Doxazosin attenuates renal matrix remodeling mediated by anti-α_1-adrenergic receptor antibody in a rat model of diabetes mellitus

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Abstract. Diabetic nephropathy is a major complication of diabetes mellitus (DM). Recent studies suggest that immunological mechanisms have a key role in the pathogenesis of DM, therefore these mechanisms may be important targets for diabetes therapy. The present study evaluated the effects of anti-α_1-adrenergic receptor antibody (α_1-R Ab) mediation and doxazosin treatment in a rat model of DM. It was observed that levels of 24-h urinary protein, serum creatinine and transforming growth factor-β_1 in DM were significantly increased after α_1-R Ab mediation (all $P<0.05$). In addition, electron microscopy identified severe damage in the renal tissue microstructures of DM rats following α_1-R Ab mediation, while only mild abnormalities were observed in that of healthy rats mediated with α_1-R Ab and of untreated DM rats. No marked abnormalities were observed in the renal tissue of healthy blank controls. Furthermore, in DM rats treated with α_1-R Ab mediation + doxazosin intervention, the expression of TGF-β_1 significantly decreased, and renal functions and renal matrix remodeling were significantly improved, relative to untreated DM controls ($P<0.01$). These results suggest that α_1-R Ab may be involved in renal matrix remodeling during DM, and that kidney protection during DM may be achieved through treatment with corresponding receptor antagonists.

Introduction

Diabetes mellitus (DM) is a disease of the endocrine system disease with a complicated pathogenesis, and is the third biggest threat to human health after cancer and cardiovascular disease (1). There are currently ~250 million worldwide cases of DM (2), and it is estimated that this number will increase to 400 million by 2030 (3,4). China currently has the largest population of DM patients, where early onset of the disease is being more prevalent (5). In addition, large vessel and capillary complications caused by DM impair the quality of life of DM patients, and are a major cause of morbidity and mortality (1,6). All organs and viscera within DM patients are injured to some extent during disease pathogenesis, with diabetic nephropathy (DN) being particularly prevalent (7). Routine treatments for DN include strict regulation and control of blood glucose and the use of renin-angiotensin system suppressants to control blood pressure (8). Although these treatments may sufficiently control blood glucose and blood pressure, few direct treatment strategies exist for the kidneys. It is insufficient to characterize the pathogenesis of DN using only blood parameters, including hemodynamic disturbance and hyperglycemia, as immunology may also have a key role in the pathogenesis and complications of DN (9).

Previous studies demonstrated that the incidence and progression rates of DN are markedly higher in patients that express autoantibodies (Auto Ab) to major receptors, including the angiotensin (AT_1), α_1 adrenergic (α_1), and β_1 adrenergic (β_1), than in those without, regardless of sufficient blood glucose and pressure control (10,11). Thus, the onset of DN may be related to levels of Auto Ab, though the involvement of Auto Abs in DN-related renal changes is not well understood. It has been suggested that DN may be an inflammatory disease that develops secondary to the metabolic disturbance that occurs during DM (12). In a pathological state, native kidney cells produce a variety of pro-inflammatory factors and inflammatory mediators, including nuclear factor-κB (NF-κB) (13), osteopontin (OPN), transforming growth factor-β_1 (TGF-β_1) and tumor necrosis factor-α (TNF-α), which may amplify inflammation initiated by autocrine and paracrine signaling and result in a cascade of inflammatory reactions (14,15). Of these factors, NF-κB is considered to have a primary modulatory role. Activated NF-κB may subsequently activate transforming growth factor-α (TGF-α), interleukin-1 (IL-1)

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and monocyte chemotactic protein-1 (MCP-1), and also lead to glomerular hypertrophy, a decreased glomerular filtration rate, a thickened glomerular basement membrane, mesangial cell proliferation, deposition of inflammatory cells and extracellular matrix (ECM) (16). In addition, active NF-κB may induce the generation of inflammatory factors, such as TGF-β_1, resulting in a cascade of reactions and enhanced inflammation (16). TGF-β_1 is recognized as a primary fibrogenic factor that promotes ECM generation while inhibiting ECM degradation through multiple pathways, which may lead to over-production of the ECM and renal matrix remodeling (17). Under normal conditions, the NF-κB heterodimer complex (composed of P50 and P65 subunits) binds to its cognate inhibitor protein (IκBα) and form the conjugate, which occurs in the cytoplasm of the majority of cells, and free P65 is rarely expressed in cell nuclei to maintain normal physiological functions (18). However, upon activation of factors, such as TGF-β_1 and protein kinase C (PKC), P65 migrates to the nuclei and induces the production of NF-κB heterodimers (17-21). PKC is a downstream transduction molecule of G protein-coupled receptor (GPCR) signaling, while serum α_1-adrenergic receptor antibodies (α_1-R Ab) are the cognate Abs of α_1-adrenergic GPCRs.

GPCRs are a group of membrane glycoproteins that bind guanosine triphosphate and include the AT_1, α_1, β_1 and M_2 receptors. GPCRs are the largest family of cell membrane receptors involved in signal transduction and mediate signals that regulate renal functions and immune responses (22,23). A typical GPCR consists of transmembrane subunits composed of seven polypeptide chains, which form a spatial configuration with three extracellular loops and three intracellular loops (24). α_1-R Abs are a class M or G immunoglobulins (IgM/G) that are specific to the 192-218 amino acid sequence of the second extracellular peptide segment of α_1-R (25). A previous study observed that, following repeated stimulation, GPCR may produce Auto Abs by an internalization mechanism, which may subsequently simulate normal physiological signals (26). In particular, these Auto Abs may stimulate angiotensin (AT) and adrenalin may be stimulated to activate corresponding GPCRs, thus inducing similar effects to angiotensin II (AT II) (27) and adrenalin (26). After binding to receptors, α_1-R Ab may modulate a number of cellular functions in renal cortex-related cells, including proliferation, differentiation and metabolism, by activating the α_1-R Ab/GPCR/PKC/NF-κB/TGF-β_1 and/or α_1-R Ab/GPCR/PKC/NF-κB/OPN/TGF-β_1 signal transduction pathways. While key steps of the immunological responses that occur in the progression of DN have been identified, the mechanisms underlying the development of DN complications and therapeutic targets for the treatment of DN remain unknown.

A previous study demonstrated that α_1-R Ab was present in patients with primary and refractory hypertension (1). α_1-R Ab also increased the beat frequency of cultured myocardial cells, in a similar way to noradrenalin, and was blocked by prazosin (28,29), indicating that α_1-R Ab may have a key role in the development of hypertension. A previous clinical study (10) identified α_1-R Ab in the sera of DN patients with albuminuria, and clinical administration of α_1-R Ab significantly reduced proteinuria, indicating that α_1-R Ab may have an important role in the development of DN. However, the effects of α_1-R Ab on renal matrix remodeling are currently unknown.

