Glomerular filtration rate determinations in conscious type II diabetic mice

Benjamin J. Bivona, Sungmi Park and Lisa M. Harrison-Bernard

Am J Physiol Renal Physiol 300:F618-F625, 2011. First published 8 December 2010;
doi:10.1152/ajprenal.00421.2010

Am J Physiol Renal Physiol 300: F618–F625, 2011.
First published December 8, 2010; doi:10.1152/ajprenal.00421.2010.

CALL FOR PAPERS | *Renal Hemodynamics*

Glomerular filtration rate determinations in conscious type II diabetic mice

Benjamin J. Bivona, Sungmi Park, and Lisa M. Harrison-Bernard
Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, Louisiana

Submitted 20 July 2010; accepted in final form 3 December 2010

Bivona BJ, Park S, Harrison-Bernard LM. Glomerular filtration rate determinations in conscious type II diabetic mice. *Am J Physiol Renal Physiol* 300: F618–F625, 2011. First published December 8, 2010; doi:10.1152/ajprenal.00421.2010.—Diabetic nephropathy is a major cause of end-stage renal disease worldwide. The current studies were performed to determine the later stages of the progression of renal disease in type II diabetic mice (BKS; *db/db*). Methodology was developed for determining glomerular filtration rate (GFR) in conscious, chronically instrumented mice using continuous intravenous infusion of FITC-labeled inulin to achieve a steady-state plasma inulin concentration. Obese diabetic mice exhibited increased GFR compared with control mice. GFR averaged 0.313 ± 0.018 and 0.278 ± 0.007 ml/min in 18-wk-old obese diabetic (n = 11) and control (n = 13) mice, respectively ($P < 0.05$). In 28-wk-old obese diabetic (n = 10) and control (n = 15) mice, GFR averaged 0.348 ± 0.030 and 0.279 ± 0.009 ml/min, respectively ($P < 0.05$). GFR expressed per gram BW was significantly reduced in 18- and 28-wk-old obese diabetic compared with control mice (5.9 ± 0.3 vs. 9.0 ± 0.3; 6.6 ± 0.6 vs. 7.8 ± 0.3 μl·min^{-1}·g body wt^{-1}), respectively ($P < 0.05$). However, older nonobese type II diabetic mice had significantly reduced GFR (0.179 ± 0.023 ml/min; n = 6) and elevated urinary albumin excretion (811 ± 127 μg/day) compared with obese diabetic and control mice (514 ± 54, 171 ± 18 μg/day), which are consistent with the advanced stages of renal disease. These studies suggest that hyperfiltration contributes to the progression of renal disease in type II diabetic mice.

renal inulin clearance; plasma volume; intravenous infusion; *db/db* mouse; Evans blue dye; indicator dilution technique

TYPE II DIABETES MELLITUS is the most common endocrine disease affecting 250 million people worldwide (1, 4). Obesity has been identified as the principal risk factor associated with the rising prevalence of type II diabetes (12), which is predicted to reach 9% of the US population by 2025 (2). Diabetic nephropathy, a major cause of end-stage renal disease, is characterized by progressive albuminuria, declining glomerular filtration rate (GFR), and increased risk for cardiovascular disease.

The obese leptin receptor-deficient type II diabetic *db/db* mouse exhibits metabolic disturbances of diabetes mellitus similar to the characteristics of humans, thus making it a valuable model of type II diabetic disease (5, 7, 16, 28). The lack of leptin receptor signaling leads to persistent hyperphagia and obesity. The *db/db* mouse exhibits hyperleptinemia, hyperinsulinemia, and develops hyperglycemia in association with insulin resistance. Most importantly, this model exhibits a robust albuminuria, renal and glomerular hypertrophy, thickening of the glomerular basement membrane, mesangial matrix expansion, focal glomerular sclerosis, arteriolar hyalinosis, and tubulointerstitial accumulation of extracellular matrix proteins (see reviews in Refs. 5, 14, and 28), which are all features of human type II diabetic nephropathy.

Creatinine clearance as a measurement of GFR is routinely used in clinical and basic science to evaluate renal function and progression of renal disease. However, creatinine clearance is not an ideal marker for experimental studies performed in the mouse. Meyer et al. (19) have reported that plasma creatinine levels in mice measured by the Jaffé alkaline picrate method yields significantly higher levels than those measured using HPLC, indicting an error in the picrate assay. Thus artifactual increases in serum creatinine may contribute to reports of decreased creatinine clearance in studies that examine renal disease in mice. Furthermore, estimation of GFR by creatinine clearance using HPLC methods for measurement of creatinine concentrations may not be reliable due to significant proximal tubular secretion of creatinine in the mouse (10). Inulin, a 5,200-Da, inert, uncharged polymer of fructose, is the only known ideal glomerular filtration marker. Therefore, it has been proposed that GFR can best be determined by inulin clearance in mice (18, 26, 27). The usefulness of FITC-inulin for the measurement of GFR has been described by Lorenz and Gruenstein (18) in anesthetized mice and by Qi et al. (27) in conscious mice. An additional benefit of the use of this methodology is that it relies on nonradioactive measurements of inulin clearance.

Since diabetic mice display polyuria, there are challenges in maintaining body fluid balance during prolonged periods of time under the influence of anesthesia. Measurement of GFR in conscious mice removes the influence of anesthesia on renal function. Also, currently utilized mouse models of type II diabetes that are leptin deficient, leptin receptor deficient, or melanocortin-4 receptor deficient exhibit significantly increased body weights (BW), which confounds the interpretation of GFR expressed per gram BW. Renal hypertrophy is observed in the early stages of diabetes in the *db/db* mouse, which may influence the interpretation of GFR expressed per gram kidney weight. Glomerular hyperfiltration leads to glomerular injury, which eventually progresses to reduced renal function, hypofiltration, and the development of diabetic glomerulopathy.

Glomerular hyperfiltration (8, 11, 13, 15, 17, 23), hypofiltration (8, 13, 32, 33), and normal filtration (8, 30, 32) have been described at various ages in a number of mouse models of diabetes. There are limited studies on the rate of glomerular filtrate formation during the progression of renal disease in diabetic mice in which creatinine is not used as an index of renal function. Therefore, the goal of the present study was to

Address for reprint requests and other correspondence: L. M. Harrison-Bernard, Dept. of Physiology, Box P7-3, Louisiana State Univ. Health Sciences Center, 1901 Perdido St., New Orleans, LA 70112 (e-mail: lharris@lsuhsc.edu).

assess the later stages of the progression of the change in GFR in a mouse model of type II diabetes using the renal clearance of inulin in the absence of the confounding influences of anesthesia on renal function. We set out to test the hypothesis that glomerular hyperfiltration progresses to hypofiltration in the later stages of diabetic renal disease in the leptin receptor-deficient, type II diabetic *db/db* mouse.

