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Mechanisms of irbesartan in prevention of renal lesion in streptozotocin-induced diabetic rats¹

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KEY WORDS transforming growth factor-beta; connective tissue growth factor; diabetic nephropathies; irbesartan

ABSTRACT

AIM: To investigate the mechanisms of angiotensin II receptor antagonist irbesartan (Irb) in prevention of renal lesion in streptozotocin (STZ)-induced diabetic rats. **METHODS:** Sprague-Dawley (SD) rats were randomly divided into three groups: normal control (group N), diabetic nephropathy (group DN), and diabetic nephropathy treated with Irb (group DNI). Diabetes was induced by injection of STZ ip after rats had received uninephrectomy. Blood glucose (BG), body weight (BW), urinary albumin excretion (Ualb), and 24-h proteinuria (24hUpro) were observed in the rats at week 4, 8, and 12, respectively. Creatinine clearance (Ccr), the kidney weight (KW), profile of kidney hypertrophy (KW/BW), renal tissue protein contents (RTP), glomerular area (A_G), glomerular volume (V_G), and width of glomerular basement membrane (GBM) were determined after the rats were sacrificed at week 12. Renal expression of connective tissue growth factor (CTGF) and transforming growth factor- β_1 (TGF- β_1) were determined by immunohistochemistry. **RESULTS:** There was no significant difference in BG between group DN and DNI ($P > 0.05$). Irb prevented the increasing of Ualb excretion, 24hUpro, and Ccr in diabetic rats ($P < 0.01$). Furthermore, Irb markedly inhibited the increasing of KW, KW/BW, RTP, A_G , and V_G shown in diabetic rats ($P < 0.05$, $P < 0.01$, respectively). Irb prevented the thickening of GBM and immunostaining of CTGF ($P < 0.01$). The extent of CTGF expression was positively correlated with the glomerular immunostaining for TGF- β_1 and size of V_G ($P < 0.01$). **CONCLUSION:** Irb exerts an early renal protective role to diabetic nephropathy, possibly through inhibition of renal hypertrophy and expression of CTGF.

INTRODUCTION

Diabetic nephropathy (DN) is characterized by

the early hypertrophy of both glomerular and tubular elements, thickening of the glomerular and tubular basement membranes, progressive accumulation of extracellular matrix components in the glomerular mesangium and tubulointerstitial fibrosis^[1]. However, the precise mechanism of DN has not been fully understood. Recently, considerable evidence suggests that the intrarenal renin-angiotensin system (RAS) plays an important role in the development of DN^[2]. Blockade of

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the RAS with either angiotensin-converting enzyme inhibitor (ACEI) or angiotensin II receptor antagonist (AIIRA) delays the progression of renal injury associated with diabetes^[3,4]. Irbesartan (Irb) is a newly approved product of AIIRA with higher bioavailability, lower plasma protein binding and longer half-life than losartan and valsartan. Irb exerted a renal protective role independently of its antihypertensive effect^[5]. Lewis *et al* reported a multicenter, randomized, and double-blind study with 1715 cases of type 2 diabetes, clearly showed that Irb was effective in protecting against the progression of nephropathy^[6]. However, the exact mechanism of Irb on renal protection is still to be clarified. Our present study focused on the effect of Irb on renal hypertrophy, glomerular expression of transforming growth factor β_1 (TGF β_1), connective tissue growth factor (CTGF), and the thickening of glomerular basement membrane (GBM) in streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Materials Adult male Sprague-Dawley (SD) rats weighing 250-300 g (Grade II, Certificate No SYXK 2001-0017, purchased from School of Medicine, Southeast University) were used in this study. They were housed at a temperature of 18-20 °C humidity of 65 %-69 %, and were submitted to a 12-h light/dark cycle. Rats had unrestricted access to tap water and standard rat chow.