In the present study, rat models of DM were used to determine the effects of α_1-R Ab on renal matrix remodeling in rats, as well as the potential effects of doxazosin, which is an α_1-adrenergic receptor blocker (30), in the attenuation of renal matrix remodeling and aberrant renal functions. The potential role of TGF-β_1 and the effects of doxazosin on the pathogenesis of DN were also evaluated. To investigate these mechanisms, α_1-R Ab was injected into rats via the caudal vein to investigate Ab mediation. Next, via the activation of GPCR, NF-κB was activated, and finally, inflammatory factors, including TGF-β_1, were activated to continually expand the inflammatory effects and result in inflammation cascade reactions. The results of this study may aid in the development of molecular targeted therapies for the treatment of DN in patients presenting with high levels of Auto Abs.

Materials and methods

Reagents and equipment. α_1-R Ab was synthesized and donated by Huazhong University of Science and Technology, as previously described (31) and streptozotocin (STZ; cat. no. S0130) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rabbit anti-mouse TGF-β_1 (cat. no. BA0290), immunohistochemical streptavidin-biotin complex (SABC) kits (cat. no. SA1025) and diaminobenzidine (DAB) colorant (cat. no. AR1022) were obtained from Boster Systems, Inc. (Plesanton, CA, USA). A glucose assay kit (Glucose Oxidase Peroxidase; cat. no. F006) was purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). A urinary albumin kit (cat. no. A028-1) for use in a radioimmunoassay was obtained from Beijing Furui Bioengineering Co., Ltd. (Beijing, China) and a serum creatinine (Scr) kit (cat. no. C011-2) was purchased from Nanjing Jiancheng Bioengineering Institute for use with a Beckman Syn Chron-LX20 full-automatic biochemical analyzer (Beckman Coulter, Inc., Brea, CA, USA). FEI Tecnai G2 20 TWIN electron microscopes were provided by the Wuhan Institute of Virology at the Chinese Academy of Sciences (Wuhan, China).

Preparation of high-fat feed. Rats used in the present study were given a high-fat feed comprised of 10% pork fat, 10% cane sugar, 25% cholesterol, 0.4% sodium cholate, and 77% basal feed (19% wheat flour, 23% corn flour, 6% sorghum flour, 10% bran, 15% soybean, 2% vegetable oil, 6.6% starch, 3.4% glycine, 0.5% calcium carbonate, 0.5% methionine, 1% saccharomyces cerevisiae, 1% natrii chloridum). The feed was prepared by thoroughly mixing and granulating all of the above ingredients.

Establishment of DM models and grouping of experimental animals. A total of 64 healthy male Wistar rats, 6 weeks old, weighing 160-170 g, were provided by the Laboratory Animal Center of Wuhan University (Wuhan, China). Following acclimation feeding for 1 week, the rats were weighted and randomized into four groups: the first experiment: i) Healthy blank control rats (n=16); ii) healthy rats with α_1-R Ab mediation (n=16); iii) DM rats without α_1-R Ab mediation (n=16);

and iv) DM rats with α_1-R Ab mediation (n=16). After being fed on a high-fat diet for 4 weeks, DM rats were fasted for 12 h and subsequently administered sterile STZ solution via intraperitoneal injection at 0.5 ml/100 g body weight. Before use, the STZ concentration was adjusted to 20 g/l with 0.1 mol/l sodium citrate buffer (pH 4.3). After 72 h, whole blood was drawn from the caudal vein and fasting blood glucose was determined using blood glucose meter purchased from Johnson & Johnson (New Brunswick, NJ, USA) according to manufacturer's protocol. Successful model establishment was defined as a fasting blood glucose of >16.7 mmol/l (3,4). Following 2 weeks, 32 rats were established DM model successfully. Following which, DM rats were fed basal feed until the end of a 16 week intervention. Rats in the control group ('healthy rat' groups) were given common feed throughout the experiment. For the duration of the experimental period, room temperature was maintained at 18-20˚C and the humidity at 69%. A 12-h light-dark cycle was used and rats were given free access to water and feed.

Establishment of an α_1-R Ab-mediated DM rat model. A total of 100 μg/100 g body weight α_1-R Ab was injected as a single dose (7) into the caudal vein of healthy and DM rats in the α_1-R Ab mediation groups at weeks 0, 4, 8, 12 and 16 after successful establishment of the DM model. An equal volume of physiological saline was injected as a single dose into the caudal vein of healthy blank control and DM rats in the non-α_1-R Ab mediation groups at weeks 0, 4, 8, 12 and 16.

Drug intervention grouping and drug administration. Another 48 healthy male Wistar rats, 6 weeks old, weighing 160-170 g, were provided by the Laboratory Animal Center of Wuhan University, and were maintained at 18-20˚C and a humidity of 69%. A 12-h light-dark cycle was used and rats were allowed *ad libitum* access to water and feed. Rats were randomized into 4 groups: i) DM rats without α_1-R Ab mediation (n=12); ii) DM rats with α_1-R Ab mediation (n=12); iii) DM rats with α_1-R Ab mediation + doxazosin intervention (n=12); and iv) DM rats with doxazosin intervention (n=12). The group of DM rats with α_1-R Ab mediation and DM rats with α_1-R Ab mediation + doxazosin intervention underwent the same treatment protocol as for α_1-R Ab rats. Doxazosin tablets (4 mg) were purchased from Pfizer, Inc., (New York, NY, USA; approval no. J20040073). A typical adult dose of doxazosin is 4 mg daily. The equivalent dose in rats, calculated by converting the body surface area ratio of laboratory animals and human beings (assuming a human adult body weight of 70 kg) was: (4 mg x 0.018x5) mg/kg=0.36 mg/kg. Doxazosin intervention was administered after the establishment of DM model, the groups of DM rats with α_1-R Ab mediation + doxazosin intervention and DM rats with doxazosin intervention were administered 0.36 mg/kg doxazosin by gavage, once/day, from the establishment of the DM model for 16 weeks.

Sample collection and preservation for all animals. At the end of the experiment, a metabolic cage was used to collect urine for 24 h. The samples were centrifuged (at 1,776 x g for 3 min at 25˚C) and the 5 ml of the supernatant was separated to detect the 24 h urinary proteins. At week 16 of intervention, rats were sacrificed and the blood and kidneys samples were harvested for measurements. Blood samples were obtained through the inferior vena cava after anesthesia with 1% pentobarbital sodium (50 mg/kg) through intraperitoneal injection, and the blood was centrifuged (at 999 x g for 10 min at 25˚C). From this, the upper serum was used to detect Scr and α_1-R Ab. Then, the kidneys were obtained from the abdominal cavity, cleared of connective tissue, washed with saline and fixed at 25˚C in 10% neutral formalin for measurements for 24 h.

