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Tubular injury markers netrin-1 is elevated early in experimental diabetes

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Abstract

Background—Netrin-1 was recently identified as an early diagnostic biomarker of acute kidney injury. However, its usefulness for early diagnosis of CKD is unknown. The current study evaluated whether these proteins are increased in urine from experimental animals with diabetes.

Methods—The current study evaluated whether netrin-1 is increased in urine from diabetic rat and mice and whether netrin-1 correlated with development of nephropathy.

Results—In rats, urinary netrin-1 excretion was significantly ($p < 0.001$) higher in diabetic group at 4 and 10 weeks after induction of diabetes as compared to control group. Similarly, netrin-1 was increased significantly ($p < 0.001$) in urine from hypertensive rats at 4 weeks as compared to controls. Likewise, urinary albumin excretion rates were increased in diabetic rats at 4 and 10 weeks as compared to controls and was increased in hypertensive rats at 4 weeks. Consistent with diabetic model in rat, netrin-1 excretion also increased early in diabetic mice urine and peak levels correlates with disease severity.

Conclusion—netrin-1 can be detected in urine from diabetic and hypertensive rats and may serve as a useful early diagnostic biomarker for development of CKD.

Keywords

Biomarker; Netrin-1; Diabetic nephropathy; Albuminuria

Introduction

Diabetes mellitus is a global epidemic that is associated with increased risk of cardiovascular disease, kidney disease, and premature death (1). Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease in the United States and its incidence is increasing (2). The effects of diabetes can cause the kidney to develop structural and functional abnormalities, which include hyperfiltration with glomerular hypertension, renal hypertrophy, increased glomerular basement membrane thickness, tubular atrophy and

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STATEMENT OF COMPETING FINANCIAL INTERESTS

Dr. Ramesh is holding a patent for the discovery of netrin-1 as an early diagnostic biomarker of disease and injury. All other authors have disclosed no competing interest.

interstitial fibrosis. These events lead to the development of proteinuria, worsened systemic hypertension, and ultimately progressive loss of kidney function. Unfortunately, currently available therapies are not effective in fully preventing progression of diabetic nephropathy. Blocking the renin-angiotensin system provides only limited protection against progression when started in the later stages of diabetic nephropathy [1–3], and consequently most cases of overt DKD lead to progressive renal failure and the need for renal replacement therapy [4;5]. Early start of treatment may be more effective. However, there is a paucity of diagnostic markers that can predict the development of progressive kidney disease in diabetes.

The diagnostic test currently used in clinical practice, i.e., increased albumin excretion rate, has significant limitations. Microalbuminuria is rarely found during the first 5 years of a patient's diabetes, suggesting that it is a sign of early glomerular damage rather than a marker for susceptibility to it. Furthermore, the presence of microalbuminuria by itself may not be an adequate indicator for disease progression in all diabetic subjects because of the observation that a number of type 1 diabetes patients revert to normoalbuminuria without treatment within a 5-year period [6]. Lastly, the occurrence of microalbuminuria in type 1 diabetes can already be associated with diabetic nephropathy (DN) lesions comparable to the ones found in overt diabetic nephropathy. Therefore, identification and validation of biomarkers for early diagnosis of DKD, i.e., before microalbuminuria develops, may help develop effective treatment for DKD. Although diabetic nephropathy is classically considered as a glomerular disease, studies suggest that tubulointerstitial injury may precede apparent glomerulopathy [7]. This view was further supported by studies that show increased levels of tubular injury biomarkers such as kidney injury molecule-1 (KIM-1) and N-acetyl- β -D-glucosaminidase (NAG) in patients with type 1 diabetes [8;9], and decreased levels of these biomarkers were associated with regression of microalbuminuria [9]. Similarly increased levels of KIM-1 and neutrophil gelatinase associated lipocalin (NGAL) were seen in patients with established diabetic nephropathy [8]. Higher levels of NGAL and KIM-1 were associated with a significant and greater decline in kidney function, but not after adjustment for other known progression factors. Furthermore, persistent albuminuria secondary to glomerular lesions may directly be harmful to renal tubular cells, leading to tubular inflammation and tubulointerstitial fibrosis [10].

Netrin-1 is guidance cues that are highly induced after acute tubular injury and excreted in urine in both mice and humans [11–13]. However, whether netrin-1 expression is increased in DKD and its utility as a biomarker for early diagnosis of DKD are unknown. The purpose of present study was to determine whether tubular injury marker netrin-1 and sema3A excretion in urine is increased early in the course of diabetes and hypertension in experimental animal model.

