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Chronic bilateral renal denervation attenuates renal injury in a transgenic rat model of diabetic nephropathy

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Yao Y, Fomison-Nurse IC, Harrison JC, Walker RJ, Davis G, Sammut IA. Chronic bilateral renal denervation attenuates renal injury in a transgenic rat model of diabetic nephropathy. Am J Physiol Renal Physiol 307: F251–F262, 2014. First published June 4, 2014; doi:10.1152/ajprenal.00578.2013.—Bilateral renal denervation (BRD) has been shown to reduce hypertension and improve renal function in both human and experimental studies. We hypothesized that chronic intervention with BRD may also attenuate renal injury and fibrosis in diabetic nephropathy. This hypothesis was examined in a female streptozotocin-induced diabetic (mRen-2)27 rat (TGR) shown to capture the cardinal features of human diabetic nephropathy. Following diabetic induction, BRD/sham surgeries were conducted repeatedly (at the week 3, 6, and 9 following induction) in both diabetic and normoglycemic animals. Renal denervation resulted in a progressive decrease in systolic blood pressure from first denervation to termination (at 12 wk post-diabetic induction) in both normoglycemic and diabetic rats. Renal norepinephrine content was significantly raised following diabetic induction and ablated in denervated normoglycemic and diabetic groups. A significant increase in glomerular basement membrane thickening and mesangial expansion was seen in the diabetic kidneys; this morphological appearance was markedly reduced by BRD. Immunohistochemistry and protein densitometric analysis of diabetic innervated kidneys confirmed the presence of significantly increased levels of collagens I and IV, α-smooth muscle actin, the ANG II type 1 receptor, and transforming growth factor-β. Renal denervation significantly reduced protein expression of these fibrotic markers. Furthermore, BRD attenuated albuminuria and prevented the loss of glomerular podocin expression in these diabetic animals. In conclusion, BRD decreases systolic blood pressure and reduces the development of renal fibrosis, glomerulosclerosis, and albuminuria in this model of diabetic nephropathy. The evidence presented strongly suggests that renal denervation may serve as a therapeutic intervention to attenuate the progression of renal injury in diabetic nephropathy.

Diabetic nephropathy is the leading cause of end-stage renal disease worldwide, and effective interventional strategies are desperately required (49). The characteristics of diabetic nephropathy manifest as albuminuria, glomerular basement membrane (GBM) thickening, mesangial hypertrophy, and tubulointerstitial fibrosis (8, 18, 44). Hypertension is often present, forming a significant factor in the progression of diabetic nephropathy (56). Elevation of sympathetic nerve activity (SNA) has been shown to occur in patients with diabetic nephropathy (47) and is established as a major contributor in the onset of hypertension and renal failure (30, 58). Furthermore, the pharmacological inhibition of SNA at doses which do not affect hypertension has been shown to effectively reduce the progression of renal damage in both clinical diabetic nephropathy (52) and in experimental diabetic nephropathy models (1). These findings appear to indicate that increased SNA may instigate and even accelerate the development of diabetic renal injury independently of its hypertensive effect.

As renal nerves play a significant role in controlling SNA (48), diseased kidneys are therefore able to exert an excitatory effect on the SNA in patients with chronic renal failure (11, 26). Earlier studies using rat models of experimental nephropathy have demonstrated that renal sensory nerve activity from the diseased kidneys to the brain is raised, resulting in an increased posterior hypothalamic noradrenaline turnover and renal SNA elevation (RSNA) (9, 10, 62–64). As the kidneys are richly supplied by renal sympathetic nerves, an increase in RSNA may instigate and even accelerate the development of diabetic renal injury independently of its hypertensive effect.

By effectively interrupting this excitatory cycle, bilateral renal denervation (BRD) has reemerged as an effective therapy shown to delay the progression of hypertension. In several clinical trials, BRD was demonstrated to provide long-term blood pressure reduction in patients with difficult-to-control and resistant hypertension (16, 31, 38, 43, 51, 53). The unexpected failings of the latest clinical study (SYMPLECTIC HTN-3) to show efficacy have, unfortunately, cast some doubt on the value of renal denervation in resistant hypertension (55). However, BRD may have renoprotective effects in patients beyond simple blood pressure control. BRD has been found to improve glucose tolerance and insulin sensitivity (43). In a rat model of early type 1 diabetes, BRD was also found to abolish glomerular hyperfiltration and prevent glomerular hypertrophy at 14 days after diabetic onset (42). Taken together, these findings suggested that renal nerves are involved in the early renal pathological changes occurring during the development of diabetic nephropathy. However, the chronic effects of BRD...
on the progression of diabetic nephropathy have not been fully examined.

