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Zhenglun Li March 28th, 2023

Efficacy of Voclosporin in Maintaining Podocyte Function and Viability in a Rat Streptozotocin Model of Diabetic Nephropathy

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Efficacy of Voclosporin in Maintaining Podocyte Function and Viability in a Rat Streptozotocin Model of Diabetic Nephropathy

By Zhenglun Li

Diabetic nephropathy is a leading cause of end-stage renal disease. Diabetes stimulates the activity and expression of calcineurin; a serine-threonine phosphatase that is a critical regulator of podocyte function and stability. Because calcineurin has been linked to podocyte apoptosis, Synaptopodin degradation, and slit pore function we investigated the effects of Voclosporin, a third-generation calcineurin inhibitor (CNI), on the progression of diabetic nephropathy. Streptozotocin (60 mg/kg) was administered to male Sprague-Dawley rats to induce diabetes. Animals were divided into three cohorts including normal animals (15), diabetic controls (30), and diabetics treated with oral Voclosporin (30, 5.0 mg/kg) by 2X per day gavage feeding. Animals were euthanized at 6, 9, and 12 weeks and the renal cortex was harvested for histologic and western analysis of calcineurin, WT-1 (Wilms tumor-1), and CTGF (connective tissue growth factor) expression. Streptozotocin-treated animals developed hyperglycemia (BG-500-600 mg/dl) within 30 days and proteinuria by Day 84. After 6 weeks, renal cortical expression of calcineurin in diabetic controls increased by 3.5X fold compared to normal controls while treatment with Voclosporin reduced the increase by 50%. Similarly, Voclosporin blocked the rise in glomerular CTGF while blocking a fall in WT-1 and Synaptopodin expression. Sirius Red staining demonstrated that Voclosporin treatment did not lead to an increase in interstitial fibrosis compared to diabetic controls. This study demonstrates that severe diabetes upregulates calcineurin expression in the renal cortex. Treatment of diabetic animals with Voclosporin reduced podocyte depletion and stabilized Synaptopodin without exacerbation of interstitial fibrosis.

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This study was supported by the Robert P. Apkarian Integrated Electron Microscopy Core (IEMC) at Emory University, which is subsidized by the School of Medicine and Emory College of Arts and Sciences. Additional support was provided by the Georgia Clinical & Translational Science Alliance of the National Institutes of Health under award number UL1TR000454. The content is solely the responsibility of the authors and does not necessarily reflect the official views of the National Institutes of Health. The data described here was collected on the JEOL JEM-1400, 120kV TEM supported by the National Institutes of Health Grant S10 RR025679. The authors thank Jeannette Taylor for her assistance with the EM studies.

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Table of Content

Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease (ESRD). While the popular use of renin-angiotensin-aldosterone system (RAAS) blockade and other conventional methods have slowed disease progression, RAAS blockade can only protect renal function for up to 1 year after dialysis initiation[1]. As a result, much effort has been devoted to understanding the mechanisms by which the diabetic condition leads to typical histopathologic changes including mesangial expansion, thickened basement membranes, and loss of podocyte density and functionality (figure 1). In this study, we concentrated on podocyte

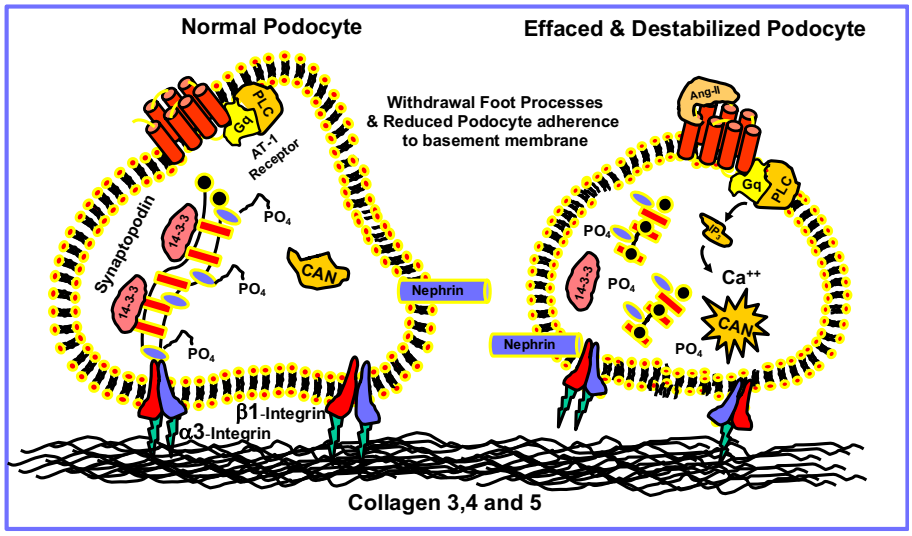

Figure 1. Graphic showing normal podocyte (left) and diabetic podocyte (right) that is effaced and destabilized in the diabetic kidney.

responses to diabetes and its potential for mitigating some of the podocytopathy in this process.

Studies have suggested that glomerular podocyte injury is central to the pathogenesis of diabetic nephropathy and that clinical treatment should be directed toward maintaining podocyte viability[2]. Podocytes are highly differentiated cells with limited differentiation or replicative capability. They function as the filtration barrier with fenestration/slit pores between their foot processes, allowing specific solutes and liquids to pass through the pores[3]. Therefore, the reduction in podocyte density either due to apoptosis or detachment contributes to DN

progression. This phenomenon is well established in Pima Indians with type II diabetes, correlating the widening of the Foot processes with the reduction in the number of podocytes[2]. Another study among type 2 diabetes patients established a negative correlation between podocyte density and albuminuria[4]. Podocyte density therefore can be used as a marker for kidney health.

In early work, Dr. Jennifer Gooch concluded that calcineurin is not only required for glomerular and whole kidney hypertrophy in diabetic rodents but also was activated in diabetes and is required for extracellular matrix accumulation[5]. In a subsequent paper, this group studied the functions of subunits of calcineurin A and determined that calcineurin A beta is required for hypertrophy but not matrix expansion in the diabetic kidney[6]. Further work showed that reactive oxygen species-generating enzymes called NADPH oxidases (Nox) shared a common signaling pathway with calcineurin. Calcineurin A alpha is constitutively active, but diabetes (high glucose levels) selectively activates Calcineurin A beta[7], meaning that Calcineurin A beta is the only subunit out of the two that mediates renal hypertrophy. Loss of calcineurin A beta reduces basal expression and blocks Nox2 and Nox4 induction[7]. This regulation is accomplished through activation or inhibition of intermediate nuclear factor of activated T cells (NFAT) whose inhibition decreases Nox2 and Nox4 expression, and its overexpression increases Nox2 and Nox4. This association is commonly referred to as the Calcineurin A beta/NFAT pathway[7]. Calcineurin A beta/NFAT pathway regulates high glucose-mediated hypertrophic responses in the kidney. Diabetic podocytopathy is positively correlated with calcineurin levels. Calcineurin activity is increased by 50% through enhanced expression of the Calcineurin A beta rather than the alpha isoform[7]. Therefore, the specific inhibition of calcineurin isoforms may provide potential therapeutic effects against podocytopathy.

The calcineurin inhibitors cyclosporine and tacrolimus have been clinically used in post-transplant patients with excellent short-term effects, but the nephrotoxic side effects prevent long-term usage of these drugs[8]. Voclosporin is a next-generation calcineurin inhibitor that is a

structural analog of cyclosporin A. While sharing a nearly identical core structure with Voclosporin, cyclosporin A has an additional double-bond carbon that alters the pharmacokinetic and immunosuppressive properties of Voclosporin. The formation of complexes between cyclophilin A and cyclosporin A or Voclosporin allows for binding to the calcineurin heterotrimer complex and allosterically inhibits its phosphatase activity, potentially increasing its affinity for the beta isoform as a selective beta antagonist. Birsan et al. compared the inhibitory effects of Voclosporin and cyclosporin. They found that Voclosporin had a stronger inhibitory effect than cyclosporin A and it also selectively inhibited phosphatase activity of the beta isoform[9]. Since the loss of calcineurin A alpha isoform is closely linked to increased transforming growth factor (TGF)-beta and fibrosis-related nephrotoxicity and renal function impairment, and the loss of calcineurin A beta isoform mediates the NFAT pathway podocytopathy[10], Voclosporin's selective inhibition of the beta isoform might limit the nephrotoxicity from inhibition of calcineurin A alpha, while still reducing podocytopathy via NFAT pathway similar to cyclosporin A.

Several proteins are chosen as renal markers to confirm the hypothesis. Calcineurin dephosphorylates Synaptopodin, undocks and signals Synaptopodin for proteolytic degradation[11]. Podocyte foot plate processes are regulated by the dephosphorylation of Synaptopodin and phosphorylation by protein kinase A (PKA) and other serine-threonine kinases. Studies in streptozotocin (STZ) treated rats shows the correlation between diabetes and the reduction in Synaptopodin expression in glomerular podocytes[12]. Further study on STZ-induced diabetic rats demonstrated a progressive reduction in Synaptopodin expression over time[13], linking Synaptopodin expression with functional loss of podocyte foot plate processes and progressive diabetic nephropathy. WT-1 staining has been proven as a reliable podocyte density indicator and previous studies have shown decreased WT-1 staining levels with diabetic nephropathy[14]. When incubated with cyclosporin A or tacrolimus, cultured mesangial cells exhibited a significant increase in CTGF levels[15]. With strong evidence supporting the positive

correlation between fibrosis and CTGF[13], CTGF can be used as a marker for fibrosis and resulting nephrotoxicity. Whether Voclosporin is non-nephrotoxic can also be assessed by comparing the level of CTGF.

The present study provides data that extends our understanding of Voclosporin's effect on podocytes in general and in the diabetic kidney specifically. We selected renal markers Synaptopodin, calcineurin, CTGF, and WT-1 to assess renal function during the progression of diabetes and whether Voclosporin attenuates the damages by diabetes and preserves podocyte density and health.

Methods

Animal model

75 male Sprague-Dawley rats between 170 and 190 g were purchased from Charles River Laboratories and the model was accomplished at Washington Biotechnology, Inc, Baltimore, MD. Rats were randomly assigned into three cohorts of 25 rats: each cohort consisted of non-diabetic control (5 rats), Diabetic (10 rats), and Diabetic with Voclosporin treatment (10 rats). The three cohorts were followed at different times with the cohorts being euthanized at 6, 9, and 12 weeks after the induction of diabetes and beginning of Voclosporin treatment. The diabetic condition was induced by tail vein injection of 60 mg/kg streptozotocin (STZ) in 0.01M citrate buffer, pH 4.5. The animal model is shown schematically in Figure 2 below. Voclosporin was solubilized in vehicle solution (VitE-TPGS, medium change triglyceride oil, Tween 40, 95% EtOH vehicle (4:2:2:1) and delivered by gavage feeding twice daily (5 mg/kg). Rats in the control and the diabetic group were fed with the same gavage vehicle without Voclosporin to reduce confounding variables. The rats were sacrificed at Washington Biotechnology, and their kidneys were collected at three-time points: 42 days; 63 days; and 84 days after the STZ injection, frozen, then shipped to Emory. The two kidneys from each rat are separated for different analysis purposes. The left kidney was sub-dissected to yield a small cortex sample and placed in a fixation buffer for electron microscopy (see below). The remainder of that kidney was placed in a 4% paraformaldehyde fixation buffer for conventional histology. The right kidney was immediately frozen in liquid nitrogen and stored at -80oC until prepared for western analyses. Blood and urine samples were collected and measured for blood glucose, total protein, serum albumin, and urinary protein losses. All animal procedures were performed according to the Washington Biotechnology IACUC.

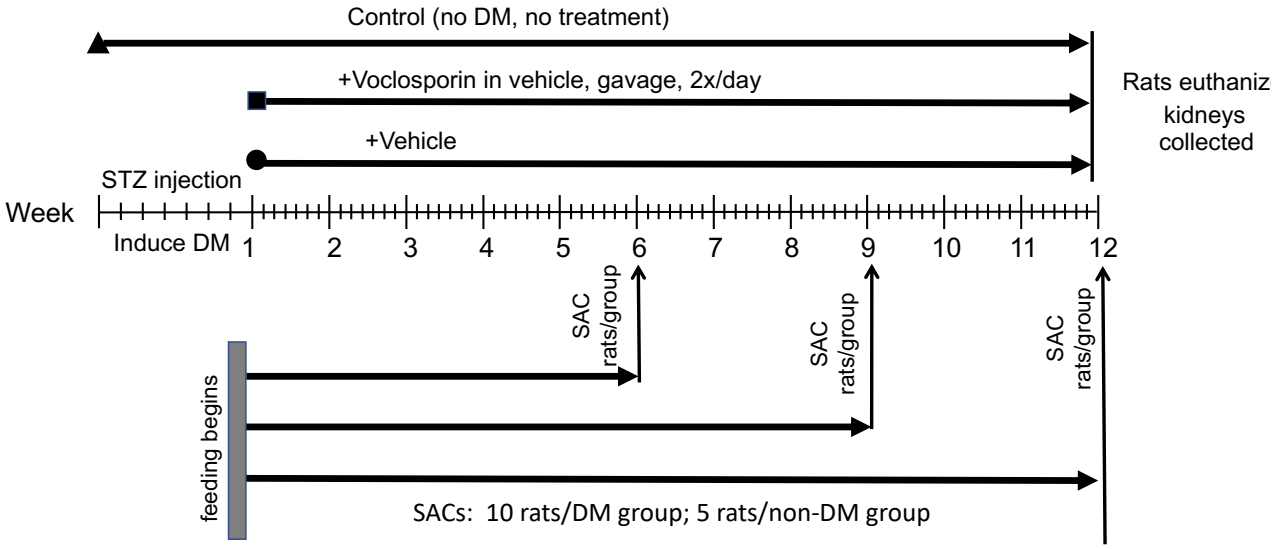

Figure 2. Schematic drawing of the animal protocol. 3 groups of rats were prepared: nondiabetic control (n=15), diabetic (DM) (n=30), and Voclo (n=30). Diabetes was induced by tail vein injection of streptozotocin (STZ) at 0 times. Voclosporin (5 mg/kg) was delivered twice daily by gavage feeding. Nondiabetic control and diabetic untreated received vehicle twice daily. Cohorts of 5 control, ten diabetic with Voclosporin treatment, and ten diabetic rats were sacrificed (SAC) and kidneys were collected at 6 weeks, 9 weeks, and 12 weeks post STZ injection.

Sample preparation

Frozen kidneys were dissected, and the kidney cortex was homogenized in a glass homogenizer on ice with 1mL isolation buffer (10 mM triethanolamine, 250 mM sucrose at pH 7.6, 0.2 μM leupeptin, 0.1 M PMSF, 1X Halt protease cocktail (ThermoFisher, LOT # XD343860), pH 7.4. After homogenizing, SDS was added to a final concentration of 1%, the sample was sheared with a 26G insulin syringe into microcentrifuge tubes and centrifuged at 4°C for 10 min. Protein concentration was determined using the BioRad DC Protein Assay (DC Protein Assay Kit II #5000112). Samples were diluted with SDS-PAGE sample buffer to 2-5 mg/mL protein concentration and boiled for 1-2 min. as previously described[16].

Western blotting

Equal amounts of kidney cortex lysate (40-50 µg/lane) were separated on SDS polyacrylamide gels as follows: Synaptopodin: 15% gel; CTGF: 10% gel; WT-1: 4-20% gradient. Protein was electroblotted to PVDF membrane, blocked, and incubated with primary antibodies overnight at 4°C and secondary antibodies for 2 hours at room temperature. as described previously[17]. Protein bands were scanned and quantified using the Li-COR Odyssey infrared scanning system (Li-COR Biosciences, Lincoln, NE, USA). Primary antibodies: Synaptopodin, 1:1000 dilution (Novus, 6049-1501), CTGF, 1:1000 dilution (RayBiotech, 81255530RAY8) (AB Clonal, cat# A11456), WT-1, 1:1000 dilution (Proteintech, 12609-1-AP), Calcineurin (AB Clonal, cat# A4346). Secondary antibody: donkey anti-rabbit IRDye 680RD, LICOR Cat. No. D20322-01 at 1:10,000 concentration. The loading controls (L.C.) were determined by staining membranes for total protein with Ponceau S.

Histology

Kidneys were sectioned into 5mm sections and fixed in 4% paraformaldehyde for 48 hours then given to the Winship Cancer Tissue and Pathology Core for paraffin embedding, slide preparation, H&E, and Sirius Red staining using their standard protocols.