METHODS

Animals

The procedures used in this study were approved by the Animal Care and Use Committee of Louisiana State University Health Sciences and conducted according to the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Experiments were performed in adult male diabetic (*db/db*; 18-wk-old, $n = 27$; 28-wk-old, $n = 16$; BKS.Cg-Dock7^{m} +/+ *Lepr^{db}*/J; no. 000642); control mouse littermates (*db/m*; 18-wk-old, $n = 30$; 28-wk-old, $n = 15$; Dock7^{m} *Lepr^{db}*); and C57BL/6J (18-wk-old, $n = 6$) mice (The Jackson Laboratory, Bar Harbor, ME). All animals were provided ad libitum access to food and water during the study with the exception of food removal for fasting blood glucose measurements.

Baseline Metabolic Parameters

Eighteen-week-old *db/db* ($n = 7$), control-*db/m* ($n = 9$), and C57BL/6J ($n = 6$) mice were housed individually in metabolic cages for a period of 6 days. After 3 days of equilibration, 24-h samples were collected for a period of 3 days. Water and food intake and fecal and urine output were measured and averaged over the final 2 days of collections. Mineral oil (1–2 ml) was placed in the urine collection flasks to prevent evaporation. Urine samples were analyzed for albumin concentration. Fasting blood glucose levels were measured in mice housed in metabolic cages following 6 h of food removal (7 a.m. to 1 p.m.) according to the standard protocol established by the National Institutes of Health (www.mmpc.org) and adapted by the Animal Models of Diabetic Complications Consortium (AMDCC; www.amdcc.org).

Measurement of Renal Inulin Clearance

GFR was determined in conscious 18- and 28-wk-old diabetic ($n = 11, 16$) and control-*db/m* ($n = 13, 15$) mice using the renal clearance of continuous intravenous infusion of FITC-inulin adapted from Tallam et al. (29).

Implantation of chronic venous catheters. Eighteen- and 28-wk-old diabetic and control mice were anesthetized with isoflurane anesthesia (2.5–3%) and prepared for aseptic surgery. A cannula pulled over heat to create a tapered tip (32-cm length, Micro Renathane MRE-040-CL-36, Braintree Scientific) was introduced into the right jugular vein, tied in place using 5.0 silk suture, heat sealed, tunneled subcutaneously, exteriorized behind the ears, and passed through PE 320 tubing (15.5 cm in length with a heat-flared button end). The PE 320 tubing served as a tether and was sutured to the skin using 5.0 silk. Mice were moved to metabolic cages after recovery from surgery.

Intravenous infusions. The venous cannula was attached to a counterbalanced arm (Solomon SMCLA, Instech) and stainless steel swivel (Solomon 375/D/22QM, Instech) mounted to the top of the metabolic cage, and sterile saline (0.9% NaCl) was infused continuously for 48 h using an infusion pump (PHD 2000 Infusion, Harvard Apparatus) at a rate of 4.5 μl/min (6.5 ml/24 h) during the recovery from surgery and equilibration to the metabolic cage. The saline syringe was replaced with one containing FITC-inulin (F3272; 15 mg/ml, Sigma) dissolved in 0.9% sterile saline and infused for 24 h. Daily water intake and urine output were recorded for the 3-day infusion protocol. Urine was stored at −20°C for determination of albumin excretion.

Blood collection and inulin assay. Blood (200 μl) was collected from conscious mice during the final stages of FITC-inulin infusion from the submandibular plexus using a lancet (GoldenRod) into microtainer tubes (20 U heparin, BD) and centrifuged. A small amount of blood was used to measure blood glucose in the fed state (OneTouch Ultra 2 glucometer) and withdrawn into a hematocrit capillary and centrifuged to measure hematocrit. Plasma (1:4) and urine samples (1:40) were diluted with 10 mM HEPES at pH 7.4. A standard curve was generated from the infused FITC-inulin (0.025, 0.05, 0.1, 0.2, 0.4, 0.5 mg/ml). FITC-inulin fluorescence was determined in samples and standards using a Spectra Max2 plate reader with excitation and emission wavelengths of 490 and 520 nm, respectively. All samples were run in duplicate.

Measurement of Plasma Volume

Plasma volume was determined in conscious 18-wk-old diabetic ($n = 9$) and control-*db/m* ($n = 8$) mice using the indicator dilution method adapted from Cole et al. (9).

Implantation of chronic venous catheters. Eighteen-week-old diabetic and control mice were anesthetized with isoflurane anesthesia (2.5–3%) and prepared for aseptic surgery. A cannula (4-cm length) was introduced into the right jugular vein, tied in place using 5.0 silk suture, heat sealed, tunneled subcutaneously, and exteriorized behind the ears. Mice were allowed 48 h for recovery from surgery in the home cage.

Intravenous injection. Plasma volume was calculated from the steady-state plasma concentration of Evans blue dye (EBD) after intravenous injection into conscious, unrestrained, quietly resting mice. On the morning of the experiment, the venous catheter was flushed with 10 μl of sterile saline (0.9% NaCl), injected with a 50-μl bolus (100-μl Hamilton syringe) of 10 mg/ml EBD (E2129, Sigma), and then flushed with 200 μl of sterile saline to ensure injection of all of the dye. Blood was sampled from the left submandibular plexus using a lancet at 2 and 15 min post-EBD injection and centrifuged. Hematocrit was recorded. The absorbance of the EBD standards (0, 0.1, 0.05, 0.025, 0.0125 mg/ml) and plasma samples (diluted 1:4) was determined by spectrophotometry at a wavelength of 615 nm in duplicate.

Tissue Harvesting

At the conclusion of the study, kidneys and hearts were harvested under continuous isoflurane anesthesia (2.5–3%). Blood was collected via cardiac puncture using a 25G needle and 1-ml syringe containing EDTA (200 mM) and centrifuged at 1,000 *g* for 10 min, and the plasma was stored at −20°C until assayed for plasma leptin and insulin concentrations. Tibias were removed for measurement. Left and right kidneys were extracted, stripped of perirenal fat, ureters, and outer connective tissue, and weights were recorded. Calculation of data relative to organ weight or BW was performed in a paired fashion. Proper placement of the jugular venous cannula was confirmed before death.