In this study, we examined the hypothesis that BRD conducted after the onset of hyperglycemia will preserve renal morphology and function and reduce the severity of hypertension and expression of renal fibrotic markers formed as a consequence of diabetic induction. Furthermore, as previous reports have shown inconsistencies in the level of intrarenal distribution of angiotensin II type 1 receptors (AT1R), particularly as renin and angiotensin-converting enzyme content in the rodent nephron may be variably altered in diabetes (35), this study also examined the effect of denervation on renal AT1R in diabetic nephropathy. The underlying features of the hypertensive transgenic (mREN-2)27 rat (TGR) model include elevated prorenin levels and high tissue renin-angiotensin system activity that are critical to the development of diabetic nephropathy in humans (4). Previous reports have shown that streptozotocin (STZ)-induced diabetes in female heterozygous TGRs results in a rapid progression of the principal pathological features of human diabetic nephropathy without the prominent effects of hypertension on renal pathology (3, 4, 32).

Consequently, diabetic nephropathy was studied in this female TGR model over a 12-wk period following hyperglycemic onset at 6 wk of age. The present studies were performed to investigate the therapeutic benefit of BRD on the progression of diabetic nephropathy in the female heterozygous, renin-overexpressing TGR model of STZ-induced hyperglycemia.

METHODS

Animal model and research design. All experimental procedures involved in this project were conducted under the approval of the University of Otago Animal Ethics Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female heterozygous TGRs were obtained from the CARA Transgenic Facility at the University of Otago. Rats received either an intravenous bolus of a citrate-buffered vehicle (0.05 M) or STZ (55 mg/kg) to induce diabetes at 6 wk of age as described (32). Blood glucose levels were monitored and maintained at 20–25 mmol/L by daily administration of isophane (sc, 1–4 units). Animals in both normoglycemic and diabetic groups were randomized to either BRD or sham denervation surgeries performed repetitively in each animal at postnatal weeks 9, 12, and 15 (Fig. 1). Systolic blood pressure (SBP) and heart rate were measured weekly following habituation, using tail-cuff plethysmography and 24-h urine collections obtained at specific intervals. Upon termination at postnatal week 18, the animals were anesthetized (halothane-N2O2-O2 mix), and the kidneys were harvested and stored for further histological and biochemical processing. In a separate set of treatment groups, the glomerular filtration rate (GFR) was measured using the inulin clearance method and corrected to the left kidney weight as previously described (24).

BRD. As renal nerves in rats have been shown to fully reinnervate the kidney within 3–6 wk after surgical denervation, it was necessary to conduct repeat denervation surgeries (34, 45). At week 3 after STZ administration, a midline dorsal lumbar incision was made bilaterally under general anesthesia (halothane-N2O2-O2 mix), and each kidney exposed by retracting the abdominal muscles. Positive identification of the renal nerves was confirmed using a stimulator (10 V/10 Hz, 1-ms square wave pulse) to produce temporary renal blanching. Subsequently, the renal arteries were stripped back of the surrounding nerve bundles surrounding the renal arteries and painted with 10% absolute ethanol to destroy any remnant nerves. Subsequent denervation surgeries were similarly conducted. Sham renal denervation in the intact animals was performed using identical procedures, with the renal nerves left intact.

Inulin clearance studies. Each animal was anesthetized with a bolus injection of pentobarbitone sodium (70 mg/kg ip; National Veterinary Supplies). The left femoral vein was cannulated and infused with 0.9% saline (18 ml kg⁻¹ h⁻¹). The left carotid artery was also cannulated to allow access for blood sampling and mean arterial pressure monitoring during the experiment. A midline laparotomy was conducted to allow cannulation of the left kidney ureter for urine collection. On completion of the preparation, the 0.9% saline infusion was replaced by a priming dose (2 ml) and then a sustained infusion (18 ml kg⁻¹ h⁻¹) of inulin (15 mg/ml; Sigma, St. Louis, MO) and pentobarbitone sodium (0.44 mg/ml) in 0.9% saline. Following a 2-h equilibration period, urine was collected from the left ureter in two 15-min periods. Blood plasma samples were collected from the carotid artery at the beginning and end of each period. Inulin concentration in the plasma and urine samples was determined using a modification of the protocol described by Bojesen (7). Plasma and urine samples (50 μl) were deproteinized in 250 μl of NaOH (0.15 M) and neutralized in 1,700 μl of an H2SO4 (3.68 mM) and ZnSO4 (850 mM) solution. The precipitated protein was removed by centrifugation (500 g, 15 min, 4°C). Subsequently, 50 μl of the supernatant was incubated (100°C for 15 min) in a final ratio of 1:2.26 (vol/vol) to 2.5 ml of the color reagent [20% diphenylamine in absolute ethanol (wt/vol)] diluted in a 3:10 (vol/vol) H2SO4 (0.125 M) solution in absolute ethanol. The absorbance of each sample was read (λ = 655 nm) at room temperature, and inulin clearance is expressed as a plasma-to-urine ratio.

