, USA). High-sensitivity C-reactive protein (hs-CRP) assay kits were purchased from USCN Life Science Inc. (Wuhan, China). NO and malondialdehyde (MDA) assay kits were purchased from Beyotime Biotechnology (Beijing, China). Urinary albumin radioimmunoassay kits were purchased from Beijing North Institute of Biological Technology (Beijing, China). VEGF antibodies and CD34 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Adenovirus expressing adiponectin (AD-AdipoQ-IRES-EGFP, AD-AdipoQ) and control AD-IRES-EGFP (AD-IRES) were constructed by Shanghai R&S Biotechnology Co., Ltd (Shanghai, China).

Experimental animals

Six-week-old male Wistar rats (specific pathogen-free quality) were purchased from Shandong Lukang Pharmaceutical Limited Company (Jining, China). The study was approved by the local ethics committee for animal studies and conducted following the Institutional Animal Care and Use Committee approval. After 1 week on a regular diet (330 kcal per 100 g), 40 rats were randomly divided into a normal control (NC) group ($n = 10$) and a diabetic group ($n = 30$). Rats in the NC group were fed with a regular diet, and rats from the diabetic group were fed a high-fat diet (50.10 % fat, mainly saturated, 493 kcal per 100 g) [9]. After 8 weeks, the diabetic group was induced by intraperitoneal injection of STZ (30 mg/kg, dissolved in 0.1 mmol/L citrate buffer, pH 4.5) while the NC group was given the same volume of vehicle citrate buffer. Blood glucose concentrations were measured 72 h after the STZ injection. Only the rats with glucose concentrations higher than 16.7 mmol/L were regarded as successful diabetic models [10, 11]. This diabetic model is similar to type 2 diabetes in humans, because the combination of high-fat intake and low-dose STZ effectively induces type 2 diabetes by altering the related gene expressions in major metabolic tissues [10, 12].

Four weeks after STZ injection, excluding dead rats and rats that failed to have a type 2 diabetes model induced, 24 diabetic mellitus rats with urinary ACR of 30–300 mg/g were accepted as the early stage of diabetic nephropathy and randomly divided into three groups: the diabetic nephropathy (DN) group ($n = 8$); the DA group, diabetic nephropathy rats injected intravenously from the tail with 0.71×10^9 IU of AD-AdipoQ ($n = 8$); and the DI group, diabetic nephropathy rats injected intravenously from the tail with the same amount of AD-IRES ($n = 8$). Rats in the NC group and the DN group were injected with the same amount of physiological saline in the same manner. All the rats were housed under standard laboratory conditions with controlled room temperature, humidity, and 12-h light–dark cycled light control.

Study protocol

Two weeks after the injection, before the rats were killed, urine was collected from each rat and stored at −20 °C after centrifugation at 3,000×g for 5 min for the assessment of urinary albumin and creatinine. The rats were killed under anesthesia by intraperitoneal injection of sodium pentobarbital (60 mg/kg). The thoracic aorta was then obtained immediately, dissected from adhesive tissue, and equilibrated in Krebs–Henseleit bicarbonate buffer (K–H solution) for measurement of endothelial function. Blood samples were collected from the left ventricular apex, and serum aliquots were stored at −20 °C after centrifugation at 3,000×g for 10 min for further use as described below. Portions of the kidney were fixed in 10 % buffered formalin for hematoxylin and eosin (HE) staining and CD34

immunohistochemistry. Isolated glomeruli were collected by the mechanical graded sieving technique (a series of screens of 80 mesh, 120 mesh, and 200 mesh) for NO and reactive oxygen species (ROS) assay. The suspension of the glomeruli was then used for protein isolation. Samples of renal tissues were partly isolated and fresh-frozen at −20 °C away from light. Light was avoided for frozen section and fluorescence microscopy to observe the expression of fluorescence of renal tissue. Abdominal fat tissue (including perirenal, epididymal, and mesenteric fat) was collected and weighed.