Experimental protocol Right kidneys of SD rats were removed under pentobarbital sodium (30 mg/kg, ip) in order to enhance the formation of renal hypertrophy^[7]. After one week, diabetes was induced by single injection of streptozotocin (STZ, Sigma, St Louis, USA) at a dose of 50 mg/kg ip, diluted in citrate buffer 0.1 mol/L (pH 4.0). Forty-eight hours after STZ injection, rats for blood glucose randomly above 16.7 mmol/L were used in the study and then randomly divided into 2 groups: diabetes (group DN, $n=6$) and diabetes treated with Irb (group DNI, $n=7$). Normal control rats (group N, $n=7$) received citrate buffer ip alone. During the experimental period, long-acting insulin was given to all diabetic rats individually by sc injection to avoid ketonemia and promote the well-being of animals. After blood glucose was stable for one week, the rats in the DNI group were treated with an AIIRA, Irb (average dose of 50 mg·kg⁻¹·d⁻¹, Sanofi, France), for 12 weeks. Every four weeks after the treatment, rats were weighed

and blood samples were collected from a tail vein. Meanwhile, 24-h urine collections from animals housed in individual metabolic cages were centrifuged at 625×g for 10 min and stored at -20 °C to subsequent examinations. Rats were killed at week 12 when the rats were deeply anaesthetized by aether and the left kidneys were weighed. Coronal sections of 2-3 mm thick through the mid-portion were fixed in 10 % neutral formalin, embedded in paraffin, and cut at 3 μ m. Sections were stained with HE, PAS, PASM, and Masson's Trichrom Stain for light microscopic evaluation. Tissue sections were fixed in 4 % glutaraldehyde for electron microscopy.

Renal functional and biochemical studies Urinary albumin (Ualb) was measured by Turbox microalbuminuria assay (Orion Corporation, Finland). The contents of total protein in left kidney were determined by Lowry method. Serum creatinine was determined using of automatic analyser (HITACH-7150, Japan).

Evaluation of renal tissue by light microscopy and electron microscopic examination Kidney sections were stained with PAS and observed under the light microscope at a magnification ($\times 400$), using CMIAS (computer manage image analysis system, Beijing Aeronautic and Aerospace University). The glomerular area (A_G) was examined by sampling 50 glomeruli of each kidney. The glomerular volume (V_G) was calculated according to the following formula: $V_G=4 \pi r^3/3$ ($\pi=3.14$, r is glomerular radius evaluated by CMIAS)^[8]. A total of 40 fields of the renal cortex of each kidney at a magnification ($\times 200$) were observed blindly as semi-quantitative assessment of immuno-histochemical staining. The degree and range of positive staining were evaluated by the value of mean light density. For electron microscopic examination, two kidneys of each group were selected. The blocks of kidney were fixed in 4 % glutaraldehyde until use. A total of 20 fields of each group at magnification ($\times 40\ 000$) were taken photographs randomly by electron microscope (HITACH-H600, Japan). Five values of GBM width were randomly measured in each photograph by CMIAS.

Immunohistochemical detection for TGF- β_1 and CTGF in the kidney Sections (3 μ m) were placed into xylene to remove the paraffin wax, hydrated in graded ethanol. Immunohistochemical detection for CTGF and TGF- β_1 was performed according to SP method (SP kit purchased from Maixin Biotechnology

Co Ltd, Fuzhou, China). The primary antibodies were polyclonal rabbit anti-human TGF- β_1 (1:100, Santa, USA) and goat anti-human CTGF C-terminal peptide antibody (1:100, R&D). The end compounds reacted with AEC reagent. The slides were counterstained with haematoxylin and mounted. As a negative control, primary antibody was replaced with PBS.

Statistical Analysis Data were expressed as mean \pm SD and analyzed by ANOVA using SPSS. $P < 0.05$ were regarded as statistically significant unless where specified.

RESULTS

General observation BG in DN and DNI groups was significantly increased and kept stable after the rats received STZ injection compared with that in normal control group ($P < 0.01$). However, there was no significant difference for BG level between group DN and group DNI ($P > 0.05$). The BW of the rats in DN group was significantly decreased compared with that in N group, but it was markedly improved after treatment with Irb compared with that in N and DN groups ($P < 0.05$ and $P < 0.01$, respectively, Tab 1).

Influence of Irb on 24hUpro, Ualb, and Ccr

Both Ualb and 24hUpro were markedly increased from

week 4 onward in DN group compared with that in N group ($P < 0.01$). Treatment with Irb for 12 weeks significantly reduced the increase in Ualb and 24hUpro in DNI group ($P < 0.01$). Ccr was significantly increased in rats of DN group compared with that in N group ($P < 0.01$). While treatment with Irb in DNI group significantly prevented the increase of Ccr compared with that in DN group ($P < 0.01$, Tab 2).

Influence of Irb on the profile of kidney hypertrophy KW, KW/BW, A_G , V_G , and RTP in DN group were significantly increased as compared to those in N group ($P < 0.01$ and $P < 0.05$, respectively). However, there was a significant reduction of all the above parameters when treatment with Irb as in group DNI compared to those in DN group ($P < 0.01$, Tab 3).