Analytic Methods

Plasma leptin, plasma insulin, and urinary albumin levels were measured using commercially available ELISA kits according to the manufacturer's instructions (Mouse Leptin ELISA Kit no. EZML-82K, Linco Research; Mercodia Ultrasensitive Mouse Insulin ELISA no. 10-1150-01; Albuwell M ELISA, code 1011 Exocell), respectively, as we have previously described (24, 25).

Calculations and Statistics

GFR was calculated based on the renal clearance of inulin: inulin infusion rate/plasma [inulin]. Plasma volume was calculated using the dye dilution technique: amount of EBD injected/plasma concentration of EBD at *time 0*. The optical density of the plasma at the initial time

Table 1. *Metabolic parameters for 18-wk-old control and diabetic mice*

	Control-C57BL/6J			Control-*db/m*			Diabetic		
	X	SE	*n*	X	SE	*n*	X	SE	*n*
BW, g	28.7	1.0	6	31.3	0.4	30†	52.7	0.9	27*
Fasted blood glucose, mg/dl	135	13	6	126	7	9	464	35	7*‡
Fed blood glucose, mg/dl	ND			135	9	21	556	19	20*
Plasma insulin, μg/l	1.3	0.5	6	3.6	1.2	6	31.9	8.7	5*
Plasma leptin ng/ml	1.3	0.2	6	2.1	0.4	6	76.9	15.5	5*
Water intake, ml/day	3.5	0.3	6	3.5	0.5	9	19.2	1.4	7*
Food intake, g/day	3.2	0.2	6	3.0	0.2	9	5.1	0.4	7*
Fecal output, g/day	0.6	0.1	6	0.5	0.0	9†	1.0	0.1	7*
Urine output, ml/day	1.0	0.2	6	1.3	0.4	9	16.1	1.7	7*
Hematocrit, %	ND			43	2	21	43	1	20

Values are means ± SE. BW, body wt; ND, not done. *$P < 0.05$ vs. control-*db/m*. †$P < 0.05$ vs. control-C57BL/6J. ‡$P < 0.05$ vs. fed.

of dye injection was determined by extrapolation. Blood volume was calculated as plasma volume/(1 − Hct). Statistical analyses were performed by one-way repeated measures (water intake and urine output during 72-h infusion) or two-way ANOVA followed by Bonferroni's test. A paired or unpaired *t*-test was used as appropriate. Statistical analyses were performed using a statistical software program (Sigma Stat 3.5, Systat Software). $P \leq 0.05$ was considered statistically significant. Values are means ± SE.

RESULTS

Baseline Metabolic Parameters

Metabolic parameters and organ weights for 18-wk-old control-C57BL/6J, control-*db/m*, and diabetic mice are shown in Tables 1 and 2, respectively. Inclusion of the 18-wk-old C57BL/6J mice (widely utilized control mouse strain) allowed us to compare these metabolic parameters with the heterozygote control-*db/m* mice. BW was significantly greater and fecal output significantly lower in control-*db/m* compared with control-C57BL/6J mice at 18 wk of age. BW, fasted and fed glucose, plasma inulin and leptin levels, water and food intake, and fecal and urine output were significantly greater in diabetic compared with control-*db/m* mice. Fed blood glucose values were significantly higher than fasted blood glucose values in diabetic mice. Hematocrit and tibia lengths were not different between diabetic and control-*db/m* mice. Kidney and heart weights were greater in control-*db/m* compared with control-C57BL/6J with the exception of heart weight expressed relative to BW. Total and right kidney weights were greater in diabetic compared with control-*db/m* mice. Absolute heart weight and heart weight relative to BW or tibia length were significantly lower in diabetic compared with control-*db/m* mice. Interestingly, kidney weight relative to BW was significantly lower, while kidney weight relative to tibia length was not different in diabetic compared with control-*db/m* mice. Right kidney weights were significantly greater than left kidney weights in control-*db/m* and diabetic mice.

Metabolic parameters and organ weights for 28-wk-old control-*db/m*, obese diabetic, and nonobese type II diabetic mice are shown in Table 3. A subset of 28-wk-old type II diabetic mice had a significantly lower BW (35.6 ± 2.0 g; range 28.4–43.0 g; $n = 6$) compared with the obese diabetic mice (52.7 ± 1.2 g; range 46.5–58.8 g; $n = 10$). BW of 28-wk-old nonobese diabetic mice were ≤1 SD of the mean of BW of all diabetic mice used in the current study. BW of 28-wk-old control-db/m mice (35.9 ± 0.6 g; $n = 15$) was significantly lower than 28-wk-old obese diabetic, but not 28-wk-old nonobese diabetic mice. Fed blood glucose levels were significantly greater in obese diabetic compared with control mice. Total kidney weights were greater in diabetic compared with control-*db/m* mice. Hematocrit of 28-wk-old diabetic and control mice were significantly lower than in 18-wk-old mice.

Water intake and urine output were significantly greater in diabetic compared with control-*db/m* mice measured throughout the 72-h continuous intravenous infusion for determination of renal inulin clearance (Fig. 1). Urine output increased significantly during the 3-day intravenous infusion period in both diabetic and control-*db/m* mice ($P < 0.05$), while water intake increased in diabetic mice. Urine output on *day 2* following surgery was not different from *day 3* in diabetic and control-*db/m* mice, suggesting that the animals were in fluid balance at the time of the measurement of renal inulin clear-

Table 2. *Organ weights for 18-wk-old control and diabetic mice*

	Control C57BL/6J			Control-*db/m*			Diabetic		
	X	SE	*n*	X	SE	*n*	X	SE	*n*
Tibia length, mm	18.1	0.1	6	18.7	0.3	6	18.4	0.4	5
Total kidney wt, mg	325	3	6	402	9	19†	441	12	16*
Right kidney wt, mg	169	5	6	208	5	19†‡	231	6	16*‡
Left kidney wt, mg	156	5	6	194	5	19†	210	8	16
Kidney wt/BW, mg/g	11.4	0.4	6	13.1	0.3	19†	8.2	0.2	16*
Kidney wt/tibia, mg/mm	17.9	0.2	6	22.2	0.7	6†	23.4	0.5	5
Heart wt, mg	144	4	6	161	2	6†	137	4	5*
Heart wt/BW, mg/g	5.0	0.1	6	5.4	0.1	6	2.4	0.1	5*
Heart wt/tibia, mg/mm	7.9	0.2	6	8.6	0.2	6†	7.4	0.2	5*

Values are means ± SE. *$P < 0.05$ vs. control-*db/m*. †$P < 0.05$ vs. control-C57BL/6J. ‡$P < 0.05$ vs. left kidney.

