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Chronically increased intrarenal angiotensin II causes nephropathy in an animal model of type 2 diabetes

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1. ABSTRACT

Diabetic nephropathy characterized by proteinuria and sclerosis is the leading cause of renal failure, but its mechanisms are not well understood. Zucker Obese (ZO) rat model of obesity, insulin resistance, and hypertension has been used to study nephropathy. We hypothesize that chronically elevated intrarenal angiotensin II (ANG II) down-regulates nephrin, a key slit-pore protein and up-regulates fibrogenic molecule transforming growth factor (TGFβ1) and thus result in progression of nephropathy in *type 2* diabetes. Untreated or angiotensin converting enzyme (ACE) inhibitor, captopril, treated ZO and control Lean (ZL) rats were used to measure intrarenal levels of ANG II, glomerular nephrin, TGFβ1, collagen and fibronectin with age using radioimmunoassay, RT-PCR and immunoblot techniques. Progression of nephropathy was established by measuring proteinuria and sclerosis. ZO rats developed obesity, hyperglycemia, hyperinsulinimia, increase in intrarenal ANG II and proteinuria. Expression of glomerular nephrin decreased while expression of TGFβ1 and matrix components increased in ZO rats. Captopril treatment prevented increase in intrarenal ANG II, and reversed expression of nephrin, TGFβ1, collagen and fibronectin. We conclude that in this model of *type 2* diabetic nephropathy, chronically elevated intrarenal ANG II causes proteinuria via decrease in nephrin and glomerulosclerosis via TGFβ1 mediated increase in matrix component.

2. INTRODUCTION

Non-insulin-dependent diabetes mellitus (NIDDM) or *type 2* diabetes accounts for 90% of all diabetic cases. Epidemiological data demonstrate an exponential increase in incidence of *type 2* diabetes associated end-stage renal disease in the United States with huge economic costs and dramatic increase in morbidity and premature mortality (1, 2). Proteinuria, glomerular hypertrophy, arteriolar hyalinosis and mesangial sclerosis characterize *type 2* diabetic nephropathy (3, 4). Zucker Obese (fa/fa) rat model has been used to study obesity, hypertension and nephropathy in *type 2* diabetes (5, 6).

A role for intrarenal angiotensin II (ANG II) in progression of diabetic nephropathy has been proposed (7,8) and beneficial effects with angiotensin converting enzyme inhibitor (ACE) and ANG II receptor blockers treatment have been shown in patients with diabetic renal failure (9) and in streptozotocin-induced diabetic rats (10,11). Role of intrarenal ANG II and beneficial effects of ACE treatment in cause and progression of nephropathy in *type 2* diabetes is not well understood (12). We hypothesize that chronically elevated intrarenal ANG II may be the cause of proteinuria and glomerulosclerosis in *type 2* diabetes. We further postulate that proteinuria may be because of ANG II mediated down-regulation of nephrin expression and glomerulosclerosis may be because of increased expression of matrix component due to increased intrarenal TGFβ1.

The purpose of this study is three folds: (i) first to document whether intrarenal ANG II levels are elevated and if so transiently or chronically, (ii) second if, there is any association between increase in intrarenal ANG II and proteinuria and sclerosis and, (iii) third to establish a link between ANG II-nephrin (a 1241-residue trans-membrane key protein of podocyte slit pore membrane) -proteinuria and ANG II-TGFβ1 (fibrogenic molecule)-sclerosis in *type 2* diabetes.

3. METHODS

3.1. Animals

Male Zucker fatty (fa/fa) rats, ZO, and age-matched male Zucker lean (fa/-) rats, ZL, (Harlan, Indianapolis, IN) were housed in animal facility of the Medical College of Wisconsin and maintained on standard rat chow, water adlibitum, 12-h light cycle and 21±2ºC temperature.

Experimental groups: A minimum of five animals at seven, 14 and 21 weeks of age were assigned to one of the four groups; (a) ZO rats without any treatment (b) ZO rats with Captopril (100mg/kg/day, Sigma Chemical Co, St Louis, MO) treatment for a period of 14 weeks, 7- 21 weeks), (c) ZL rats without any treatment and (d) ZL rats with Captopril (100mg/kg/day, Sigma Chemical Co, St Louis, MO) treatment for a period of 14 weeks, (7- 21 weeks). We selected angiotensin converting enzyme inhibitor, captopril because of its endothelial-independent effects (13).

3.2. Urine collection

Rats were placed in metabolic cages (Harvard Bioscience, South Natick, MA) without food, but free access to water for 24 hrs and urine was collected over 50 µl of 1 % NaN_3 to avoid bacterial contamination. Collected urine was centrifuged to remove debris, aliquoted and frozen at -70 ºC for subsequent analysis of total urinary protein (Bio-Rad protein assay; Bio-Rad, Richmond, CA) and creatinine using calorimetric assay and in some samples, albumin using radioimmunoassay.