Materials & Methods

Animals

Experiments utilized male Sprague-Dawley rats (200–225 g) from Charles River Laboratories and male C57BL/6 mice from Jackson Laboratories. All protocols were approved by the Institutional Animal Care and Use Committee of the Georgia Health

Sciences University (Approval number: BR10-10-369) and followed the American Physiological Society Guidelines for the Care and Use of Laboratory Animals. Rats and mice were housed under conditions of constant temperature and humidity and exposed to a 12:12-hour light-dark cycle. Animals were allowed free access to drinking water and normal chow.

STZ-Induced Diabetes

Hyperglycemia was induced by streptozotocin (STZ) (65 mg/kg dissolved in sterile saline, Sigma-Aldrich, St. Louis, MO) injected into the penile vein (n = 9). Diabetes was confirmed 48 hours after injection and was subsequently monitored by measuring glucose concentration in tail blood samples using an Accu-Chek Glucometer (Roche Diagnostic, USA). A group of control rats were injected with sterile saline (n = 7). To prevent complications from severe hyperglycemia, rats were treated with timed-release insulin implants (Linplant, Scarborough, ON, Canada) at a dose of approximately 1 unit per day to maintain blood glucose below 500 mg/dl. Sham rats were implanted with palmitic acid blanks. Implants were sterilized with 2% povidone-iodine solution and inserted by a 16-gauge hypodermic needle under the dorsal skin of the neck. Rats were subsequently followed for 10 weeks during which time body weight, non-fasting blood glucose levels, and severity of diabetic-related symptoms were monitored bi-weekly.

The diabetes induction protocol we followed for mice was described by the Animal Models of Diabetic Complication Consortium (AMDCC) (<http://www.diacomp.org/shared/protocols.aspx>) [14–16]. Eight-week-old C57BL/6J mice (Jackson Laboratories) was given streptozotocin (STZ) (100 mg/kg BW/day in citrate buffer) as a single dose. Blood glucose and urine albumin were measured at 1 week after STZ injection and every three weeks thereafter.

DOCA-Salt Induced Hypertension

Rats were anesthetized with isoflurane (2% by inhalation; Baxter Healthcare, Deerfield, IL, USA), the right kidney was removed via a flank incision, and a 60-day timed-release pellet of DOCA 200 mg (Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously (n = 7). The drinking water of the DOCA-treated rats was replaced with 0.9% NaCl solution with 1% KCl (to prevent severe potassium depletion). Rats were subsequently followed for 4 weeks.

Metabolic Cages

Rats and mice were placed in metabolic cages (Nalgene) for a 24 h acclimation period, followed by a 24 h period where food and water intake and urine was collected. Metabolic cage studies were performed at baseline (prior to STZ or DOCA treatment), at 4 weeks (all animals) and 10 weeks (sham and STZ groups) for rat and baseline, 3, 6 and 12 weeks for mouse.

Tail-cuff Blood Pressure

Blood pressures were measured weekly using the tail-cuff method. In brief, rats were warmed in a restraining chamber and occluding cuffs and pneumatic pulse transducers were

placed on the rats' tails. A programmed electrosphygmomanometer automatically inflated and deflated the tail cuff while signals from the transducer were automatically collected using a MacLab (ADInstruments, Milford, CT) connected to a Macintosh Power PC. Six readings were taken for each rat and then averaged to determine systolic arterial pressure. Any measurements associated with excess noise or animal movements were discarded and the pressures were repeated.

Necropsy Procedure and Sample Collection

After 4 weeks (DOCA) and 10 weeks (sham and STZ) respectively, rats were anesthetized (65 mg/kg, i.p., sodium pentobarbital), and a terminal blood sample was collected from the abdominal aorta in a tube containing EDTA and was centrifuged at 4°C 3000g for 10 minutes. Plasma was removed and frozen at -80°C for later analysis. The left kidney was removed and immersion-fixed in 10% neutral buffered formalin for histological analysis.

Biochemical Analyses

Urine and serum creatinine were measured at 4 weeks, 8 weeks, and 10 weeks using picric acid method adapted for microtitre plates. Biomarkers were measured by enzyme-linked immunosorbent assay (ELISA); rat netrin-1 (Biotang, Waltham, MA, USA), mouse netrin-1 (CUSABIO, Wuhan, China) rat KIM-1 (Kamiya Biomedical Medical Company, Seattle, WA, USA) and albumin (AssayPro, St. Charles, MO). Color change was measured using a plate reader (Synergy 2, Bio Tek Inc.,) at a wavelength of 450 nm. Concentrations were determined by comparing the O.D. of the samples to a standard curve. All assays were performed in duplicate. Urinary netrin excretion is expressed pg/24hr urine for rat and picogram per mg of creatinine for human.