α_1-R Ab assay. Autoantibodies were detected using an enzyme-linked immunoabsorbent assay (ELISA). Anti-α_1-R autoantibodies were detected as previously described (28-30,32). Peptide segments of the second extracellular loop of the α_1-R amino acid sequence were synthesized that comprised of residual segments of amino acids at sites 192-218 of α_1-R (amino acid residue sequence, G-W-K-E-P-V-P-P-D-E-R-F-C-G-I-T-E-E-A-G-Q-A-V-F-S-S-V). The purity of synthesized peptides, analyzed by high-performance liquid chromatography, was >95%. Blank (nothing added), positive (serum, antibody and the solution of antibody added) and negative controls (serum and the solution of antibody added) were used in the experiment. When measuring the absorbance (A), zero adjustment was performed using the blank control to ensure the validity of the test results. The antibody assay was defined as positive when the absorbance ratio of the study serum to the negative serum was >2.1, according to the following formula: Absorbance ratio = (Value of specimen - A value of blank control)/(A value of negative control - A value of blank control). For α_1-R Abs, the intra-batch coefficient of variation was 7.26% and the inter-batch variation was 10.1%.

Immunohistochemical assay of TGF-α_1 expression in renal tissue. Renal tissue was fixed at 25 C in 10% neutral formalin for 24 h and paraffin sections (3 μm) were prepared. Following routine deparaffination of sections and addition of rabbit anti-mouse TGF-β_1 (1:200), sections were incubated at 4˚C overnight. Sections were subsequently incubated with 1:400 goat anti-rabbit immunoglobulin G (cat. no. KS002; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 30 min at 4˚C, followed by incubation with SABC for 20 min at 4˚C, and washed 4 times for 3 min with PBS. Sections were then stained with DAB colorant and counterstained with hematoxylin and sealed using gum.

Image analysis of renal tissue. Renal tissues stained with anti-TGF-β_1 were analyzed using Image-Pro plus software, version 6.0 (Media Cybernetics, Rockville, MD, USA) under a light microscope (Olympus Corporation, Tokyo, Japan), using average luminosity and positive units as representatives, as described previously (33-35). Ten random images were captured of the renal cortex (including the renal glomerulus and renal tubule) of each renal section, and the average luminosity value was used as the result of the specimen for statistical analysis.

Statistical analysis. Experimental data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). All measurement data satisfied a normal distribution and homogeneity of variances. A paired-samples t-test was used

Table I. Comparison of 24-h Upro and Scr levels among the experimental groups.

Group	N	24-h Upro, mg/24 h	Scr, mmol/l
Healthy blank control rats	16	0.32±0.18	24.35±2.25
Healthy rats with α_1-R Ab mediation	16	0.47±0.13[a]	25.15±2.81
t	-	2.735	0.887
P-value	-	0.011	0.383
DM rats without α_1-R Ab mediation	16	1.59±0.26	27.02±2.71
DM rats with α_1-R Ab mediation	16	2.13±0.26[b]	33.26±2.25[b]
t	-	5.845	7.078
P-value	-	<0.001	<0.001

Data are presented as the mean ± standard deviation and were compared using an independent-samples t-test. [a]P<0.05 vs. healthy blank control rats; [b]P<0.05 vs. DM rats without α_1-R Ab mediation. Upro, urinary protein; Scr, serum creatinine; DM, diabetes mellitus; α_1-R Ab, anti-α_1-adrenergic receptor antibody.

to compare the body weights of rats in each group before and after the experiment. An independent-samples t-test was used to compare the levels of 24-h urinary protein (Upro; mg/24 h), Scr (mmol/l) and average luminosity and positive units of renal tissue TGF-β_1 among the healthy and intervention groups. After ruling out the degree of pure immunohistochemistry and smear of background factors, the degree of immunohistochemical reaction was divided into differing grades according to the gray level image, and each grade was defined as 1 positive unit. One-way analysis of variance was used to compare the 24-h Upro and Scr of rats in each group after α_1-R Ab intervention and doxazosin, and a Student-Newman-Keuls-q test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Changes in 24-h Upro and Scr of DM rats following α_1-R Ab mediation. As depicted in Table I, levels of 24-h Upro and Scr of DM rats with α_1-R Ab mediation were significantly increased when compared to DM rats without α_1-R Ab mediation (both P<0.001), indicating a degree of renal function impairment following α_1-R Ab mediation. In addition, the level of 24 h Upro of healthy rats with α_1-R Ab mediation were significantly increased when compared to healthy blank control rats (P<0.001; Table I).

Pathological changes in the renal tissues of rats following α_1-R Ab mediation. Proximal convoluted tubules, distal convoluted tubules and renal glomeruli of the renal tissue from healthy blank control rats (Fig. 1), healthy rats following α_1-R Ab mediation (Fig. 2), DM rats without α_1-R Ab mediation (Fig. 3) and DM rats with α_1-R Ab mediation (Fig. 4)

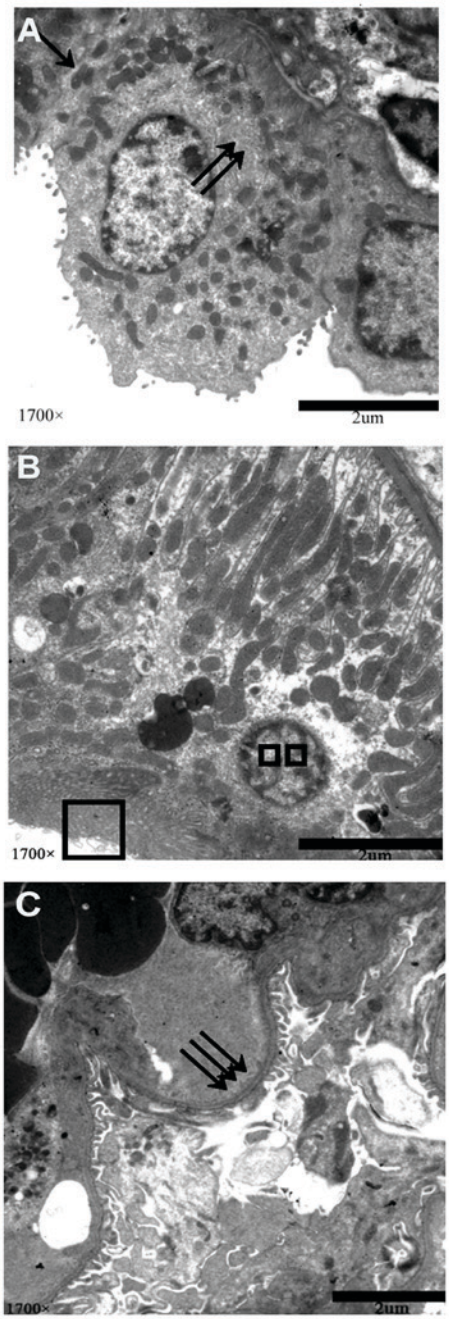

Figure 1. Electron microscopy of the renal cortex in healthy blank control rats. No apparent abnormalities were observed in the (A) proximal convoluted tubules, (B) distal convoluted tubules or (C) renal glomeruli. Magnification, x1,700. Lesions are demonstrated by arrow(s) or box(es); scale bar, 2 μm. Two arrows, cytoplasm; three arrows, basilar membrane; one box, lumen; two boxes, nucleus.