3.3. Blood collection and kidneys removal

Rats were anesthetized with pentobarbital (Mallinckrodt Veterinary, Inc. Mundelein, IL). Blood was collected by cardiac puncture and 1 ml was added to 9 ml of chilled methanol for ANG II measurement and the remaining was used for plasma collection. Immediately after removal of blood, left kidney was removed (<30 sec), weighed and placed into chilled methanol and homogenized rapidly (10% wt/vol) for ANG II measurement. A small piece from right kidney was fixed in buffered formaline for histology and rest of the right kidney was used for the isolation of glomeruli.

3.4. Determination of plasma and intra-renal ANG II

Plasma and kidneys at seven, 14 and 21 weeks of age were collected and ANG II was measured using a modified (SPI-BIO, Massycedox, France) radioimmunoassay method (14, 15).

3.4.1. Extraction of ANG peptides by Solid Phase Extraction Chromatography (SPEC)

The blood sample and kidney homogenates in chilled methanol were centrifuged at 4°C for 10 minutes and supernatant from the blood and kidney homogenate was dried overnight in a vacuum centrifuge, (Savant, Hicksville, NY) and reconstituted in 6 ml of 50mM Sodium phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin. All samples were kept on ice throughout the extraction procedure. Reconstituted plasma and kidney extract were applied to a pre-washed (3ml methanol followed by 6 ml water) phenyl-bonded Solid Phase Extraction (SPE) column (Bond-Elut, Analytichem, Harbor City, CA). After application of the samples, each SPE column was washed sequentially with water (3 ml), hexane (1.5 ml), and chloroform (1.5 ml) to remove salts, and other polar substances, lipids, and hydrophobic materials from the column. ANG peptides eluted from the SPE column were washed twice with 1 ml of 90% methanol in water, and the eluates were collected and stored at –20°C.

3.4.2. Quantification of ANG II levels by radioimmunoassay

All stored samples (-20°C) were dried under vacuum and reconstituted in 1 ml of assay buffer. Each assay tube contained 50 to 300µl of reconstituted samples or ANG II standard (Sigma Chemical Co, St. Louis, MO), and 0 or 100µl of diluted rabbit anti-ANG II serum (Arnel, New York, NY) and 100µl of diluted mono-iodinated ^{125}I-labeled ANG II (Amersham, Arlington Heights, IL), the volume was adjusted to 500 µl with assay buffer. Assay tubes were incubated for 48 hrs at 4°C and the bound and

Table 1. The sequences of primers and product size

Gene	Sense	Antisense	Size (bp)
TGFβ1	5′ GCTTGCTCCAGATTCAGATC 3'	5′ TCACATGCTCGGTAGAAAACGG 3'	312
Rat collagen IV	5′ TAG GTG TCA CGA ATT AGG CAG G 3′	5′ CGG ACC ACT ATG CTT GAA GTG 3'	484
Fibronectin	5′ ATG ATG AGG TGC ACG TGT GT 3′	5′ GAT GGG GTC ACA TTT CCA TC 3′	210
β-actin	5′ AAC CCT AAG GCC AAC CGT GAA AAG 3′	5′ TCA TGA GGT AGT CTG TCA GGT 3′	240

free ANG II were separated by dextran-coated charcoal (10 mg charcoal and 1mg dextran in 1ml of assay buffer/tube). After centrifugation, the supernatant was removed and counted for 5 minutes in a gamma counter. The ratio of binding to maximum binding (B/Bo) of each sample was corrected for non-specific binding and expressed as percent of maximum binding and read against the standard curve (log-logit transformation) using a least squares regression analysis. Measurable ANG II levels were defined as displacement of radio labeled binding by greater than twice the standard deviation from the mean maximum binding.

3.5. Measurement of functional parameters

Body weight and tail cuff blood pressure were measured repeatedly. Proteinuria was determined from timed urine collections prior to sacrifice (metabolic cages for 14 to 18 hrs over 50 µl of 1% NaN3 added to avoid bacterial contamination). Quantitative determination of urinary albumin was done using rat albumin ELISA kits. Serum creatinine, urine protein and creatinine levels were determined using commercially available clinical lab kits. Kidney and body weight were taken at time of sacrifice

3.6. Isolation of glomeruli and preparation of RNA and protein lysate

After removing the capsule, the harvested kidney were cut into fine fragments and passed through consecutive screens of 80, 120 and 200-mesh size. Glomeruli were retained and recovered from the top of the 200-mesh screen. Isolation was carried out at room temperature in phosphate buffered saline (pH 7.4).