Immunohistochemistry

Kidney section slides from the histology core were dewaxed (ethanol washes), treated with trilogy for antibody retrieval (Millipore-Sigma, Cat. #920P-09), incubated overnight at 4°C with primary antibodies, and labeled with the ImmPRESS Horse Anti-Rabbit IgG (Vector Laboratories Cat.# MP-6401-15) as previously described[16]. The slides were then stained with a DAB Substrate Kit (Vector Laboratories, Cat. # SK-4100). The following dehydration and mounting Permount mounting medium (Fisher Scientific Company, Cat #SP15-100). The slides were visualized using Hamamatsu Nanozoomer HT 2.0 at 40x. Protein staining was visualized

using NDP.view2 software (figure 4,5,9,11,12,14) and analyzed with QuPath (figure 9,11,12,14). QuPath analyzed Synaptopodin and CTGF by measuring the staining levels within the glomerulus, the staining level was also measured for CTGF tubules, and WT-1 was measured by the percentage of area stained within the glomerulus region.

Electron microscopy

Electron microscopy was performed at the Robert P. Apkarian EM core. Samples were fixed with Karnovsky's fixative (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The sample material was washed and embedded in Ted Pella's Eponate 12™ Epoxy resin. The polymerized sample blocks were sectioned using a Diatom diamond knife with a Leica/Reichert Ultra Cut S ultramicrotome. The thin sections (~80nm) were collected onto 200 mesh copper grids and post-stained in 5% Uranyl Acetate and Lead Citrate. Sections were imaged using a JEOL JEM-1400 LaB_6TEM at 80 kV with Gatan Ultra Scan 1000 2K x 2K CCD camera (figure 7).

Statistics

All data are presented as mean ± SE. To test for statistical significance between the 2 groups, we used a student's t-test. To test more than 2 groups, we used an analysis of variance, followed by Fisher's least significant difference (protected t-test)[18] to determine which groups were significantly different. The criterion for statistical significance was $P < 0.05$.

Results

Diabetic rats were used as the animal model to study the effect of Voclosporin on the diabetic kidney. As detailed in the methods section, 60 rats were injected with 60 mg/kg of STZ and blood glucose was measured after 3 days to verify hyperglycemia. Groups were established with the control group never receiving STZ, and the diabetic group (receiving 1 STZ injection)

split into the diabetic control (no further treatment) and the Voclo group (receiving Voclosporin by gavage feeding twice a day after successful induction of diabetes). Multiple physiological measurements were made at Washington Biotechnology at the beginning of the model and at the 3 termination points (6 weeks, 9 weeks, and 12 weeks) over the 12-week duration of the model as shown in figure 2.

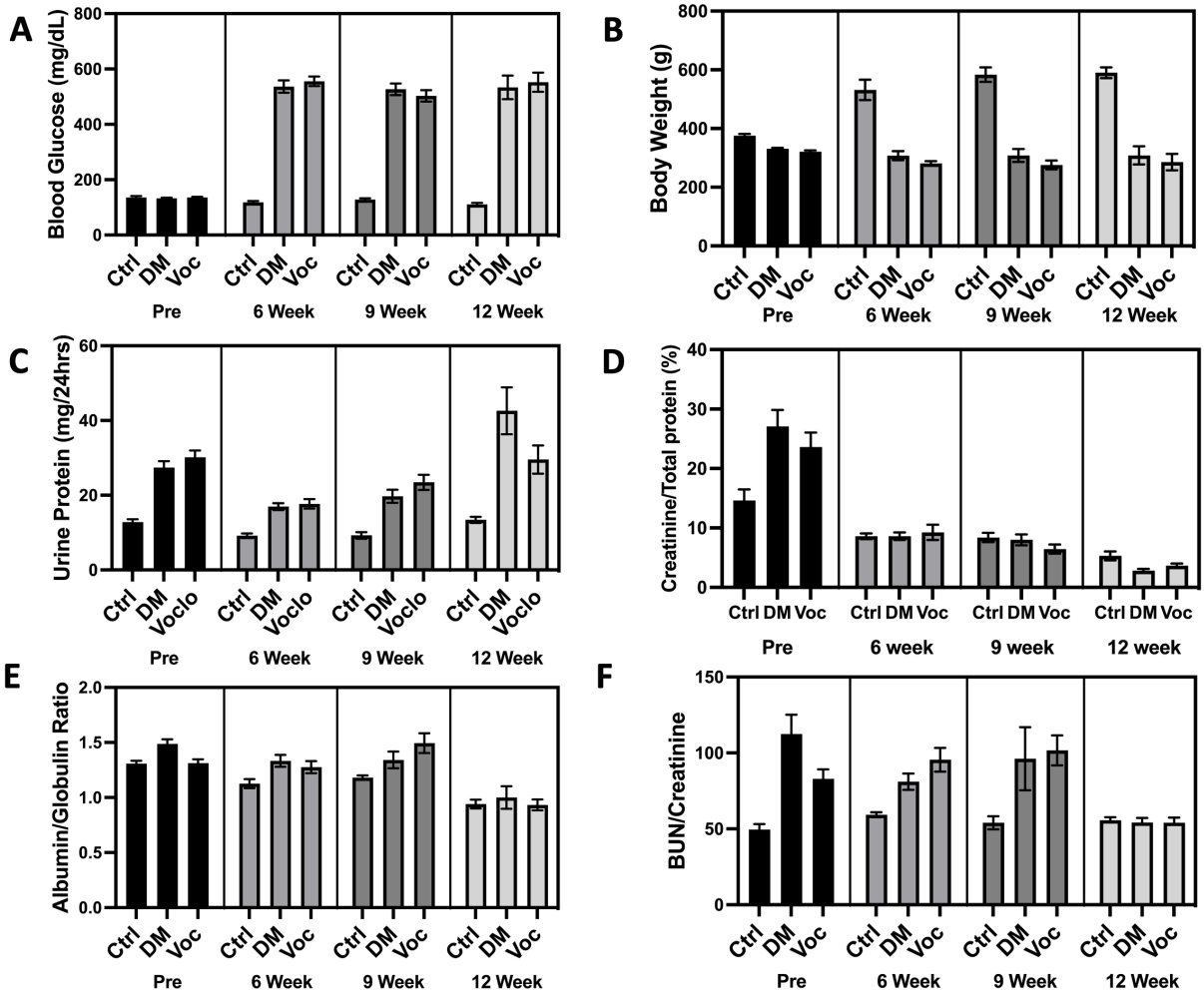

Figure 3. Physiological parameter measurements of diabetic rat groups over time. The rats were divided into 9 groups, groups 1 (control, C, n=5, white), 4 (diabetic, DM, n=10, black) and 7 (DM + voclosporin treatment, V, n=10, gray) were terminated at 6 weeks; groups 2 (C, n=5), 5 (DM, n=9) and 8 (V, n=8) were terminated at week 9; groups 3 (C, n=5), 6 (DM, n=6) and 9 (V, n=6) were terminated at week 12 after the initiation of diabetes with streptozotocin injection. The initial values at time of STZ injection is shown as “Pre” and provides levels for all rat treatment groups (C, n=15; DM, n=30; V, n=30). Data=mean +/- s.e. Parameters: (A) Blood glucose levels provided in mg/dL; (B) body weight in grams; (C) urinary protein as mg excreted per 24 hours; (D) percentage of urine creatinine (E) Albumin/Globulin ratio; (F) BUN/Creatinine ratio.

Blood Glucose, Total Protein, Albumin, and Proteinuria at Baseline

As shown in Figure 3A, STZ treatment resulted in blood glucose levels ranging between 500 and 600 mg/dL. Rat blood glucose levels were measured prior to the experiment and at each termination time point. There was a significant increase when comparing the Voclosporin-treated (Voclo) group (531 ± 10 to 552 ± 34) or diabetic group (531 ± 9 to 534 ± 42) with the control group (113.3 ± 4 to 111 ± 5), but there was no significant difference between the diabetic and the Voclo groups. This level of hyperglycemia remained stable for the duration of the 12-week treatment period. A trend of a similar reduction in DM and Voclo groups when the body weights of rats were compared with the control group was also observed (figure 3B). To determine whether there was evidence of renal failure, urine protein, and urine creatinine levels were assessed (figure 3C). Urine protein levels rose within 15 days of STZ treatment and remained 2X above baseline levels at 6 weeks. The addition of Voclosporin reduced urinary protein levels at 12 weeks but did not change levels either in weeks 6 or 9. Urine creatinine percentage remains relatively stable across different groups at all 3 collection times (figure 3D). Figure 3E shows the change in total protein and albumin levels in the serum. Total protein levels were significantly lower in the Diabetic and Voclosporin animals by week 6 and tended to increase in the Voclosporin-treated animals by week 12. Both total serum protein and serum albumin were significantly different from control levels at all three collection points. Although diabetic and Voclo groups appear to diverge at the 12 week point, the difference did not reach statistical significance. Figure 3F illustrates BUN to creatinine ratios in the serum. The ratio is significantly higher in the diabetic or Voclo groups when compared with the control group in weeks 6 and 9, but Voclo group does not differ from DM group. And there were no differences in BUN to creatinine levels in any group over the 12-week study period.

Cortical Glomerular and Interstitial Fibrosis: Effect of Voclosporin in Renal Scarring

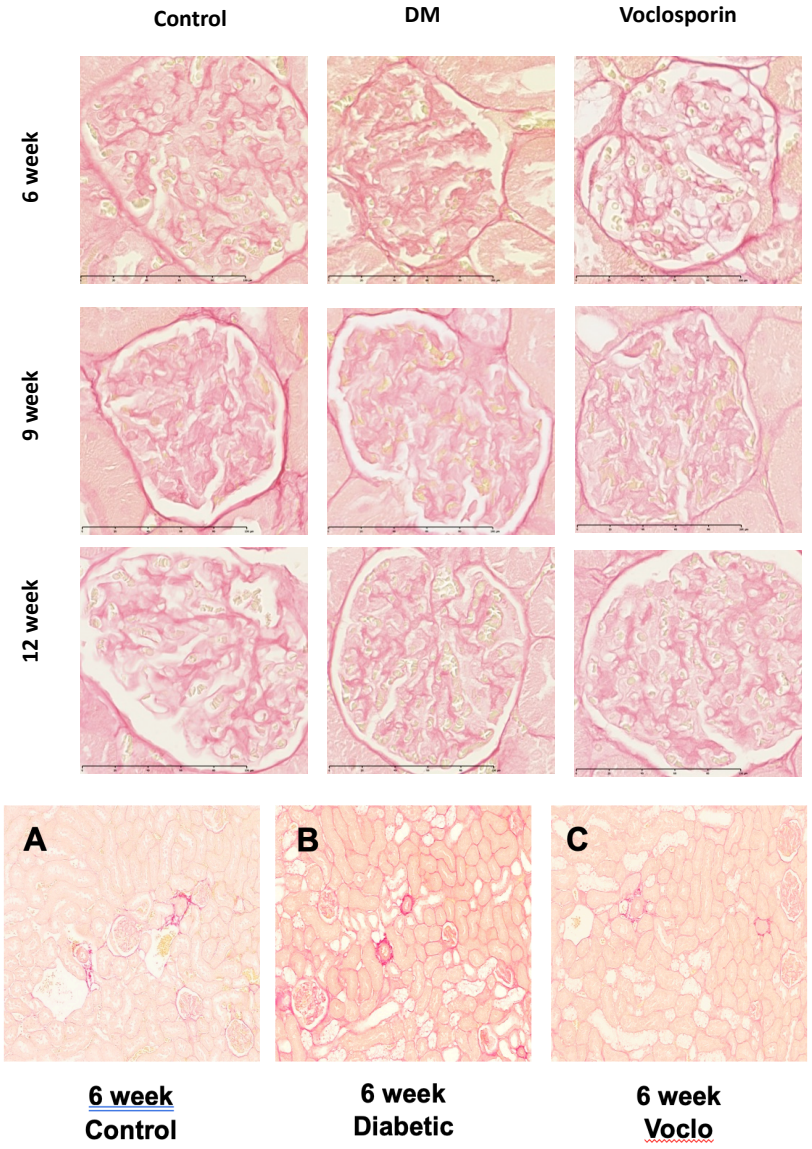

Figure 4. Staining for indications of collagen deposition. Top panel of 9 shows single glomeruli at each timepoint. The bottom panel shows a lower magnification of the early treatment timepoint (6 weeks). Shown are Sirius Red-stained sections from control (A), diabetic (B) and Voclosporin treated (C) rats. Red stain indicates collagen deposition. Scale bars indicate image size in μm.

Figure 4 is a representative sample of the whole glomeruli stained for collagen deposition using Sirius Red. There was minimal collagen deposition within Bowman's capsule and the peri-glomerular space or within the tubulointerstitium. The middle column of the DM group is a representative sample of glomeruli obtained from animals with uncontrolled diabetes. After 6 weeks, there is an increase in collagen staining in the peri-glomerular areas as well as the

tubulointerstitium and basement membranes of the tubular epithelium. The right image demonstrates glomeruli at week 6 from the Voclo group. There was a reduction in collagen deposition within all regions of the glomeruli when compared to the diabetic group in the middle column.

Calcineurin Expression Following Induction of Diabetes: Effect of Voclosporin

To determine whether the onset of diabetes altered the expression of calcineurin, we isolated the renal cortex at week 6 and performed a western analysis of the protein expression of calcineurin.

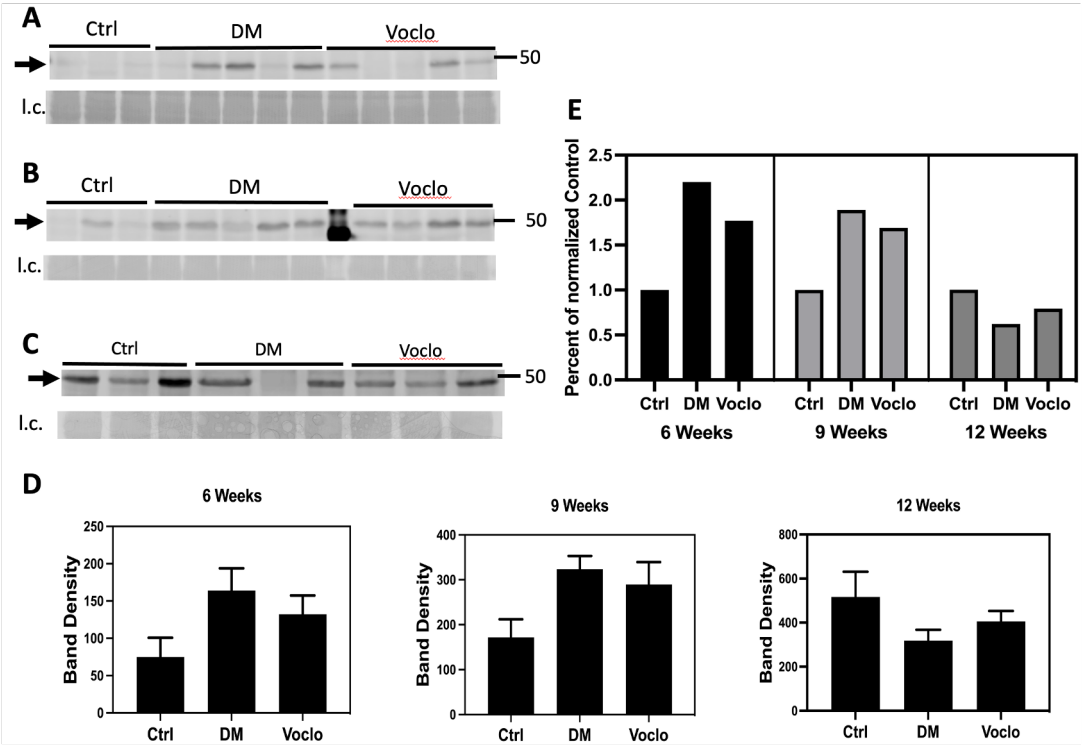

Figure 5. Representative Western blot results showing levels of calcineurin in kidney cortex. (A) Left: Western blot of rats treated for 6 weeks with, from left to right, control (Ctrl), diabetic (DM), and voclosporin treated DM (Voclo) samples. Beneath each western blot is the loading control (l.c.). Each lane is a sample from a different rat. Right: bar graph of band densities normalized for loading controls. Control n=5, DM n=10, Voclo n=10. Data = mean +/- s.e. (B) Western blot of rats treated for 9 weeks. Bars: Control n=5, DM n=9, Voclo n=7. (C) Western blot of rats treated for 12 weeks. Bars: Control n=5, DM n=6, Voclo n=6 (D) Levels of Calcineurin in each group over time compared to normalized control value expressed as a percent of control.