Table 3. *Metabolic parameters and organ weights for 28-wk-old control and diabetic mice*

	Control-*db/m*			Obese Diabetic			Nonobese Diabetic		
	X	SE	*n*	X	SE	*n*	X	SE	*n*
BW, g	35.9	0.6	15‡	52.7	1.2	10*	35.6	2.0	6†
Fed blood glucose, mg/dl	122	7	12	528	20	10*	579	16	6*
Total kidney wt, mg	420	10	15	514	20	10*‡	461	17	6*
Right kidney wt, mg	227	8	15§	264	12	10*‡	220	23	6
Left kidney wt, mg	198	6	15	249	8	10*‡	245	27	6
Kidney wt/BW, mg/g	11.7	0.3	15‡	9.7	0.5	10*‡	13.1	0.7	6†
Hematocrit, %	38	2	14‡	37	2	10‡	34	4	6

Values are means ± SE *$P < 0.05$ vs. control-*db/m*. †$P < 0.05$ obese diabetic vs. nonobese diabetic. ‡$P < 0.05$ vs. 18-wk-old. §$P < 0.05$ vs. left kidney.

ance. Water intake and urine output in diabetic and control mice reflected the 6.5 ml of fluid infused each day.

Eighteen- and 28-wk-old diabetic mice had significantly greater urinary albumin excretion compared with the age-matched control-*db/m* mice (Fig. 2). Urinary albumin excretion was significantly lower in 18-wk-old control-C57BL/6J (38 ± 8 μg/day) compared with 18-wk-old control-*db/m* (90 ± 8 μg/day) mice. Urinary albumin excretion was significantly higher in 18-wk-old diabetic (555 ± 73 μg/day) compared with 18-wk-old control mice. Urinary albumin excretion was significantly higher in 28-wk-old nonobese diabetic (811 ± 127 μg/day) compared with 28-wk-old obese diabetic (514 ± 54 μg/day) mice, suggesting that nonobese diabetic mice have a more progressive renal disease. Urinary albumin excretion was significantly higher in 28-wk-old control-*db/m* (171 ± 18 μg/day) compared with 18-wk-old control-*db/m* mice.

Measurement of Renal Inulin Clearance

Renal inulin clearance was significantly greater in diabetic compared with control-*db/m* mice at 18 wk of age (Fig. 3*A*). Renal inulin clearance averaged 278 ± 7 and 313 ± 18 μl/min in 18-wk-old control and diabetic mice, respectively. Also, renal inulin clearance was significantly greater in obese diabetic compared with control-*db/m* mice at 28 wk of age (Fig. 3*B*). Renal inulin clearance was significantly reduced in 28-wk-old nonobese diabetic compared with obese diabetic and control-*db/m* mice. Renal inulin clearance averaged 279 ± 9, 348 ± 30, and 179 ± 23 μl/min in 28-wk-old control, obese diabetic, and nonobese diabetic mice, respectively. Renal inulin clearance expressed per gram BW was significantly lower in obese diabetic compared with control mice, but not when expressed per gram kidney weight (Table 4). The average plasma inulin concentration was 0.246 ± 0.005 mg/ml (n = 28) in control-*db/m* and 0.215 ± 0.011 mg/ml (n = 21) in obese diabetic mice (P = 0.01). The average plasma inulin concentration was 0.424 ± 0.067 mg/ml (n = 6) in 28-wk-old nonobese diabetic mice (P = 0.002 vs. obese diabetic). There was a significant correlation between urinary albumin excretion and renal inulin clearance in 18- and 28-wk-old mice (y = 1,821x − 254; r^2 = 0.244; n = 43; $P < 0.001$). Twenty-eight-week-old nonobese diabetic mice were excluded from this analysis. Urinary FITC-inulin excretion was determined to document patency of the venous catheter and adequate intravenous infusion of the marker. FITC-inulin was infused at a steady rate of 0.0675 mg/min for 24 h (no bolus administered). Urinary FITC-inulin excretion averaged 0.046 ± 0.002 mg/

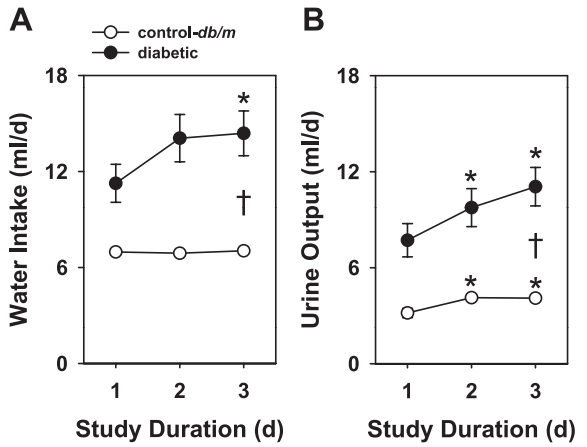

Fig. 1. Water intake (*A*) and urine output (*B*) in conscious control-*db/m* (○, n = 28) and diabetic (●, n = 27) mice studied during intravenous infusions of saline (48 h; *day 1*, day 2) or FITC-labeled inulin (24 h; *day 3*). Data are combined for 18- and 28-wk-old mice. Urine output increased significantly during the 3-day intravenous infusion period in both diabetic and control-*db/m* mice ($P < 0.05$). Water intake and urine output were significantly greater in diabetic compared with control mice. Urine output increased during the study, although values were not different between *days 2* and *3*, suggesting that the mice were in balance during the measurement of renal inulin clearance. Water intake represents fluid intake from drinking and intravenous infusions (6.5 ml/day). The SE bars are plotted for the control groups, but are too small to be visualized on the graphs. *$P < 0.05$ vs. *day 1*. †$P < 0.05$ *db/m* vs. *db/db*.

