Modulation of single-nephron GFR in the db/db mouse model of type 2 diabetes mellitus

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IT HAS LONG BEEN RECOGNIZED that there is hyperfiltration in patients with early diabetes, and this hyperfiltration, or its associated increased glomerular capillary pressure, may play a role in progression to diabetic nephropathy (4, 5). In type 2 diabetes mellitus (DM2), progression to end-stage renal disease is sufficiently prevalent to account for the largest number of patients on life support dialysis, and the role of hyperfiltration in DM2 continues to be the subject of clinical trials (3, 15).

However, virtually all of our experimental knowledge of hyperfiltration and the modulation of single-nephron GFR (SNGFR) in DM derives from streptozotocin-induced type 1 diabetes mellitus (STZ DM1) in the rat. Reviews and recent studies on this STZ DM1 model have focused on the nitric oxide (NO) system (7–10, 23), alterations in the tubuloglomerular feedback (TGF) system (7, 9, 10, 23, 24, 26, 27, 30), and the role of enhanced proximal reabsorption in sustaining hyperfiltration (22, 24). Although reports have suggested that STZ, known to be clinically nephrotoxic, may have renal effects separate from the diabetic state (6, 12, 16), this STZ DM1 rat model has nevertheless provided most of the framework of our current understanding of single-nephron function in diabetes.

Despite the importance of DM2, little is known about SNGFR modulation in this condition, and, as stated by Komers and Anderson (8), “... considering the epidemic increases in type 2 diabetes-associated nephropathy, studies should focus on evaluating these systems in models of type 2 diabetes.” Welch (29) has recently stressed the importance of using in vivo micropuncture techniques to provide the most direct analyses of renal segmental function in genetically engineered mouse models of disease, notwithstanding the fact that the mouse micropuncture technique is much more difficult than in the rat (18). This was our experience in the present study using a fragile diabetic mouse preparation—B6.Cg-m1/+LeprdbJ (db/db), the classic obese mouse model of DM2. These mice do not develop renal failure (2) despite the presence of significant glomerular changes (19) found in human diabetic nephropathy. Nevertheless, we share the view of others (19) that this classic diabetes model may provide valuable insights into human diabetic nephropathy, and we set out to document hyperfiltration in single nephrons and to gain insight into its underlying mechanisms. Our studies show for the first time, in untreated hyperglycemic db/db mice, SNGFR is elevated and responsive to changes in extracellular fluid volume (ECV), TGF effects can be demonstrated and modulated by DM2 and changes in ECV, and SNGFR can be reduced by neuronal NO synthase (nNOS) inhibition.

METHODS

Wild-type, db/m, and db/db mice (stock number 000697) were purchased from Jackson Laboratories (Bar Harbor, ME). As noted in the Jackson Laboratories database (http://jaxmicejax.org/jaxmice-cgi/jaxmicedb.cgi), the nomenclature of these db/db mice has changed. They are now identified as B6.Cg-m1/+LeprdbJ or Leprdb, but they formerly were designated as C57BL/6J-m1/+Leprdb. The heterozygote (Leprdb+) will be referred to as db/m and the wild type (Lepr++) as WT. The three groups were allowed free access to chow and water. The db/db mice, at 8–10 wk of age, manifested hyperglycemia and glycosuria, as well as striking weight gain (db/db mice at 10 wk weigh about 45 g, with blood glucose ~14 mM) vs. the WT and db/m mice weighing about 33 and 25 g, respectively, with blood glucose ~6 mM.

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(With the Technical Assistance of Christiano T. de Souza)
All studies were approved by the Animal Care Committee of the University of Ottawa. For statistical analyses ANOVA, paired and unpaired Student’s t-testing, was used.