Histopathology. Freshly harvested kidneys were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, and sectioned using standard protocols. Periodic acid-Schiff’s staining was used to identify changes in basement membrane architecture and glycogen deposition in the kidneys. The glomerulosclerotic index (GSI) for each animal was determined by examining 150–200 glomeruli in each grade of sclerosis.

Immunohistochemistry. Staining was performed on 5-μm microwave-fixed, paraffin-embedded sections using standard protocols. After a 2-h incubation with an animal-free blocker (Vector Laboratories, Burlingame, CA), the sections were incubated (15 h at 4°C) with the following primary antibodies diluted in Tris-buffered saline: mouse anti-rat α-smooth muscle actin (SMA; 1:400; Sigma), rabbit anti-rat collagen I (1:100; Rockland, Gilbertsville, PA), rabbit anti-rat colla-

Fig. 1. Experimental design and confirmation of successful diabetic induction and chronic bilateral renal denervation (BRD). Streptozotocin (STZ; iv) was administered to 6-wk-old female transgenic rats (TGRs) allocated to the diabetic group. BRD or sham surgeries were performed, repetitively, at 9, 12, and 15 wk of age. Weekly systolic blood pressure recordings (4) and 24-h urine collections (5) were performed. At 18 wk of age, animals were euthanized, and kidneys were collected for immunohistochemical and Western blot analysis.
Sample preparation, gel loading, electrophoresis, and transblotting procedures were carried out using a Tris-glycine-buffered system (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Immunoblotting was performed using standard protocols with the following primary antibodies: rabbit anti-rat collagen I (1:1,000; Rockland), and rabbit anti-rat collagen IV (1:1,000; Abcam), and rabbit anti-rat TGF-β1 (1:400; Santa Cruz Biotechnology). To confirm equal protein loading, blots were reprobed with rabbit anti-rat β-tubulin (1:5,000; Sigma). A horseradish peroxidase-conjugated secondary antibody (1:1,000; Pierce) was used to label the protein band, and blots were visualized using chemiluminescence (SuperSignal West Dura Extended Duration Substrate; Pierce). Densitometric scanning of protein bands of interest was performed using a GS-710 Calibrated Imaging Densitometer (Bio-Rad) and quantified by Quantity One software (Bio-Rad). Each specific protein band density was ratioed against the β-tubulin protein density quantified in the same sample.

**RENAL BIOCHEMICAL STUDIES.** The Na⁺ concentration of the urine samples was analyzed using a FP20 flame photometer (SEAC, Florence, Italy) against a 100 mM NaCl standard solution, diluted (1:200) in a Li₂CO₃ (15 meqV) reference solution. Urinary albumin concentration was measured using an Albumin Blue Fluorescent Assay Kit (Active Motif, Carlsbad, CA) performed according to the manufacturer’s instructions. Renal cortical norepinephrine levels were quantified at the terminal study point (12 wk post-diabetic induction) using

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**Fig. 2.** Physiological parameters in (mRen-2)27 TGRs from 5 to 18 wk of age. Values are means ± SE. Vertical dotted line represents the time point when BRD/sham surgery was performed. ○: Normoglycemic sham (NGInx); ●: diabetic sham (DBInx); □: normoglycemic denervated (NGDnx); ■: diabetic denervated (DBDnx). A: mean weekly blood glucose levels. B: absolute body weight change. C: absolute body weight change. D: heart rate. E: absolute Na⁺ excretion (U₄0). F: urine flow. In A, B, D, E, F: ***P < 0.01 or ****P < 0.001 DBInx vs. NGInx, and *P < 0.05 or **P < 0.01 DBDnx vs. NGDnx. In C: ***P < 0.001 NGInx vs. NGDnx, ****P < 0.001 DBInx vs. DBDnx and ***P < 0.01 DBDnx vs. NGDnx. *P < 0.01, **P < 0.01, or +++P < 0.001 vs. systolic blood pressure at baseline (8.5 wk) within the group.
a commercial norepinephrine ELISA kit (BA E-5200, Labor Diagnostika Nord) performed according to the manufacturer’s instructions.