As shown in Figure 5A, a 51 kDa molecular weight protein binding to a monoclonal primary antibody to Calcineurin was identified. Among the diabetic animals (N = 10, 10, 6 for 6, 9, and 12 weeks, respectively), calcineurin expression was increased compared to controls (N=5 for 6, 9, and 12 weeks). Animals that were treated with Voclosporin demonstrated calcineurin levels that were comparable to normal control animals. Densitometry analysis of the representative western blots (figure 5D) demonstrated that the onset of diabetes increased calcineurin expression by approximately 3.5-fold. Concomitant treatment with oral Voclosporin reduced calcineurin expression by 50% versus diabetic levels.

Effect of Diabetes on Podocyte Foot Plate Processes: Effect of Voclosporin

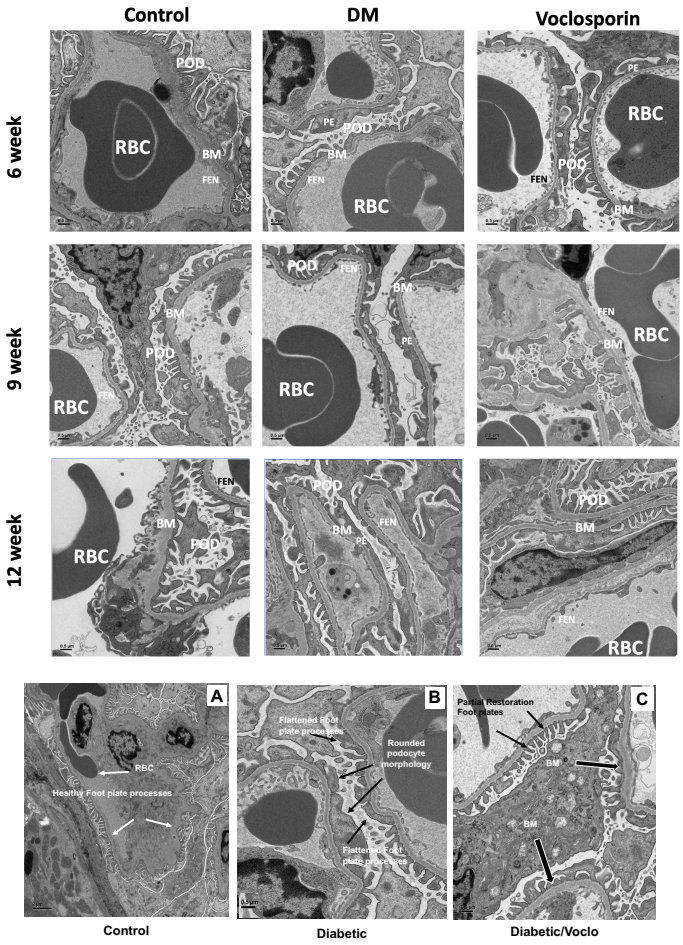

Figure 6. Representative electron micrograph images for control, diabetic (DM), and Voclosporin-treated diabetic (Voclosporin) rat kidneys from 6-week, 9-week, and 12-week cohorts. Representative key structures are identified as RBC (red blood cell), POD (podocyte), PE (podocyte effacement), BM (basement membrane) and FEN (endothelial cell fenestrations). Scale bar = 0.5 µm.

To assess diabetic effects on footplate processes, we performed transmission electron microscopy on cortical glomeruli from control, Diabetes, and Voclosporin groups.

The top panel of 9 images in Figure 6 shows representative electron micrographs of glomeruli from the control, diabetic, and Voclo groups at each of the 3 time points studied. Figure 6A is a representative image of a normal glomerular capillary loop at 6 weeks. Arrows point to normal-appearing podocyte foot plate processes. In contrast, Figure 6B demonstrates a capillary loop from a diabetic animal at 6 weeks. Black arrows highlight areas of footplate effacement with rounded cellular morphology. There were also areas notable for focal

thickening of the glomerular basement membrane (heavy arrow). A glomerulus from a Voclo group kidney treated for 6 weeks is shown in Figure 6C. Footplate processes have partially normalized with interdigitation between cells and more approximates control animals.

Synaptopodin Expression Following Induction of Diabetes: Effect of Voclosporin

Early studies by others in cultured podocytes demonstrated that Synaptopodin is a calcineurin substrate and that dephosphorylation of serine and threonine moieties can accelerate its protein degradation[19]. Because diabetes increased the protein expression of calcineurin, we investigated whether the onset of diabetes and marked hyperglycemia would reduce the protein expression of Synaptopodin.

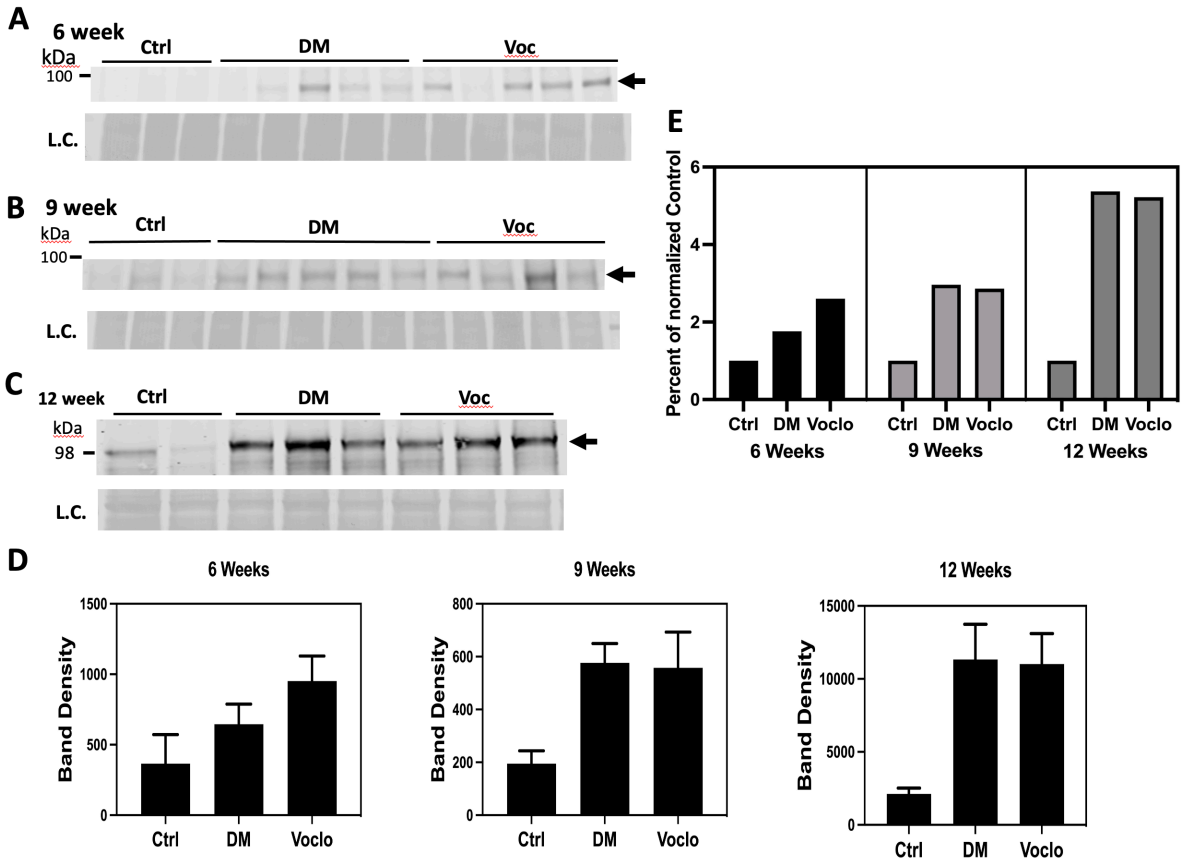

Figure 7. Representative Western blot results showing levels of synaptopodin in kidney cortex. (A) Left: Western blot of rats treated for 6 weeks with, from left to right, control (Ctrl), diabetic (DM), and Voclosporin treated DM (Voclo) samples. Beneath each western blot is the loading control (l.c.). Each lane is a sample from a different rat. Right: bar graph of band densities normalized for loading controls. Control n=5, DM n=10, Voclo n=10. Data = mean +/- s.e. (B) Western blot of rats treated for 9 weeks. Bars: Control n=5, DM n=9, Voclo n=7. (C) Western blot of rats treated for 12 weeks. Bars: Control n=5, DM n=6, Voclo n=6. (D) Levels of Synaptopodin in each group over time compared to normalized control value expressed as a percent of control.

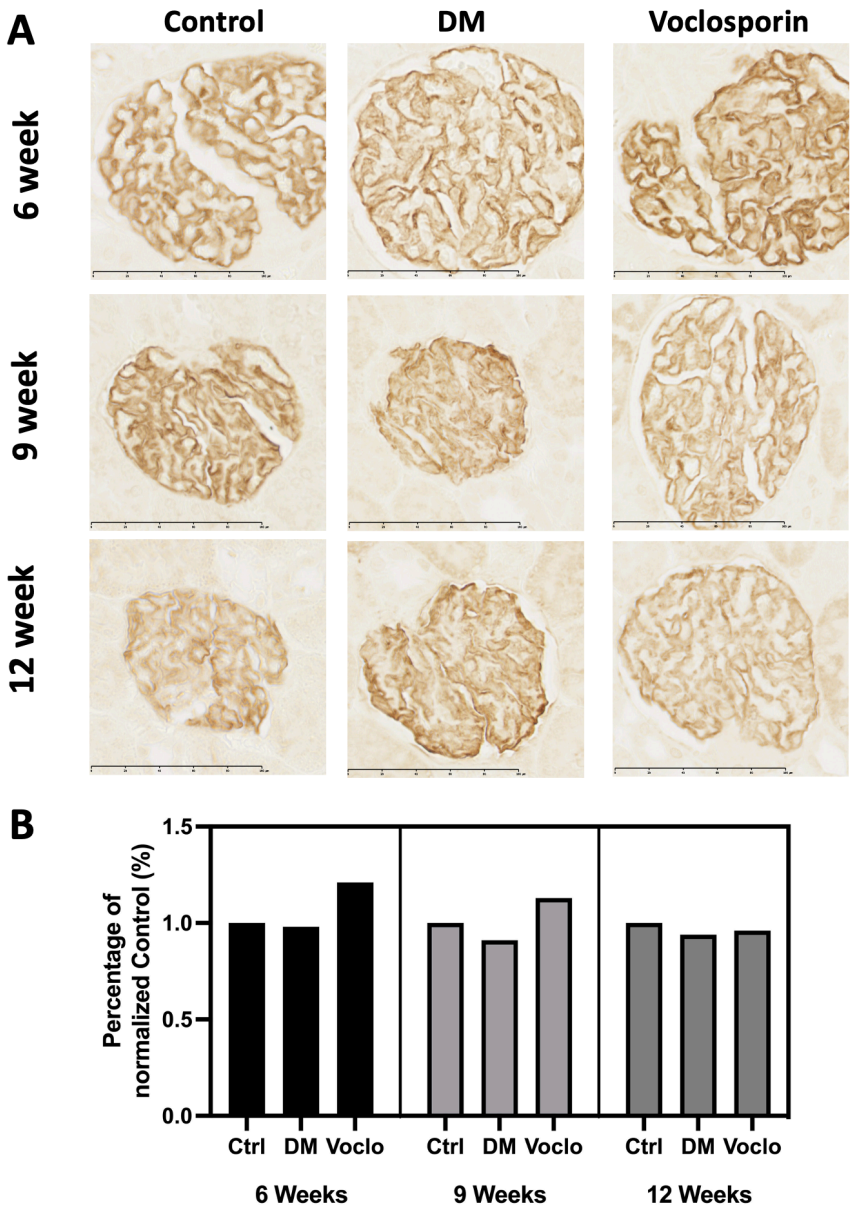

Figure 8. Synaptopodin staining in glomeruli from control, diabetic, and Voclosporin-treated diabetic rats. (A) Images of paraffin embedded kidney sections stained with Synaptopodin antibody followed by HRP-linked secondary antibody and visualized with diaminobenzidine (DAB). Images were acquired using the Hamamatsu Nanozoomer HT 2.0. Representative images of immunohistology for control, diabetic (DM), and Voclosporin-treated diabetic (Voclosporin) rat kidneys from 6-week (top row), 9-week (middle row), and 12-week (bottom row) cohorts are shown. (B) Bar graph shows stain level per glomerulus per kidney (n=25 glomeruli per kidney) for each group at each treatment duration (6, 9, and 12 weeks) as a percent of staining/glomerulus in the normalized control, measured by QuPath and ImageJ. Data = mean ± s.e.; * = $P < 0.05$.

Even though western blotting fails to demonstrate the decreased synaptopodin that is a known consequence of diabetes, we looked for differences histologically using immunoperoxidase staining of cortical glomeruli. Figure 8 demonstrates representative

immunoperoxidase staining of synaptopodin in cortical glomeruli isolated at weeks 6, 9, and 12 from control, diabetic, and Voclo groups. Immunoperoxidase staining for Synaptopodin in glomeruli isolated from the diabetic animals appeared slightly reduced. This did not reach statistical significance. Further analysis of this protein in the histology images will be required to determine if it is reduced and therefore usable in our analysis of voclosporin effects.

Effect of Diabetes on Glomerular CTGF Expression: Effect of Voclosporin

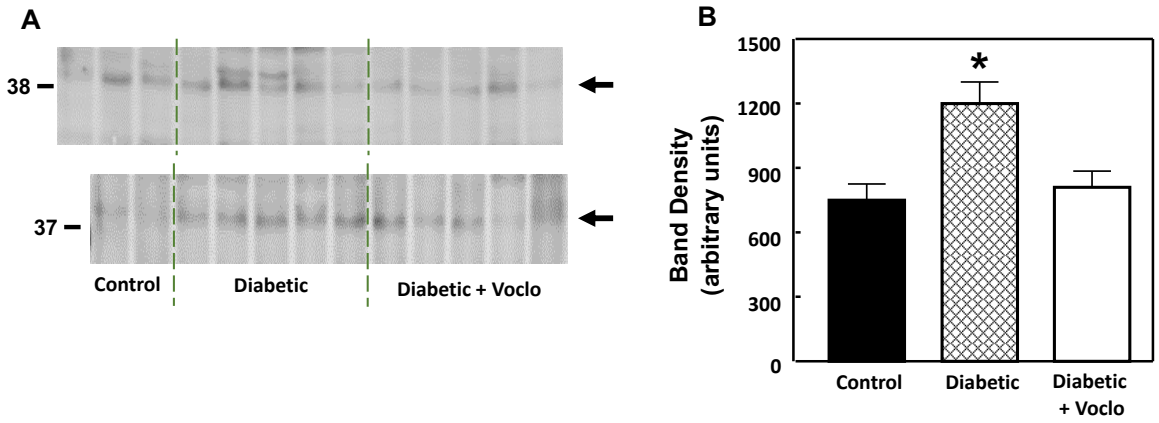

Figure 9A. Western Blots of renal cortical homogenates from control (n=5), diabetic (n=10), and Voclosporin treated (n=10) animals were probed with polyclonal CTGF antibody. Animals treated for 6 weeks are presented here; each lane represents a different animal. Ponceau staining of total protein was used as a loading control (not shown). A faint 38 kDa band consistent with the molecular weight of CTGF was detected in the cortex of normal controls. The CTGF band increased in the diabetic controls but was normalized following treatment with oral Voclosporin. B. Densitometry of the CTGF bands from all samples collected at 6 weeks, expressed in arbitrary units. The bars are Control: Black (n=5); Diabetic: Thatched Lines (n=10); Diabetic + Voclosporin: White (n=10). Data are presented as the mean ± se; $P < 0.05$ is significant.

Previous studies have documented that expression of connective tissue growth factor (CTGF) both in the heart and the kidney is regulated by calcineurin-NFAT dependent pathways[20]. Figure 9 is a western blot of renal cortical homogenates and demonstrates a faint 38 kD band was observed in the cortex of control animals. A similar but enhanced 38 kD band was noted in the diabetic animals, while concomitant treatment with Voclosporin blocked CTGF enhancement. Figure 9D (6 weeks) demonstrates that renal cortical expression of CTGF expression among diabetic animals increased by approximately 40% and was completely normalized by Voclosporin treatment.