**Micropuncture preparation.** Mice were anesthetized with 100 mg/kg ip thiobutabarbital sodium (Inactin; Sigma-Aldrich, St. Louis, MO) and 100 mg/kg im with ketamine hydrochloride (Bimeda-MTC, Cambridge, ON, Canada). After being anesthetized, the mouse was placed on a heated mouse operating table; a tracheostomy was performed with polyethylene (PE)-90 tubing inserted. With the use of pulled PE-10 tubing, the left carotid artery was cannulated for continuous blood pressure measurement, blood sampling for glucose, and insulin measurements. The left jugular vein was cannulated with pulled PE-10 tubing, providing two lines for infusion of fluid and thiobutabarbital sodium anesthesia. The bladder was cannulated with PE-50 tubing. The left kidney was exposed by a flank incision, separated from the surrounding fat, and carefully dissected away from the adrenal gland while the ureter remained attached to the kidney. In the db/db mice, fat accumulation in the neck, and especially in the abdomen, made the surgical procedure most difficult. Because of the excess intra-abdominal fat, a longer flank incision was made to the linea alba to provide space for the kidney cup, and special care was taken to prevent excess heat and fluid losses from the larger wound by using Surgicel (Ethicon). The Surgicel was placed just below the kidney cup, covering the entire incision, thereby preventing the abdominal wound from widening excessively. This operative approach allowed sufficient fat to be shifted so that the kidney could be positioned appropriately after being immobilized in a Lucite cup (10–12 mm). The kidney was set in 0.9% NaCl agar, with a surface opening created to hold a shallow layer of heavy mineral oil.

All groups were infused intravenously with 2.25% albumin (Sigma-Aldrich) in 0.9% saline for 30 min and then with 0.9% saline at either 3.3 or 4.0 ml·100 g body wt⁻¹·h⁻¹ for the remainder of the experiment. When the preparation was considered to be stable, a 0.3 ml bolus of [H]inulin (Perkin Elmer) was injected (equivalent to 5–10 Ci), followed by a sustaining infusion containing the same amount of inulin per hour. During the experimental period, groups 5 and 6, described in Experimental groups, received an infusion containing S-methylthiocitrulline (SMTC; Sigma-Aldrich), at 1.2 mg·kg⁻¹·h⁻¹ as part of the same 3.3 ml·100 g body wt⁻¹·h⁻¹. We found SMTC solubility in saline at this dose satisfactory for intravenous infusion as used in rats (28). Micropuncture samples were collected from 30–60 min after the bolus in the control period and 30–90 min after the start of the SMTC experimental period. For proximal tubule-determined SNGFR, the late proximal site was identified by injecting tiny boluses of 1% Lissamine green in upstream segments using a pipette with a 3–4 μm tip diameter.

**Clearance studies.** For GFR and other whole animal parameters, mice were prepared as described, but without the left kidney isolated for micropuncture. The protocol was designed to replicate the time, infusion, and blood sampling of the micropuncture experiments. Blood samples were taken for hematoctrit, glucose, and insulin. Urine was collected under oil for determination of inulin concentration and urine flow from both kidneys. The mice were infused with a priming of 5–10 μCi of [H]inulin and a sustaining infusion of an equal amount per hour in 0.9% saline.

**Microalbuminuria.** Individual 10- to 11-wk-old mice (db/db, n = 11; db/m, n = 4; and WT, n = 4) were placed in separate metabolic cages for 24-h urine collections. Urine albumin concentrations were analyzed using an Albuwell Murine Microalbuminuria ELISA kit (Exocell, Philadelphia, PA), and absorbance was measured with a Fluostar Galaxy Luminometer.

**Experimental groups.** Group 1 was db/db mice infused paired proximal-distal SNGFR determinations (10 mice/11 tubules) with an infusion of 3.3 ml·100 g body wt⁻¹·h⁻¹ and 4.0 ml·100 g body wt⁻¹·h⁻¹ in groups 2, 3, 5, 6, and 7 as well. Some of the group 1 samples were also derived from the control period for group 5, below. The other groups were group 2 (11 db/m mice, 13 tubules); group 3 (8 WT mice, 18 tubules); group 4 (5 db/db mice, 8 tubules) infused at 4.0 ml·100 g body wt⁻¹·h⁻¹; group 5 (7 db/db mice, 7 SNGFR’s representing the mean, per period, of 15 tubules from individual mice) were infused with SMTC after a control period; group 6 (4 db/m mice, 4 SNGFRs representing the mean, per period, of 18 tubules from individual mice) were infused with SMTC after a control period; group 7 (2 db/db mice, 11 tubules) were treated with vehicle and punctured as time controls for group 5. An additional group of 9 db/m mice (9 tubules), were infused with saline at 2.0 ml/h (–8.0 ml·100 g body wt⁻¹·h⁻¹) to assess the effects of obvious ECV expansion on SNGFR in these nondiabetic heterozygotes.