3.6.1. Preparation of glomerular RNA

Total RNA from glomeruli was washed twice with PBS (pH 7.4) and isolated using phenol chloroform method (RNAzol, Tel-Test, Friendswood, TX) described by Chomczynski and Sacchi (16). The relative purity of RNA was determined by a 260/280 ratio of spectrophotometric readings and was stored at –80°C.

3.6.2. Preparation of glomerular protein lysate

The glomeruli were sonicated for 1 min at power 3 in the lysis buffer (50 mM HEPES, pH 7.5; 150 nm NaCl; 1.5 mM $MgC1_2$; 1mM EGTA; 10% glycerol; 1% Triton X-100; 1µg/ml aprotinin; 1 µg/ml leupeptin; 1mM phenylmethylsulfonyl fluoride (PMSF) and 200 µM sodium orthovanadate) and centrifuged at 14,000g for 30 min at 4°C. The supernatant was collected (protein lysate), and the protein concentrations were measured using Bio-Rad protein assay kit and stored at -80°C.

3.7. Assessment of changes in the levels of mRNA for nephrin by RT-PCR

1 µg of total RNA was reverse transcribed using Pharmacia, First-Strand cDNA synthesis kit, Piscataway, NJ. Specially designed primer for rat nephrin were used, the PCR products were analyzed on a 1% agarose gel, and visualized by ethidium bromide staining and ultraviolet illumination

3.8. Assessment of glomerular sclerosis

Glomerular message and protein levels of collagen IV and fibronectin were carried out by RT-PCR and immunoblot respectively. Stained sections of the renal cortex were used for assessment of sclerosis.

3.8.1. Assessment of changes in the levels of mRNA for collagen types IV and fibronectin by RT-PCR

1 µg of total RNA reverse was transcribed using reverse transcriptase from Maloney murine leukemia virus (Pharmacia, First-Strand cDNA synthesis kit, Piscataway, NJ) in a reaction buffer (950mM Tris pH 8.3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT) containing 0.02 units/µL Rnasin (Promega). Reaction mixture was heated at 68°C for 5 minutes to stop the reaction. 1 mM of specially designed primers for $TGF\beta_1$, collagen IV, fibronectin and GAPDH were amplified with 0.05units/µl of Ampli Taq DNA polymerase (Perkin Elmer, Foster City, CA) in a total volume of 50 µl PCR buffer. The PCR profiles were as follows: Thirty cycles at 95^0C (1 minute), 55^0C (1 minute) and 72^0C (3 minutes) and a final extension at 72^0 C for 10 minutes. PCR products were separated on a 1 % agarose gel, visualized with ethidium bromide staining and by ultraviolet illumination and photographed. RT-PCR has the advantage of permitting identification of mRNA signals even when they are present in low amounts. Sequence and product size of various primers used in this study are listed in table 1.

3.8.2. Assessment of changes in protein levels of collagen types IV and fibronectin

Equal amount of glomerular protein lysate were added with polyclonal goat anti-mouse type IV collagen (Southern Biotech, Birmingham, AL), polyclonal rabbit anti-rat fibronectin (Chemi-Con, Temecula, CA), and a monoclonal murine antibody specific for laminin-s chain (Developmental Studies Hybridoma Bank, Baltimore, MD) at 4°C overnight. Precipitated antigen-antibody complex was solubilized and analyzed on a 4-20% gradient polyacrylamide gels (100V, constant voltage), then electrophoretically transferred to nitrocellulose paper and blocked with 5% non-fat dry milk in T-TBS (Tri-buffered saline; 10mm Tris pH 7.5, 100 mm NaCl, 0.1% tween-20) for one hour at room temperature. The nitrocellulose membrane was probed for one hour at room temperature with the anti-collagen IV, anti-fibronectin and anti-laminin b antibody at 1µg/ml in T-TBS, followed by incubation with secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse/rat IgG at 1:5000), then washed with T-

Table 2. Body weight, Mean Arterial Blood Pressure, Serum glucose and serum insulin in Zucker Obese and control Lean rats

Parameters	Zucker Lean rats			Zucker Obese rats		
	7-weeks	14-weeks	21-weeks	7-weeks	14-weeks	21-weeks
Body Weight (gm)	149 ± 6 N=7	359 ± 13 N=8	454 ± 28 N=6	195 ± 8 N=7	540 ± 24[1] N=8	685 ± 40[1] N=6
Mean arterial pressure (mm of Hg)	ND	116±3 N=5	ND	ND	118±2 N=5	ND
Serum Insulin (ng/ml)	0.31 ± 0.1 N=4	0.97 ± 0.1 N=5	1.5 ± 0.9 N=6	1.9 ± 0.8[1] N=4	1.4 ± 0.4[1] N=5	7.3 ± 2.1[1] N=6
Serum Glucose (mg/dl)	139 ± 19 N=4	75 ± 24 N=4	155 ± 7 N=6	144 ± 21 N=4	228 ±58[1] N=4	190 ±15 [1] N=6

Values are mean ± SEM, N= number of rats, [1]p<0.05 vs. Zucker Lean rats at same age.