Next we performed immunohistochemistry on whole renal cortical tissue and western blot analysis on cortical homogenates using a monoclonal antibody against CTGF examining the effects of diabetes and treatment with Voclosporin.

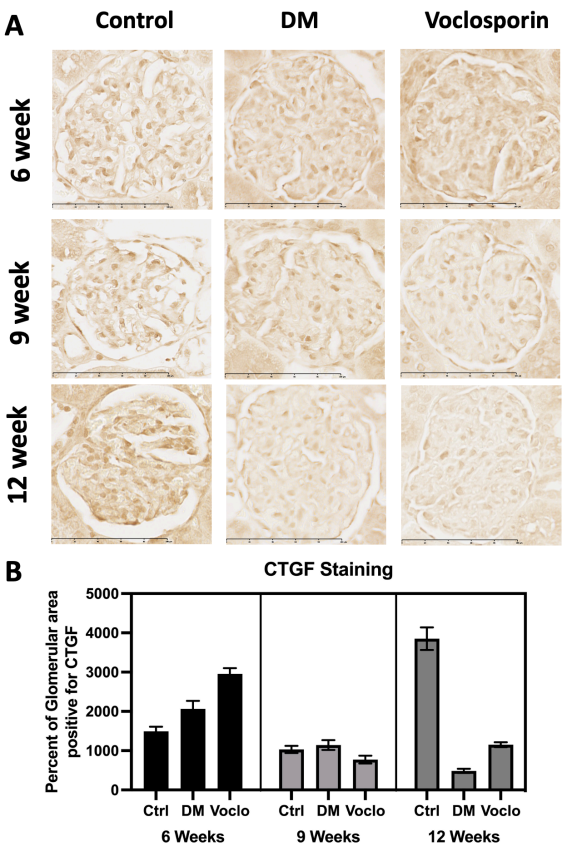

Figure 10. CTGF staining in glomeruli from control, diabetic, and Voclosporin-treated diabetic rats. (A) Images of paraffin embedded kidney sections stained with CTGF antibody followed by HRP-linked secondary antibody and visualized with diaminobenzidine (DAB). Images were acquired using the Hamamatsu Nanozoomer HT 2.0. Representative images of immunohistology for control, diabetic (DM), and Voclosporin-treated diabetic (Voclosporin) rat kidneys from 6-week (top row), 9-week (middle row), and 12-week (bottom row) cohorts are shown. (B) Bar graph shows the average stain density per glomerulus per kidney (n=25 glomeruli per kidney) for each group at each treatment duration (6, 9, and 12 weeks), measured by QuPath and ImageJ. Data = mean ± s.e.; * = $P < 0.05$.

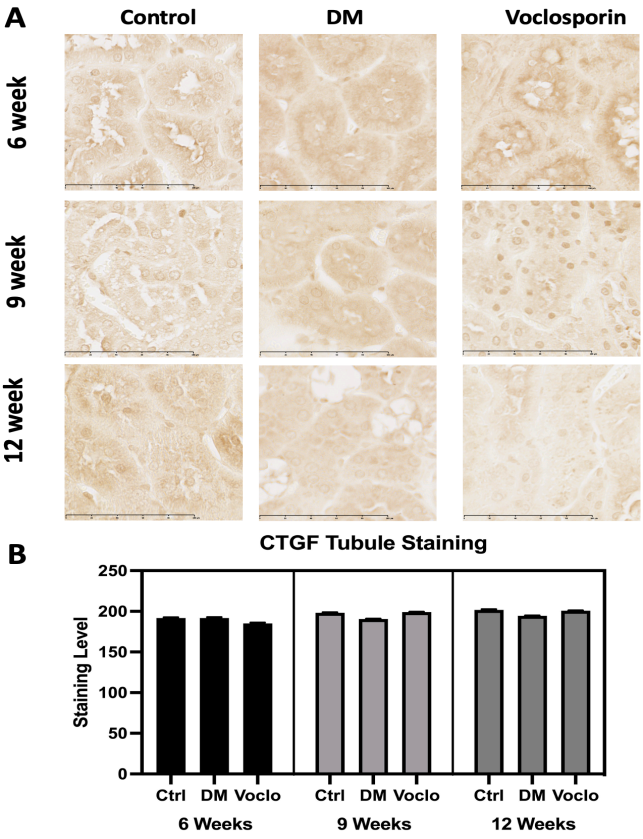

Figure 11. CTGF staining in tubule from control, diabetic, and Voclosporin-treated diabetic rats. (A) Images of paraffin embedded kidney sections stained with CTGF antibody followed by HRP-linked secondary antibody and visualized with diaminobenzidine (DAB). Images were acquired using the Hamamatsu Nanozoomer HT 2.0. Representative images of immunohistology for control, diabetic (DM), and Voclosporin-treated diabetic (Voclosporin) rat kidneys from 6-week (top row), 9-week (middle row), and 12-week (bottom row) cohorts are shown. (B) Bar graph shows the average stain level per rectangular tubular area of 4000 μm^2 per kidney (n=25 rectangular areas per kidney) for each group at each treatment duration, measured by QuPath and ImageJ. Data = mean ± s.e.; * = $P < 0.05$.

As shown in Figures 10A and 11A, after 6 weeks, the expression of CTGF was found in the periglomerular areas and along the basement membranes of the renal tubular epithelium. For the diabetic group, CTGF staining at 6 weeks was enhanced in both the periglomerular areas

and tubular basement membranes compared to the control group. Treatment with Voclosporin showed reduced CTGF staining in all renal cortical areas.

Effect of Diabetes on Glomerular WT-1 Expression: Effect of Voclosporin

Previous studies have used expression of Wilms's Tumor-1 (WT-1) on the surface of glomerular podocytes as a maker for podocyte density and to assess the effectiveness of therapeutic maneuvers to reduce podocyte apoptosis and detachment[13]. We examined the levels of WT-1 by western analysis and followed with assessment by immunohistochemistry.

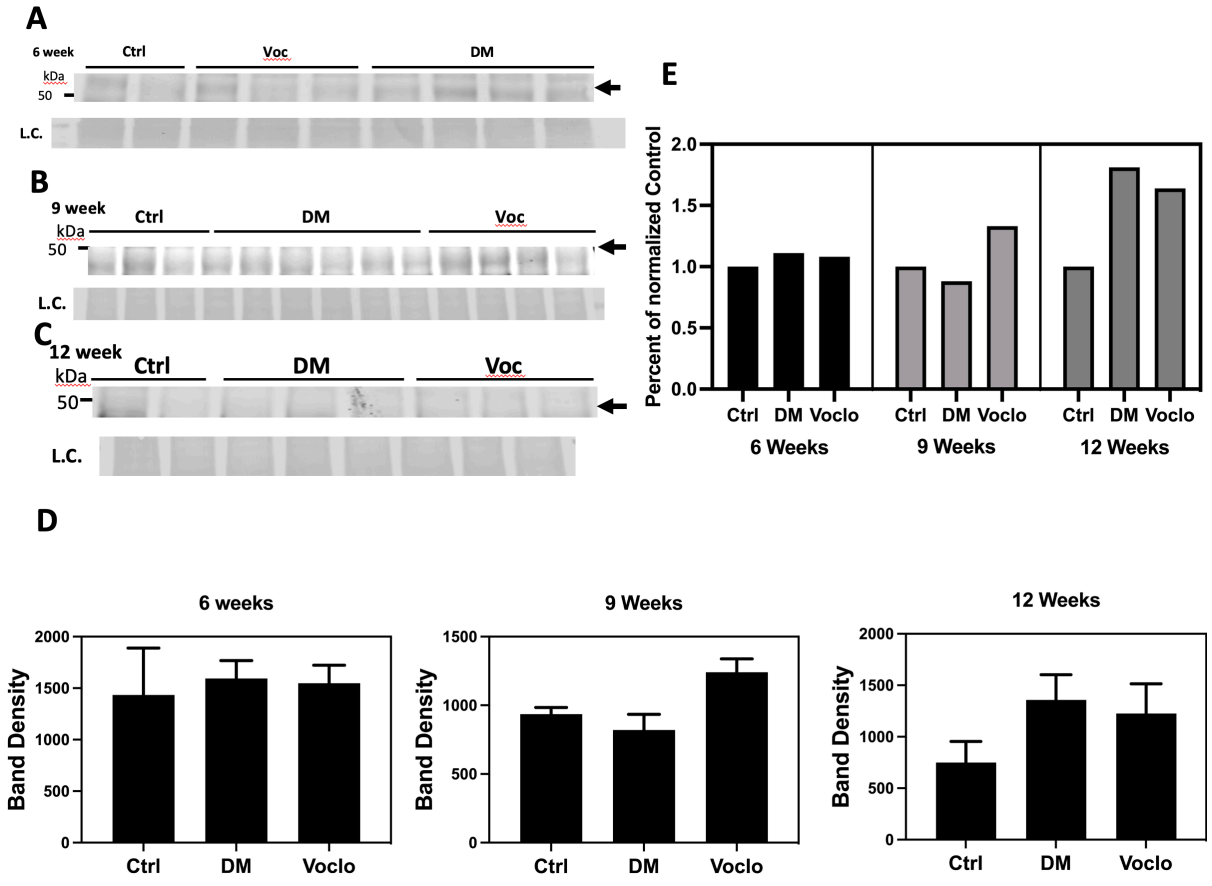

Figure 12. Representative Western blot results showing levels of WT-1 in kidney cortex. (A) Left: Western blot of rats treated for 6 weeks with, from left to right, control (Ctrl), diabetic (DM), and voclosporin treated DM (Voclo) samples. Beneath each western blot is the loading control (l.c.). Each lane is a sample from a different rat. Right: bar graph of band densities normalized for loading controls. Control n=5, DM n=10, Voclo n=10. Data = mean +/- s.e. (B) Western blot of rats treated for 9 weeks. Bars: Control n=5, DM n=9, Voclo n=7. (C) Western blot of rats treated for 12 weeks. Bars: Control n=5, DM n=6, Voclo n=6 (D) Levels of WT-1 in each group over time compared to normalized control value expressed as a percent of control.

Figure 12 demonstrates fluctuation of WT-1 expression. There is a suggestion of decreased WT-1 in diabetic kidney at 9 weeks (not statistically significant) but increased WT-1 in voclo treated animals at the same time. By 12 weeks both diabetic groups show increased WT-1 which appears to disqualify these results from the analysis as an increase in podocytes is not possible under these conditions.

Using a monoclonal antibody specific for the WT-1 protein and immune-peroxidase staining, we examined the density of podocytes in glomeruli isolated from control or Voclo groups.

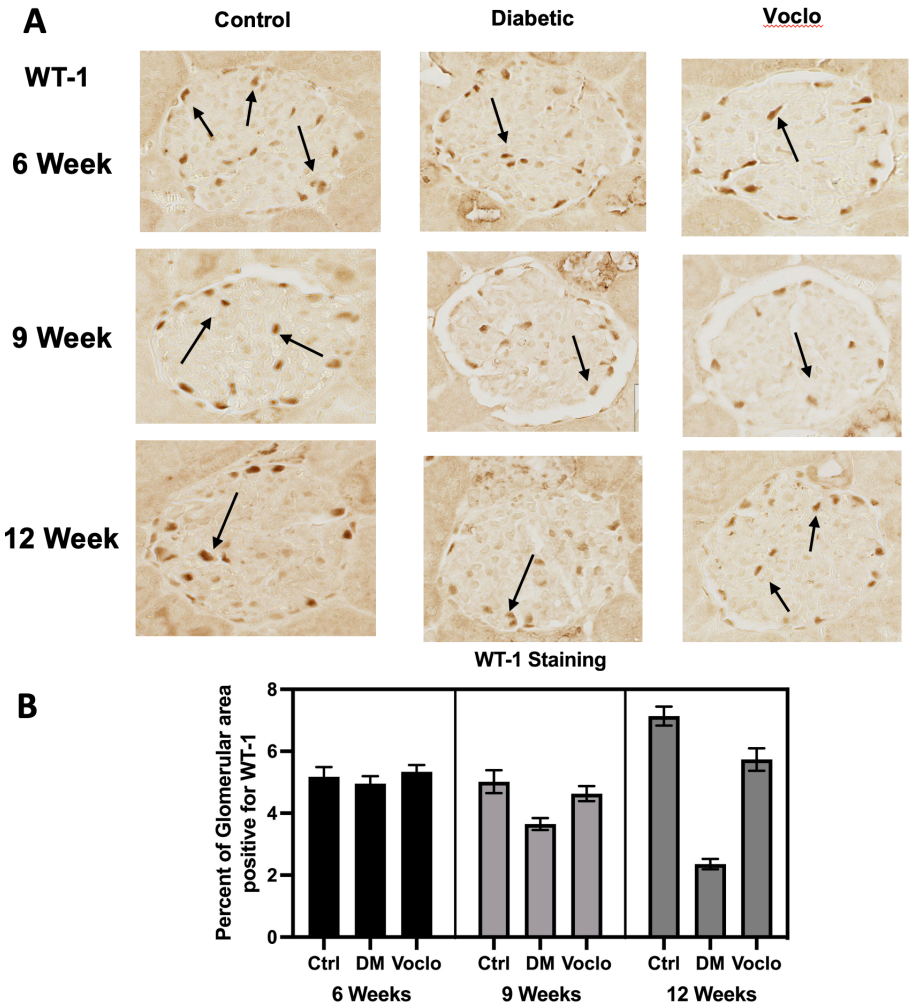

Figure 13. WT-1 staining in glomeruli from control, diabetic, and Voclosporin-treated diabetic rats. (A) Images of paraffin embedded kidney sections stained with WT-1 antibody followed by HRP-linked secondary antibody and visualized with diaminobenzidine (DAB). Images were acquired using the Hamamatsu Nanozoomer HT 2.0. Representative images of immunohistology for control, diabetic (DM), and Voclosporin-treated diabetic (Voclosporin) rat kidneys from 6-week (top row), 9-week (middle row), and 12-week (bottom row) cohorts are shown. (B) Bar graph shows the average stain density per glomerulus per kidney (n=25 glomeruli per kidney) for each group at each treatment duration (6, 9, and 12 weeks), measured by QuPath and ImageJ. Data = mean ± s.e.; * = $P < 0.05$.

Figure 13A is a representative immunoperoxidase stain of glomeruli from control, diabetic, and Voclo groups. Black arrows highlight positive WT-1 staining at glomerular podocytes. The number of podocytes per unit area was counted. The addition of Voclosporin stabilized the number of WT-1 positive cells and maintained cellular density. Figure 13B demonstrates the number of podocytes per square μm for weeks 6, 9, and 12. By week 9, podocyte density per glomerulus among the diabetic animals was less than the control in line with published data. The addition of Voclosporin protected podocyte density at near-control levels.

Although the western blots could not be used to evaluate voclosporin effects due to the failure of the technique to follow known responses of this protein to diabetes, since we know that the animal model was successfully established (hyperglycemia established and maintained), we decided to evaluate the kidneys by immunohistochemistry. These images were analyzed by stain quantification, and we believe this is valid due to the large signal-to-noise ratio.

Discussion

In our pilot evaluations of the kidney marker proteins, most antibodies throughout this study were underperforming. When we encountered antibodies that did not have a major or sometimes even minor band at the protein's reported molecular weight, we ordered replacement antibodies from different vendors and repeat this trial process. Optimal protein loading amounts were also determined with repeated experimentation. The three proteins reported in this document were the only assessable ones with an identifiable band in the correct region.

When western blot comparison between the control and the diabetic group failed to show differences corresponding to recognized/published responses of that specific antibody in comparable models, we considered the antibody inadequate for further analysis of Voclosporin treatment's effects[21].

Overall, the 12-week diabetic and Voclosporin groups were subject to sampling bias due to advanced diabetic-induced catabolism, as only half of the 12-week diabetic group survived to the planned collection time for data collection, making the data less generalizable or representative. We tried analyzing 12-week kidneys for each of the proteins presented but finally concluded that they were too unreliable to assess the effect of Voclosporin.

Calcineurin inhibitors (CNI) have been used to treat a broad array of primary glomerulopathies including idiopathic membranous glomerulonephropathy, focal segmental

glomerulosclerosis, and Lupus nephritis[22-24]. Treatment with CNIs are best known as treatments to reduce transplant rejection. They have proven to have protective success rates exceeding 60% and a reduction in the urinary protein of over 75%[25]. The mechanisms by which CNIs lower proteinuria are complex but involve stabilization of podocyte foot plate processes, inhibiting the degradation of slit port proteins such as Nephrin and disrupting pathways leading to podocyte apoptosis. Despite these podocyte protective properties, few studies have investigated the effects of CNIs in reducing proteinuria and stabilizing renal function in diabetic animal models.