**Histological evaluation of kidneys.** Sections were examined from db/db, db/m, and WT mice at ages from 30 to 40 wk. Whole left kidneys were bivalved, fixed in buffered formalin, embedded in paraffin, and sectioned at 3 μm. Each section was stained with hematoxylin and eosin, Jones silver methenamine, periodic acid-Schiff, Masson trichrome, and Sirius red. One full section was assessed on hematoxylin and eosin stains for glomerular diameter. All glomeruli in that section were measured using an ocular grid micrometer at ×400 magnification. Approximately 60–100 glomeruli were examined per section. Vessels were assessed using the Masson trichrome stain, and the mesangial matrix by the silver methenamine, PAS, and Sirius red. The interstitial areas were examined by the Masson trichrome stain only. A total of 23 mice were examined: six 30-wk-old WT, five 30-wk-old db/db mice, two 40-wk-old db/db mice, and ten 40-wk-old db/m mice. The glomerular diameters from all mice were quantified and statistically analyzed by ANOVA and Student’s t-test. To quantitate the degree of mesangial expansion, 50 db/m and 50 db/db glomeruli were stained with Sirius red, photographed, and analyzed by Image Pro 4.0 using color segmentation of the Sirius red mesangial staining. Quantitative results are expressed as the ratio of red-stained pixels over total glomerular pixel surface area.

**RESULTS**

**Microalbuminuria.** The mean albumin excretion rate for the db/db mice was 303 ± 32 μg/24 h and for the db/m and WT mice was 62 ± 21 and 83 ± 16 μg/24 h, respectively, with db/db mice values significantly higher than db/m and WT mice (P < 0.02).

**Histopathology.** It was immediately obvious on inspection that the db/db glomeruli at all time points examined were larger in diameter when compared with either the WT group or even the older db/m mice. The mean glomerular diameter for the 30-wk-old WT mice was 75.6 ± 2 μm vs. 95.4 ± 1 μm and 80.4 ± 1 μm in the db/db and db/m mice, respectively. ANOVA was significant (P < 0.001) for the three groups, with the glomerular diameter in the db/db mice significantly greater than either the WT or db/m (P < 0.001) with two-tailed Student’s t-test. Furthermore, the 40-wk-old db/db mice (see methods), showed no significant difference from the 30-wk glomeruli, with the same strong significant differences (P < 0.001) vs. db/m and WT groups. Although measurements focused on subcapsular and midcortical glomeruli, the juxtaglomerular glomeruli appeared larger, and this might account for the higher percentage increase in whole kidney GFR vs. the SNGFR increase in db/db mice vs. WT (see Tables 1 and 2). Mesangial expansion was noted only in db/db mice, and characterized by segmental increases as seen with the Silver methenamine and Sirius red stains expressed as the ratio of red-stained pixels over total glomerular pixel surface area. Control db/m mean was 0.183 (0.015 SD) vs. db/db mean of 0.323 (0.010 SD), t = 6.7, P < 0.001. Fig. 1, A and B, show these obvious changes. Fig. 1C (silver stain) shows db/db mesangial expansion segmentally accentuation, and Fig. 1D
shows db/db mouse arteriolar hyalinization. Accordingly, although the mesangial changes were marked, and almost nodular in configuration, true nodules associated with aneurysmal capillary loop dilation as seen in human diabetic nephropathy were not present. Clear vascular changes, again only in the db/db mice as noted above, were restricted to arteriolar hyalinization and luminal narrowing. This was highlighted on the PAS and Masson trichrome stains. Interstitial fibrosis or inflammation was not apparent.