were observed under an electron microscope. No apparent abnormalities were observed in the healthy blank control rats. However, in the healthy rats with α_1-R Ab mediation, the proximal convoluted tubule exhibited cell swelling, liberated and suspended cellular organs, a dissolved cytoplasmic matrix, a loss of membrane structure and separation and disorder in the microvilli. Furthermore, the distal convoluted tubule exhibited concentrated cells and accumulation of residue in the lumen and the renal glomerulus exhibited uneven thickening of the basement membrane. The lesions were more severe in DM rats with α_1-R Ab mediation, compared with the slight lesions in

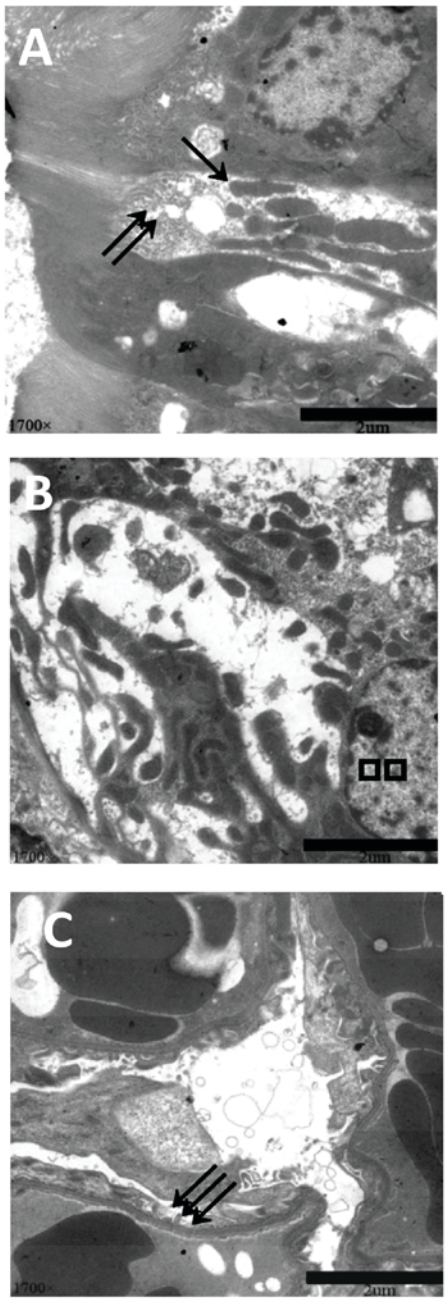

Figure 2. Electron microscopy of the renal cortex in healthy rats following anti-α_1-adrenergic receptor antibody mediation. (A) The proximal convoluted tubule exhibited cell and microvilli swelling, a dissolved cytoplasmic matrix and partially dissolved endoplasmic reticulum, a slightly increased nuclei concentration and irregular morphologies. (B) The distal convoluted tubule exhibited cell membrane destruction, a dissolved cytoplasmic matrix, rough endoplasmic reticulum and smooth endoplasmic reticulum, and partial destruction of chondriosomes. (C) The renal glomerulus exhibited increased interstitial space between foot cells and slightly increased endothelial cell concentration near the basement membrane. Figures are representative of a group of rats. Lesions are demonstrated by arrow(s) or box(es). Magnification, x1,700; scale bar, 2 μm. One arrow, organelle; two arrows, cytoplasm; three arrows, basilar membrane; two boxes, nucleus.

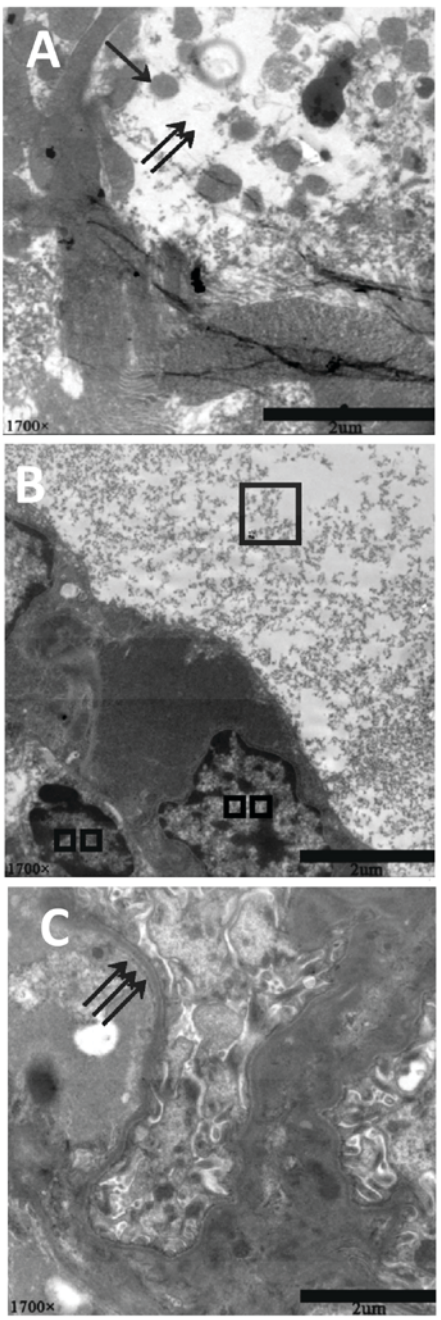

Figure 3. Electron microscopy of the renal cortex in rat models of diabetes mellitus lacking anti-α_1-adrenergic receptor antibody mediation. (A) The proximal convoluted tubule exhibited cell swelling, liberated and suspended cellular organs, a dissolved cytoplasmic matrix, a loss of membrane structure and separation and disorder in the microvilli. (B) The distal convoluted tubule exhibited concentrated cells and accumulation of residue in the lumen. (C) The renal glomerulus exhibited uneven thickening of the basement membrane. Figures are representative of a group of rats. Lesions are demonstrated by arrow(s) or box(es). Magnification, x1,700; scale bar, 2 μm. One arrow, organelle; two arrows, cytoplasm; three arrows, basilar membrane; one box, lumen; two boxes, nucleus.