Voclosporin Blocks the Diabetes Induced Rise in Calcineurin Expression

Therefore, we examined the effects of Voclosporin, a next generation CNI, in an STZ rat model of diabetic nephropathy. Voclosporin is a cyclic undecapeptide with a structure similar to cyclosporin A. The two agents differ only in the addition of a single double-carbon bond on amino acid-1 along the immunophilin binding domain of the molecule. This minor change altered the quaternary structure of the Voclosporin-immunophilin-calcineurin complex resulting in increased inhibition of calcineurin and more potent immunosuppression[26, 27]. The observation that calcineurin could be a therapeutic target in the treatment of diabetic nephropathy arose from observations of Gooch et.al and others who found that hyperglycemia (>450 mg/dl) can rapidly (10 minutes) increase calcineurin phosphatase activity[7]. In subsequent studies, Gooch et.al demonstrated that prolonged hyperglycemia (14 days) leads to increased protein expression of calcineurin via a calcineurin-NFAT-dependent pathway. We extended these observations and demonstrated that protein expression of calcineurin in the renal cortex could be detected out to 6 weeks. When diabetic animals were treated with oral Voclosporin (Voclo group), the rise in calcineurin expression was blocked. These data are similar to the findings of Qi et.al who found that Tacrolimus was also able to block a hyperglycemia-induced rise in calcineurin expression after 56 days[28].

Voclosporin Blocks Diabetes Induced Reductions in Synaptopodin Diabetic Glomeruli

Early work investigating the mechanisms by which CNIs reduce glomerular proteinuria found that calcineurin regulates key components of the podocyte cell cytoskeleton. Faul et.al demonstrated in cultured podocytes that the actin-associated cell cytoskeletal protein Synaptopodin is a calcineurin substrate[11]. Under steady-state conditions, Synaptopodin is highly phosphorylated leading to the formation of tight complexes with the cytosolic docking protein 14-3-3. Upon activation of calcineurin, Synaptopodin is dephosphorylated leading to "uncoupling" of the protein from 14-3-3 binding sites. This disassociation leads to enhanced degradation by Cathepsin L and a subsequent retraction of footplate processes[11]. Menini et.al extended these experiments in an STZ rat model of diabetic nephropathy and demonstrated glomerular expression of Synaptopodin could be detected within 56 days[13]. In our study, we found that glomerular Synaptopodin levels were reduced in diabetic animals by week 9 and were maximally depleted by week 12. However, the degradation of Synaptopodin was partially blocked and corrected by Voclosporin treatment with electron microscopic data demonstrating stabilization of podocyte footplate processes.

Voclosporin Blocks Diabetes Upregulation of Connective Tissue Growth Factor (CTGF)

A significant impediment to the broader use of CNIs in diabetic nephropathy and other podocytopathies has been the progressive development of tubulointerstitial fibrosis and tubular atrophy. Serial biopsy studies find that CNI toxicity and increased interstitial fibrosis can be detected within 6 months of renal transplantation[29]. While the mechanism of CNI nephrotoxicity is incompletely known, both cyclosporin and tacrolimus have been shown to increase the pro-fibrotic cytokines of TGF-beta and CTGF[20, 30-32]. To determine the effects of Voclosporin on the

renal cortical expression of CTGF diabetic nephropathy, we performed immunohistochemistry on western blot analysis of cortical tissue isolated from all three animal groups. CTGF expression was increased in the diabetic animal but restored to control levels with Voclosporin treatment. When tissue from the same cortical samples was examined for collagen deposition using Sirius Red staining, an increase in periglomerular and interstitial fibrosis was only observed among the diabetic animals. Previous studies in rat models of diabetes found activation of TGF-beta-induced collagen synthesis through a SMAD-CTGF dependent pathway. Akool et.al noted that cultured mesangial cells exhibited a marked increase in CTGF production when incubated with cyclosporin A or tacrolimus[15]. In contrast, we found that in whole tissue or cortical homogenates, treatment with Voclosporin blocked the production of CTGF. These observations coupled with the normalization of periglomerular and interstitial fibrosis among the diabetic animals receiving Voclosporin suggest that the minor structural changes to Voclosporin may have yielded a CNI with reduced capacity to induce tubulointerstitial fibrosis. Long-term studies using surveillance biopsies will need to perform to confirm this, but the potential development of a podocyte protective and non-fibrosing CNI could open a new therapeutic pathway for treating diabetic nephropathy.

In summary, we have conducted a prospective study of a novel next-generation CNI in a rat model of diabetes and demonstrated a protective effect on glomerular podocytes as early as week 6. We additionally demonstrated that Voclosporin was able to block diabetes-induced degradation of the cell cytoskeletal protein Synaptopodin and a stabilization of the morphology of podocyte foot plate processes. We also showed that, in contrast to cyclosporin and tacrolimus, Voclosporin was able to block the production of CTGF; an important effector molecule for TGF-beta-induced interstitial fibrosis. Future clinical studies with Voclosporin in patients with diabetic nephropathy will need to be performed to determine whether Voclosporin can decrease diabetes-induced proteinuria by stabilizing glomerular and tubulointerstitial fibrosis.

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Manuscript in progress attached, planning on submission to Kidney International by April 1st, 2023

Manuscript in Progress

Efficacy of Voclosporin in Maintaining Podocyte Function and Viability in a Rat Streptozotocin Model of Diabetic Nephropathy

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Short title: Voclosporin effects in diabetic kidney

Key words: podocytes, synaptopodin, WT-1, CTGF, glomeruli

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Conflict-of-interest statement: ZL, JDK, JMS, PG, RAH declare no conflict of interest exists. LR, JV, JC and RH are associated with Aurinia Chemicals, makers of Voclosporin. JAT is a consultant with Aurinia Pharmaceuticals This research was funded by an unrestricted educational grant by the Aurinia Corp.

Abstract

Diabetic nephropathy is a leading cause of end-stage renal disease. Diabetes stimulates the activity and expression of calcineurin; a serine-threonine phosphatase that is a critical regulator of podocyte function and stability. Because calcineurin has been linked to podocyte apoptosis, synaptopodin degradation and slit pore function we investigated the effects of Voclosporin, a third generation calcineurin inhibitor (CNI), on the progression of diabetic nephropathy. Streptozotocin (60 mg/kg) was administered to male Sprague-Dawley rats to induce diabetes. Animals were divided into three cohorts including normal animals (15), diabetic controls (30) and diabetics treated with oral Voclosporin (30, 5.0 mg/kg) by 2X per day gavage feeding. Animals were euthanized at 6, 9, and 12 weeks and renal cortex was harvested for histologic and western analysis of calcineurin, WT-1 (Wilms turmor-1) and CTGF (connective tissue growth factor) expression. Streptozotocin treated animals developed hyperglycemia (BG-500-600 mg/dl) within 30 days and proteinuria by Day 84. After 6 weeks, renal cortical expression of calcineurin in diabetic controls increased by 3.5X fold compared to normal controls while treatment with Voclosporin reduced the increase by 50%. Similarly, Voclosporin blocked the rise in glomerular CTGF while blocking a fall in WT-1 and synaptopodin expression. Sirius Red staining demonstrated that Voclosporin treatment did not lead to an increase in interstitial fibrosis compared to diabetic controls. This study demonstrates that severe diabetes upregulates calcineurin expression in the renal cortex. Treatment of diabetic animals with Voclosporin reduced podocyte depletion, and stabilized synaptopodin without exacerbation of interstitial fibrosis.

Introduction: 527

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease in the United States and other developed countries. While the wide-spread use of RAAS blockade and other maneuvers have slowed disease progression, approximately one third of patients with diabetic nephropathy from Type II diabetes will progress to end-stage renal disease (ESRD) within 10 years[1]. As a result, much effort has been devoted to understanding the mechanisms by which the diabetic condition leads to the typical histopathologic changes including mesangial expansion, thickened basement membranes and loss of podocyte density and functionality.

There is accumulating evidence that injury to the glomerular podocyte is central to the pathogenesis of diabetic nephropathy and that clinical treatments should be directed toward maintaining podocyte viability. Podocytes are highly differentiated cells with limited capacity for cell division and replacement. They are central to the support and maintenance of glomerular capillary networks and function as the final barrier in glomerular filtration. Evidence from experimental models of diabetic nephropathy suggests that reduction in podocyte density either through apoptosis or detachment contributes to the development of sclerotic lesions within the glomerular tuft. Indirect evidence suggests that exposure of the basement membrane in podocyte depleted situations can result in expanded basement membrane to Bowman's capsule ratios and subsequent expansion of sclerotic lesions[2,3]. When podocyte density falls below threshold levels (40%), there is increasing evidence that the glomerular sclerotic process becomes progressive. These observations suggest that clinical treatments should focus on maintaining podocyte viability and reducing processes that drive apoptosis and cell detachment[4].

Calcineurin is a heterotrimeric protein composed of two isoforms of the alpha catalytic unit (a & b) (61kD), a calcium binding beta subunit (19kD) and calmodulin. The association of these two calcium binding proteins allows for tight regulation of its phosphatase activity with marked stimulation of enzyme activity following even nanomolar changes in intracellular calcium[5]. Under diabetic conditions (blood glucose of 450 mg/dl), calcineurin activity is increased by 50%[6]. Moreover, podocytes exposed to prolonged diabetic conditions increase calcineurin activity through upregulation of both the alpha and beta subunits[7,8]. A growing body of evidence suggests that calcineurin may play a key role in regulating podocyte function and viability. The first observations linking calcineurin phosphatase activity to apoptotic pathways performed by Wang et al. demonstrated in T cells that the pro-apoptotic protein BAD is dephosphorylated and activated by calcineurin. Under steady state conditions, BAD is phosphorylated and bound to the cytosolic docking protein 14-3-3. Under diabetic conditions, the down-stream activation of calcineurin leads to dephosphorylation of BAD and subsequent uncoupling of the BAD-14-3-3 complex. The subsequent deposition of BAD-BCLx or BAD-BAX complexes on the mitochondrial surface results in release of cytochrome C and subsequent cellular apoptosis[9]. Calcineurin also regulates podocyte apoptosis through the Dynamin-related Protein-1 (DrP-1) pathway. Cereghetti demonstrated that calcineurin dephosphorylates specific serine residues on DrP-1 leading to translocation to mitochondrial membranes and subsequent fragmentation[10]. Because the CNIs of cyclosporin and tacrolimus can block this apoptotic pathway, we chose to examine the

efficacy of a third generation CNI voclosporin on podocyte apoptosis, foot plate function and generation of cortical fibrosis through CTGF and other pro-fibrotic cytokines.

Methods:

Animal model: 75 male Sprague-Dawley between 170 and 190 g were purchased from Charles River Laboratories and the model was accomplished at Washington Biotechnology, Inc., Baltimore, MD. Rats were randomly assigned into three cohorts of 25 rats: each cohort consisted of non-diabetic control (5 rats), Diabetic (10 rats), and Diabetic with Voclosporin treatment (10 rats). The three cohorts were followed for different times with the cohorts being euthanized at 6, 9, and 12 weeks after the induction diabetes and beginning of voclosporin treatment. The diabetic condition was induced by tail vein injection of 60 mg/kg streptozotocin (STZ) in 0.01M citrate buffer, pH 4.5. Voclosporin was solubilized in vehicle solution (VitE-TPGS, medium change triglyceride oil, Tween 40, 95% EtOH vehicle (4:2:2:1) and delivered by gavage feeding twice daily (5 mg/kg). Rats in the control and the diabetic group were fed with the same gavage vehicle without Voclosporin to reduce confounding variables. The rats were sacrificed at Washington Biotechnology, and their kidneys were collected at three time points: 42 days; 63 days; and 84 days after the STZ injection, frozen, then shipped to Emory. The two kidneys from each rat are separated for different analysis purposes. The left kidney was sub-dissected to yield a small cortex sample and placed in a fixation buffer for electron microscopy (see below). The remainder of that kidney was placed in a 4% paraformaldehyde fixation buffer for conventional histology. The right kidney was immediately frozen in liquid nitrogen and stored at -80oC until prepared for western analyses. Blood and urine samples were collected and measured for blood glucose, total protein, serum albumin and urinary protein losses. All animal procedures were performed according to the Washington Biotechnology IACUC.

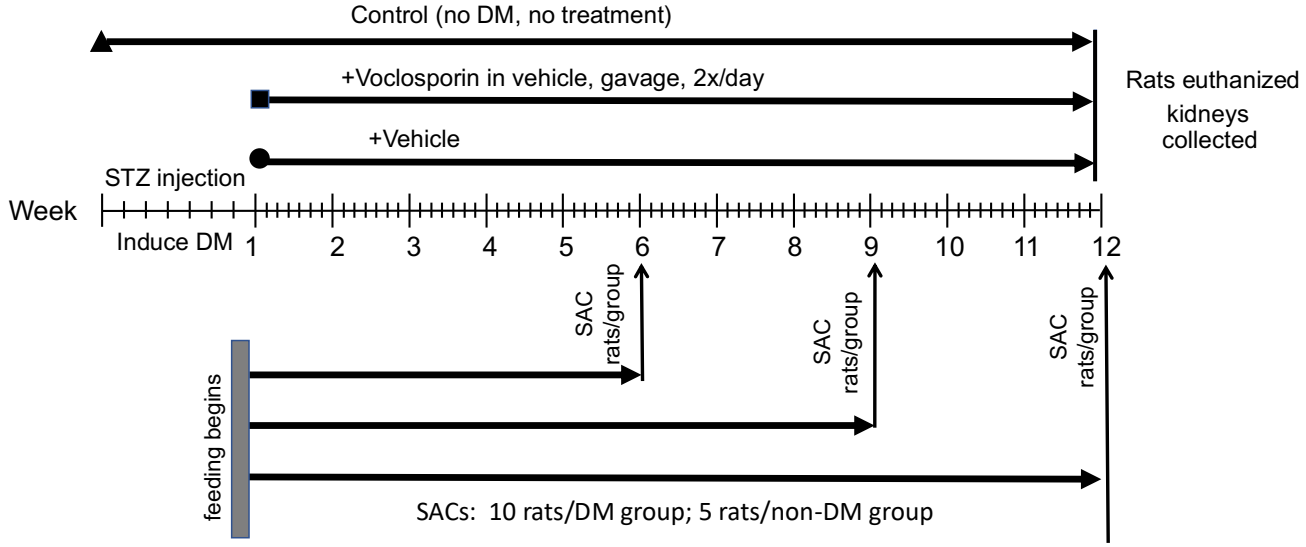

Diagram 1. Schematic drawing of the animal protocol.

Protein analysis:

Sample preparation

Frozen kidneys were dissected, and the kidney cortex homogenized in a glass homogenizer on ice with 1 ml isolation buffer (10 mM triethanolamine, 250 mM sucrose at pH 7.6, 0.2 µM leupeptin, 0.1 M PMSF, 1X Halt protease cocktail (ThermoFisher, LOT # XD343860), pH 7.4. After homogenizing, SDS was added to a final concentration of 1%, the sample was sheared with a 26G insulin syringe into microcentrifuge tubes and centrifuged at 4°C for 10 min. Protein concentration was determined using the Bio-Rad DC Protein Assay (DC Protein Assay Kit II #5000112). Samples were diluted with SDS-PAGE sample buffer to 2-5 mg/mL protein concentration and boiled for 1-2 min. as previously described [16].

Western blotting

Equal amounts of kidney cortex lysate (40 - 50 µg/lane) were separated on SDS polyacrylamide gels as follows: Synaptopodin: 10 % gel; CTGF: 10 % gel; WT-1: 4-20% gradient. Protein was electroblotted to PVDF membrane, blocked and incubated with primary antibodies overnight at 4°C and secondary antibodies for 2h at room temperature. as described previously [17]. Protein bands were scanned and quantified using the Li-COR Odyssey infrared scanning ensystem (Li-COR Biosciences, Lincoln, NE, USA). Primary antibodies: Synaptopodin, 1:1000 dilution (Novus, 6049-1501), CTGF, 1:1000 dilution (RayBiotech, 81255530RAY8) (AB Clonal, cat# A11456), WT-1, 1:1000 dilution (Proteintech, 12609-1-AP). Secondary antibody: donkey anti-rabbit IRDye 680RD, LICOR Cat. No. D20322-01 at 1:10,000 dilution.