Statistics. All values are expressed as means ± SE of n = 7–8 individual experiments/group. Statistical analysis was conducted to examine differences in responses between the four groups: normoglycemic innervated (NGInx), normoglycemic denervated (NGDnx), diabetic innervated (DBInx), and diabetic denervated (DBDnx). For systolic blood pressure (SBP), heart rate, body weight, urine flow, and absolute sodium secretion (UNaV), a two-way repeated measures ANOVA was used to provide a comparison between each of the two treatment paradigms: NGInx vs. NGDnx, DBInx vs. DBDnx, NGInx vs. DBInx, and NGDnx vs. DBDnx. Significant interaction between two groups in SBP and heart rate comparisons was further analyzed using one-way repeated measures ANOVA followed by a Dunnett post hoc comparison against an internal baseline measured at 8.5 wk post-STZ administration (presurgical values). All other comparisons were made with two-way ANOVA followed by a Bonferroni post hoc comparison. Statistics were performed using SigmaStat v3.5 software (Systat Software, San Jose, CA). Differences in group means were considered statistically significant if P < 0.05.

RESULTS

Physiological parameters. Diabetic onset resulted in elevated blood glucose levels (Fig. 2A) and reduced body weight gain (DBInx vs. NGInx, P < 0.01; DBDnx vs. NGDnx, P < 0.05, Fig. 2B) in both sham-operated and denervated TGRs. Repeated BRD had no impact on these parameters in either normoglycemic or diabetic groups.

As expected, SBP was elevated in the TGRs after week 6 with no significant difference seen between the sham-operated normoglycemic and diabetic groups over the duration of the experiment. As shown in Fig. 2C, BRD intervention in both normoglycemic and diabetic animals produced a progressive, significant decrease in SBP compared with the sham-operated groups, which was maintained over the 9-wk denervation period (NGInx vs. NGDnx, P < 0.001; DBInx vs. DBDnx, P < 0.001, Fig. 2C). Denervation, however, produced a significantly greater decrease in SBP in diabetic animals compared with the normoglycemic group (DBDnx vs. NGDnx, P < 0.01). Diabetic TGRs also had a lower heart rate compared with normoglycemic groups in both sham-operated (DBInx vs. NGInx, P < 0.001) and denervated groups (DBDnx vs. NGDnx, P < 0.001, Fig. 2D). Both diabetic groups exhibited an increase in UNaV (Fig. 2E) and urine flow (Fig. 2F) following diabetic induction which persisted until termination (DBInx vs. NGInx, P < 0.001; DBDnx vs. NGDnx, P < 0.001). Repeated BRD had no impact on these parameters in either normoglycemic or diabetic animals.

Fig. 3. Confirmation of successful chronic BRD. A: confirmation of surgical denervation in kidneys isolated from diabetic TGRs. Left: innervated kidney section from diabetic TGR subjected to sham surgery showing tyrosine hydroxylase-positive fluorescent immunostaining (green) indicated by arrows within the adventitia of intrarenal arterioles, tubules, and glomeruli. Middle: denervated kidney section from a diabetic TGR subjected to repeated chronic BRD. Right: a negative control kidney section without tyrosine hydroxylase primary antibody. Nuclei were stained with 4,6-diamidino-2-phenylindole (blue). Scale bars = 50 μm. a, arteriole; g, glomerulus. B: renal cortical tissue norepinephrine levels analyzed by ELISA. Values are means ± SE of n = 7. ***P < 0.001 vs. NGInx, **P < 0.01 vs. DBInx.

Fig. 4. Renal morphological changes in TGRs. A: morphological changes in cortex and inner stripe of outer medulla in kidney sections stained with periodic acid-Schiff. In NGInx and NGDnx kidneys, glomerular appearance and tubular structure in the cortex and medulla appeared normal with no remarkable features. In the kidneys from the DBInx TGRs, most glomeruli showed a severely thickened glomerular basement membrane (GBM) and mesangial hypertrophy; many cortical tubules were dilated with fibrotic casts deposited in the lumen; inflammatory cell infiltration was seen in some areas of the kidney (arrows). In the medulla, severe fibrosis was observed extensively across the interstitium and tubules (arrow). In DBDnx kidneys, the occurrence of glomeruli with a thickened GBM and mesangial hypertrophy was reduced to normal levels; a few cortical tubules were still dilated but with significantly less fibrotic casts appearing in the lumen; inflammatory infiltration was not noticeable. In the medulla, some interstitial fibrosis was still seen; however, the frequency and the severity of fibrosis were reduced. B: glomerulosclerotic index in kidneys from TGRs subjected to diabetes and renal denervation. Values are means ± SE. ***P < 0.001 vs. NGInx, *P < 0.05 vs. DBInx.
Confirmation of renal denervation. Positive tyrosine hydroxylase staining was found within cortical sections of sham-operated TGR kidneys indicating the presence of sympathetic innervation (Fig. 3A). Successful denervation was confirmed by the absence of tyrosine hydroxylase staining and by the reduction of total norepinephrine levels in denervated renal cortical tissues (Fig. 3B). Denervation reduced cortical norepinephrine content significantly from 23 ± 1.6 to 1.0 ± 0.1 ng/mg protein in the NGInx vs. NGDnx groups, respectively (P < 0.001), and from 37 ± 2.6 to 1.7 ± 0.6 ng/mg protein in the DBInx vs. the DBDnx groups, respectively (P < 0.001). Renal norepinephrine content was also raised as a consequence of diabetic induction in the sham-operated animals (P < 0.001).