**Whole animal data.** Table 1 summarizes groups 1–3 whole animal data from db/db, db/m, and WT mice infused at 3.3 ml·100 g body wt⁻¹·h⁻¹. As described previously (12, 18), the anesthetized mouse micropuncture preparation is fragile and, with time, whole kidney GFR may fall. In both the db/db and the db/m mice (groups 1 and 2), there was a significant difference (P < 0.01) between the first period GFR, and the mean of all three periods, but not in the WT mice (group 3). Group 1 db/db mice showed a significantly (P < 0.02) higher GFR (mean of 3 periods) vs. db/m and WT mice (P < 0.01). With the lower infusion (3.3 ml·100 g body wt⁻¹·h⁻¹), SNGFR in group 1 db/db mice, determined at both proximal and distal sites, was significantly higher vs. group 2 db/m mice (P < 0.01, P < 0.04, respectively), but not different vs. SNGFR in group 3 WT mice. Finally, in the group of nine db/m mice infused at 8.0 ml/100 g body wt⁻¹·h⁻¹, paired proximal and distal SNGFR obtained in nine nephrons were 8.0 ± 0.6 and 6.7 ± 0.5 nl/min, respectively, with a significant difference (P < 0.05), indicating intact TGF responsiveness (data not shown in Table 2). Importantly, SNGFR values are almost identical to that obtained from group 2 db/m mice infused at less than half the rate (3.3 ml·100 g body wt⁻¹·h⁻¹).

**Absolute fluid reabsorption and ECV changes.** In the volume-replete db/db mice (group 4), absolute net fluid reabsorption, determined at the late proximal site was significantly higher than that of the db/m mice (P < 0.03; Table 2). Similarly, absolute net fluid reabsorption measured at the distal site, reflecting the net reabsorption of fluid from the proximal tubule, loop of Henle, and early distal tubule, was more than 50% higher than in group 2 db/m mice (P < 0.01). This enhanced reabsorption is consistent with the view that group 1 db/db mice were ECV contracted as a result of the glycosuric volume losses. (The TGF responses, described below, are also consistent with ECV changes.) Of interest, despite this increased fluid reabsorption, group 4 distal flow rate past the macular densa region was almost twice that measured in db/m and WT mice (groups 2 and 3; P < 0.01).

**TGF responses.** TGF responses were determined under free-flow conditions as differences between SNGFR determined at the late-proximal tubule site, with flow to the macula densa region interrupted, and that from the distal tubule. SNGFR and

### Table 2. Micropuncture results of paired fluid collections at proximal and distal tubular sites from db/db, db/m, and WT mice

<table>
<thead>
<tr>
<th></th>
<th>Group 1, db/db</th>
<th>Group 2, db/m</th>
<th>Group 3, WT</th>
<th>Group 4, db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prox</td>
<td>Dist</td>
<td>Prox</td>
<td>Dist</td>
</tr>
<tr>
<td>Collected flow, nl/min</td>
<td>7.4 ± 0.8</td>
<td>3.3 ± 0.5*</td>
<td>4.4 ± 0.4‡,†</td>
<td>2.0 ± 0.3*‡,†</td>
</tr>
<tr>
<td>TF/Pin</td>
<td>1.64 ± 0.12</td>
<td>3.07 ± 0.31*</td>
<td>1.87 ± 0.1‡</td>
<td>4.02 ± 0.40*†</td>
</tr>
<tr>
<td>SNGFR, nl/min</td>
<td>11.6 ± 0.8</td>
<td>9.3 ± 1.0*</td>
<td>8.0 ± 0.8‡</td>
<td>7.2 ± 0.6*‡</td>
</tr>
<tr>
<td>Abs.reab., nl/min</td>
<td>4.2 ± 0.5</td>
<td>6.0 ± 0.7*</td>
<td>3.6 ± 0.53</td>
<td>5.0 ± 0.4‡</td>
</tr>
<tr>
<td>Fractional reabsorption</td>
<td>0.36 ± 0.04</td>
<td>0.65 ± 0.03*</td>
<td>0.44 ± 0.04</td>
<td>0.72 ± 0.03*†</td>
</tr>
<tr>
<td>Prox-dist SNGFR, nl/min</td>
<td>2.1 ± 0.6</td>
<td>0.9 ± 0.4‡</td>
<td>1.7 ± 0.6</td>
<td>1.7 ± 1.6</td>
</tr>
<tr>
<td>Dist/Prox SNGFR (%)</td>
<td>73 ± 9</td>
<td>91 ± 4*</td>
<td>90 ± 5*</td>
<td>120 ± 13†</td>
</tr>
<tr>
<td>Number of mice/tubules</td>
<td>10/11</td>
<td>11/13</td>
<td>8/18</td>
<td>5/8</td>
</tr>
</tbody>
</table>