Histology

Kidneys were sectioned into 5mm sections and fixed in 4% paraformaldehyde for 48 hours then given to the Winship Cancer Center Tissue and Pathology Core at Emory for paraffin embedding, slide preparation, and H&E staining using their standard protocols.

Immunohistochemistry

Kidney section slides from the histology core were dewaxed (ethanol washes), Treated with trilogy for antibody retrieval (Millipore-Sigma, Cat. #920P-09), incubated overnight 4°C with primary antibodies and labelled with the Impress Horse Anti-Rabbit IgG (Vector Laboratories Cat.# MP-6401-15) as previously described [16]. The slides were then stained with a DAB Substrate Kit (Vector Laboratories, Cat. # SK-4100). Following dehydration and mounting (Permount mounting medium (Fisher Scientific Company, Cat #SP15-100). The slides were visualized using Hamamatsu Nanozoomer HT 2.0 at 40x. Protein staining was visualized using NDP.view2 software and analyzed with QuPath (figure 7-9). QuPath analyzed Synaptopodin and CTGF by measuring the staining levels within the glomerulus, staining level is also measured for CTGF tubules, and WT-1 is measured by the percentage of area stained within the glomerulus region.

Electron microscopy

Electron microscopy (EM) was performed at the Robert P. Apkarian EM core at Emory. Samples were fixed with Karnovsky's fixative (4% paraformaldehyde and 2.5%

glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). The sample material was washed and embedded in Ted Pella's Eponate 12™ Epoxy resin. The polymerized sample blocks were sectioned using a Diatom diamond knife with a Leica/Reichert Ultra Cut S ultramicrotome. The thin sections (~80nm) were collected onto 200 mesh copper grids and post-stained in 5% Uranyl Acetate and Lead Citrate. Sections were imaged using a JEOL JEM-1400 LaB_6TEM at 80 kV with Gatan Ultra Scan 1000 2K x 2K CCD camera.

Statistics

All data are presented as mean ± SE. To test for statistical significance between 2 groups, we used a Student's t-test. To test more than 2 groups, we used an analysis of variance, followed by Fisher's least significant difference (protected t-test) (Snedecor and Cochran, 1980) to determine which groups were significantly different. The criterion for statistical significance was $P < 0.05$.

Results:

Blood Glucose, Total Protein, Albumin, and Proteinuria at Baseline, 48, 63 and 84 Days

Streptozotocin treatment resulted in blood glucose levels ranging between 500 and 600 mg/dl (**Figure 1A**). This level of hyperglycemia remained stable for the whole of the 84-day treatment period. Urine protein levels rose within 15 days of streptozotocin treatment and remained 2X above baseline levels at 42 days (**Figure 1B**). The addition of Voclosporin reduced urinary protein levels at 84 days but did not change levels at Day 42 or 63. **Figure 1C** illustrates the change in total protein and albumin levels in the serum. Total protein levels were significantly lower in the Diabetic and Diabetic + Voclosporin animals by Day 42 and tended to increase in the voclosporin treated animals by Day 84. There were no differences in serum albumin levels in any group over the 84-day study period.

Calcineurin Expression Following Induction of Diabetes: Effect of Voclosporin

To determine whether the onset of diabetes altered the expression of calcineurin in the renal cortex, we isolated renal cortex at 42 days and performed a western analysis of calcineurin protein expression. As shown in **Figure 2A**, a 51 kDa band binding to a monoclonal antibody to calcineurin was identified. Among the diabetic animals, calcineurin expression was increased compared to normal controls. Animals that were gavage fed Voclosporin demonstrated calcineurin levels that were comparable to normal control animals. Laser densitometry analysis of the representative western blots demonstrated that the onset of diabetes increased calcineurin expression by approximately 3.5-fold higher. Concomitant treatment with oral voclosporin reduced calcineurin expression by 50%.

Synaptopodin Expression Following Induction of Diabetes: Effect of Voclosporin

Early studies in cultured podocytes demonstrated that synaptopodin is a calcineurin substrate and that dephosphorylation of serine and threonine moieties can

accelerate its protein degradation[10]. Because diabetes increased protein expression of calcineurin, we investigated whether the onset of diabetes and marked hyperglycemia would reduce protein expression of synaptopodin. **Figure 3** demonstrates a representative immunoperoxidase staining of cortical glomeruli isolated at 42, 63 and 84 days from control, diabetic and diabetic animals treated with oral voclosporin. Immunoperoxidase staining for all three groups was equivalent at 42 days, whereas synaptopodin staining in glomeruli isolated from the diabetic animals was reduced at 63 days. This reduction in synaptopodin expression was further reduced at 84 days, while staining among the diabetic animals treated with voclosporin was comparable to the control animals.

Effect of Diabetes on Podocyte Foot Plate Processes: Effect of Voclosporin

To determine whether the reduction in synaptopodin expression resulted in a corresponding loss of foot plate processes, we performed transmission electron microscopy on cortical glomeruli from control animals with uncontrolled diabetes and diabetic animals treated with oral voclosporin. Figure 4 is a representative electron micrograph of glomeruli from control, diabetic and diabetic animals treated with oral voclosporin. **Figure 4A** is a representative image of a normal glomerular capillary loop at 42 days. Arrows point to normal appearing podocyte foot plate processes. In contrast, **Figure 4B** demonstrates a capillary loop from animals 42 days after the induction of diabetes. Arrows highlight areas of foot plate effacement with rounded cellular morphology. There were also areas notable for focal thickening of the glomerular basement membrane (heavy arrows). Glomeruli from a diabetic animal treated with oral Voclosporin are shown in **Figure 4C**. Foot plate processes have partially normalized with interdigitation between cells and that more approximates control animals.

Effect of Diabetes on Glomerular CTGF Expression: Effect of Voclosporin

Previous studies have documented that expression of connective tissue growth factor (CTGF), both in the heart and the kidney, is regulated by calcineurin-NFAT dependent pathways[12]. We therefore performed western blot analysis on cortical homogenates and immunohistochemistry on whole renal cortical tissue using a polyclonal antibody and examined the effects of uncontrolled diabetes on the cortical expression of CTGF. **Figure 5A** shows western blots of all renal cortical homogenates from the day 42 treatment group and demonstrates a faint 38 kDa band was observed in the cortex of control animals. A similar but enhanced 38 kDa band was noted in the diabetic animals, while concomitant treatment with voclosporin blocked CTGF enhancement. **Figure 5B** shows the averaged band density of the western blots and quantitation of the bands demonstrates that renal cortical expression of CTGF expression among the diabetic animals increased by approximately 40% and was completely normalized by Voclosporin treatment. While the western blots revealed changes in abundance of CTGF at 42 days, this was not apparent in the histological staining at that time point. As shown in **Figure 5C**, however, after 63 days, peroxidase staining shows the expression of CTGF was found in the periglomerular areas and along the basement membranes of the renal tubular epithelium. For animals with uncontrolled diabetes, CTGF staining at 63 days was enhanced in both the

periglomerular areas and tubular basement membranes compared to control animals. Treatment of the diabetic animal with oral voclosporin reduced CTGF staining in all renal cortical areas.

Cortical Glomerular and Interstitial Fibrosis: Effect of Voclosporin in Renal Scarring

Tubulointerstitial fibrosis as measured by Sirius Red staining was increased by day-42 in the diabetic animals compared to controls. **Figure 6A** demonstrates Sirius Red staining in control animals while **Figure 6B and 6C** are control diabetic and diabetic animals treated with oral voclosporin respectively. **Figure 6B** under 20X microscopy demonstrates increased fibrosis in the periglomerular area and tubulointerstitial basement membranes. As shown in **Figure 6C**, the addition of voclosporin return Sirius Red staining to near control levels.

Effect of Diabetes on Glomerular WT-1 Expression: Effect of Voclosporin

Previous studies have used expression of Wilms's Tumor-1 (WT-1) on the surface of glomerular podocytes as a maker for podocyte density and to gage the effectiveness of therapeutic maneuvers to reduce podocyte apoptosis and detachment [13]. Using a polyclonal antibody specific for the WT-1 protein, we used immunoperoxidase staining to examine the density of podocytes in glomeruli isolated from control, diabetic, and voclosporin treated diabetic rats. **Figure 7A** is a representative immunoperoxidase stain of glomeruli from control, diabetic and diabetic animal treated with oral voclosporin. Black arrows highlight glomerular podocytes. The number of podocytes per unit area was counted for control animals, diabetics and voclosporin treated rats at 42, 63 and 84 days. The addition of voclosporin stabilized the number of WT-1 positive cells and maintained podocyte density. **Figure 7B** demonstrates the number of a podocytes per square μm for the three time points. By day 63 and 84, podocyte density per glomerulus among the diabetic animals was less than the control at any time point. The addition of voclosporin restored podocyte density to near control levels by day 84.

Discussion:

Calcineurin inhibitors (CNI) have been used to treat a broad array of primary glomerulopathies including idiopathic membranous glomerulonephritis (GN), focal segmental glomerulosclerosis and Lupus nephritis[14,15,16]. Treatment with CNIs is effective with complete or partial response rates exceeding 60% and reduction in urinary protein of over 75%. The mechanisms by which CNIs lower proteinuria are complex but involve stabilizing podocyte foot plate processes, inhibiting the degradation of slit port proteins such as Nephrin and disrupting pathways leading to podocyte apoptosis. Despite these podocyte protective properties, few studies have investigated the effects of CNIs in reducing proteinuria and stabilizing renal function in diabetic animal models.

Voclosporin Blocks Diabetes Induced Rise in Calcineurin Expression

Therefore, we examined the effects of Voclosporin, a 3rd generation CNI, in a streptozotocin rat model of diabetic nephropathy. Voclosporin is a cyclic undecapeptide with a structure that is similar to cyclosporin A. The two agents differ only in the addition of a single double-carbon bond on amino acid-1 along the immunophilin binding domain of the molecule. This minor change altered the quaternary structure of the voclosporin-immunophilin-calcineurin complex resulting in increased inhibition of calcineurin and more potent immunosuppression[17,18]. The observation that calcineurin could be a therapeutic target in the treatment of diabetic nephropathy arose from observations of Gooch et al. and others who found that hyperglycemia (>450 mg/dl) can rapidly (10 minutes) increase calcineurin phosphatase activity[20]. In subsequent studies, Gooch et al. demonstrated that prolonged hyperglycemia (14 days) lead to increased protein expression of calcineurin via a calcineurin-NFAT dependent pathway. We extended these observations and demonstrated that protein expression of calcineurin in the renal cortex could be detected out to 42 days. When diabetic animals were treated with oral Voclosporin, the rise in calcineurin expression was blocked. These data are similar to the findings of Qi et al. who found that Tacrolimus was also able to block a hyperglycemia induced rise in calcineurin expression after 56 days[21].

Voclosporin Blocks Diabetes Induced Reductions in Synaptopodin Diabetic Glomeruli

Early work investigating the mechanisms by which CNIs reduce glomerular proteinuria found that calcineurin regulates key components of the podocyte cell cytoskeleton. Faul et al. demonstrated in cultured podocytes that the actin-associated cell cytoskeletal protein synaptopodin is a calcineurin substrate. Under steady state conditions, synaptopodin is highly phosphorylated leading to the formation of tight complexes with the cytosolic docking protein 14-3-3. Upon activation of calcineurin, synaptopodin is dephosphorylated leading to “un-coupling” of the protein from 14-3-3 binding sites. This disassociation leads to enhanced degradation by Cathepsin L and subsequent retraction of foot plate processes[22]. Menini et al. extended these experiments in a streptozotocin rat model of diabetic nephropathy and demonstrated that glomerular expression of synaptopodin could be detected within 56 days[23]. In our study, we found that glomerular synaptopodin levels were reduced in diabetic animals by Day 63 and were maximally depleted by Day 84. However, the degradation of synaptopodin in diabetic animals treated with Voclosporin was partially blocked and correlated with electron microscopic data demonstrating stabilization of podocyte foot plate processes.

Voclosporin Blocks Diabetes Upregulation of Connective Tissue Growth Factor (CTGF)

A significant impediment to the broader use of CNIs in diabetic nephropathy and other podocytopathies has been the progressive development of tubulointerstitial fibrosis and tubular atrophy. Serial biopsy studies find that CNI toxicity and increased interstitial fibrosis can be detected within 6 months of renal transplantation[24]. While the mechanism of CNI nephrotoxicity is incompletely known, both cyclosporin and tacrolimus have been shown to increase the pro-fibrotic cytokines of TGF-beta and CTGF [12,25,26,27]. To

determine the effects of Voclosporin on renal cortical expression of CTGF in diabetic nephropathy, we performed immunohistology and western blot analysis of cortical tissue isolated from all three animal groups. CTGF expression was increased in the diabetic animal but restored to control levels in those animals treated with voclosporin. When tissue from the same cortical samples were examined for collagen deposition using Sirius Red staining, an increase in periglomerular and interstitial fibrosis was only observed among the diabetic animals. Previous studies in rat models of diabetes found that activation of TGF-beta induced collagen synthesis through a SMAD-CTGF dependent pathway. Akool et al. noted that cultured mesangial cell exhibited a marked increase in CTGF production when incubated with cyclosporin A or tacrolimus[28]. In contrast, we found that in whole tissue or cortical homogenates, treatment with voclosporin blocked the production of CTGF. These observations coupled with the normalization of periglomerular and interstitial fibrosis among the diabetic animals receiving voclosporin suggests that the minor structural changes to voclosporin may have yielded a CNI with reduced capacity to induce tubulointerstitial fibrosis. Long-term studies using surveillance biopsies will need to be performed to confirm this, but the potential development of a podocyte protective and non-fibrosing CNI could open a new therapeutic pathway for treating diabetic nephropathy.

In summary, we have conducted a prospective study of a novel 3rd generation CNI in a rat model of uncontrolled diabetes and demonstrated a protective effect on glomerular podocytes as early as 42 days. We additionally demonstrated that voclosporin was able to block diabetes induced degradation of the cell cytoskeletal protein synaptopodin and correspondingly a stabilization of the morphology of podocyte foot plate processes. Lastly, in contrast to cyclosporin and tacrolimus, voclosporin was able to block the production of CTGF; an important effector molecule for TGF-beta induced interstitial fibrosis. Future clinical studies with voclosporin in patients with diabetic nephropathy will need to be performed to determine whether the lowering of proteinuria can be accomplished with the prospect of stabilizing glomerular and tubulointerstitial fibrosis.