Effect of Irb on the renal expression of CTGF, TGF- β_1 , and width of GBM Expression of CTGF was mainly within the glomeruli in diabetic kidney, while there was no marked expression of CTGF in the normal kidney (Fig 1A, 1B). Expression of TGF- β_1 could be detected in both glomeruli and tubular area in normal rat kidney (Fig 2D). Semi-quantitative assessment of the immunostaining for CTGF and TGF- β_1 showed that glomerular expression of CTGF and TGF- β_1 in group DN were significantly increased as compared with group

Tab 1. Changes of blood glucose and body weight in different experimental groups. Mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs group N. ^e $P < 0.05$ vs group DN.

Group	n	Blood glucose/mmole·L ⁻¹			Body weight/g		
		4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
N	7	5.4 \pm 0.6	5.3 \pm 0.5	5.9 \pm 0.8	410 \pm 41	426 \pm 33	461 \pm 47
DN	6	28 \pm 4 ^c	24.9 \pm 2.9 ^c	24.6 \pm 2.3 ^c	321 \pm 48 ^c	333 \pm 24 ^c	304 \pm 33 ^c
DNI	7	25.5 \pm 1.4 ^c	22 \pm 3 ^c	24 \pm 5 ^c	351 \pm 53 ^b	382 \pm 59	393 \pm 72 ^e

N: normal group; DN: diabetes group; DNI: diabetes treated with Irb group.

Tab 2. Changes of 24 hUpro, Ualb, and Ccr in different experimental groups. Mean \pm SD. ^c $P < 0.01$ vs group N. ^f $P < 0.01$ vs group DN.

Group	n	Ualb/mg·d ⁻¹			24 hUpro/g·mmolC r ⁻¹			Ccr/ mL·min ⁻¹ ·kg ⁻¹
		4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks	12 weeks
N	7	0.14 \pm 0.05	0.15 \pm 0.04	0.16 \pm 0.04	0.13 \pm 0.03	0.14 \pm 0.04	0.16 \pm 0.05	0.68 \pm 0.13
DN	6	2.8 \pm 0.7 ^c	5.0 \pm 0.5 ^c	7.2 \pm 1.3 ^c	3.8 \pm 2.1 ^c	5.1 \pm 1.2 ^c	7.10 \pm 1.7 ^c	1.24 \pm 0.19 ^c
DNI	7	1.83 \pm 0.21 ^{c,f}	2.6 \pm 0.4 ^{c,f}	4.4 \pm 0.7 ^{c,f}	2.3 \pm 0.7 ^{c,f}	3.4 \pm 0.6 ^{c,f}	4.3 \pm 1.2 ^{c,f}	0.95 \pm 0.15 ^{c,f}

N: normal group; DN: diabetes group; DNI: diabetes treated with Irb group.

Tab 3. Changes of the renal hypertrophy related parameters. Mean±SD. ^aP<0.01 vs group N; ^bP<0.05, ^cP<0.01 vs group DN.

Group	<i>n</i>	KW/g	KW/BW (%)	10 ⁻³ ×A _G /μm ²	10 ⁻⁴ ×V _G /μm ³	RTP/mg
N	7	1.49±0.17	3.3±0.4	6.5±0.6	43±6	63±10
DN	6	3.3±0.4 ^c	11.0±1.6 ^c	10.7±1.0 ^c	98±12 ^c	215±25 ^c
DNI	7	2.83±0.24 ^{c,e}	7.4±1.4 ^{c,f}	8.4±1.2 ^{c,f}	65±14 ^{c,f}	153±23 ^{c,f}

N: normal group; DN: diabetes group; DNI: diabetes treated with Irb group; KW: kidney weight; KW/BW: profile of kidney hypertrophy.