Renal morphological and functional parameters. Glomerular structure appeared normal in the normoglycemic kidneys (Fig. 4A). In contrast, most glomeruli were sclerosed in kidneys from diabetic sham-operated animals. This appearance was attenuated in denervated diabetic kidneys. The glomerular pathology was reflected by the significantly higher GSI recorded in kidneys isolated from sham-operated diabetic TGRs compared with the normoglycemic groups (1.94 ± 0.47 DBInx vs. 0.56 ± 0.10 NGInx, P < 0.001, Fig. 4B). This elevated GSI was attenuated in the diabetic TGRs subjected to BRD (0.68 ± 0.14 DBDnx vs. 1.94 ± 0.47 DBInx, P < 0.001). Normal renal medullary structure was observed in the normoglycemic TGR kidneys (Fig. 4A). In contrast, in diabetic sham-operated TGR kidneys, matrix expansion, GBM thickening, and lumen expansion were observed among the renal tubules distributed at the outer medullary region. Denervation of the diabetic TGR kidneys greatly reduced the extent of structural injury.

Podocin staining was diminished within the glomeruli of sham-operated diabetic animals compared with the normoglycemic groups (Fig. 5, A and B). Diabetic-induced podocin loss was prevented in TGRs subjected to BRD. Denervation did not have any effect, however, on podocin staining in these hypertensive TGRs animals in the absence of diabetic insult. The functional significance of this podocyte injury was demonstrated by the presence of albuminuria in the diabetic TGRs. Urinary absolute albumin levels assessed after 12 wk of hyperglycemia were raised in the diabetic sham-operated group compared with the normoglycemic groups (10.7 ± 2.5 vs. 1.6 ± 0.2 mg·24 h⁻¹·kg⁻¹, respectively, P < 0.001, Fig. 5A). Diabetic animals subjected to BRD showed a significantly reduced urinary albumin level compared with the diabetic sham-operated group (5.0 ± 1.1 vs 10.7 ± 2.5 mg·24 h⁻¹·kg⁻¹, respectively, P < 0.05).

Left kidney weight recorded at termination was raised in the diabetic TGRs compared with the normoglycemic TGRs (P < 0.001, Fig. 6A). GFR (corrected for kidney weight) in the diabetic TGRs was significantly lower (P < 0.001, Fig. 6B) compared with the normoglycemic TGRs. BRD had no impact on kidney weight or GFR in either normoglycemic or diabetic TGRs.

Renal fibrotic and profibrotic markers. Weak collagen I immunolabeling was seen distributed in both renal cortex and medulla in the normoglycemic TGRs (Fig. 7A). In the diabetic sham-operated group, elevated collagen I expression was found extracellularly at the GBM and in the interstitial space of the outer medulla. Intervention with BRD after the onset of diabetes reduced collagen I expression in both the cortex and medulla. Differences in collagen I expression were confirmed by Western blot analysis showing a twofold higher expression in the renal cortex of diabetic sham-operated TGRs (Fig. 7B), and this elevation was reduced in diabetic TGRs subjected to BRD. Collagen IV was weakly labeled in the normoglycemic TGR kidneys (Fig. 8A). Kidneys from the diabetic sham-operated group show elevated collagen IV expression within the glomeruli and in the interstitial space throughout the cortex and medulla. BRD intervention also successfully attenuated the extent of collagen IV expression found in diabetic TGRs. Protein analysis confirmed increased collagen IV expression in the cortex of diabetic sham-operated TGRs compared with the normoglycemic groups (Fig. 8B), and this elevated expression was also reduced in the denervated diabetic TGRs.

Very low levels of α-SMA were detected in the medulla of normoglycemic TGR kidneys (Fig. 9, A and B). In the diabetic sham-operated group, α-SMA was extensively expressed.
within the renal tubule and interstitial space at the outer medullary region (Fig. 9, C and E), and this diabetic-induced elevation was attenuated in the medulla of diabetic TGRs subjected to BRD (Fig. 9, D and F).

TGF-β1 was elevated in both the cortex and medulla of the sham-operated diabetic TGR kidneys compared with normoglycemic TGRs (Fig. 10A). This expression was reduced in the diabetic TGR kidneys subjected to BRD. Western blot analysis showed a twofold higher expression in the renal cortex of the diabetic sham-operated group compared with normoglycemic TGRs (Fig. 10B), and this elevation was reduced in diabetic TGRs subjected to BRD.