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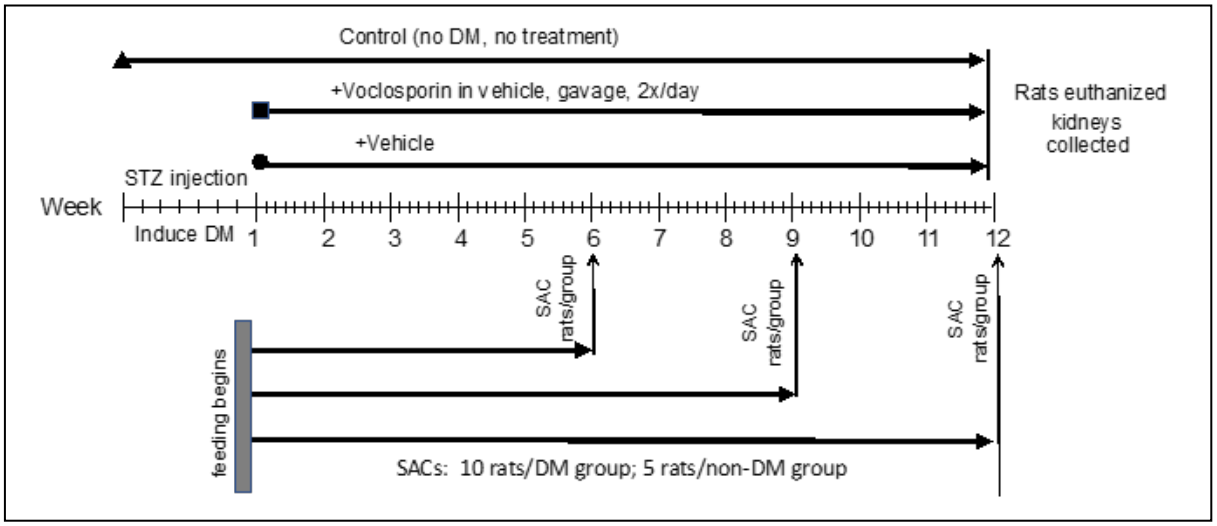
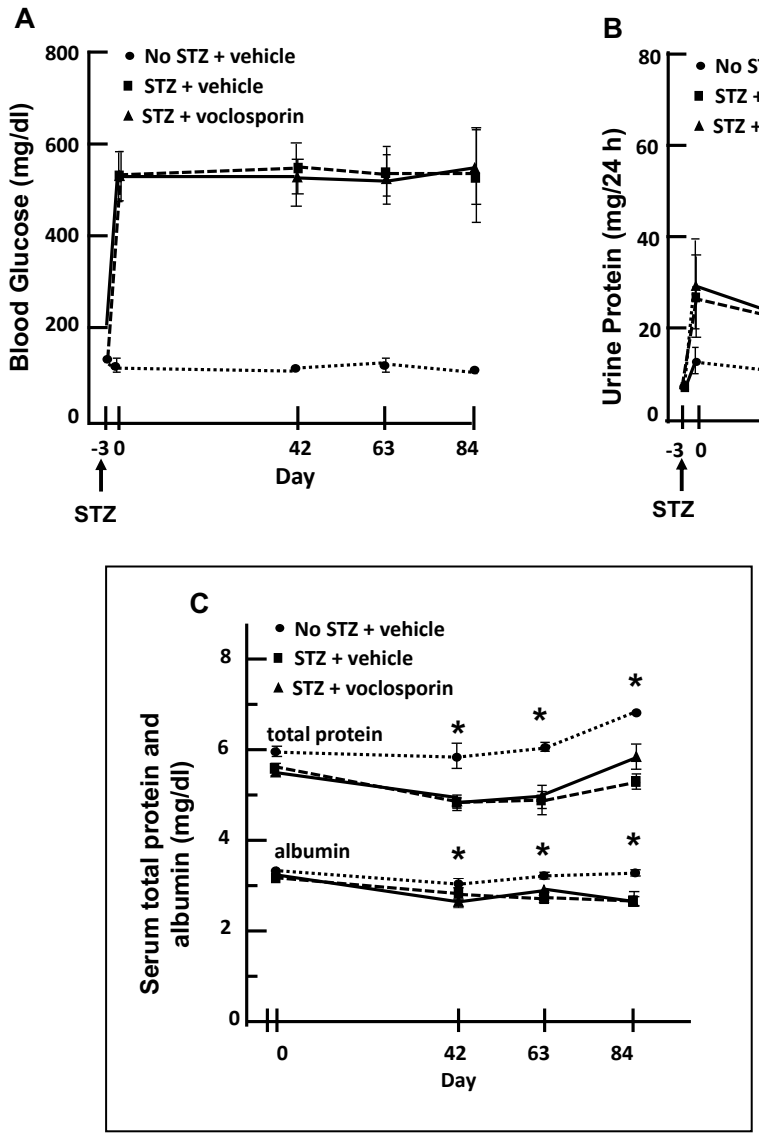
Diagrams:



Schematic drawing of the animal protocol. Three groups of rats were prepared at Washington Biolabs: nondiabetic control (n=15), diabetic (DM) untreated (n=30), diabetic treated with Voclosporin (n=30). Diabetes was induced by tail vein injection of streptozotocin (STZ) at 0 time. Voclosporin (5 mg/kg) was delivered twice daily by gavage feeding. Nondiabetic control and diabetic untreated received vehicle twice daily. Cohorts of 5 control, ten diabetic with Voclosporin treatment, and ten diabetic rats were sacrificed (SAC) and kidneys collected at 6-weeks, 9-weeks, and 12-weeks post STZ injection.

Figures and Legends:

Figure 1



Physiological parameter measurements of diabetic rat groups over time. The rats were divided into 9 groups, groups 1 (No STZ, n=5), 4 (STZ + vehicle, n=10) and 7 (STZ + Voclosporin, n=10) were terminated at 42 days; groups 2 (No STZ, n=5), 5 (STZ + vehicle, n=9) and 8 (STZ + Voclosporin, n=8) were terminated at 63 days; groups 3 (No STZ, n=5), 6 (STZ + vehicle, n=6) and 9 (STZ + Voclosporin, n=6) were terminated 84 days after the initiation of diabetes with streptozotocin (STZ) injection. Data are presented as the mean ± se; $P < 0.05$ is significant. No STZ (circle, dotted line), STZ + vehicle (square, dashed line), and STZ + Voclosporin (5.0 mg/kg, 2X daily; triangle, solid line).
A Blood glucose levels (mg/dL) plotted vs. time (days). Blood glucose reached a maximum of approximately 500 mg/dl and remained stable throughout the 84-day study.

B Urinary protein (mg/24 hours) plotted vs. time (days). Levels began to increase within 15 days of STZ injection. Urinary protein levels were elevated above controls in the diabetic and diabetic plus Voclosporin treated animals. By Day 84, urinary protein levels in the diabetic plus Voclosporin treated animals was lower that control diabetics.
C Serum albumin (g/dl, bottom lines) and total protein (g/dl, top set of lines) levels plotted vs. time (days). Serum albumin and total protein levels in diabetic or diabetic + Voclosporin (Voclo) treated animals were statistically different from non-diabetic control rats from the 42 days throughout the 84-day study. Serum total protein was not different in Voclosporin treated animals compared to the untreated diabetic rats at any collection time point. The same is true for serum albumin levels. * = $P<0.05$ vs control.

Figure 2

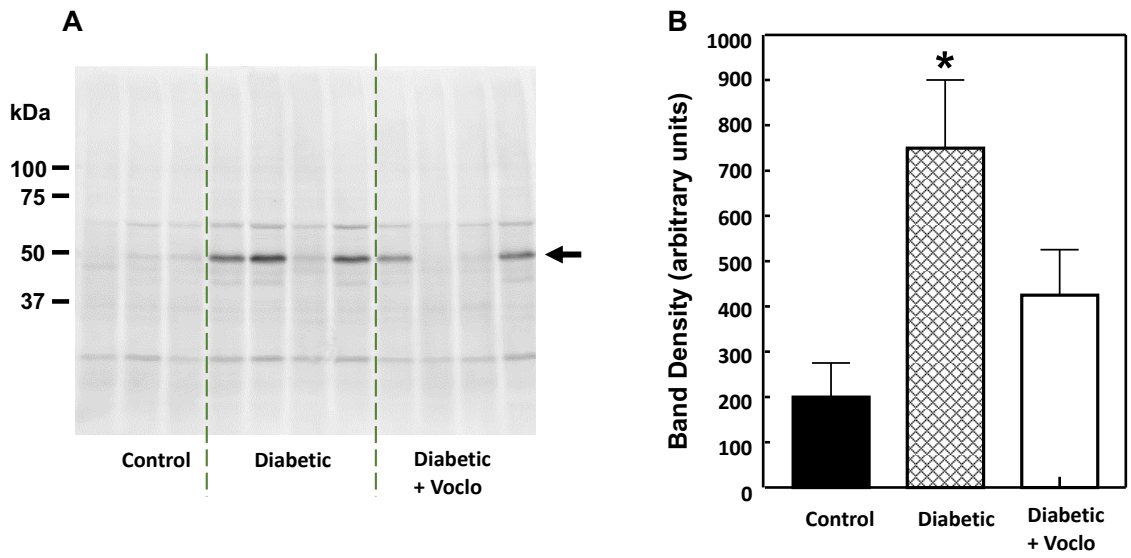


Figure 2: Renal cortical Expression of Calcineurin.
A Representative Western Blot of renal cortical homogenates from control (n=5), diabetic (n=10), and diabetic + Voclosporin treated (n=10) after 42 days of treatment. Each lane represents a different animal. Ponceau S staining of total protein was used as a loading control (not shown). A 50kDa band (denoted by arrow) binding to a monoclonal antibody against calcineurin was identified in all three groups. At 42 days, there was a significantly increased expression of the calcineurin in the diabetic rat kidney versus levels in the control rat kidneys. There was a significant ($P < 0.05$) reduction in calcineurin expression in kidneys from diabetic rats that received Voclosporin compared to the diabetic animals that were gavage fed with vehicle.
B Densitometry of the calcineurin bands from all samples collected at 42 days, expressed in arbitrary units. The bars are Control: Black (n=5); Diabetic: Thatched Lines(n=10); Diabetic + Voclosporin: White (n=10). Data are presented as the mean ± se; $P < 0.05$ is significant.

Figure 3

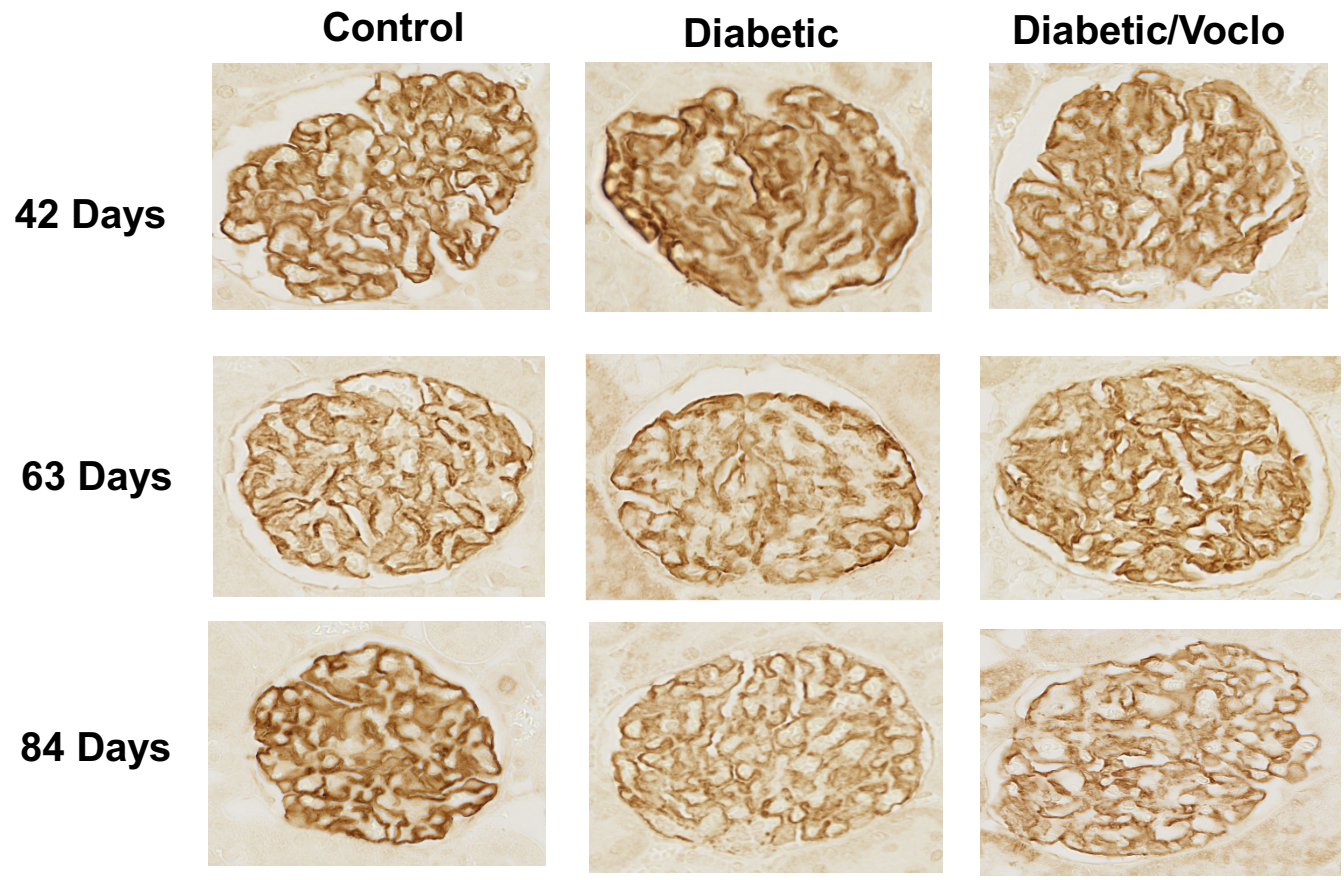
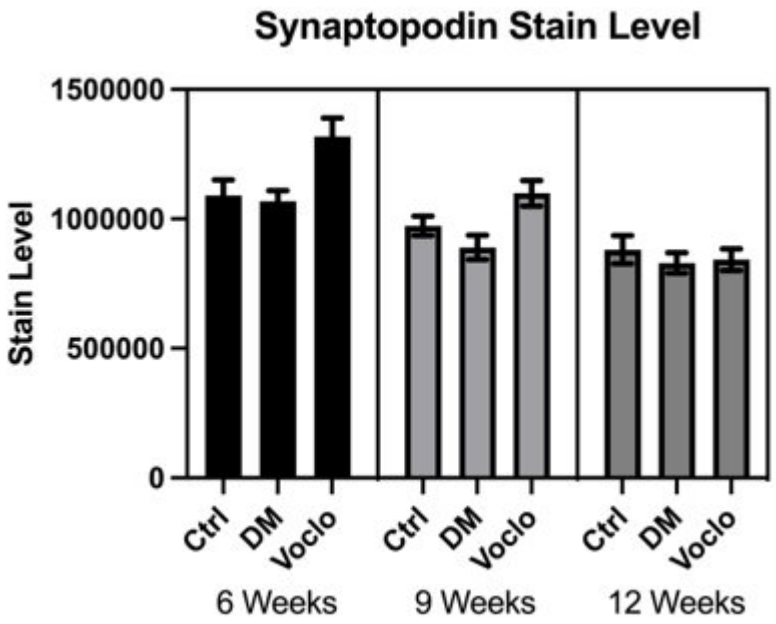


Figure 3. ***Glomerular Expression of Synaptopodin.***
Images of paraffin embedded kidney sections stained with polyclonal Synaptopodin antibody followed by HRP-linked secondary antibody and visualized with diaminobenzidine (DAB). Images were acquired using the Hamamatsu Nanozoomer HT 2.0. Representative images of immunohistology for control, diabetic, and Voclosporin-treated diabetic (Diabetic/Voclo) rat kidneys from 42 Day (top row), 63 Day (middle row), and 84 Day (bottom row) cohorts are shown. Compared to control animals, a progressive decrease in synaptopodin expression can be observed in the diabetic animals beginning at day 63 and progressed through Day 84. Synaptopodin expression among animals receiving oral Voclosporin was maintained at Days 63 and 84.



Note, this is the quantitation for the synaptopodin density. I don't think it is helpful. Your thoughts?

Figure 4

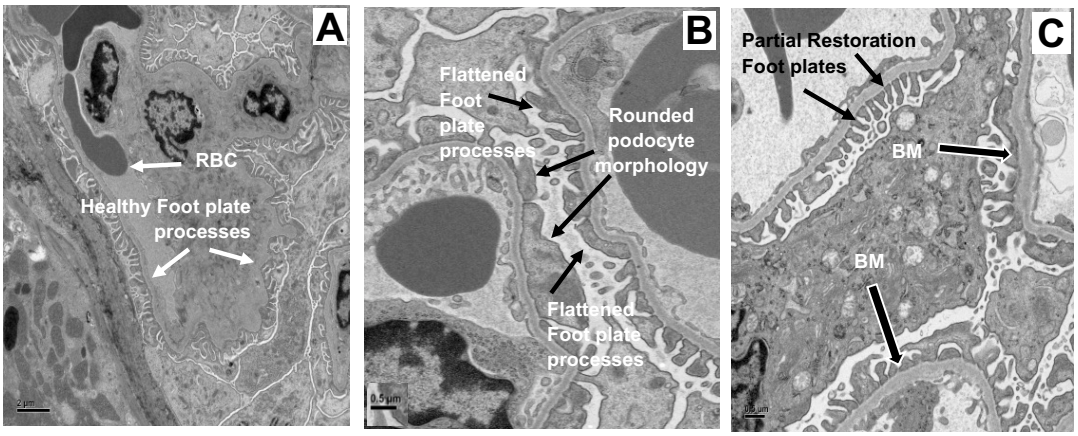


Figure 4. Transmission Electron Microscopy of 42 Day Samples.
Transmission electron microscopy was performed on control (A), diabetic (B) and diabetic animals treated with oral Voclosporin (C; Diabetic/Voclo). All images presented are from animals treated for 42 days. Arrows indicate corresponding labels. BM: Basement Membrane; RBC: Red Blood Cell. Scale bar = 2 µm (A), 0.5 µm (B, C). In control animals **(A)**, basement membranes demonstrated normal width (300 nm) with visible fenestrations. Foot plate processes are of normal configuration and spacing. Among the diabetic animals **(B)**, there is patchy thickening of the basement membrane and partial effacement of podocyte foot plates. Podocytes are flattened with fewer foot processes. Endothelial fenestrations in this group appeared unchanged. Treatment with oral Voclosporin. (**C)** Shows partially normalized foot plate effacement but did not alter basement membrane thickness.