DM rats without α_1-R Ab mediation (Figs. 1-4). All samples presented with a degree of degeneration following α_1-R Ab mediation. Renal structures of DM rats with α_1-R Ab mediation were markedly damaged, as indicated by thickening of the basement membrane, the formation of an interlayer and subsequent renal matrix remodeling.

TGF-a_1 expression in the renal cortex of rats following α_1-R Ab mediation. Using immunohistochemistry, the expression of TGF-β_1 was evaluated based on average luminosity and positive units. As depicted in Table II, it was observed that healthy rats and DM rats exhibited a higher degree of TGF-β_1 expression following α_1-R Ab mediation. In particular, markedly high levels of average gray value and number of positive

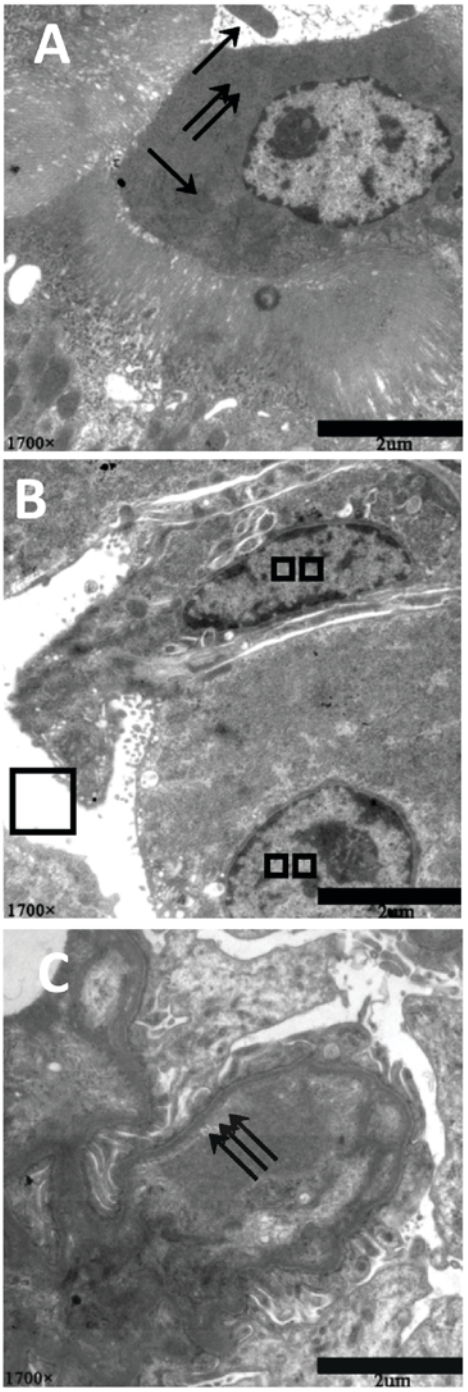

Figure 4. Electron microscopy of the renal cortex in in rat models of diabetes mellitus following anti-α_1-adrenergic receptor antibody mediation. (A) The proximal convoluted tubule exhibited a concentrated and homogenic cytoplasm, cellular organs were masked, swelling of the smooth endoplasmic reticulum and shedding of into the lumen. (B) The distal convoluted tubules exhibited concentrated cells, cellular organs are partially covered by the degeneration of intracellular glycogens, thickening of the outer nuclear envelope and widening of intercellular spaces. (C) The renal glomerulus exhibited thickening of the basement membrane and formation of interlayers. Lesions are demonstrated by arrow(s) or box(es). Magnification, x1,700; scale bar, 2 μm. One arrow, organelle; two arrows, cytoplasm; three arrows, basilar membrane; one box, lumen; two boxes, nucleus.

units of TGF-β_1 were identified in DM rats with α_1-R Ab mediation, which was deemed to be significant relative to DM rats without mediation (all P<0.01). In addition, the mean gray value and number of positive units of TGF-β_1 in healthy rats

Table II. Expression of TGF-e_1 in the renal cortex in each experimental group.

Group	N	Mean gray value	Number of positive units
Healthy blank control rats	16	114.88±9.33	21.56±3.40
Healthy rats with α_1-R Ab mediation	16	123.62±8.72[a]	25.17±3.54[b]
t	-	2.737	2.945
P-value	-	0.01	0.006
DM rats without α_1-R Ab mediation	16	127.24±10.55	32.34±6.36
DM rats with α_1-R Ab mediation	16	181.18±11.92[c]	40.66±6.22[d]
t	-	13.554	3.74
P-value	-	<0.001	0.001

Data are presented as the mean ± standard deviation and were compared using an independent-samples t-test. [a]P<0.05 and [b]P<0.01 vs. healthy blank control rats; [c]P<0.001 and [d]P<0.01 vs. DM rats without α_1-R Ab mediation. TGF, tumor growth factor; DM, diabetes mellitus; α_1-R Ab, anti-α_1-adrenergic receptor antibody.

with α_1-R Ab mediation were significantly increased when compared to healthy blank control rats (P<0.05; Table II). Observations by electron microscopy also identified markedly higher levels of TGF-β_1 expression in DM rats with α_1-R Ab mediation, relative to DM rats without α_1-R Ab mediation and each of the healthy rat groups (Fig. 5).

Changes in 24-h Upro and Scr of DM rats following doxazosin intervention. Levels of 24-h Upro in rats in each of the DM groups treated with doxazosin (DM + doxazosin intervention and DM + α_1-R Ab mediation + doxazosin intervention) were significantly decreased when compared to the DM groups without doxazosin intervention (DM rats without α_1-R Ab mediation and DM rats with α_1-R Ab mediation; P<0.01). Scr in rats with DM + α_1-R Ab mediation + doxazosin intervention group were significantly decreased when compared to DM rats with α_1-R Ab mediation (P<0.01). These results indicate that renal function improved following doxazosin intervention (Table III).

TGF-a_1 expression in the renal cortex of rats following doxazosin intervention. Following doxazosin intervention, levels of TGF-β_1 expression in DM rats with α_1-R Ab mediation + doxazosin intervention were significantly decreased when compared with the other groups that underwent α_1-R Ab mediation (P<0.01; Fig. 6 and Table IV), indicating that TGF-β_1 expression significantly improved in DM rats following α_1-R Ab mediation + doxazosin intervention.