Immunohistochemical Analysis

Kidneys were perfused with 4% paraformaldehyde in 100 mmol/L dibasic sodium phosphate buffer and immersed in 4% paraformaldehyde solution overnight at room temperature, transferred to 70% ethanol for 24 h, and paraffin embedded. The kidneys were sectioned at a thickness of 4 µm onto Superfrost plus slides and processed. Slides were incubated in the absence or presence of primary antibodies to netrin -1 (Santacruz Biotechnology Inc., CA) or sema 3A (Abcam) in humidified chambers overnight at 4°C, followed by incubation with biotin-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hr at room temperature. Color was developed after incubation with ABC reagent (Vector Lab). The stained sections were photographed with an Olympus BX40 microscope (Olympus America, Melville, NY) on bright-field setting fitted with a digital camera (Olympus DP12; Olympus America) (magnification ×600). To determine fibrosis, kidney section was stained with Masson's Trichrome staining. For assessment of injury, 5 µm sections were stained with periodic acid-Schiff (PAS) followed by hematoxylin. Tubular injury was assessed in PAS-stained sections using a semiquantitative scale [17] in which the percentage of cortical tubules showing dilation, epithelial cell necrosis, brush-border loss, cast formation, and apoptotic bodies in the cortex was assigned a score: 0 = normal; 1 = <10%; 2 = 10–25%; 3 = 26–75%; 4 = >75%. 10 fields of 40× magnification were examined and averaged. The individual scoring of the slides was blinded to the genotype of the animal.

Analysis of Netrin-1 by western blot analysis—A volume of urine containing 1 µg of creatinine for each mouse sample and 15 µg creatinine for each human sample was subjected to Western blot analysis of Netrin-1. Urine sample was loaded onto 4–12% polyacrylamide gels, separated, and then transferred onto a PVDF membrane. The membrane was probed with rabbit anti-netrin-1 antibody (Calbiochem cat # PC344) or goat anti-netrin-1 antibody (Everest Biotech, cat # EB07007). Proteins were detected using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Inc.).

Statistical Analyses

Data are presented as the mean $\pm$ SEM. $p < 0.05$ was considered significant. Statistical analyses were performed using Graphpad Instat 3 (GraphPad Software, San Diego, CA, USA). Data were analyzed at the same time points with comparisons between groups using the Student's *t*-test.

Results

Characteristics of the Experimental Animals

Initially, all rats had similar weights and blood glucose levels that averaged 96 ± 5 mg/dl. Twenty four hours after STZ injection, blood glucose levels were elevated in the STZ rats, but not in controls (CTL) or in the DOCA rats (DOCA), as shown in Table 1. STZ and DOCA rats gained less weight and displayed hyperphagia, polydipsia and polyuria. Diabetic rats developed glomerular hyperfiltration as estimated by creatinine clearance. In contrast and as expected, DOCA rats had reduced creatinine clearance.

Blood Pressures

As shown in Figure 1, systolic blood pressures as measured by tail-cuff were elevated in DOCA rats compared to CTL. With the exception of week 2, CTL and STZ rats maintained consistent and similar blood pressures throughout the 10-wk period.

Netrin-1, a biomarker of tubular injury, is elevated in diabetic rat urine

Twenty-four-hour urinary excretion of netrin-1 was measured at baseline, and 4 and 10 weeks in control (vehicle-treated) and diabetic rats. As shown Figure 2A, netrin-1 excretion in control rats was not changed significantly. However, in the diabetic rat, netrin-1 excretion rate (pg/24hr urine) was increased more than 7 fold at 4 weeks and 10 fold at 10 weeks after STZ administration. The increase of netrin-1 was seen even after normalized to creatinine (12477 ± 2980 pg/mg of creatinine at 4 weeks and 12491 ± 4808 pg/mg of creatinine at 12 weeks as compared to 1091 ± 247 pg/mg of creatinine at baseline or sham). In contrast, KIM-1, another proximal tubular injury marker, is not increased significantly at 4 weeks of STZ administration (Figure 2C) but moderate increase was seen at 12 weeks after STZ administration as compared to control.