Figure 5

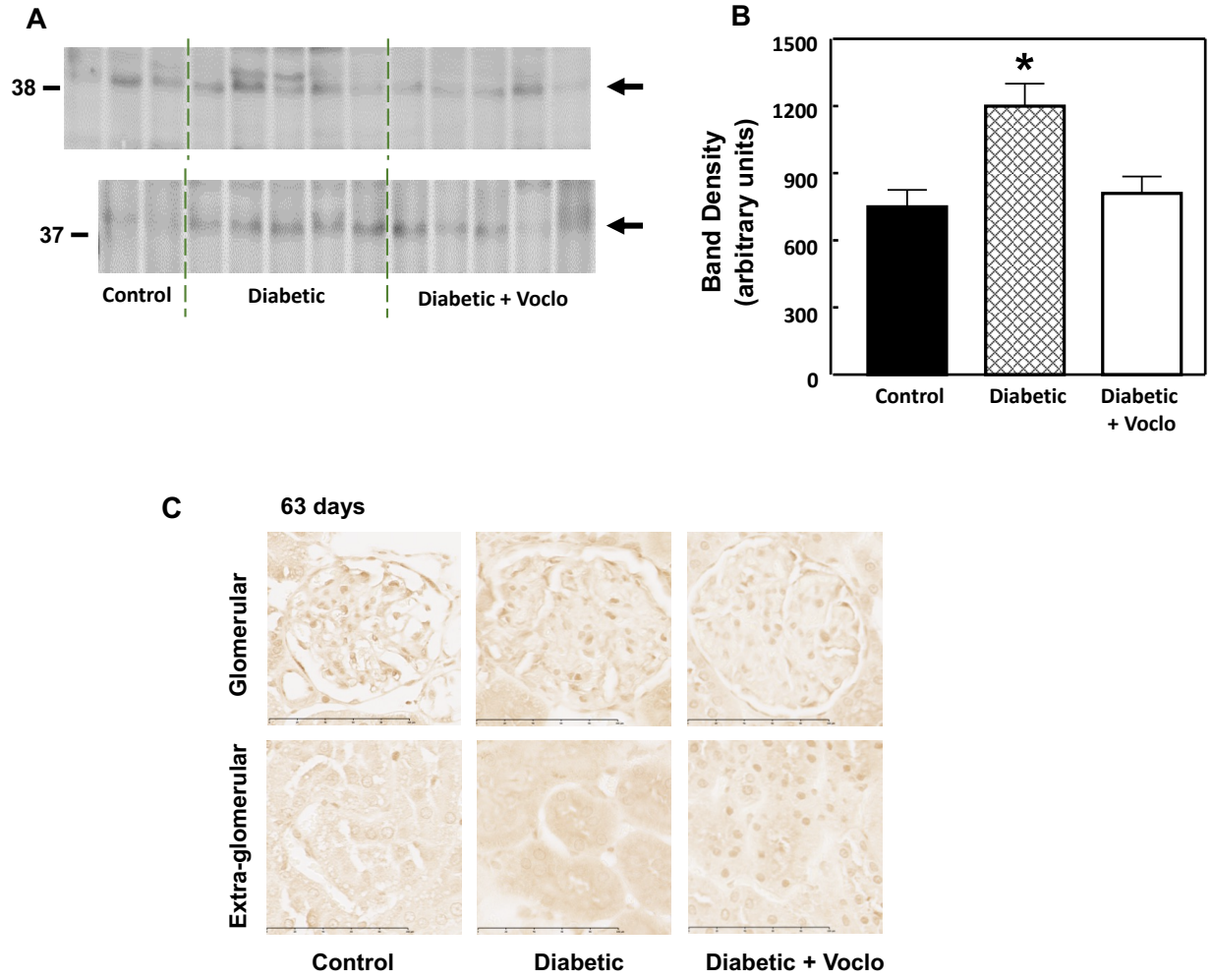
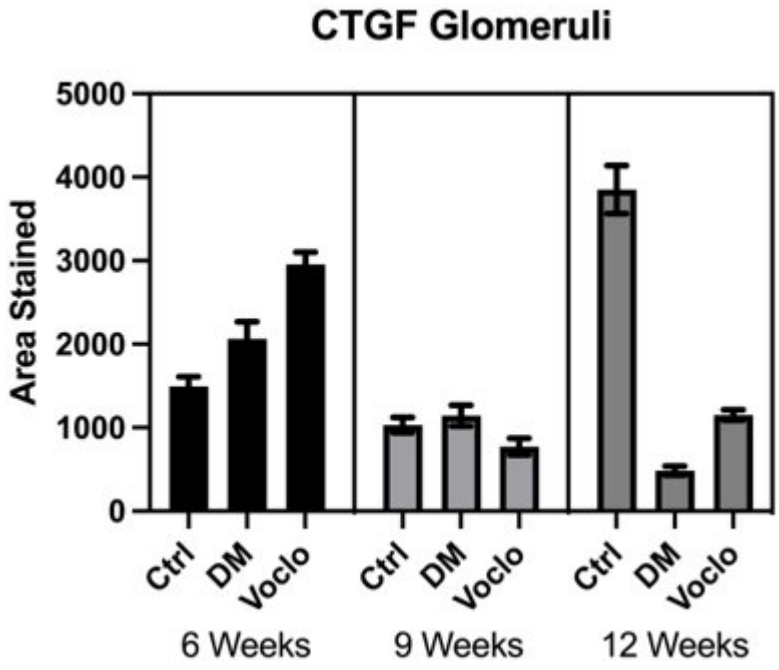


Figure 5. Renal cortical Expression of CTGF. **A.** Western Blots of renal cortical homogenates from control (n=5), diabetic (n=10), and diabetic + Voclosporin treated (n=10) animals were probed with polyclonal CTGF antibody. Animals treated for 42 days are presented here; each lane represents a different animal. Ponceau S staining of total protein was used as a loading control (not shown). A faint 38 kDa band consistent with the molecular weight of CTGF was detected in the cortex of normal controls. The CTGF band increased in the diabetic controls but was normalized following treatment with oral Voclosporin. **B.** Densitometry of the CTGF bands from all samples collected at 42 days, expressed in arbitrary units. The bars are Control: Black (n=5); Diabetic: Thatched Lines (n=10); Diabetic + Voclosporin: White (n=10). Data are presented as the mean ± se; $P < 0.05$ is significant. **C** Images of paraffin embedded kidney sections stained with CTGF antibody followed by HRP-linked secondary antibody and visualized with diaminobenzidine (DAB). Images were acquired using the Hamamatsu Nanozoomer HT 2.0. Representative images of immunohistology for control, diabetic, and Voclosporin-treated diabetic (Diabetic/Voclo) rat kidneys from 63 Day treatment are shown. Top row shows glomerular expression and bottom row shows extra-glomerular expression.

Staining was increased in the diabetic control animals compared to Voclosporin treated animals and normal controls.



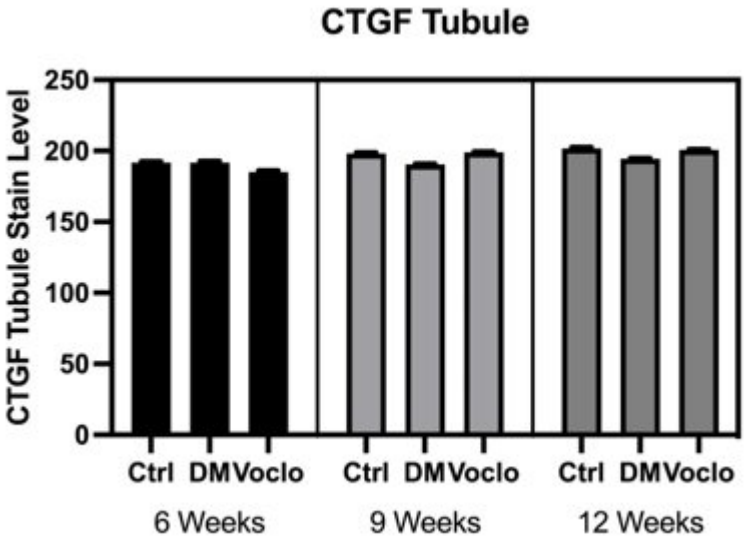


Figure 6:

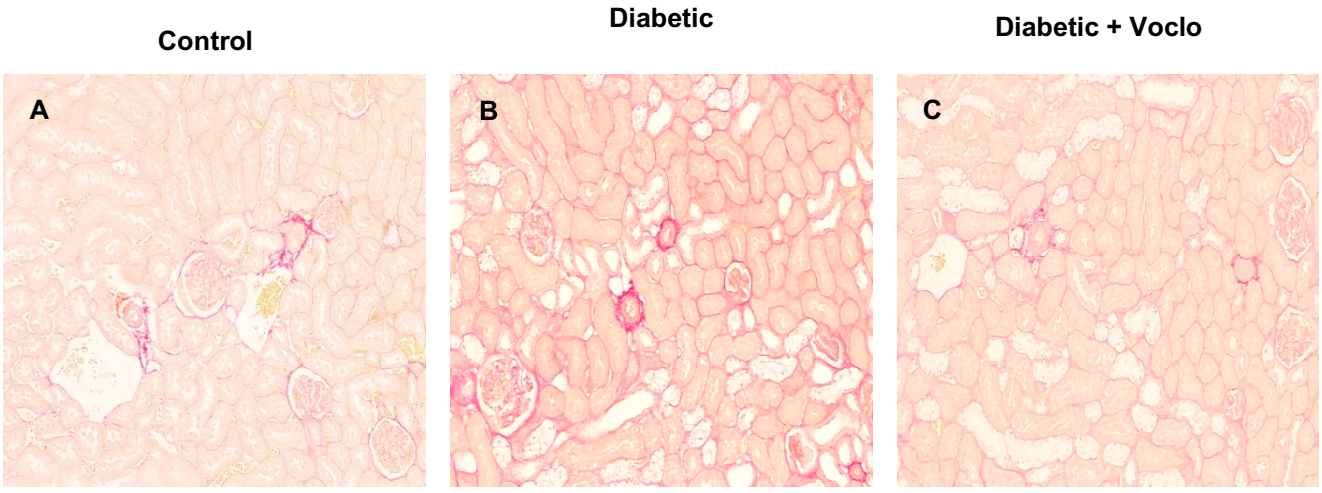


Figure 6. Indications of collagen deposition. Representative samples of whole cortex in control, Diabetic, and Diabetic + Voclosporin treated (Diabetic + Voclo) rats that was stained for collagen deposition using Sirius Red staining. All samples shown are from rats treated for 42 days. **A** shows that there is minimal collagen deposition within Bowman's capsule and the peri-glomerular space or within the tubulointerstium of control animals. **B** is a representative sample of renal cortex obtained from animals with uncontrolled diabetes. After 42 days, there is an increase in collagen staining in the peri-glomerular areas as well as the tubulointerstium and basement membranes of the tubular epithelium. **C** demonstrates renal cortex at Day 42 from diabetic animals receiving oral Voclosporin. There is a reduction in collagen deposition within all regions of the cortex when compared to the diabetic controls in **B**.

Figure 7

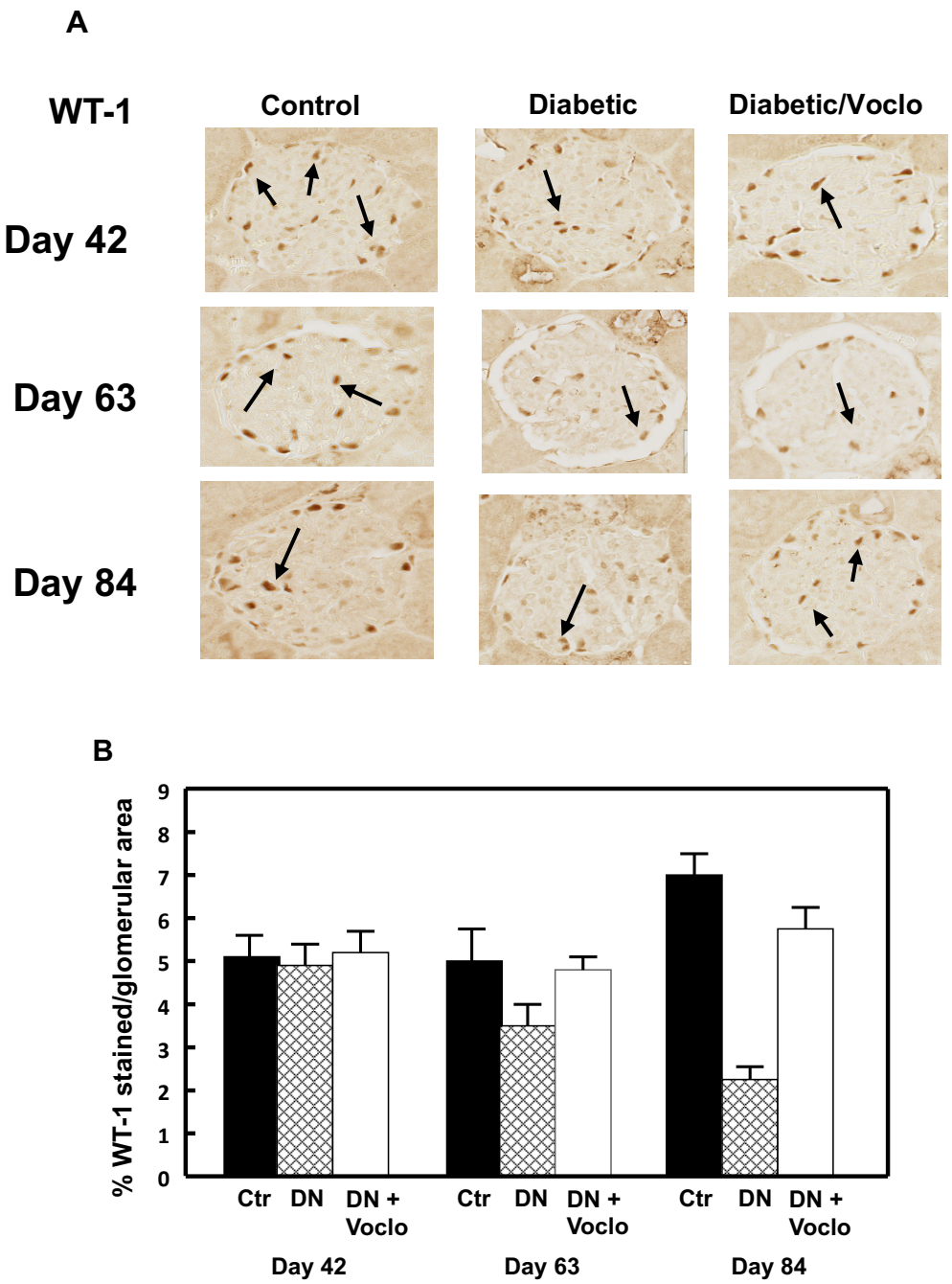


Figure 7. Glomerular Expression of WT-1. **A** Representative images of paraffin embedded kidney sections stained with polyclonal WT-1 antibody followed by HRP-linked secondary antibody and visualized with diaminobenzidine (DAB). Images were acquired using the Hamamatsu Nanozoomer HT 2.0. Representative images of immunohistology for control, diabetic, and Voclosporin-treated diabetic (Diabetic/Voclo) rat kidneys from 42

Day (top row), 63 Day (middle row), and 84 Day (bottom row) cohorts are shown. As shown in the diabetic controls there is a progressive loss of WT-1 podocytes by Days 63 and 84. There was partial stabilization of podocyte depletion in the animals receiving Voclosporin. Arrows denote positive WT-1 staining. **B** Histogram showing the percent of the glomerular area stained positive with WT-1 per glomerulus (n=25 glomeruli per kidney) for each treatment group (42, 63, and 84 days) in control (Ctr; black), Diabetic Nephropathy (DN; crosshatched), and Diabetic rats treated with Voclosporin (DN + Voc; white). Data = mean ± s.e.; * = $P < 0.05$. Among control animals, podocyte density was unchanged over the 84 days of the study. The was a significant 20% podocyte reduction by Day 63 and 60% reduction by Day 84 among the animals with uncontrolled diabetes. Animal concomitantly treated with oral Voclosporin demonstrated no change in podocyte density compared to control animals.