Structural changes in the renal tissue of rats following doxazosin intervention. In DM rats with α_1-R Ab mediation + doxazosin intervention, cellular swelling and structural damage to the membrane of proximal convoluted tubules

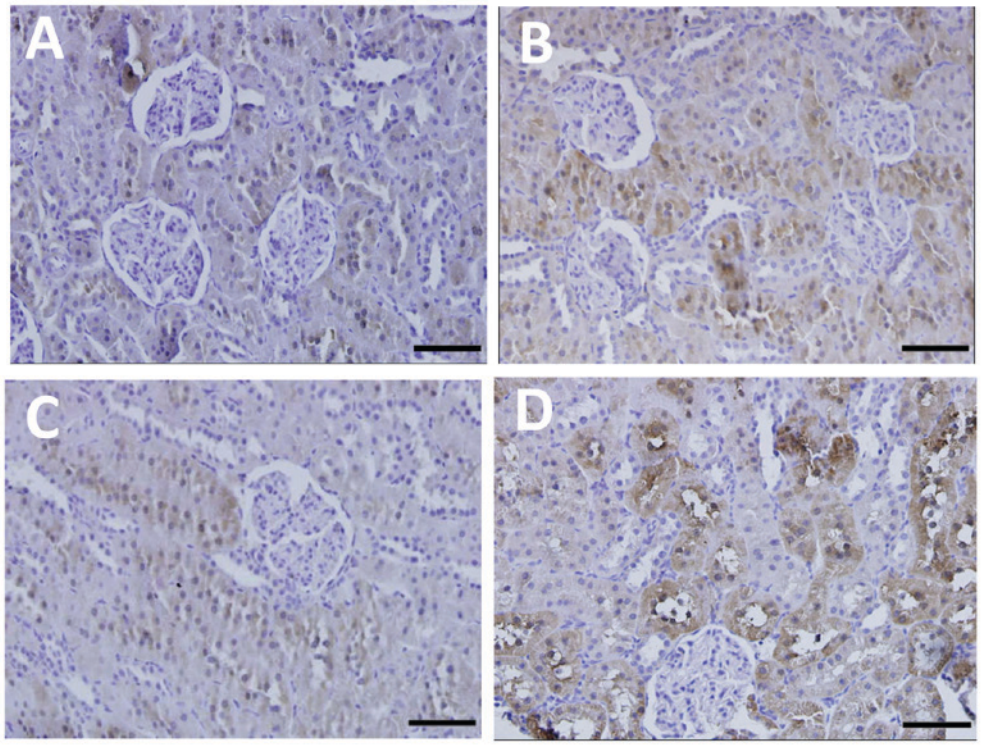

Figure 5. Expression of TGF-β_1 in the renal cortex. (A) Healthy blank control group. (B) Healthy rats with with α_1-R Ab mediation. (C) DM rats lacking α_1-R Ab mediation. (D) DM rats with α_1-R Ab mediation. Streptavidin-biotin complex stain (purple) indicates TGF-β_1 expression. Upon comparison of the average luminosity values in each group, it was observed that TGF-β_1 expression in DM rats significantly increased following α_1-R Ab mediation ($P<0.001$; Table II). TGF-β_1 expression in healthy rats with α_1-R Ab mediation significantly increased compared to that in healthy blank control rats ($P=0.010$; Table II). Comparison of positive unit values in each group: TGF-β_1 expression in DM rats with α_1-R Ab mediation significantly increased compared to that in DM rats without α_1-R Ab mediation ($P=0.001$). TGF-β_1 expression in healthy rats with α_1-R Ab mediation significantly increased compared to that in healthy blank control rats ($P=0.006$; Table II). Magnification, x400; scale bar, 50 μm. TGF, tumor growth factor; DM, diabetes mellitus; α_1-R Ab, anti-α_1-adrenergic receptor antibody.

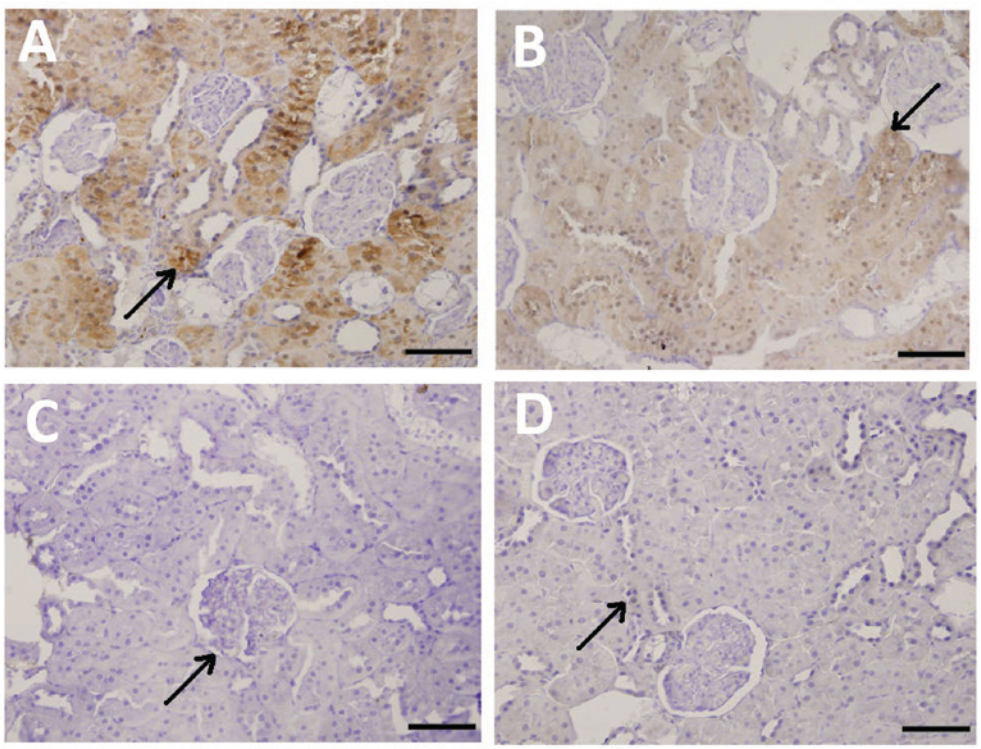

Figure 6. Expression of TGF-β_1 receptor antibody of rats with diabetes mellitus. (A) α_1-R Ab-mediated group; the arrow shows strongly positive units of the tubule. (B) Non-mediated group; the arrow shows medium positive units of the tubule. (C) Doxazosin alone group; the arrow shows none positive unit of the glomerulus. (D) Doxazosin + α_1-R Ab-mediated group; the arrow shows a non-positive unit of the tubule. Streptavidin-biotin complex stain (purple) indicates TGF-β_1 expression. Magnification, x400; scale bar, 50 μm. TGF, tumor growth factor; α_1-R Ab, anti-α_1-adrenergic receptor antibody.

Table III. Comparison of 24-h Upro and Scr levels among the experimental groups.