Netrin-1 is elevated in hypertensive rats

To determine whether netrin-1 is increased in hypertension-mediated kidney injury, netrin-1 excretion was quantified in 24 hr urine. As shown in Figure 2B, netrin-1 excretion was

unchanged in control rats. However, netrin-1 excretion was significantly elevated at 4 weeks in the DOCA salt model of hypertension. Unlike STZ model, KIM-1 excretion is significantly increased in DOCA induced hypertensive rats urine at 4 weeks (Figure 2D).

Netrin-1 protein induction is localized in the kidney proximal tubular epithelial cells in both diabetic and hypertensive models

To determine whether increased excretion of netrin-1 in urine is associated with induction in the proximal tubular epithelial cells, netrin-1 was immunolocalized as described in Materials and Methods. As shown in Figure 3, no staining was seen in control rat kidney (A & B). However, intense staining was seen in proximal tubular epithelial cells in the cortex of diabetic (C) and DOCA salt sensitive hypertensive rats (D). No staining was seen in the medulla in both diabetes and DOCA salt-treated animals. However, some staining was seen in the distal tubule in the cortex in the DOCA salt-sensitive rat kidney (data not shown). These results suggest that the source of netrin-1 in diabetes is the proximal tubular epithelial cells. In the case of the DOCA salt-sensitive hypertensive rat, the major source is proximal tubular epithelial cells but a minor contribution was also seen in the distal tubules.

Increased albumin excretion in urine was seen in both diabetic and hypertensive rats

Albuminuria is a hallmark of diabetic nephropathy. To determine whether diabetes and hypertension are associated with increased excretion of albumin, urinary albumin excretion was determined in our animal models. As shown in Figure 4, albumin excretion was significantly elevated in the diabetic rat at 4 and 10 weeks compared to sham controls. Similarly, urinary albumin excretion was also increased in DOCA salt-treated rats. The combination of increased albumin and netrin-1 in both diabetes and hypertensive rats suggest that tubular injury could be a contributing factor in the development of proteinuria, but further studies will be needed to test this hypothesis.

Netrin-1 excretion is increased in diabetic mice urine—To determine the observation made in rat is also true in diabetic mice, we have quantified netrin-1 and albumin excretion at different time after induction of diabetes. As shown in figure 5A, netrin-1 is not detectable at baseline. Administration of STZ induced hyperglycemia (360 ± 30 mg/dl) within 5 days after injection. However, netrin-1 was still undetectable on day 5 after STZ administration suggesting that direct tubular epithelial injury by STZ is not a cause for increased levels of netrin-1 in urine. Netrin-1 is elevated at 3 weeks after induction of diabetes. Elevated levels were seen at 6 and 12 weeks after induction of diabetes as well. Although Western blot analysis of urine albumin showed no increase till 6 weeks, ELISA based quantification showed a significant increase at 2 weeks after induction of diabetes and later time points (Figure 5B). ELISA based quantification of netrin-1 in urine showed a significant increase on day 10 much before urine albumin increase and increase was sustained as the severity of the disease progressed (Figure. 5C). Moreover, highest levels of netrin-1 correlated with albuminuria, increased fibrosis and tubular injury (Figure F and G) suggesting urine increased and concentration reflects the severity of disease.

Discussion

This is the first study to evaluate netrin-1 as an early diagnostic biomarker of CKD and hypertensive renal injury in rat and mouse models. Our results demonstrate that netrin-1 excretion is increased in experimental models of both type 1 diabetes and hypertension. We show that increased expression was identified in the proximal tubular epithelial cells. Moreover, netrin-1 is significantly elevated early in patients without microalbuminuria and all later stages of diabetic nephropathy as compared to control. Netrin-1 was recently identified as a biomarker of acute kidney injury. Both animal model and human studies demonstrated that netrin-1 level is elevated in urine in many forms of acute kidney injury [11;12]. However, its usefulness for early diagnosis of chronic kidney injury is unknown. Our current studies demonstrated that netrin-1 excretion is elevated in chronic kidney injury disease models in rats, including progressive diabetic and hypertension-induced injury. Despite the common views that diabetic nephropathy is primarily a glomerular disease, we find high levels of the tubular injury marker netrin-1 in urine in patients without microalbuminuria. These results suggest that tubular damage may precede glomerular injury in chronic kidney injury and warrant further study. In contrast to netrin-1, KIM-1 excretion significantly increased at later stages of diabetes. A similar observation has been seen in human diabetic nephropathy where KIM-1 expression was elevated at late stages of diabetic nephropathy and did not predict the development of nephropathy early in the course of disease [8].