Evaluation of endothelial function

As has been described previously [9], each thoracic aorta vascular ring was then cut into 3-mm ring segments and immersed in individual organ chambers filled with 10 mL K–H solution. The rings were mounted on two steel wires: one steel wire was fixed and the other was inserted into the lumen and attached to the chamber and to an isometric force displacement transducer (PowerLab, AD Instruments) to record force changes. The solution was continuously gassed with 95 % O_2 and 5 % CO_2 and maintained at 37 °C. Rings were equilibrated for 60–90 min with resting tension of 1.0 g. During this time, tissues were washed every 30 min with the K–H solution. Before adding the Ach (10^{-8}–10^{-4} mol/L) or SNP (10^{-8}–10^{-4} mol/L) to the solution to assess the EDV or endothelium-independent vasodilatation response of the artery rings, NE (1 μM) was added to induce the precontraction. Data were used to prepare concentration–relaxation response curves.

Plasma measurements

Plasma glucose level was measured using the glucose oxidase method. Plasma insulin was measured by radioimmunoassay according to the manufacturer's instructions. The concentrations of total cholesterol, triglyceride, and malondialdehyde (MDA) were measured with colorimetric assays. Serum hs-CRP was assayed with an enzyme-linked immunosorbent assay kit. Serum creatinine concentrations were measured by the Jaffe method. All tests were done according to the manufacturer's instructions.

Measurement of urinary albumin excretion

Albumin-to-creatinine ratio (ACR) was used as the index of urinary albumin excretion. Urinary albumin was measured using a commercially available radioimmunoassay kit. Urinary creatinine was measured using the Jaffe method, according to the manufacturer's instructions.

Histology and immunohistochemistry

The renal tissues were fixed in 10 % neutral-buffered formalin, embedded in paraffin, cut into 4-μm-thick slices, and stained with HE for renal morphologic examination. Glomerular CD34 (endothelial cell marker) protein was measured with immunohistochemistry.

Measurements of NO and ROS in the isolated glomeruli

NO and ROS concentrations in the glomeruli were measured using the Griess and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) methods, respectively [5, 13]. NO is unstable under physiologic conditions; it oxidizes easily to nitrite (NO_2^-) and further to nitrate (NO_3^-). Levels of NO_2^-/NO_3^- were detected using a colorimetric assay following the manufacturer's instructions. For ROS determination, isolated glomeruli were incubated in RPMI-1640 containing 0.5 mM DCFH-DA (excitation 490 nm, emission 530 nm) for 60 min. The mean fluorescence intensity of the isolated glomeruli was analyzed using a fluorescence microplate reader [9].

Western blot analysis

Portions of the isolated glomeruli protein were electrophoresed using 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were then transferred onto nitrocellulose membranes. The filters were blocked with a solution of Tris-buffered saline containing 5 % fat-free milk and 0.1 % Tween-20 (TBST) at room temperature for 60 min, and then, the membranes were probed with anti-VEGF, anti-eNOS, or anti-phospho-eNOS antibody and washed three times with TBST. We incubated the membranes with the appropriate secondary antibodies for 60 min at room temperature and washed them three times with TBST. We stained the products with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. A Pro-plus 5.0 image-processing system was used to quantify the proteins.

Statistical analysis

Date were analyzed statistically using SPSS 13.0 statistical package. All data were presented as mean ± standard deviation (SD) for normally distributed variables and median (interquartile range) for skewed variables. Statistical analysis was performed. One-way ANOVA was used to perform the statistical analysis. The number of intergroup differences was detected using Student–Newman–Keuls (SNK) method. Level of significance was set at a P value of <0.05.

Table 1 Biometric and blood parameters of rats in the studied groups

Group	NC ($n = 8$)	DN ($n = 8$)	DA ($n = 8$)	DI ($n = 8$)
Weight (g)	509.3 ± 26.6	467.9 ± 44.5*	494.6 ± 96.7	495.2 ± 94.9
Visceral fat (g)	13.81 ± 1.69	27.79 ± 4.24*	32.02 ± 8.13*	27.30 ± 10.85*
Visceral fat/weight (10^{-3})	27.10 ± 2.39	60.60 ± 14.98*	60.80 ± 11.95*	51.40 ± 15.36*
Glucose (mmol/L)	5.6 ± 0.7	21.2 ± 3.3*	11.3 ± 5.5$^{\#}$	22.7 ± 4.0*
Insulin (μU/ml)	10.19 ± 0.89	20.91 ± 2.78*	16.70 ± 2.40*,$^{\#}$	22.42 ± 2.02*
Serum creatine (μmol/L)	45.56 ± 4.53	49.53 ± 4.89	50.10 ± 3.55	51.12 ± 3.25
Hs-CRP (mg/L)	0.69 ± 0.10	3.13 ± 0.38*	1.85 ± 0.56*,$^{\#}$	2.96 ± 0.31*
MDA (μmol/L)	1.43 ± 0.10	4.80 ± 0.59*	2.44 ± 0.28*,$^{\#}$	4.46 ± 0.58*
Adiponectin (μg/L)	190.99 ± 32.80	94.97 ± 25.12*	273.50 ± 21.20*,$^{\#}$	108.93 ± 15.83*