Group	N	24-h Upro, mg/24 h	Scr, μmmol/l
DM rats without α_1-R Ab mediation	12	1.58±0.09	28.08±1.00
DM rats with α_1-R Ab mediation	12	2.50±0.08[a]	37.81±1.42[a]
DM rats with α_1-R Ab mediation + doxazosin intervention	12	1.27±0.13[b]	27.21±1.72[b]
DM rats with doxazosin intervention	12	1.21±0.13[a]	27.88±1.06
F	-	1.708	1.305
P-value	-	0.179	0.285

The F and P-values are comparing the homogeneity of variance for the data. Data are presented as the mean ± standard deviation and were compared using a Student-Newman-Keuls-q test. [a]P<0.01 vs. DM rats without α_1-R Ab mediation; [b]P<0.01 vs. DM rats with α_1-R Ab mediation. Upro, urinary protein; Scr, serum creatinine; DM, diabetes mellitus; α_1-R Ab, anti-α_1-adrenergic receptor antibody.

Table IV. Expression of TGF-β_1 in the renal cortex in each experimental group.

Group	N	Mean gray value	Number of positive units
DM rats without α_1-R Ab mediation	12	131.77±6.25	29.97±4.99
DM rats with α_1-R Ab mediation	12	183.20±9.09[a]	44.08±4.91[a]
DM rats with α_1-R Ab mediation + doxazosin intervention	12	47.42±7.20[b]	6.25±4.30[b]
DM rats with doxazosin intervention	12	31.06±6.72[a]	6.68±3.76[a]
F	-	0.931	0.137
P-value	-	0.434	0.937

The F and P-values are comparing the homogeneity of variance for the data. Data are presented as the mean ± standard deviation and were compared using an SNK-q test. [a]P<0.01 vs. DM rats without α_1-R Ab mediation; [b]P<0.01 vs. DM rats with α_1-R Ab mediation. One-way analysis of variance was used to compare values of average luminosity and positive units of renal tissue TGF-β_1 between each group, and the variance was determined to be homogenous. An SNK-q test was used for multiple comparisons. TGF, tumor growth factor; DM, diabetes mellitus; α_1-R Ab, anti-α_1-adrenergic receptor antibody; SNK-q, Student-Newman-Keuls-q.

were markedly reduced when compared with DM rats treated with α_1-R Ab alone. In addition, mitochondrial cristae and reduced damage to the microvilli and smooth endoplasmic reticulum of the distal convoluted tubules was observed in the DM + α_1-R Ab mediation + doxazosin intervention group. Damage to foot cells, foot processes and basal membranes of the renal glomeruli were markedly improved in DM rats treated with α_1-R Ab mediation + doxazosin when compared to DM rats treated with α_1-R Ab alone (Fig. 7). In DM rats treated with doxazosin alone, damage to the proximal convoluted tubules, distal convoluted tubules and renal glomeruli were improved when compared to DM rats alone, though improvements were less marked than in DM rats treated with α_1-R Ab + doxazosin (Fig. 8).

Discussion

The activity of kidney cells, like other cells, involves the modulation of cellular signal transduction systems comprised of multiple components, including membrane and nuclear receptors (36,37). GPCRs account for the majority of membrane receptors (1). GPCRs, including the AT1, α_1, β_1 and M_2 receptors, are the largest family of cell membrane receptors involved in signal transduction. Signals mediated by GPCRs are critical in numerous cellular activities, including those related to the regulation of vision, renal functions and immune responses (38).

Regarding renal function, the present study observed that levels of 24-h Upro (mg/24 h) and Scr (μmmol/l) increased in DM rats following α_1-R Ab mediation; an effect deemed to be significant when compared with healthy rats and DM rats lacking α_1-R Ab mediation. These data indicated that α_1-R Ab mediation may have induced renal impairment in DM rats. In addition, microstructural changes in the proximal and distal convoluted tubules and collecting tubes of the kidneys of DM rats with α_1-R Ab mediation were observed. Other indicators of microstructural damage included increased cellular swelling and concentration, liberation of cellular organs, thickening of the basement membrane and formation of interlayers. By contrast, no structural abnormality was identified in the renal tissues of healthy blank control rats, and only slight and mild abnormalities were observed in healthy rats with α_1-R Ab mediation and DM rats without α_1-R Ab mediation, respectively. These data suggest that damage to the renal structures may have been associated with α_1-R Ab mediation.

Regarding renal TGF-β_1 expression, DM rats with α_1-R Ab mediation exhibited high expression of TGF-β_1, with significant increases in the mean gray value and number of positive units when compared with DM rats without α_1-R Ab mediation. As TGF-β_1 acts as a renal fibrogenic factor (39), high expression of renal TGF-β_1 in DM rats with α_1-R Ab mediation indicates that α_1-R Ab may promote renal matrix remodeling and subsequent renal dysfunction by mediating the expression of TGF-β_1. Furthermore, these data indicate that renal matrix remodeling in DN may be associated with Ab-mediated autoimmunity. It was also observed that TGF-β_1 expression in the renal cortex of DM + α_1-R Ab rats treated with doxazosin was significantly lower than that in DM + α_1-R Ab rats lacking doxazosin intervention, indicating that doxazosin blocked the stimulatory effects of α_1-R Ab on TGF-β_1 expression. Therefore, inhibition of TGF-β_1 may aid in preventing the development of renal fibrosis and matrix remodeling in DN.

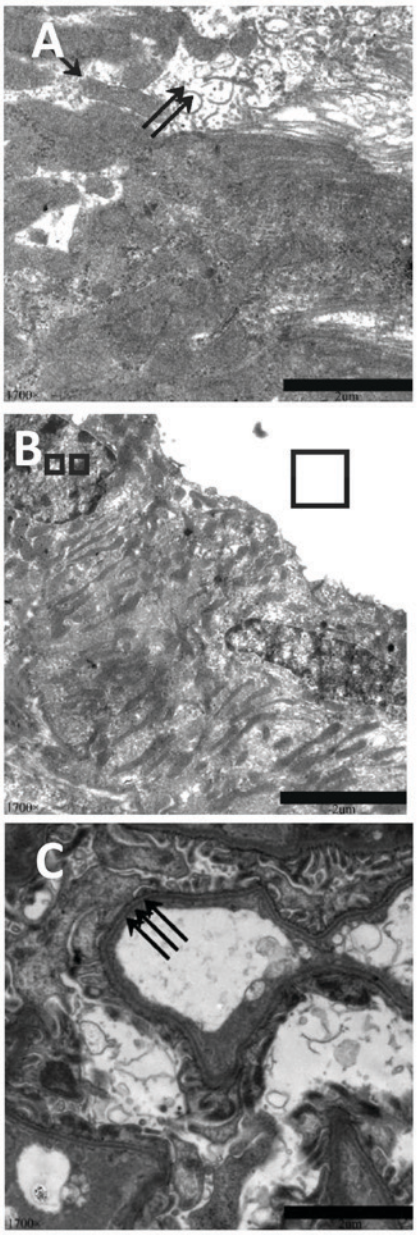

Figure 7. Electron microscopy of the renal cortex in rat models of DM following α_1-R Ab mediation and doxazosin intervention. (A) The proximal convoluted tubule exhibited cells swelling; however structural damage to the membrane and microvilli was reduced and cristae were more visible relative to the DM + α_1-R Ab alone group. (B) The distal convoluted tubule exhibited reduced damage to the smooth endoplasmic and mild expansion. (C) The renal glomerulus exhibited reduced damage in the foot cells, foot processes and basement membranes, though slightly larger interstitial spaces were observed between foot cells. Magnification, x1,700; scale bar, 2 μm. DM, diabetes mellitus; α_1-R Ab, anti-α_1-adrenergic receptor antibody. Lesions are demonstrated by arrow (s) or box (es). One arrow, organelle; two arrows, cytoplasm; three arrows, basilar membrane; one box, lumen; two boxes, nucleus.