The role of netrin-1 in the pathogenesis of diabetic nephropathy is unknown. There are some data suggest that netrin-1 may have a role in diabetic neuropathy and retinopathy. Earlier studies suggest that administration of recombinant netrin-1 or overexpression of netrin-1 improved conductance velocity of nerves and increases angiogenesis in diabetic mice [18]. More recently, diabetic retinopathy was associated with increased levels of netrin-1 in the vitreous fluid [19;20]. However, the exact role of netrin-1 in diabetic complications is unknown. Our recently published data shows that that netrin-1 overexpression in the proximal tubular epithelial cells protects kidney from hyperglycemia induced nephropathy by suppressing proteinuria and inflammation [21].

In the kidney, the signal for the induction of netrin-1 in the tubular epithelial cells of the diabetic rat is also unknown. Our *in vitro* studies have determined that hyperglycemia down-regulates netrin-1 production, whereas high levels of albumin induced netrin-1 production from proximal tubular epithelial cells [22]. This protein-induced increase in netrin-1 production was mediated through the ERK and Akt kinase pathways by enhancing translation [22]. Western blot analysis of mouse urine shows that large amount albumin is excreted even under basal condition as well in diabetes suggesting increased albumin traffic in tubules due to defective reduced capacity of proximal tubules in early diabetes may trigger netrin-1 production as a compensatory mechanism. Netrin-1 is shown to increase albumin uptake in proximal tubular epithelial cells [21]. In addition, overexpression of netrin-1 in proximal tubular epithelial cells suppressed albuminuria [21]. Therefore, both tubular epithelial injury and increased albumin may induce netrin-1 expression in epithelial to protects epithelial cells from injury and to increase albumin uptake.

Earlier studies showed that the source of netrin-1 in acute kidney injury is proximal tubular epithelial cells [11]. Our current studies show that netrin-1 expression may be localized in the proximal tubular epithelial cells in both diabetic and hypertensive rats, consistent with previous reports that proximal tubular epithelial cells are a major source for urine netrin-1 [23].

Our results are consistent with a recent report that several proximal tubular epithelial markers are increased in DKD [8;24]. Kidney injury molecule-1 and *N*-acetyl- β -D-glucosaminidase were elevated in urine of diabetic subjects without micro- or macroalbuminuria [8;9]. Similarly, we recently reported that netrin-1 is already increased in diabetic subjects with normoalbuminuria when compared to non-diabetic control subjects [25], suggesting that an early tubular defect may precede glomerular defects in developing micro- and macroalbuminuria. This view is further supported by recent studies demonstrating that the severity of DKD depends not only on the severity of glomerular lesions but also on the degree of tubulointerstitial damage. In fact, a histological study [26] showed that proximal tubular basement membrane width already is thickened in normoalbuminuric diabetic patients compared with healthy control subjects, and another study [27] in microalbuminuric diabetic patients with similar eGFR showed that only 29% had typical histological glomerular features of diabetic nephropathy, whereas 42% had severe tubulointerstitial lesions disproportional to the mild glomerular involvement. Even urinary albumin excretion, long believed to be a consequence of glomerular damage is now also recognized to be a tubular injury biomarker secondary to nephrotoxins by the US Food and Drug Administration and the European Medicines Agency [28]. Therefore, it is important to recognize that albuminuria is a marker of tubular injury in addition to increase in glomerular permeability. The importance of tubular epithelial cells in developing increased levels of albuminuria is reinforced by the recent observation that a mutation in cubilin, a receptor for albumin to mediate its endocytosis, caused severe proteinuria in humans [29]. In addition, a proximal tubular epithelial cell-specific cubilin knockout mice also developed albuminuria [30].

In conclusion, we have demonstrated for the first time that urinary excretion of the proximal tubular marker netrin-1 is elevated early in diabetes and hypertension in animals, and that netrin-1 may be a useful early biomarker predicting development of renal injury in diabetes.

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Figure 1.

Systolic blood pressure in conscious rats during the 10-week course of study after administration of STZ and DOCA salt. Values are means $\pm$ SEM at 2-week time intervals. * $p < 0.05$ compared to CTL at the same time period.

Figure 2.