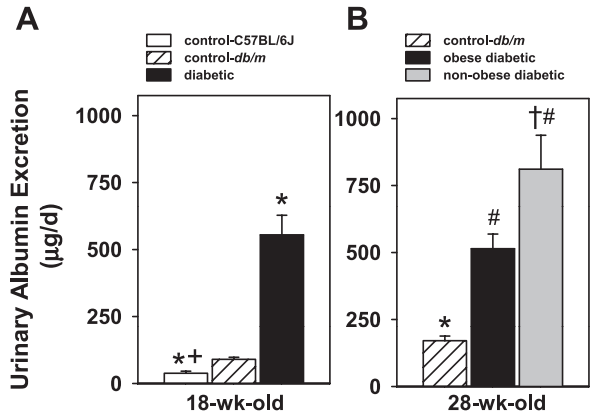

Fig. 2. Urinary albumin excretion (μg/day) in 18- (*A*; n = 5, 20, 17) and 28-wk-old (*B*; n = 14, 8, 6) control (C57BL/6J, *db/m*) and diabetic mice, respectively. Albumin excretion is significantly higher in diabetic compared with control mice at 18 and 28 wk. Nonobese type II diabetic mice have significantly higher urinary albumin excretion compared with obese diabetic and control-*db/m* mice at 28 wk. *$P < 0.05$ vs. 18-wk-old control-*db/m*. +$P < 0.05$ vs. 18-wk-old diabetic. #$P < 0.05$ vs. 28-wk-old control-*db/m*. †$P < 0.05$ obese diabetic vs. nonobese diabetic.

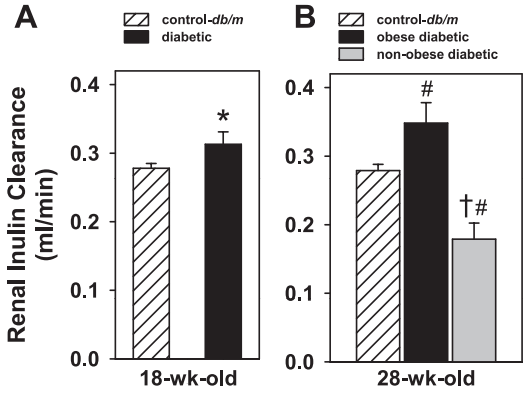

Fig. 3. Renal inulin clearance (ml/min) in 18- (*A*; $n = 13, 11$) and 28-wk-old (*B*; $n = 15, 10, 6$) control-*db/m* and diabetic mice. Renal inulin clearance is significantly higher in obese diabetic compared with control-*db/m* mice at 18 and 28 wk of age. Nonobese diabetic mice have significantly lower renal inulin clearance compared with obese diabetic and control-*db/m* mice at 28 wk. $*P <$ 0.05 vs. 18-wk-old control-*db/m*. $\#P < 0.05$ vs. 28-wk-old control-*db/m*. $\dagger P <$ 0.05 obese diabetic vs. nonobese diabetic.

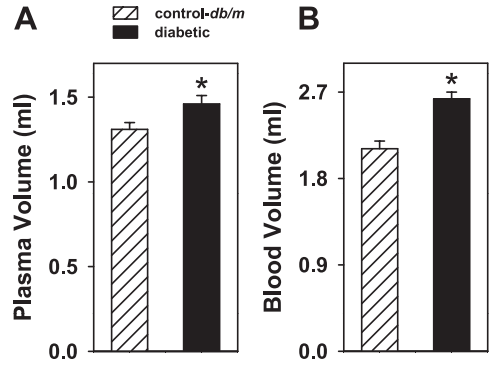

Fig. 4. Plasma volume (ml; *A*) and blood volume (ml; *B*) in conscious 18-wk-old control-*db/m* ($n = 8$) and diabetic ($n = 9$) mice. Plasma volume was measured using the indicator dilution method. Plasma volume and blood volume are significantly higher in diabetic compared with control-*db/m* mice. $*P < 0.05$ vs. 18-wk-old control.

min ($n = 54$), which represents 68% of the infusion rate of inulin. These data suggest that ~8 h were required to reach steady-state plasma inulin concentrations.

Measurement of Plasma Volume

Plasma volume was significantly elevated in 18-wk-old diabetic compared with control-*db/m* mice (Fig. 4*A*). Plasma volume was 1.31 ± 0.04 and 1.46 ± 0.05 ml in control and diabetic mice, respectively. Plasma volume expressed per gram BW was significantly lower in diabetic compared with control mice, averaging 30.3 ± 1.4 and 43.4 ± 2.1 μl/g BW, respectively. Blood volume was significantly elevated in 18-wk-old diabetic compared with control-*db/m* mice (Fig. 4*B*). Blood volume was 2.11 ± 0.08 and 2.63 ± 0.07 ml in control and diabetic mice, respectively. Blood volume expressed per gram BW was significantly lower in diabetic compared with control mice, averaging 54.7 ± 2.0 and 69.5 ± 3.2 μl/g BW, respectively.

DISCUSSION

The prevalence of diabetic nephropathy is increasing and is now the number one cause of end-stage renal disease in the industrialized world. Increased glomerular capillary pressure and hyperfiltration have been identified as important determinants of the development of diabetic nephropathy. The aim of the present study was to determine the GFR during the later stages of the progression of diabetic renal disease in an animal model of type II diabetes.

We show that adult male diabetic mice exhibit significantly elevated urinary albumin excretion at 18 and 28 wk of age compared with control-*db/m* mice. Interestingly, we found that the 18-wk-old control-*db/m* mice have a significantly higher urinary albumin excretion compared with C57BL/6J mice, suggesting that heterozygous *db* mice exhibit mild albuminuria. Urinary albumin excretion was significantly higher in 28-wk-old compared with 18-wk-old control-*db/m* mice. The degree of albuminuria did not increase with the duration of diabetes in obese diabetic mice. Interestingly, 28-wk-old nonobese diabetic mice had significantly higher urinary albumin excretion compared with 28-wk-old obese diabetic mice, suggesting that reduced BW is indicative of worsening diabetic disease. In the initial report of the discovery of the *db/db* mouse by Hummel et al. (16), it was noted that a decrease in body weight occurs as the *db/db* mice begin to succumb to the disease. It is plausible that there is biological variation in the progression of the late stages of the diabetic disease in *db/db* mice.