AT1R immunohistochemical studies in the normoglycemic kidneys produced faint staining within the proximal tubules and cortical and medullary collecting ducts (Fig. 11A). AT1R immunostaining became more pronounced within epithelial cells of the dilated cortical and outer medullary collecting tubules in the diabetic kidneys, producing a significant increase in AT1R immunostaining intensity relative to normoglycemic innervated kidneys (Fig. 11B). Conversely, AT1R staining in the rest of the nephron was reduced, particularly within regions of extracellular matrix expansion. While chronic renal denervation failed to affect the low AT1R staining intensity within the normoglycemic group, the procedure resulted in an abrupt loss of AT1R intensity in the diabetic animals.

**DISCUSSION**

The current study is the first to demonstrate that renal nerves play an important role in the long-term development of structural damage seen in diabetic nephropathy. BRD intervention after pancreatic β-cell destruction and diabetic onset attenuated the progression of diabetic nephropathy in this transgenic model of renin-overexpressing hypertensive rats. As expected, BRD reduced the severity of hypertension and renal catecholamine levels in the normoglycemic animals. These parameters were also successfully attenuated within the diabetic animals. However, while BRD had no effect on renal morphology or injury markers in normoglycemic hypertensive groups, this denervation protocol clearly provided protection when hypertension was compounded by the presence of diabetes. Both diabetic-induced albuminuria and podocin loss but not hyperglycemia were decreased by BRD intervention. BRD also reduced intrarenal AT1R expression within the collecting ducts and attenuated renal fibrosis in diabetic TGRs by reducing the expression of the profibrotic inducer TGF-β1. This reduction was supported by the attendant reduction in α-SMA and collagens I and IV. This study assesses for the first time the
effect of renal denervation on the individual contribution of diabetes as well as hypertension in diabetic nephropathy.

The induction of diabetes in the hypertensive TGR animals resulted in a reduced rate of growth compared with the normoglycemic animals. This pattern of body weight difference recorded in the intact normoglycemic and diabetic TGRs is consistent with earlier published data gained from the same animal (32). While this reduction in body weight gain has also been observed in other diabetic rat models following STZ administration, our results indicate that the surgical intervention procedure itself had minimal impact on the animals’ health and growth status (12). The 12-wk time course of this experimental model was carefully selected to ensure that the development of renal injury did not result solely from the effects of chronic hypertension but as a consequence of the synergistic effects of hyperglycemic induction. Applehoff and coworkers (3) clearly demonstrated that both diabetes and the ren-2 gene may contribute to the glomerular pathology described at 4 mo in the male rat. Conversely, female TGRs develop lower levels of hypertension (30–40 mmHg less) than their male littermates and show no evidence of morphological changes in the nephron before 4–6 mo of age (4). Consequently, while SBP was elevated in the intact female heterozygous TGR animals over the 12-wk period, no evidence of renal morphological injury or proteinuria was seen in the normoglycemic female TGRs. We found that BRD intervention reduced SBP and renal cortical catecholamine levels in the normoglycemic renin-overexpressing hypertensive rats. While heart rate was unaffected by denervation in either normo- or hyperglycemic groups, BRD produced a more pronounced decrease in SBP in the diabetic than in the normoglycemic animals, suggesting that BSNA is highly important in maintaining elevated blood pressure in the diabetic TGRs with renal injury. Indeed, renal catecholamine levels were significantly raised in the diabetic fibrosed kidneys compared with the hypertensive only group, strongly supporting the hypothesis that diabetic renal injury can elevate BSNA. In support of this premise, afferent renal nerve activity has also been shown to be elevated in the rat renal injury model (10). Consequently, in innervated diabetic hypertensive TGRs, the amplified afferent signals originating from the kidneys may serve to sustain the elevation in systemic SNA and maintain an increased blood pressure, possibly to compensate for a potential fall in cardiac output resulting from diabetic cardiomyopathy. In contrast, in denervated diabetic TGRs, permanent removal of renal afferent nerves suppressed SNA, resulting in a fall in SBP. Studies conducted by Campese and colleagues (5, 9, 63) in the nephrectomized hypertensive rat renal injury model have shown that selective renal afferent denervation can abolish the severity of hypertension and reduce posterior hypothalamic noradrenaline content. Similarly, BRD was found to reduce blood pressure and attenuate the hypothalamic norepinephrine turnover in a neurogenic hypertensive rat model (62–64). These prior studies support the suggestion that elevated afferent impulses from injured kidneys can stimulate central sympathetic activity at the hypothalamic level, thereby evoking hypertension and may explain the antihypertensive effects of BRD observed in the present study. Further work on this model should help to clarify the specific role of afferent renal nerve activity in the progression of hypertension and renal failure.