Data are shown as mean ± SD

Hs-CRP high-sensitivity C-reaction protein, *MDA* malondialdehyde

* $P < 0.01$ versus NC group;
$^{\#}$ $P < 0.01$ versus DN group

Results

Biometric and blood parameters

Table 1 illustrates the biometric and blood parameters of the rats in the study groups. The rats in the DN group, the DA group, and the DI group had increased visceral fat mass compared with the rats in the NC group. STZ-injected rats in the DN group had severe hyperglycemia and high levels of insulin ($P < 0.05$). This was reduced by intravenous injections of AD-AdipoQ ($P < 0.05$). Serum creatinine levels were not different between any of the treatment groups ($P > 0.05$). Serum hs-CRP and MDA levels increased and serum adiponectin levels decreased significantly in the DN group compared with the NC group ($P < 0.05$). Intravenous injections of AD-AdipoQ reduced serum hs-CRP and MDA levels and increased serum adiponectin levels in diabetic rats ($P < 0.05$). There were no significant differences in these parameters between the DN and DI groups.

Urinary albumin excretion

ACR was significantly higher in rats in the DN group than those in the NC group [180.35 (113.54–233.29) vs 13.15 (8.93–17.95) mg/g, $P < 0.01$]. After 2 weeks of adenovirus injections, ACR was significantly reduced in rates in the DA group compared with those in the DN group [72.0 (46.18–99.41) vs 180.35 (113.54–233.29) mg/g, $P < 0.01$], indicating an improvement in early diabetic nephropathy. There was no difference between rats in the DI group and those in the DN group [184.50 (116.98–275.99) vs 180.35 (113.54–233.29) mg/g, $P > 0.01$].

Endothelial function

Concentration-dependent vasorelaxation in response to Ach was attenuated in the rats in the DN group compared with those in the NC group, indicating significant endothelial dysfunction in diabetic rats. Treatment of diabetic rats with AD-AdipoQ enhanced Ach-induced vasorelaxation and improved endothelial function (Fig. 1). However, treatment of diabetic rats with AD-IRES did not improve endothelial function. Concentration-dependent vasorelaxation in response to SNP remained unchanged in these four groups (Fig. 1).