There are difficulties in maintaining body fluid status in anesthetized diabetic mice due to the significant polyuria resulting from glucosuria. Therefore, the present studies were conducted in conscious mice in a euhydrated condition. It is well established that anesthesia and surgery have profound influences on vasopressin release, which affects the period of

Table 4. *Renal inulin clearance in conscious 18- and 28-wk-old control and diabetic mice*

	μl/min			μl·min^{-1}·g KW^{-1}			μl·min^{-1}·g BW^{-1}		
	X	SE	*n*	X	SE	*n*	X	SE	*n*
				18-wk-old					
Control-*db/m*	278	7	13	711	35	13	9.0	0.3	13
Diabetic	313	18	11*	703	34	11	5.9	0.3	11*
				28-wk-old					
Control-*db/m*	279	9	15	666	19	15	7.8	0.3	15
Obese diabetic	348	30	10*	696	64	10	6.6	0.6	10*
Nonobese diabetic	179	23	6*†	397	58	6*†	5.3	0.9	6*

Values are means ± SE. KW, kidney weight. $*P < 0.05$ vs. control-*db/m*. $\dagger P < 0.05$ obese diabetic vs. nonobese diabetic.

recovery where the animals experience temporary reduced drinking and eating behavior. Therefore, mice were provided a 48-h recovery period following surgical implantation of chronic venous catheters. GFR and plasma volume measurements were obtained during the period of reestablished water intake and urine output.

The state of GFR in diabetic mice has been difficult to interpret based on the problems associated with the use of creatinine picric acid assay (5, 7) and the contribution of proximal tubule secretion of creatinine (10) in mice. In mice, cross-reacting chromogens contribute substantially more to the picrate-based measurement of serum creatinine than in humans. Eisner et al. (10) reported that 35–50% of the renal excretion of creatinine is a result of renal tubular secretion in mice. The contribution of tubular secretion of creatinine to its clearance may be increased in renal disease, which is accompanied by elevated plasma creatinine concentrations (6). Since inulin is considered the gold standard for GFR measurements in both clinical and animal studies, the current study was performed using the renal clearance of FITC-inulin. Steady-state infusion of inulin was employed so that a single blood sample could be obtained in conscious mice. This is the first report of the use of a continuous intravenous infusion of a nonradioactive form of inulin for the determination of GFR in conscious mice.

Previous studies in which GFR was measured using creatinine clearance based on the picric acid method have reported significantly elevated GFR in 8-wk-old [48 μl/min, (11); 150 μl/min (8)] and decreased GFR in 16-wk-old [43 μl/min (8); 33 μl/min, (33)] *db/db* diabetic mice (Table 5). Utilizing creatinine concentrations measured by HPLC, Park et al. (23) reported significantly increased GFR (1,140 μl/min) in 8-wk-old diabetic mice. A consistent finding has been a significantly elevated GFR in young (6–10 wk of age) type II diabetic compared with control mice (Table 5) (8, 11, 13, 17, 23). GFR is vastly different (10–20 times) depending on the methods used for measuring creatinine concentration in type II diabetic mice.

Table 5. *Comparison of methodologies and GFR values measured in conscious or anesthetized type II diabetic mice*

Clearance Method	Strain	Model	GFR			Gender	Age, wk	BW, g	Reference
			μl/min	μl·min^{-1}·g KW^{-1}	μl·min^{-1}·g BW^{-1}				
			Conscious state						
Continuous FITC-inulin infusion	BKS *db/db*	D	313*	703	5.9*	M	18	53*	
			348*	696	6.6*		28	53*	
			179*†	397	5.3			36*†	
	BKS *db/m*	C	278	711	9.0		18	31	
			279	666	7.8		28	36	
Single bolus FITC-inulin	BKS *db/db*	D	366	—	6.2	—	26	59*	32
	eNOS−/− BKS *db/db*		265*	—	2.5		—	23	
	BKS *db/m*	C	331	—	~14.1	—		58*	
Single bolus [^{51}Cr]-EDTA	B6 and BKS *db/db*	D	~400*	—	~16	F	6	~25	13
			~700*	—	~16		14–17	~45	
			~470	—	~9		24	~50	
	B6 and BKS *db/m*	C	~300	—	~15		6–14	~20	
Creatinine clearance (HPLC)	BKS db/db	D	1,140*	—	~22	M	8	51*	23
	BKS *db/m*	C	330	—	~11			31	
Creatinine clearance (colorimetric)	*db/db*	D	~33*	~170	~0.9	—	16	37	33
	db/m	C	~58	~457	~2.9	—		20	
	db/db	D	~150*	—	~3.8	M	8	39*	8
			74	—	~1.8		12	42*	
			~43*	—	~1.1		16	39*	
	db/m	C	~83	—	~4.1		8	20	
			67	—	~2.5		12	27	
			~62	—	~2.2		16	28	
			Anesthetized state						
Continuous [^{3}H] inulin infusion	B6 *db/db*	D	501*	977*	~8.2	—	8–10	51*	17
	B6 *db/m*	C	223	606	~7.4	—		24	
Continuous FITC-inulin infusion	B6 *db/db*	D	—	650	—	M	—		30
	B6.V *ob/ob*	D	—	830	—			—	
	B6 *db/m*	C	—	620	—			—	
	B6.V *ob* /+	C	—	770	—			—	
Creatinine clearance (colorimetric)	BKS *db/db*	D	48*	—	~1.0	F	8	47*	11
	BKS *db/m*	C	25	—	~1.2			21	

Values are means. GFR, glomerular filtration rate; BKS, background strain BLKS; B6, background strain C57Bl/6J; eNOS−/−, endothelial nitric oxide synthase null mice; M, male; F, female; ~, data estimated from published report; -, data not reported or estimated. *$P < 0.05$ diabetic vs. control. †$P < 0.05$ vs. obese diabetic.

Various methodologies which do not rely on renal creatinine clearance have utilized ^{51}Cr-EDTA, [^{3}H]inulin, or FITC-inulin to measure GFR in type II diabetic mice (Table 5). The first reports were made by Gartner (13) in 1978 using the renal clearance of ^{51}Cr-EDTA in conscious female *db/db* mice. Significant renal hyperfiltration (~ 600 μl/min) was observed in *db/db* mice from 6 to 17 wk of age (13). GFR measured using FITC-inulin in 8- to 10-wk-old leptin-deficient type II diabetic, *ob/ob* (830 $\mu l \cdot min^{-1} \cdot g$ kidney wt^{-1}), and *db/db* diabetic mice (650 $\mu l \cdot min^{-1} \cdot g$ kidney wt^{-1}) were similar compared with littermate control mice studied under anesthesia (30). Levine et al. (17) have reported that single-nephron and whole kidney GFR (500 μl/min) are significantly elevated in anesthetized 8- to 10-wk-old *db/db* (B6.Cg-m$^{+/+}$*Lepr^{db}*/J model) compared with *db/m* mice using the renal clearance of [^{3}H]inulin. Previous reports of GFR measured in diabetic mice have demonstrated numerically, but not significantly higher, GFR in diabetic C57BLKS *db/db* (366 μl/min) compared with *db/m* mice (331 μl/min) at 26 wk of age using a single-bolus FITC-inulin injection method in conscious mice (32). Gurley et al. (15) reported glomerular hyperfiltration in conscious 6-mo-old type I diabetic Akita mice (range 16–24 $\mu l \cdot min^{-1} \cdot g$ BW^{-1}) using bolus administration of FITC-inulin. Taken together, most published studies have reported elevated GFR in diabetic mice.