BS and visualized using enhanced chemiluminiscence (ECL kit; Amersham Corp) according to the manufacturer's instructions. Fluor-imager was used to scan and obtain quantitative data.

3.8.3. Histology

Kidney slices from 21 weeks old ZO and ZL rats were fixed in 3.7% buffered formaldehyde, washed with phosphate buffer and embedded in paraffin. After being embedded in paraffin, 2μm thin sections were cut, mounted on poly-L-lysine-coated slides (Polysciences, Inc., Warrington, PA) and stained with Trichrome, Hematoxylin-Eosin (H&E), and Periodic Acid Stiff's (PAS) reagents. The severity of the glomerular injury was assessed using light microscopy. A minimum of 20 glomeruli in each specimen were examined in randomly selected two sections from at least 3 different rats. An injury score from 0-5 was assigned to each glomeruli (0= no sclerotic changes, 1=minor changes, 2=mild changes, 3 moderate and 4= severe sclerotic changes) and tubular dilation, increase in interstitial volume and cellularity as tubular dysfunction was also observed.

3.9. Statistics

All values expressed are as mean ± SEM. N represents the number of rats. Statistical analysis carried out with the Sigma Stat program (Systat Inc. software, version 5, Evanston, IL) using one-way ANOVA followed by the Tukey test for post hoc comparisons to determine significance. p value <0.05 accepted as significant.

4. RESULTS

4.1. Measurement of body weight, mean arterial blood pressure, serum insulin and glucose

As outlined in Table 2, average weight increased significantly in Zucker Obese rats by 14 and 21-weeks of age. There was no difference in the mean arterial blood pressure at the 14 weeks of age, whereas serum insulin and glucose levels were significantly higher at 14 and 21 weeks in age in Obese as compared to Lean rats. In this study ZO rats were not hypertensive as compared to ZL rats, because we only studied these rats up to 21 weeks of age and ZO rats are known to develop mild to severe hypertension only after 40 weeks of age (11).

4.2. Measurement of proteinuria

As shown in Figure 1A, urinary protein (mg/ mg creatinine) significantly increased in ZO rats as compared to their lean control littermates at 14 weeks of age and further increased at 21 weeks of age. Since concentration of urinary albumin is the true measure of proteinuria, we also measured the urinary albumin in ZO and ZL Lean rats at 21 weeks of age using radioimmunoassay. As shown in Figure 1B, urinary albumin significantly increased in all ZO rats as compared to control Lean littermates. Treatment with ACE inhibitor, captopril diminished proteinuria (Figure 1C).

4.3. Measurement of plasma and intrarenal ANG II

We measured concentration of ANG II in plasma and kidneys of ZO and ZL rats at seven, 14 and 21 weeks of age using modified radioimmunoassay method. As shown in Figure 2, there was no difference in plasma levels of ANG II up to 21 weeks of age (not shown), but the intrarenal concentration of ANG II (p gm/gm of kidney) increased significantly (p<0.05) at 14 weeks and further increased at 21 weeks of age in Zucker Obese rats. This increase in intrarenal ANG II coincided with the increase in urinary albumin (Fig 1B). A smaller increase in intra-renal ANG II with aging in ZL rats was not significant. Ascompared to untreated ZO rats (25.39±1.58, N=3, picogram/gm of kidney), captopril treatment (from 7 to 21 weeks) significantly (p<0.01) slowed increase in intrarenal ANG II concentration (13.98 ± 1.31 N=3). Intrarenal ANG II levels of captopril treated ZO rats were not significantly different from ZL control rats (13.98 ± 1.31 N=3 vs. 11.60 ± 2.27, N=3).

4.4. Expression of Nephrin by RT-PCR

As shown in Figure 3 (a) nephrin gene expression significantly reduced at 21 weeks of age in ZO rats compared to ZL rats. Treatment with captopril (100mg/kg/day) for 14 weeks in ZO rats improved the decrease in nephrin expression. Nephrin to β-actin ratio for untreated and captopril treated ZL rats are 0.4129 ± 0.0495 (N=3), 0.4231 ± 0.1188 (N=3), where as ratio for untreated and captopril treated ZO rats are 0.3268 ± 0.0675 (N=3), 0.3836 ± 0.0381 (N=3) respectively. As shown in densitometry analysis graph in Figure 3 (b), there is a significant reduction in nephrin expression in ZO rats compared to ZL rats (0.3268 ± 0.0675 vs. 0.4129 ± 0.0495, p<0.05) and captopril treatment though increased nephrin expression in ZO rats it did not reach to significance level.