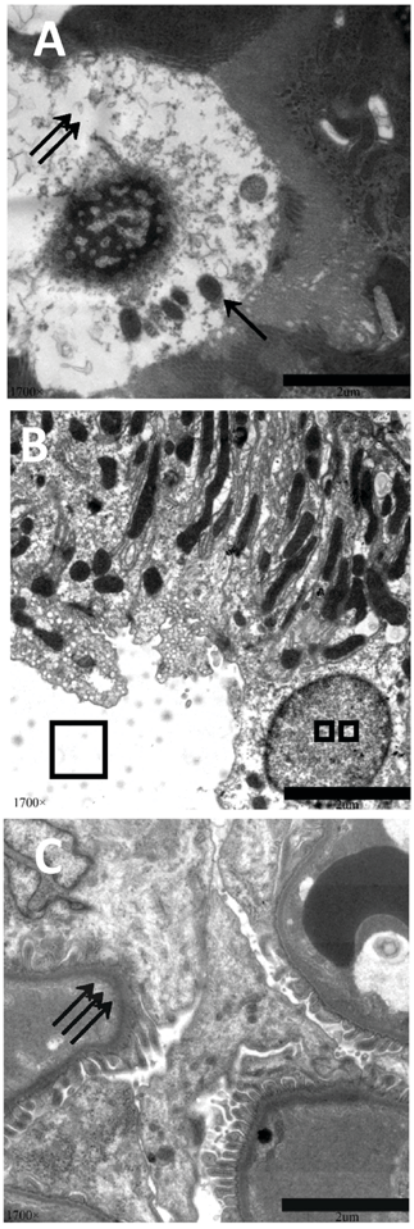

Figure 8. Electron microscopy of the renal cortex in rat models of diabetes mellitus following doxazosin intervention. (A) Proximal convoluted tubules exhibited cells swelling, dissolved cellular organs, a small quantity of concentrated and dense chondriosomes, and a small quantity of concentrated microvilli. (B) Distal convoluted tubules exhibited mild swelling of the smooth endoplasmic reticulum. (C) The renal glomerulus exhibited local, uneven thickening of the basement membrane. Lesions are demonstrated by arrow (s) or box (es). Magnification, x1,700; scale bar, 2 μm. One arrow, organelle; two arrows, cytoplasm; three arrows, basilar membrane; one box, lumen; two boxes, nucleus.

Following intervention with doxazosin, the present study demonstrated that levels of 24-h Upro and Scr significantly decreased in rats treated with α_1-R Ab + doxazosin, whereas these levels were significantly increased in DM + α_1-R Ab rats lacking intervention with doxazosin. These results support the hypothesis that α_1-R Ab mediation impairs renal function and that targeted intervention with receptor antagonists improves renal function. Observations by electron microscopy also identified marked improvements in the renal microstructures of DM + α_1-R Ab rats following doxazosin intervention. By contrast, the renal cortex structure of DM + α_1-R Ab rats lacking drug intervention remained damaged, and the direct changes of the proximal convoluted tubules, distal convoluted tubules and collecting tubes indicated severe microstructural damage. In particular, the glomerular basement membranes were substantially thickened and exhibited interlayer formation. These pathological changes verify that α_1-R Ab mediation may lead to renal matrix remodeling. The recovery of renal cortex components following doxazosin intervention

also suggests that renal impairment and matrix remodeling associated with α_1-R Ab mediation may be attenuated by early treatment with receptor antagonists.

It is possible that α_1-R Ab influences renal matrix remodeling in DM rats through the stimulation of TGF-β_1 protein synthesis and secretion, possibly by activating the α_1-R Ab/GPCR/PKC/NF-κB/TGF-β_1 signal transduction pathway (40). This may lead to glomerular hypertrophy, an increased glomerular filtration rate, thickening of the glomerular basement membrane and mesangial cell proliferation. Activated NF-κB may also result in a cascade of reactions and enhanced inflammation by inducing the generation of inflammatory factors, including OPN and in particular, TGF-β_1, as a key fibrogenic factor (33,41-43). Auto Abs may be produced by auto receptors via an internalization mechanism upon repeated stimulation, and are considered to have pathological agonist-like activity that leads to pathological effects via corresponding receptors, resulting in autoimmune responses and tissue injury (1,16,44,45). These GPCR auto Abs may also simulate normal physiological signals, including those mediated by ATII and adrenalin, by activating the corresponding GPCRs. This may induce ATII and adrenalin-like effects, including upregulation of TGF-β_1 expression and fibrogenesis of renal tubular interstitial substances, resulting in ECM deposition and renal matrix remodeling (17,19,46).

The primary ingredient of doxazosin is doxazosin mesylate, an α_1-R antagonist, which may outcompete α_1-R Ab in receptor binding. Doxazosin mesylate also selectively blocks postganglionic α_1-adrenergic receptors (16,47). Furthermore, doxazosin may prevent the direct attack of renal tissue cells by Ab and reduce pathological agonistic effects, thereby interrupting renal matrix remodeling. In addition, receptors binding to α_1-R Ab belong to the GPCRs, including AT_1, α_1, β_1, and M_2 receptors (24,26). Furthermore, structures of GPCR are relatively regular, while ligands are highly specific in binding to receptors (15). This structural characteristic may be useful in the design of targeted drug therapy (48). α_1-R auto Abs were likely responsible for the majority of TGF-β_1 upregulation in model rats in the current study; however, Abs corresponding to other receptors, including AT1, β_1 and M_2, may also mediate the expression of TGF-β_1.

Collectively, these results indicate that doxazosin may be useful in the treatment of DN. Though the effects of doxazosin in a clinical setting remain unknown, its potential ability to downregulate the expression of the fibrogenic factor TGF-β_1, as indicated in the present experiment, suggests that doxazosin may be a novel agent for the treatment of DN patients presenting with high expression of auto Abs. Doxazosin, as a 'diabetic receptor' antagonist, may also have a therapeutic role in the development of molecular and individualized targeted therapy for the treatment of DN. Future studies are warranted to elucidate the underlying mechanisms regarding the effects of doxazosin on renal function and fibrogenic factor expression.

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