Numerous indexes are used for reporting GFR which include absolute terms, expressed per gram kidney weight, or expressed per gram BW. The *db/db* mouse exhibits significantly elevated BW and renal hypertrophy compared with control-*db/m* mice. Since the tibia lengths were not different between diabetic and control mice, we focused our attention on GFR values expressed in absolute terms. It is of interest to note that the data presented in the current paper on the GFR in 18- and 28-wk-old obese diabetic and control mice lead to a conclusion of hyperfiltration based on GFR data reported as microliters per minute, no change in renal function for data reported as microliters per minute per gram kidney weight, and hypofiltration for data reported as microliters per minute per gram BW. Therefore, it is important to keep these concepts in mind when one makes conclusions about renal function in obese animals.

The goal of the present study was to determine whether the glomerular hyperfiltration persists or reverts to hypofiltration in older C57BLKS/J *db/db* mice since severe susceptibility to diabetes appears to be present on the BKS background. GFR averaged 0.313 and 0.278 ml/min in 18-wk-old obese diabetic and control mice, respectively. In 28-wk-old obese diabetic and control mice, GFR averaged 0.348 and 0.279 ml/min, respectively. However, older nonobese diabetic mice had significantly reduced GFR (0.179 ml/min) and elevated urinary albumin excretion compared with obese diabetic and control mice, which are consistent with the advanced stages of renal disease. GFR was 50% lower in 28-wk-old nonobese compared with 28-wk-old obese diabetic mice and 36% lower compared with 28-wk-old control mice. In the present study, 38% of the 28-wk-old diabetic mice progressed to significantly reduced BW and GFR and increased urinary albumin excretion. In type II diabetes, 20–40% of patients progress to overt nephropathy, and ~20% of those with overt nephropathy will progress to end-stage renal disease within 20 yr (20). Thus the *db/db* mouse appears to replicate the pattern of disease progression in humans.

Evans Blue is a vital dye which is quickly bound to plasma proteins and an ideal indicator for determination of plasma volume by the dye dilution technique. Barbee and colleagues (3) observed that plasma volume determinations in conscious mice were 21% higher than those obtained from anesthetized mice; therefore, the present study was conducted in control and diabetic mice studied in the conscious state. Plasma volume (1.3 ml) in control-*db/m* mice was similar to values reported by others in conscious mice (3, 9, 22). Blood volume of diabetic mice is 25% greater than control-*db/m* mice, although their BW is 60% higher. Therefore, blood volume is not increased relative to BW in the obese mouse since the excess BW is mainly due to increased adipose tissue which is not highly vascular. In the present study, blood volumes in the control-*db/m* and diabetic mice were similar to those measured by Yen et al. (31) using ^{51}Cr-labeled blood. Plasma volumes were not measured by Yen et al. (31). We show that diabetic mice exhibited significantly higher plasma volume compared with control mice, suggesting an elevated extracellular fluid volume in the obese diabetic model.

It is well known that hyperfiltration contributes to renal disease in diabetes. Our previous work (24, 25) demonstrated significantly dilated juxtamedullary afferent arterioles in kidneys of 18-wk-old diabetic mice. We have expanded upon these initial findings and now demonstrate that dilated afferent arterioles could contribute to increased glomerular capillary pressure, hyperfiltration, and renal injury in the *db/db* model. Dilated juxtamedullary afferent arterioles have also been reported in the type 1 diabetic rat with hyperfiltration (21). A significant finding of the present study is that hyperfiltration occurs in the diabetic mouse, which may contribute to the progression of renal damage.

ACKNOWLEDGMENTS

The authors acknowledge the valuable technical advice of Drs. R. Wayne Barbee, John N. Lorenz, Michael J. Ryan, and Annie M. Whitaker. Portions of this work have been published in abstract form (*FASEB J* 23: 804.21, 2009).

Present address of S. Park: Dept. of Pharmacology, University of Iowa, Iowa City, IA.

GRANTS

This work was supported by National Institutes of Health Grants DK62003, 2R56DK62003, and P20RR018766, American Heart Association (AHA) Grant-In-Aid 2250875, and the Research Enhancement Fund from the LSUHSC School of Medicine (to L. M. Harrison-Bernard), and AHA Predoctoral Grant 0715445B (to S. Park).

DISCLOSURES

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

REFERENCES

1. **Anonymous.** *National Diabetes Statistics.* Bethesda, MD: National Diabetes Information Clearinghouse, 2007.
2. **Anonymous.** American Society of Nephrology. http://www asn-online org/facts_and_statistics/2010.
3. **Barbee RW, Perry BD, Re RN, Murgo JP.** Microsphere and dilution techniques for the determination of blood flows and volumes in conscious mice. *Am J Physiol Regul Integr Comp Physiol* 263: R728–R733, 1992.
4. **Bays HE, Bazata DD, Clark NG, Gavin JR, III, Green AJ, Lewis SJ, Reed ML, Stewart W, Chapman RH, Fox KM, Grandy S.** Prevalence of self-reported diagnosis of diabetes mellitus and associated risk factors