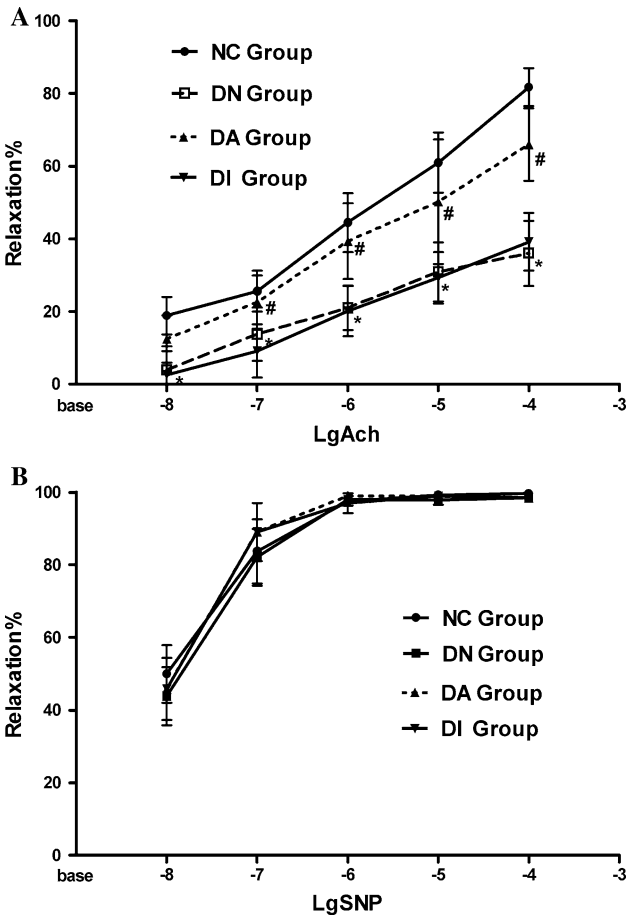

Fig. 1 Vasorelaxation response of thoracic aorta segment to Ach and SNP in the studied groups. **a** Concentration-dependent vasorelaxation of aortic segments in response to Ach. **b** Vasorelaxation response of aortic segments in response to SNP. Data are shown as mean ± SD. *$P < 0.05$ versus NC group. $^{\#}P < 0.05$ versus DN group

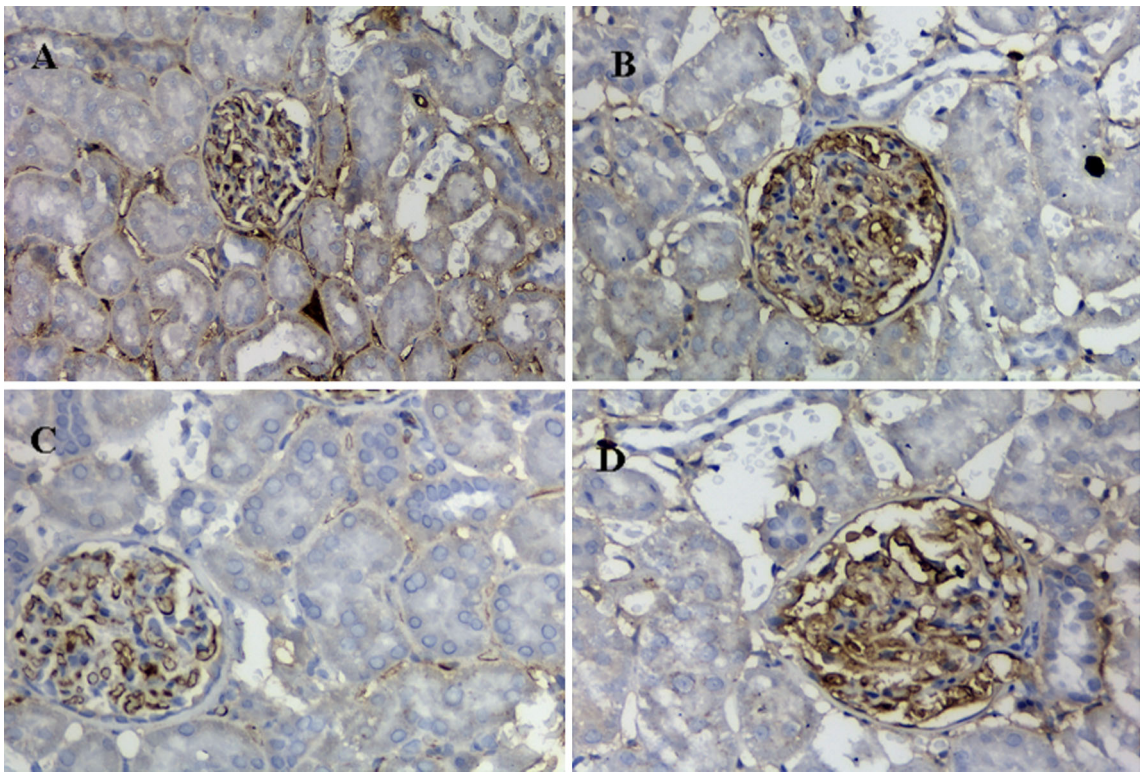

Fig. 2 CD34 expressions in the glomeruli in the studied groups (magnifications: ×400). *Brown color* indicates CD34 staining as a marker of endothelial cell in NC group (**a**), DN group (**b**), DA group (**c**), and DI group (**d**)

Histology and immunohistochemistry

Morphological observation of kidney tissues from diabetic rats revealed glomerulomegalia-increased interstitial inflammatory cells and Bowman's capsular expansion. CD34 expression in the glomeruli was also enhanced in diabetic rats, indicating increased proliferation of glomerular endothelial cells [mean optical density (IOD/area): 0.145 ± 0.015 vs 0.073 ± 0.007, $P < 0.05$]. However, treatment with AD-AdipoQ reduced the glomerular hypertrophy and partly improved the increased proliferation of endothelial cells in glomeruli (IOD/area: 0.113 ± 0.012 vs 0.145 ± 0.015, $P < 0.05$) (Fig. 2d–f). There was no significant improvement in morphological changes or CD34 expression in glomeruli in rats in the DN and DI groups.