4.5. Documentation of sclerotic changes

Sclerosis was measured by two methods, one by the expression of fibrogenic molecule, matrix components and also by histological changes as outlined below;

Message expression of TGFß1, fibronectin and collagen IV by RT-PCR: As shown in Figure 4 (a), message of TGFß1, fibronectin and collagen IV significantly increased in ZO rats as compared to those of ZL control rats. TGFß1, fibronectin and collagen IV to β-actin ratio for ZL rats are 0.36 ± 0.03 (N=6), 0.59 ± 0.049 (N=6) and 1.11 ± 0.16 (N=6) respectively, where as ratio for ZO rats are 0.97 ± 0.04 (N=6), 1.61 ± 0.067 (N=6), and

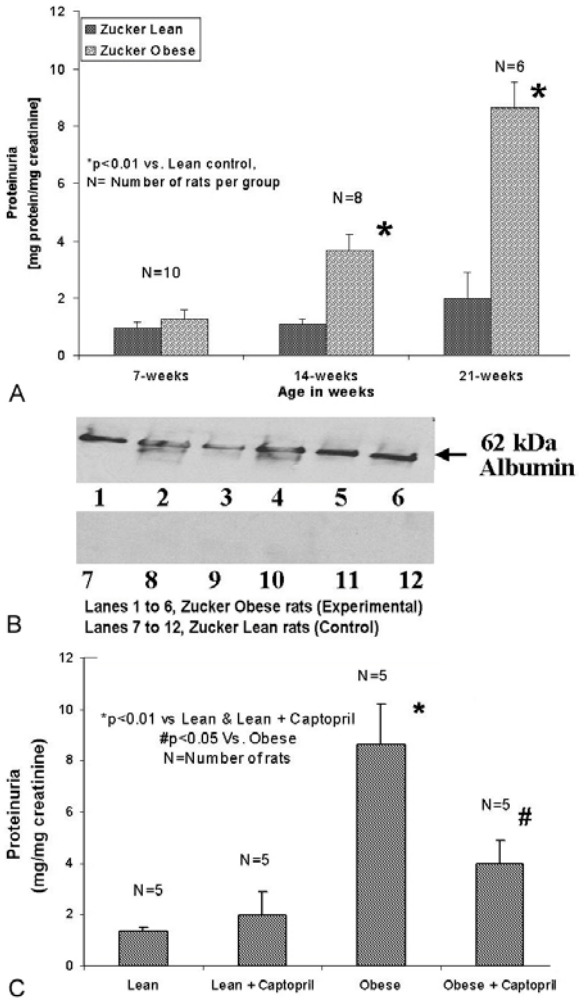

Figure 1. A. Proteinuria in Zucker rats with age. Zucker Obese and control Lean rats at seven, 14 and 21 weeks of age during the progression of the disease kept in metabolic cages for 16-18 hrs with no food but water adlibitum. Urine collected over 1% NaN3 to avoid contamination and proteinuria (urinary protein mg/mg creatinine) measured. Values represent mean ± SEM. N represents number of rats studied in each group. *p<0.01, significantly different from control Zucker Lean rats of same age.B. Albuminuria in Zucker rats at 14 weeks of age. Documentation of albumin in urine of six Zucker Obese and six Lean control rats at 14 weeks of age in the urine of by immunoblot analysis. C. Proteinuria in Zucker rats after captopril treatment. Proteinuria (mg protein/mg of creatinine) in Zucker Obese and Lean control rats at 21 weeks of age after treatment with or without Angiotensin converting enzyme inhibitor, captopril (100mg/kg/day) in drinking water for a period of 14 weeks.

Histology: Trichrome, H&E and PAS stained thin sections of kidney from ZL and ZO rats were used for evaluation of histological changes including early tubular changes, glomerular hypertrophy, interstitial and glomerulosclerosis. As shown by arrows in Figure 5A, early changes of tubular and glomerular hypertrophy, interstitial and early glomerulosclerosis are evident in the kidneys from ZO rats compared to ZL rats. Light micrographs of a kidney cortex with glomeruli from a ZL-control and diabetic ZO-rats with trichrome stain (ZL-1, ZL-2, ZO-1 and ZO-2), H&E stain (ZL-3, ZL-4, ZO-3 and ZO-4) at 100x and 200X magnifications are shown. Figure 5B shows a representative PAS Stained glomeruli at 400X magnification from ZO (A) and ZL rats (B). Prominent glomerular cell growth, glomerulosclerosis and glomerular hypertrophy is evident in diabetic ZO-rats, in contrast to control ZL rats with normal glomerular morphology and without any signs of glomerular hypertrophy or glomerulosclerosis. Severity score for glomeruli from ZO rats (3.75 ± 0.50, N=85) was significantly higher ($p<0.01$) as compared to glomeruli from ZL rats (1.25 ± 0.25, N=60). Tubules of ZL kidney (B) are tightly packed were as tubules of ZO kidney are dilated with a significant interstitial infiltrate shown by arrow in Figure 5B, panel A.