Following diabetic onset, marked hyperglycemia and albuminuria, combined with a reduction in the podocyte-specific protein podocin, were observed in the innervated diabetic TGRs. The association between glomerular podocin loss and albuminuria has also been found in both experimental models and in human diabetic nephropathy (37, 61). The reduction of the diabetes-induced albuminuria with BRD can be partially explained by the reduction in blood pressure consequent with this intervention (54). However, studies in cultured podocytes have shown that the α-adrenoceptor agonist phenylephrine can increase intracellular Ca²⁺ concentration to alter podocyte shape and reduce foot processes, thus directly regulating podocyte functions (29). These in vitro studies strongly suggest that elevated cortical norepinephrine levels recorded in the diabetic nephropathy animals may directly mediate podocyte injury, consequently permitting the development of albuminuria. This supports and may partially explain the mechanism through which BRD attenuated podocyte injury in the diabetic kidneys. Indeed, BRD also reduced the severity of glomerulosclerosis.

Fig. 8. Collagen IV localization and expression in the cortex and inner stripe of outer medulla of kidney sections immunolabeled with collagen IV antibody and counterstained with hematoxylin. A: in sections from normoglycemic innervated and denervated kidneys, collagen IV was weakly labeled in the glomeruli and medullary tubules. In the diabetic innervated kidney sections, intense collagen IV staining was found within the glomeruli; strong collagen IV staining was also found extensively in the interstitium of the medullary tubules. BRD in the diabetic TGRs reduced collagen IV labeling to normoglycemic levels in the glomeruli and medullary tubules. Scale bars = 50 μm. B: Western blot analysis indicates increased collagen IV protein expression in the diabetic innervated TGR kidneys. This elevation was attenuated in the diabetic TGR kidneys subjected to renal denervation. Values are means ± SE. *P < 0.05 vs. NGInx. #P < 0.05 vs. DBInx.
and preserved glomerular structure in diabetic TGRs. Selective renal afferent denervation was shown to reduce blood pressure and glomerulosclerosis severity in earlier studies of a hypertensive rat model of chronic renal failure conducted by Campese and colleagues (10). While glomerular protection may in part stem from the reduction in blood pressure consequent to BRD intervention, it is important to note, however, that in the absence of hyperglycemia, none of the hypertensive animals used in this study showed any evidence of proteinuria or renal morphological damage. Evidence in support of this antihypertensive-independent effect comes from other studies by Nagasu and coworkers (46) showing that removal of sympathetic influence by acute renal denervation can directly protect glomerular structure in the absence of a blood pressure-lowering effect. Furthermore, the inhibition of RSNA using pharmacological sympatholytic agents such as prazosin, timolol, guanethidine, and moxonidine at subantihypertensive concentrations has also been shown to preserve glomerular structure in rat models of hypertensive renal failure (2, 22). The presence of $\beta_1$-adrenoceptors within glomerular mesangial cells (6) suggests that the RSNA may play a direct role in mesangial dysfunction. Consequently, removal of this elevated sympathetic influence on diabetic kidneys may protect the structural and functional integrity of the glomerular mesangium.

The presence of renal cortical and medullary fibrosis as evidenced by the extensive deposition of glycogen and accompanied by tubule dilation in the diabetic kidneys was confirmed by the elevated expression of renal fibrotic markers TGF-$\beta_1$, $\alpha$-SMA, and collagens I and IV. Repeated BRD at 3 wk following diabetic onset suppressed the levels of these fibrotic markers. Multiple mechanistic pathways may be involved in mediating the protective effect of BRD against diabetic renal fibrosis and warrants further investigation. Previous work conducted in other models of renal injury has shown that suppression of renal sympathetic innervation by surgical denervation or moxonidine administration may be effective in suppressing renal fibrotic markers such as TGF-$\beta_1$ (2, 23, 57). Renal fibrogenesis induced by ureteric obstruction in a rat model has similarly been shown to be attenuated by denervation and by selective $\alpha_2$-adrenoreceptor agonist administration; this protection was reversed, however, by the infusion of a calcitonin gene-related peptide and norepinephrine, elegantly demonstrating that both renal afferent and efferent nerves are involved in the formation of renal fibrosis (33). Inflammatory responses are also involved in mediating the progression of diabetic renal injury (19). As renal denervation in the anti-Thy-1.1-induced glomerulonephritis model was shown to produce anti-inflammatory and antifibrotic effects (57), it is conceivable that the protection afforded by BRD against diabetic nephropathy in the TGRs could partially be due to an anti-inflammatory effect.