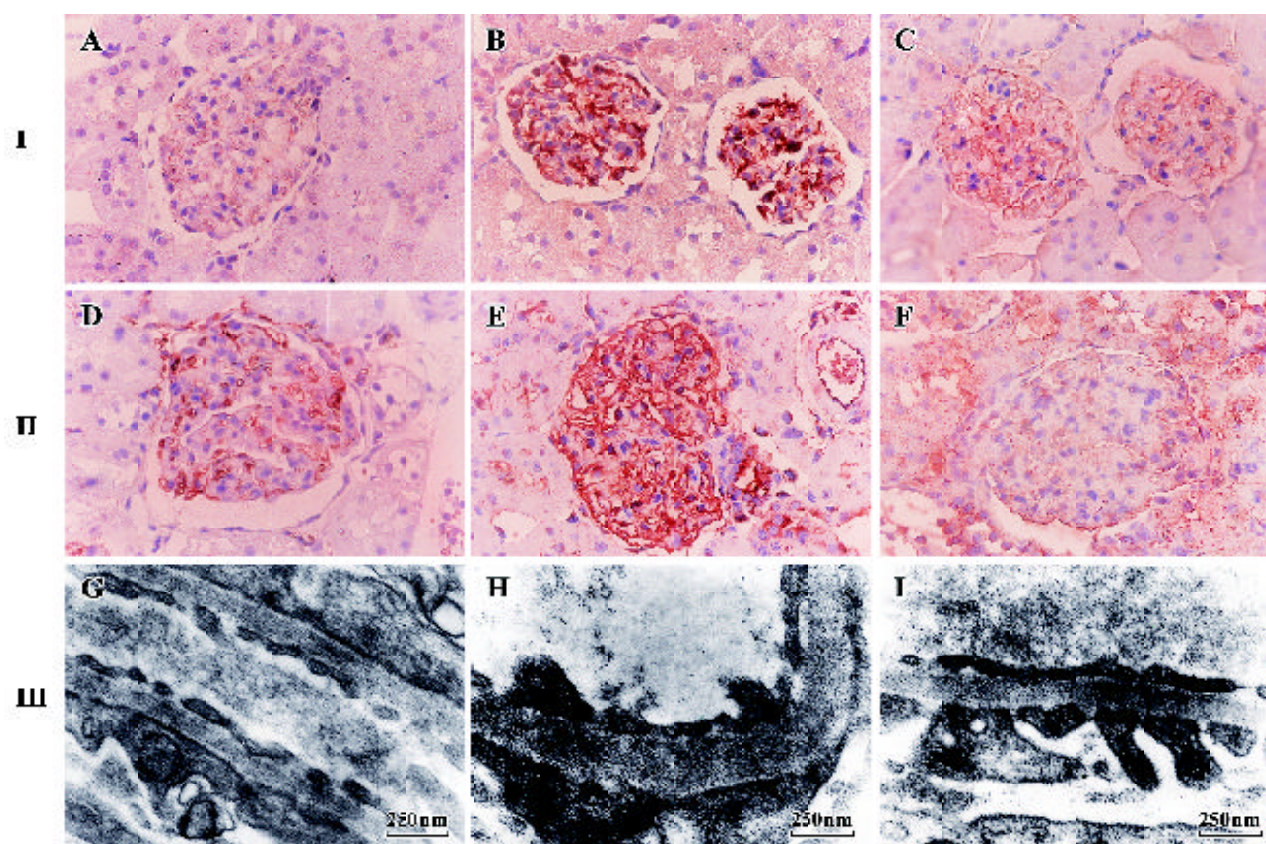


Fig 1. Renal immunostaining for CTGF in different groups (SP method, ×400, I); Immunostaining for TGF-β₁ in different groups (SP method, ×400, II); Electron microphotographs showed the appearance of GBM in different groups (×40 000, III). A, D, G: normal rat kidney; B, E, H: diabetic rat kidney; C, F, I: the kidney in diabetic rat treated with irbesartan.

N ($P<0.01$, Fig 1B, 1E), and the expression of CTGF and TGF-β₁ was much lower in DNI group as compared with group DN ($P<0.01$, Tab 4, Fig 1C, 1F). GBM in DN group was thicker than that in group N ($P<0.01$), and Irb significantly restrained the thickening of GBM ($P<0.01$, Fig 1G, 1H, and 1I).

Correlations of the expression of CTGF with V_G and TGF-β₁ The glomerular expression of CTGF was significantly correlated with the increasing of V_G ($Y=12.98X+3.54$, $r=0.83$, $P<0.01$), and glomerular expres-

Tab 4. Changes of the width of GBM, glomerular immunostaining for CTGF and TGF-β₁, Mean±SD. ^bP<0.05, ^cP<0.01 vs group N; ^dP<0.05, ^eP<0.01 vs group DN.