Netrin-1 excretion is increased in diabetic and hypertensive rats. Urine netrin-1 level was quantified by ELISA. A. Diabetes induced a large increase in netrin-1 excretion in urine, which was not changed in non-diabetic control animal urine. #, $p < 0.001$ vs. control. $n = 9$. B. Hypertension induced a large increase in netrin-1 excretion in urine, which was not changed in vehicle-treated control animal urine. #, $p < 0.001$ vs. control. $n = 7$. C. KIM-1 excretion is not significantly increased at 4 weeks after diabetes but increased significantly at 12 weeks. *, $p < 0.005$. $n = 9$. D. Hypertension induced a large increase in KIM-1 excretion in urine, which was not changed in vehicle-treated control animal urine. #, $p < 0.001$ vs. control. $n = 7$

Figure 3.

Immunohistochemical localization of netrin-1 expression in kidneys from diabetic and hypertensive rats, harvested at 10 and 4 weeks after administration of STZ and DOCA salt, respectively. A and B. Sham rat kidney section shows no staining for netrin-1. C. Diabetic rat kidney showing intense staining for netrin-1 in the proximal tubular epithelial cells (PT). D. Hypertensive rat kidney section showing intense staining for netrin-1 in the proximal tubular epithelial cells (PT) and distal tubular epithelial cells (DT).

Figure 4.

Albumin excretion rate in hypertensive and diabetic rats. Albumin excretion urine was quantified by ELISA. A. DOCA salt-sensitive hypertension induced seven-fold increase in the excretion of albumin over 24 hrs as compared to control. *, $p < 0.001$ vs. sham. $n = 7$. B. Diabetes induced fifteen-fold increase in the excretion of albumin over 24 hrs as compared to control at 4 and 10 weeks after STZ administration. *, $p < 0.001$ vs. sham. $n = 7$.

Figure 5.

Netrin-1 excretion is increased in diabetic mice. Urine netrin-1 level was quantified by Western blot analysis. Urine samples from individual animals. A. Diabetes induced a large increase in netrin-1 excretion in urine by 3 week and remains elevated at 6 and 12 weeks of diabetes. Albumin excretion was not increased till week 6 when urine was normalized to creatinine. B. Albumin excretion rate in diabetic mice is significantly higher at 2, 3, 6 and 12 weeks after induction of diabetes. *, $p < 0.05$ vs. baseline. C. Quantification of netrin-1 in urine by ELISA. Netrin-1 excretion is increased significantly at 10 days after induction of diabetes and further increased in subsequent time points. *, $p < 0.05$ vs. baseline. D. Blood glucose elevated throughout study period. *, $p < 0.01$ vs. baseline. E. Trichrome stained control kidney showing normal morphology and no fibrosis. F. Trichrome

stained 12 weeks diabetic animal kidney showing tubular dilation, brush border damage and interstitial fibrosis. G. Tubular injury was significantly increased in diabetic animal as compared to control animal at 12 weeks after diabetes. *, $p < 0.001$ vs. control. n=8.

Characteristics of sham rats (CTL n = 7), diabetic rats (STZ n = 9), and hypertensive rats (DOCA n = 7) at 4 and 10 weeks after induction. Excretory data were derived from 24-h urine collections in metabolic cages within 24 h of sacrifice. Plasma was obtained under anesthesia immediately prior to sacrifice.

Table 1

	CTL 4 wks	STZ 4 wks	DOCA 4 wks	CTL 10 wks	STZ 10 wks
Weight (g)	337.3 ± 5.8	308.5 ± 13.1 *	271.0 ± 8.0 *	399.9 ± 9.4	319.6 ± 12.8 **
Glucose	99 ± 4.0	456 ± 29.5 *	X	96 ± 5.1	485 ± 22.7 **
Food intake (g)	25.6 ± 0.8	39.1 ± 2.3 *	16.0 ± 0.6 *	22.3 ± 0.6	45.5 ± 1.9 **
H ₂ O in (ml)	40.1 ± 1.7	165.5 ± 19.3 *	126.2 ± 14.6 *	35.1 ± 0.8	221.1 ± 13.7 **
Urine Flow (ml)	15.5 ± 0.7	153.6 ± 20.9 *	114.3 ± 13.0 *	17.7 ± 1.1	200.8 ± 15.1 **
CrCl (ml/min)	X	X	0.62 ± 0.1 **	1.39 ± 0.2	1.82 ± 0.3
U protein (mg)	25.1 ± 7.7	29.0 ± 7.6	315.3 ± 51.8 *	32.3 ± 6.4	77.5 ± 13.4 **

Values are means ± SEM;

* $P < 0.05$ compared to 4 weeks CTL;

** $P < 0.05$ compared to 10 weeks CTL