Detection of NO and ROS in the glomeruli

The diabetic rats had low NO levels and high ROS production in the glomeruli compared with the control group (Fig. 3). In contrast, AD-AdipoQ intervention significantly increased NO production and decreased ROS production in the glomeruli of diabetic rats. There was no significant difference of NO or ROS levels between rats in the DN group and those in the DI group.

Western blot for VEGF level in the glomeruli

Diabetic rats showed increased glomerular VEGF levels compared with normal control rats ($P < 0.05$). However, AD-AdipoQ intervention prevented the increase in VEGF levels ($P < 0.05$) (Fig. 4).

Discussion

We demonstrated that adiponectin reduces the degree of microalbuminuria and has renal protective effects by improving endothelial dysfunction and uncoupling the glomerular VEGF–NO axis in early diabetic nephropathy.

Adiponectin, secreted by adipocytes, has been shown to be lower in patients with diabetes and to have beneficial effects in improving glucose metabolism in patients with diabetes [14]. We developed a chronic adiponectinemic state by giving AD-AdipoQ to diabetic rats. AD-AdipoQ targets the liver, with 90 % of hepatocytes being infected and secreting adiponectin into the circulation, as previously reported [7]. We found that serum adiponectin levels in diabetic rats increased nearly threefold after injection of AD-AdipoQ, indicating successful induction of a chronic adiponectinemic state in diabetic rats.

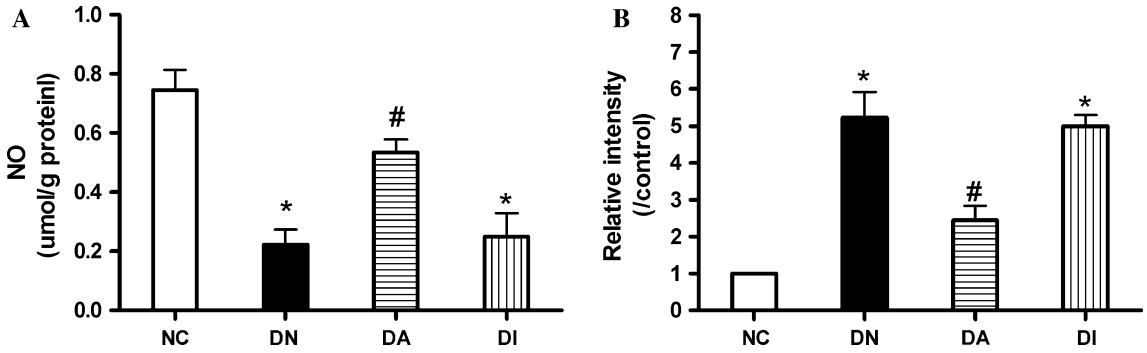

Fig. 3 Production of NO (**a**) and ROS (**b**) in glomeruli in the studied groups

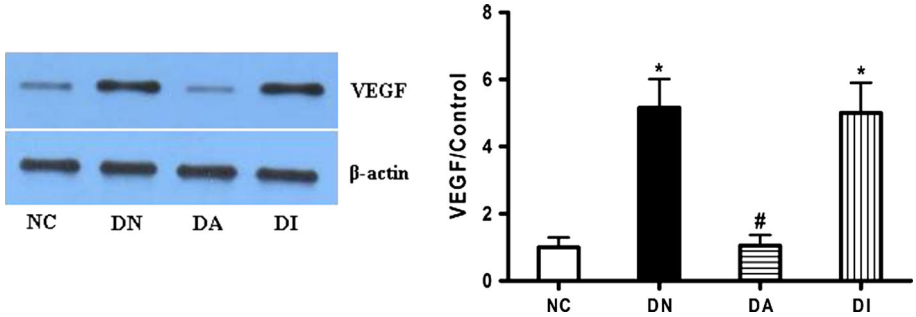

Fig. 4 Western blot analysis for the levels of VEGF proteins in glomeruli. In the DN group, the levels of VEGF protein were significantly higher than those in the control. AD-AdipoQ treatment tended to decrease the levels of VEGF protein compared with those in DN group