5. DISCUSSION

Our results document that intrarenal ANG II is chronically elevated and is associate with decreased expression of nephrin, increased expression of TGFß1, collagen IV and fibronectin in this model of *type2* diabetes. Our data chronicle the onset and progression of renal disease in ZO rats and demonstrate that inhibition of intrarenal ANG II by ACE treatment markedly reduces renal injury. Results further demonstrate that treatment with captopril significantly reduces increased intrarenal ANG II, reverses expression of a key slit pore protein, nephrin and fibrogenic molecule TGFß1 and matrix components, collagen IV and fibronectin along with proteinuria and sclerosis. Results of this study document that chronic increase in intrarenal ANG II is the cause of development of diabetic nephropathy in this animal model.

ZO rats develop glomerular hypertrophy with mesangial expansion by 12 weeks, albuminuria around 14 weeks and glomerulosclerosis by 28 weeks of age (17). Antihypertensive drugs including calcium channel blockers, ACE inhibitors, angiotensin receptor blockers (ARBs) alone or in combination have been proven to be effective in slowing the progression of nephropathy, but ACE inhibitors have proven to be relatively more effective in diminishing glomerulosclerosis in this model of type 2 diabetes (18,19). Studies using either angiotensin-converting enzyme inhibitors or type 1 receptor (AT_1) blockers indicate that ANG II is a mediator of progressive injury in diabetic nephropathy. Evidence suggests that intrarenal RAS within glomeruli and proximal tubules may be activated with hyperglycemia, leading to stimulation of local ANG II production. Our studies document that there is a significant increase in the level of intrarenal ANG II that persists and further increased with the age up to 21 weeks of age. Once formed, intrarenal ANG II exerts most of its well-described effects through binding to AT_1 receptors that are abundantly present in glomerular cells, tubules, vasculature, and interstitium. Thus, AT_1 receptor activation increases vascular resistance, reduces renal blood flow, and stimulates production of extracellular matrix in the mesangium and tubulointerstitium (20, 21). Podocytes

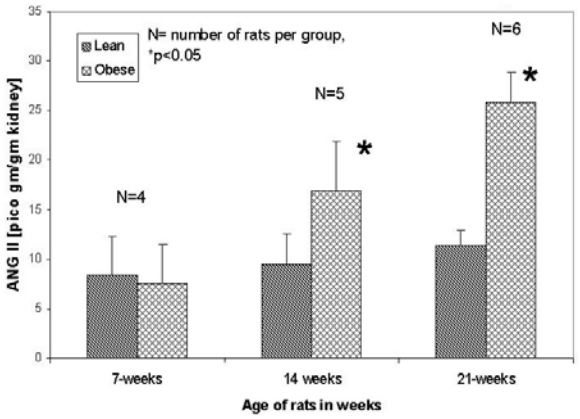

Figure 2. Intrarenal levels of ANG II. Intrarenal Angiotensin II in Zucker Obese and Lean control rats at seven, 14 and 21 weeks of age during progression of the disease measured using modified radioimmunoassay. ANG II levels are expressed in pico gram/gram kidney weight. Values represent mean ± SEM. N represents number of rats studied in each group. *p<0.01, significantly different from control Zucker Lean rats of same age.

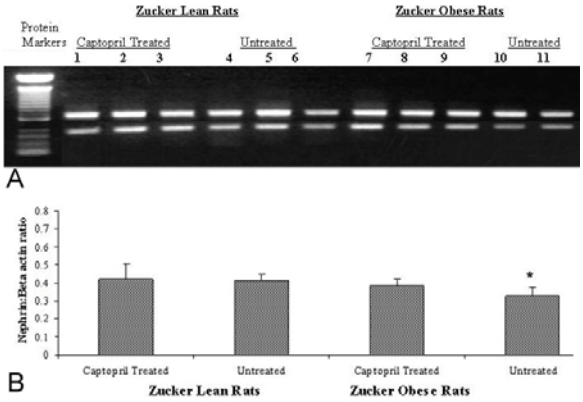

Figure 3. Glomerular expression of nephrin. A. Expression of Nephrin measured in glomeruli of Zucker Obese (ZO) and Lean control (ZL) rats with or without captopril treatment (100mg/kg/day) for 14 weeks by RT-PCR. Lanes 1-3 represents ZL rats treated with captopril, lanes 4-6 represent untreated ZL rats, lanes 7- 9 represents ZO rats treated with captopril and lanes 10-11 represent untreated ZO rats. Upper band is of house keeping gene β-actin and lower band represents nephrin. B. Graph shows densitometry analysis (mean ± SEM, groups of 3 animals) of the ratio of nephrin to β-actin. ZO rats have significant less (*P<0.05) nephrin expression as compared to ZL rats (untreated or captopril treated).