Systemic renin angiotensin system activity can also be effectively reduced by a BRD abrogation of renin release from the juxtaglomerular apparatus (20). Elevated norepinephrine release from renal sympathetic nerve terminals as seen in the diabetic TGRs group can induce TGF-$\beta$ expression, thereby enhancing the extracellular matrix accumulation and profibrotic actions of intrarenal angiotensin II at the renal tubules (13). BRD intervention in this study may consequently inhibit the involvement of the intrarenal renin-angiotensin system in the TGF-$\beta$-mediated profibrotic pathways observed in the diabetic kidneys. Unfortunately, this study was not set up to tease out the effects of denervation on the catabolic action of proinflammatory matrix metalloproteinases and other proteolytic enzymes on pathogenic extracellular matrix accumulation (40). The potential impact of BRD on metalloproteinases specific to each stage of albuminuria development remains to be clarified.

While various studies have demonstrated the interstitial and intratubular localization of the intrarenal renin-angiotensin system, only a few studies have attempted to resolve the activation status of this pathway in diabetes. Induction of moderate hyperglycemia using STZ in a rat model has been shown to result in elevated systemic angiotensin II and increased AT$_2$R immunostaining in the renal collecting ducts within 20 days (25, 35). Examination of our animal kidneys subjected to a longer time course of hyperglycemia (12 wk post STZ) shows that diabetic induction also had the effect of increasing AT$_2$R expression in the cortical and outer medullary collecting ducts of these renin-overexpressing TGRs. No
Diabetic induction resulted in the development of renal hypertrophy and a decreased GFR after 12 wk in the present model, indicating that renal function was at the declining phase of the diabetic disease progression. However, both GFR reduction (32) and elevation (59) have been reported at this same time point following diabetic onset in the same diabetic TGR model. Type 1 diabetic induction in the TGR increased GFR at 4 wk but reduced it by >50% after 12 wk of diabetes in TGRs. These studies suggest that the initial point of GFR decline is variable, occurring at slightly different time points during the disease progression in different batches of animals. In contrast to the renal morphological protection afforded by BRD in diabetic TGRs, no similar effect was observed with the GFR indices. It should be noted that GFR in the normoglycemic hypertensive TGRs was also unaffected by BRD intervention. Studies conducted in a type 1 diabetic rat have indicated that BRD can reverse GFR changes if conducted early (2 wk) within the disease progression (42). While this discrepancy is hard to explain, our observations are consistent with a recent clinical study, which found that GFR was unchanged by BRD treatment in patients with moderate to severe chronic kidney disease (27).

In conclusion, the present study conducted in renin-overexpressing TGRs substantiates the significance of renal sympathetic nerves in the pathogenesis of diabetic nephropathy. While denervation clearly had no effect on renal morphology or fibrotic marker status in hypertensive only animals, BRD substantially reduced albuminuria and attenuated the renal fibrosis and glomerulosclerosis consequences of hyperglycemia. BRD was conducted over a 12-wk period starting 3 wk after the onset of early diabetic injury (36). Further work will be needed to clarify whether this procedure can revert established diabetic nephropathy and show benefit over current pharmacological interventions. Early experimental studies using mesenchymal cell transplantation in type 1 diabetic mice have proved promising, indicating that both microalbuminuria and renal structural injury may be reversed; however, this therapy is still experimental (17). While reducing the progress of the disease, angiotensin-converting enzyme inhibitors fail to prevent angiotensin escape pathways from eventually provoking the renal manifestations of hyperglycemia. By attenuating the development of diabetic nephropathy, renal denervation may consequently prove valuable as an additive to the current practice of renin-angiotensin-aldosterone blockade. The superiority of this combination therapy over monotherapy with quinapril has already been demonstrated in a renal ablation model (23). We conclude that the protection of renal indices and structure reported in this study provides strong scientific support for the application of renal denervation procedures in diabetic nephropathy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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Fig. 11. A: ANG II type 1 receptor (AT1R) immunolabeling and localization in the cortex and outer medulla of normoglycemic and diabetic sections counterstained with hematoxylin. Faint AT1R labeling (brown DAB stain) was observed within the proximal and distal tubules of normoglycemic or diabetic TGRs. AT1R labeling was also weak within the collecting duct segments and connecting tubules of normoglycemic kidney sections but appeared prominent (see arrows) within the epithelial layer of the collecting tubules obtained from the intact DBInx group. Note the lack of DAB staining in the abnormally expanded matrix around the dilated collecting tubules of the DBInx. AT1R labeling was strikingly reduced by denervation in the diabetic animals. Scale bars = 50 μm. B: percentage of tissue positively stained for DAB against background, recorded in 10 randomly selected cortical and medullary fields. Values are means ± SE (n = 7 - 8 individual animals/group). ***P < 0.0001 vs. NGInx.