Adiponectin improves diabetic nephropathy [7]. We also found that ACR was significantly higher in rats in the DN group than in those of the NC group. After 2 weeks of adenovirus injection of AD-AdipoQ, the ACR of diabetic rats was significantly reduced, indicating an improvement in early diabetic nephropathy. This is consistent with other studies [7].

Patients with diabetic nephropathy have the highest chance of developing cardiovascular disease. The mechanism may be generalized endothelial dysfunction, which is usually seen in diabetic patients [15]. Endothelial dysfunction precedes and predicts the onset of microalbuminuria [16, 17]. Because the glomerular endothelium is part of the generalized endothelium, it is highly likely that this is also dysfunctional. This raises the question of how glomerular endothelial dysfunction could lead to microalbuminuria. Glomerular endothelial cell function is regulated by the glomerular VEGF–NO axis [18]. VEGF, a potent angiogenic cytokine synthesized in podocytes, has vascular permeability and stimulates endothelial cell proliferation by binding to VEGF receptors in glomerular endothelial cells. VEGF could promote eNOS phosphorylation through the PI3 K/AKT pathway, stimulate endothelial NO release and production, and thus maintain normal glomerular endothelial cell function [19]. Elevated NO could inhibit the excessive production of VEGF to protect the endothelium cells. In addition, reduced NO levels could increase VEGF levels and an uncoupling of VEGF from NO [20]. We found that in rats in the DN group, EDV of thoracic aorta was impaired and, despite higher glomerular VEGF levels, the glomerular NO level was lower and was accompanied by increased CD34 expression in glomeruli compared with normal control rats. This indicates that the DN group had generalized endothelial dysfunction and uncoupling of the glomerular VEGF–NO axis, which is consistent with previous studies in early DN [4, 6, 20]. However, adiponectin improved EDV, increased glomerular NO levels, and reduced glomerular VEGF and CD34 expression, which indicates that adiponectin could improve endothelial function, reduce the proliferation of glomerular endothelial cells and improve the uncoupling of the glomerular VEGF–NO axis.

There are some mechanisms to explain how adiponectin could improve endothelial function and uncoupling of the glomerular VEGF–NO axis in early diabetic nephropathy. First, NO is essential for endothelial function, and hyperglycemia in diabetes could inhibit AKT-mediated eNOS phosphorylation, which is essential for the generation of NO. In our study, adiponectin could improve glucose metabolism, and injections of adiponectin resulted in a significant reduction in glucose, which has the potential to increase endothelial NO levels. In addition, ROS induced by hyperglycemia and inflammation could quench NO to form peroxynitrite and reduce NO bioavailability. We found that serum MDA and hs-CRP and glomerular ROS production significantly increased in diabetic rats. Injection

of AD-AdipoQ reduced serum MDA and hs-CRP levels and also reduced ROS production in glomeruli. These results indicate that adiponectin could improve the glomerular VEGF–NO axis by reducing inflammation and oxidative stress. Finally, decreased production and/or bioactivity of NO impaired endothelial function and caused uncoupling of the VEGF–NO axis in diabetic rats. Some studies have shown that adiponectin also increases the phosphorylation of eNOS and NO production in endothelial cells through the AMPK signal pathway, which may be an alternate means of regulating endothelial function [8, 21]. Our findings also showed that adiponectin significantly enhanced total production of NO from glomeruli in diabetic rats, compared with diabetic rats that were not given adiponectin.

In summary, we have shown that adiponectin reduces the degree of microalbuminuria and has renal protective effects by improving endothelial dysfunction and uncoupling the glomerular VEGF–NO axis in early diabetic nephropathy. Together, these results indicate that adiponectin treatment could serve as therapy for patients with early diabetic nephropathy of type 2 diabetes.

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Conflict of interest The authors declare that they have no conflict of interest.

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