Group	<i>n</i>	CTGF	TGF-β ₁	Width of GBM/nm
N	7	0.30±0.05	0.32±0.03	125±22
DN	6	0.48±0.07 ^c	0.61±0.04 ^c	280±53 ^c
DNI	7	0.39±0.07 ^{b,e}	0.42±0.04 ^{c,f}	144±25 ^{c,f}

sion $\text{TGF-}\beta_1$ ($Y=0.455X+0.395$, $r=0.83$, $P<0.01$).

DISCUSSION

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease in Western world. Glomerular hypertrophy and expansion of extracellular matrix (ECM) have been regarded as the early feature of DN, which eventually leads to proteinuria and glomerulosclerosis^[9]. Our preliminary study clearly showed that early using of Irb could reduce proteinuria, albuminuria, and increasing of Ccr. Specifically, Irb could significantly prevent the early renal hypertrophy.

The precise mechanism of renal hypertrophy in diabetes is still unclear. Several growth factors have been proposed to be involved in mediating the development of diabetic renal hypertrophy, which including angiotensin II (Ang II), TGF β , insulin like growth factor-1 (IGF-1), platelet derived growth factor (PDGF), and hepatocyte growth factor (HGF)^[10-13]. Among them, Ang II is regarded as one of the most important factors contributing to renal hypertrophy and subsequent renal fibrosis^[14]. Arresting cells in the G₁ phase of the cell cycle through induction of cyclin-dependent kinase (Cdk) inhibitors such as p27^{kip1} and p21 may be the molecular mechanism of the development of renal hypertrophy^[15]. Both Ang II and TGF- β can induce the production of p27^{kip1} and p21^[16]. Treatment of diabetic rats with ACEI attenuates glomerular expression of the Cdk-inhibitors p16 and p27, indicating that the cell cycle arrest can be therapeutically influenced by blocking the local RAS^[17]. Our previous work suggested that the blockade effect of valsartan on Ang II could reduce the renal production of TGF- β_1 in diabetic patients^[18]. The present study demonstrated that Irb significantly inhibited the glomerular expression of TGF- β_1 and renal hypertrophy, further suggesting that inhibition of renal production of TGF- β_1 might be the potential mechanism of Irb in exerting its renal protective role on diabetic nephropathy.

Connective tissue growth factor (CTGF), a member of the CNN (CTGF/ Fisp 12, Cyr61/CEF-10, Nov) immediate early gene family of proteins, is newly recognized growth factor, involving in the tissue fibrosis such as scleroderma^[19], liver fibrosis^[20], IgA nephropathy, crescentic glomerulonephritis, and diabetic nephropathy^[21,22]. It may serve as a downstream mediator of TGF- β , activation through SMAD3- and SMAD4-dependent pathway^[23]. Riser *et al* demonstrated that the induction of CTGF protein mediated by

TGF- β occurred early in the course of DN when mesangial expansion was mild, and interstitial disease and proteinuria were absent^[24]. Wahab *et al*^[25] also demonstrated that CTGF could be detected in the glomeruli in non-obese diabetic mice 14 d after the onset of diabetes. Culture of primary human mesangial cells for 14 d in high glucose or in low glucose supplemented with TGF- β_1 markedly increased CTGF mRNA levels and fibronectin synthesis. All these data clearly suggested that CTGF played an important role in mediating diabetic nephropathy. Our study firstly demonstrated that Irb markedly inhibited the increasing immunohistochemical expression of CTGF in STZ-induced diabetic rats. Specifically, we found that the expression of CTGF was positively correlated with the expression of TGF- β_1 and the extent of renal hypertrophy. These findings suggested that inhibiting the expression of CTGF might be the mechanism for the renoprotective role of blocking RAS in diabetes as well.

The thickening of GBM is also the early feature of diabetic nephropathy. Some studies indicated that non-enzymatic glycation of collagen IV and the effect of TGF- β_1 contributed to the thickening of GBM^[26]. In this study, Irb could significantly restrain the early thickening of GBM, further supported the primary preventive role of Irb in diabetic nephropathy.

Taking together, our study demonstrated that Irb exerted a renal protective effect in STZ-induced diabetic rats through reducing proteinuria, albuminuria, and preventing renal hyperfiltration. Specifically, our results suggested that the renoprotective effect of Irb might be related to its inhibition of renal hypertrophy and renal expression of newly recognized growth factor, CTGF. These data further provided an evidence for preventing the development of diabetic nephropathy by blocking intrarenal renin angiotensin system.

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