in a national survey in the US population: SHIELD (Study to Help Improve Early evaluation and management of risk factors Leading to Diabetes). *BMC Public Health* 7: 277, 2007.
5. **Breyer MD, Bottinger E, Brosius FC, III, Coffman TM, Harris RC, Heilig CW, Sharma K.** Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 16: 27–45, 2005.
6. **Breyer MD, Qi Z.** Better nephrology for mice—and man. *Kidney Int* 77: 487–489, 2010.
7. **Brosius FC, III, Alpers CE, Bottinger EP, Breyer MD, Coffman TM, Gurley SB, Harris RC, Kakoki M, Kretzler M, Leiter EH, Levi M, McIndoe RA, Sharma K, Smithies O, Susztak K, Takahashi N, Takahashi T.** Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 20: 2503–2512, 2009.
8. **Cohen MP, Lautenslager GT, Shearman CW.** Increased urinary type IV collagen marks the development of glomerular pathology in diabetic d/db mice. *Metabolism* 50: 1435–1440, 2001.
9. **Cole JM, Khokhlova N, Sutliff RL, Adams JW, Disher KM, Zhao H, Capecchi MR, Corvol P, Bernstein KE.** Mice lacking endothelial ACE: normal blood pressure with elevated angiotensin II. *Hypertension* 41: 313–321, 2003.
10. **Eisner C, Faulhaber-Walter R, Wang Y, Leelahavanichkul A, Yuen PS, Mizel D, Star RA, Briggs JP, Levine M, Schnermann J.** Major contribution of tubular secretion to creatinine clearance in mice. *Kidney Int* 77: 519–526, 2010.
11. **Flyvbjerg A, Gnaes-Hansen F, De Vriese AS, Schrijvers BF, Tilton RG, Rasch R.** Amelioration of long-term renal changes in obese type 2 diabetic mice by a neutralizing vascular endothelial growth factor antibody. *Diabetes* 51: 3090–3094, 2002.
12. **Ford ES, Giles WH, Dietz WH.** Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *JAMA* 287: 356–359, 2002.
13. **Gartner K.** Glomerular hyperfiltration during the onset of diabetes mellitus in two strains of diabetic mice (c57bl/6j db/db and c57bl/ksj db/db). *Diabetologia* 15: 59–63, 1978.
14. **Guo M, Ricardo SD, Deane JA, Shi M, Cullen-McEwen L, Bertram JF.** A stereological study of the renal glomerular vasculature in the db/db mouse model of diabetic nephropathy. *J Anat* 207: 813–821, 2005.
15. **Gurley SB, Mach CL, Stegbauer J, Yang J, Snow KP, Hu A, Meyer TW, Coffman TM.** Influence of genetic background on albuminuria and kidney injury in Ins2(+/C96Y) (Akita) mice. *Am J Physiol Renal Physiol* 298: F788–F795, 2010.
16. **Hummel KP, Dickie MM, Coleman DL.** Diabetes, a new mutation in the mouse. *Science* 153: 1127–1128, 1966.
17. **Levine DZ, Iacovitti M, Robertson SJ, Mokhtar GA.** Modulation of single-nephron GFR in the *db/db* mouse model of type 2 diabetes mellitus. *Am J Physiol Regul Integr Comp Physiol* 290: R975–R981, 2006.
18. **Lorenz JN, Gruenstein E.** A simple, nonradioactive method for evaluating single-nephron filtration rate using FITC-inulin. *Am J Physiol Renal Physiol* 276: F172–F177, 1999.
19. **Meyer MH, Meyer RA Jr, Gray RW, Irwin RL.** Picric acid methods greatly overestimate serum creatinine in mice: more accurate results with high-performance liquid chromatography. *Anal Biochem* 144: 285–290, 1985.
20. **Molitch ME.** Management of early diabetic nephropathy. *Am J Med* 102: 392–398, 1997.
21. **Ohishi K, Okwueze MI, Vari RC, Carmines PK.** Juxtamedullary microvascular dysfunction during the hyperfiltration stage of diabetes mellitus. *Am J Physiol Renal Fluid Electrolyte Physiol* 267: F99–F105, 1994.
22. **Oppermann M, Gess B, Schweda F, Castrop H.** Atrap deficiency increases arterial blood pressure and plasma volume. *J Am Soc Nephrol* 21: 468–477, 2010.
23. **Park CW, Kim HW, Ko SH, Lim JH, Ryu GR, Chung HW, Han SW, Shin SJ, Bang BK, Breyer MD, Chang YS.** Long-term treatment of glucagon-like peptide-1 analog exendin-4 ameliorates diabetic nephropathy through improving metabolic anomalies in db/db mice. *J Am Soc Nephrol* 18: 1227–1238, 2007.
24. **Park S, Bivona BJ, Feng Y, Lazartigues E, Harrison-Bernard LM.** Intact renal afferent arteriolar autoregulatory responsiveness in *db/db* mice. *Am J Physiol Renal Physiol* 295: F1504–F1511, 2008.
25. **Park S, Bivona BJ, Kobori H, Seth DM, Chappell MC, Lazartigues E, Harrison-Bernard LM.** Major role for ACE-independent intrarenal ANG II formation in type II diabetes. *Am J Physiol Renal Physiol* 298: F37–F48, 2010.
26. **Qi Z, Breyer MD.** Measurement of glomerular filtration rate in conscious mice. In: *Methods in Molecular Biology*, edited by Hewitson TD and Becker GJ. Totowa, NJ: Humana, 2009, p. 61–72.
27. **Qi Z, Whitt I, Mehta A, Jin J, Zhao M, Harris RC, Fogo AB, Breyer MD.** Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. *Am J Physiol Renal Physiol* 286: F590–F596, 2004.
28. **Sharma K, McCue P, Dunn S.** Diabetic kidney disease in the *db/db* mouse. *Am J Physiol Renal Physiol* 284: F1138–F1144, 2003.
29. **Tallam LS, da Silva AA, Hall JE.** Melanocortin-4 receptor mediates chronic cardiovascular and metabolic actions of leptin. *Hypertension* 48: 58–64, 2006.
30. **Wang W, Poole B, Mitra A, Falk S, Fantuzzi G, Lucia S, Schrier R.** Role of leptin deficiency in early acute renal failure during endotoxemia in ob/ob mice. *J Am Soc Nephrol* 15: 645–649, 2004.
31. **Yen TT, Stienmetz J, Simpson PJ.** Blood volume of obese (ob-ob) and diabetic (db-db) mice. *Proc Soc Exp Biol Med* 133: 307–308, 1970.
32. **Zhao HJ, Wang S, Cheng H, Zhang MZ, Takahashi T, Fogo AB, Breyer MD, Harris RC.** Endothelial nitric oxide synthase deficiency produces accelerated nephropathy in diabetic mice. *J Am Soc Nephrol* 17: 2664–2669, 2006.
33. **Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K.** Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci USA* 97: 8015–8020, 2000.