2.09 ± 0.017 (N=6) respectively. As shown in densitometry analysis graph in Figure 4(b), there is a significant increase in expression of TGFß1 (** p<0.01), fibronectin (** p<0.01) and collagen IV (* p<0.05) in ZO rats compared to ZL rats.

are highly specialized glomerular epithelial cells that make up a major portion of the glomerular filtration barrier and play a pivotal role in the glomerular function (22, 23). Nephrin, a 1241-residue trans-membrane cytoskeletal key protein localized to the podocyte slit pore membrane and a major contributor of the glomerular filtration barrier play a role in proteinuria (24). Mutations in the nephrin coding gene NPHS1 are responsible for the Finnish-type congenital nephrotic syndrome (25). Nephrin specific promotor has been characterized (26). Diabetic nephropathy, one of the major causes of end-stage renal disease, is associated with substantial proteinuria and in experimental models with a reduction in slit pore density. The link between permutations in nephrin expression and proteinuria has been shown in an experimental model of diabetes and hypertension (27-29). Dr. Langham and colleagues documented recently that decrease in glomerular expression of nephrin in human kidney tissue correlates with development of diabetic nephropathy and reversal of decrease in nephrin similar to that in the non-diabetic controls levels following perindopril treatment diminishes proteinuria in diabetic patients (30). The results of this study support our hypothesis that proteinuria in diabetic nephropathy is associated with decrease in nephrin levels mediated by an increase in intrarenal ANG II levels.

In human and experimental diabetes, early renal involvement is characterized by hypertrophy of both glomerular and tubuloepithelial elements, thickening of the glomerular and tubular basement membranes, and accumulation of extracellular matrix (ECM) components in mesangium (31). As the disease progresses, mesangial expansion leads to obliteration of the glomerular capillary lumen, proteinuria, and loss of glomerular filtration (32). Altered production of fibrogenic cytokine TGFβ1 has been implicated in diabetic nephropathy (33, 34). Treatment with anti-TGFß1 antibody has been shown to attenuate renal hypertrophy and enhanced extracellular matrix gene expression in STZ-induced diabetic mice (35). In this study, we have shown induction of TGFß1 and type IV collagen gene expression as early as 14 weeks of age in Zucker rats. Our findings suggest that agents that block TGFß1 locally may be useful for preventing matrix accumulation at a very early stage of diabetic nephropathy.

One of the hallmarks of diabetic nephropathy is progressive mesangial expansion, eventually leading to a decrease in filtration surface area and a reduction in glomerular filtration rate (36, 37). Mesangial expansion is due to an accumulation of ECM components mediated by elevated levels of glucose and/or TGFß1 (38- 41). Though type V and VI collagens, heparin sulfate proteoglycan, laminin, and fibronectin are present, the glomerular extracellular matrix is primarily composed of type IV collagen (31). Type IV collagen consists of at least five distinct chains: "classical" [alpha 1(IV) and alpha 2(IV)] and "novel" [alpha 3(IV), alpha 4(IV), and alpha 5(IV)] chains (31). Several studies have determined the expression of type IV collagens in human and experimental models of diabetes (42-46). Results of this study also confirm published observation of increase in IV collagen expression and renal pathology with respect to glomerulosclerosis, increased ECM deposition tubular dilation and interstitial fibrosis (47, 48).

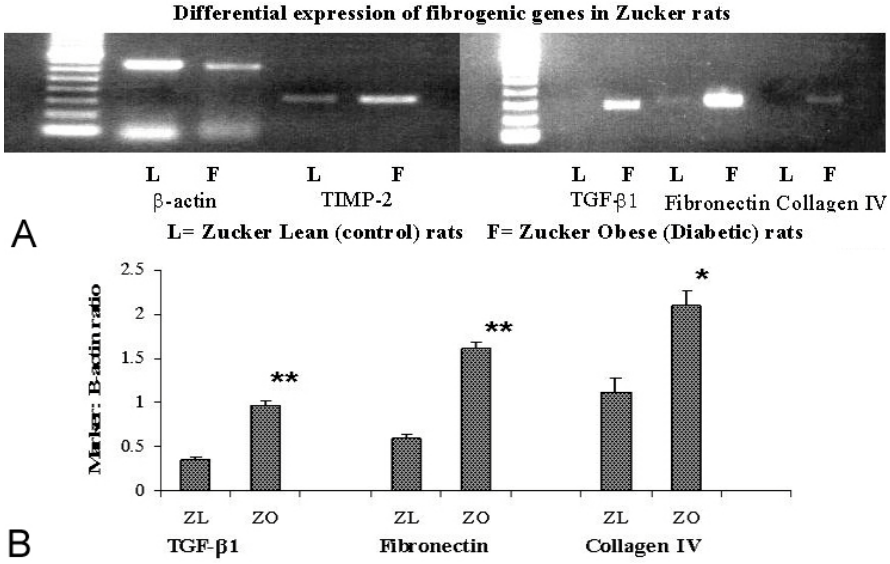

Figure 4. Glomerular expression of TGFβ1, collagen type IV and fibronectin. A. Differential expression of TGFβ1, collagen type IV and fibronectin and tissue specific inhibitor of matrix degrading proteases (TIMP2) measured in glomeruli from Zucker Obese (ZO) and control Lean (ZL) rats at 14 weeks of age by RT-PCR. L bands represents from Lean control rat, where as F bands represents from ZO rats. B. Graph shows densitometry analysis (mean ± SEM, groups of 3 animals) of the ratio of nephrin to β-actin. ZO rats have significant less (*P<0.05) nephrin expression as compared to ZL rats (untreated or captopril treated).

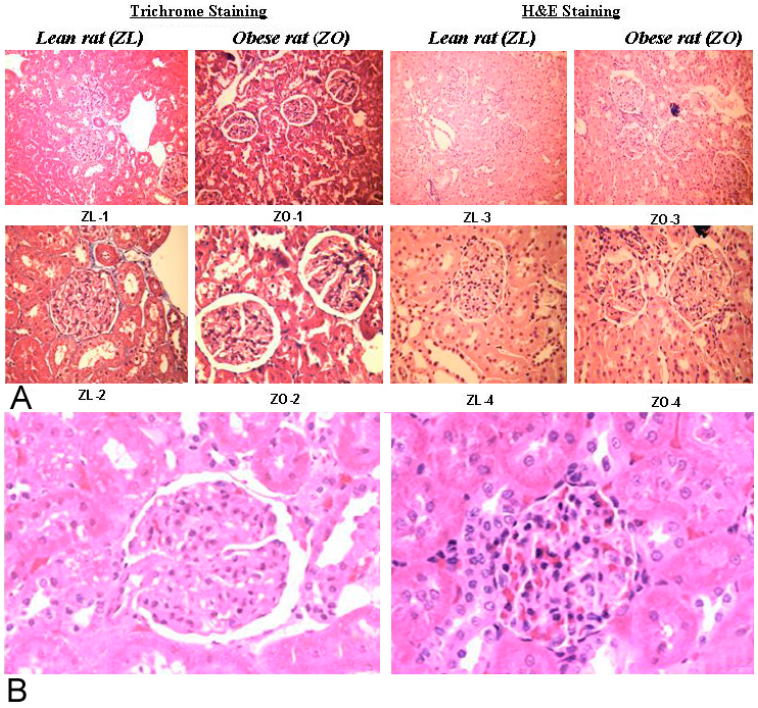

Figure 5. A. Histological changes in kidneys. Representative Trichrome and H&E stained thin section of kidneys from Zucker Obese (ZO) and Zucker Lean (ZL) rats at 21 weeks of age are shown. ZL-1, ZL-2, ZO-1 and ZO-2 are representative trichrome stained thin section, whereas ZL-3, ZL-4, ZO-3 & ZO-4 are representative H&E stained thin section of kidney cortex at 100X and 200X magnification respectively. In comparison to ZL control rat normal kidney cortex and glomerular morphology, ZO rat exhibits moderate degree of chronic nephropathy at 100 X (ZO-1 & ZO-3), and global glomerular sclerosis at 200X magnification. B. Histological Changes in glomeruli and tubules. Representative PAS stained thin section of kidneys from Zucker Obese (B. Left) and Zucker Lean (B. Right) rats at 21 weeks of age. (B. Left) ZO rat glomerulus at 400X magnification shows global glomerular sclerosis, tubular dilation and increased interstitial cellularity. B. Right. ZL control rat glomerulus at 400X magnification exhibit normal "open-lattice" morphology and tightly packed tubules.

In summary, our results document that intrarenal ANG II is chronically increased and mediates proteinuria via decrease in expression of nephrin and sclerosis via an increase in expression of TGFβ1. Treatment with ACE blocks intrarenal ANG II increase and reverses expression of nephrin and TGFβ1 and thus results in decrease in proteinuria and sclerosis. Understanding the mechanism by which Intrarenal ANG II activates TGFβ1 and modulates the expression of key cytoskeletal protein such as nephrin will open new avenues for treatment of *type 2* diabetic nephropathy.

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