Values (means ± SE) for Groups 1–3 (3.3 ml·100 g body wt⁻¹·h⁻¹) and for Group 4 (4.0 ml·100 g body wt⁻¹·h⁻¹); Prox, proximal; Dist, distal; TF/Pin, tubular fluid to plasma [H]inulin concentration ratio; SNGFR, single-nephron GFR; Abs.Reab, absolute net fluid reabsorption to the point of collection. * paired P < 0.05; † unpaired vs. db/db, 3.3 ml·100 g body wt⁻¹·h⁻¹; P < 0.05; ‡unpaired vs. db/db, 4.0 ml·100 g body wt⁻¹·h⁻¹, P < 0.05.

AJP-Regul Integr Comp Physiol • VOL 290 • APRIL 2006 • www.ajpregu.org
associated measurements are shown in Table 2 and Fig. 3 for groups 1-4. The TGF response in db/db group 1 mice shows the largest proximal-distal difference, whereas the TGF response is absent in the volume-replete db/db group 4 mice. Both db/m and WT mice had significant responses, although in the WT mice, significance was borderline (P < 0.049).

Effects of systemic SMTC infusion in groups 5, 6, and 7. Figures 4 and 5 show the results of the SMTC infusion without alteration of systemic blood pressure. In db/db group 5 mice, SNGFR determined at the proximal site fell from 11.0 ± 1.1 to 7.4 ± 1.0 nl/min (P < 0.01), with no significant fall in SNGFR measured at the distal tubule site (Fig. 4). In contrast, in the db/m mice (Fig. 5), both proximal and distal SNGFR dropped significantly after SMTC infusion (P < 0.04 and P < 0.01, respectively). Because of the possibility of SNGFR declining as a consequence of elapsed time and anesthesia, a vehicle control group was also undertaken (Fig. 4, group 7), and samples were meticulously obtained at a time corresponding to those taken in the SMTC period. As seen in Fig. 4, there was no significant difference between the vehicle and the control periods.

DISCUSSION

Preparation. Schnermann and colleagues (13, 18) have detailed the inherent difficulties in studying the anesthetized mouse with micropuncture techniques. As previously observed in NHE3 knockout mice (18), we noted a fall in whole kidney GFR as the period of anesthesia extends to 3 h. Moreover, as already emphasized, the micropuncture preparation of these db/db mice, weighing twice as much as their db/m heterozygotes at the same age, is difficult because of excessive fat accumulation both in the neck and abdomen. Indeed, the abdominal incision and freeing the kidney from surrounding fat can be a formidable task. Furthermore, in this study, hyperglycemia was present and not treated in the db/db groups to simulate the clinical phenotype. These data provide the first information on single-nephron GFR in the db/db mouse model of DM2, which has been the subject of more than 1,800 publications.

Hyperfiltration and TGF feedback. SNGFR of the db/db mice was significantly greater than db/m mice even when an equivalent infusion per 100 g body weight was used, despite the increased urine losses. The db/db mice distal SNGFR increased by about 30% with the higher infusion to offset the
urine losses of ~0.3 ml·100 g body wt⁻¹·h⁻¹. (See also below.)

Rather than vary loop perfusion to examine TGF activity by changes in early proximal stop-flow pressure (Pₛ₊), we measured, in a paired fashion, proximal-distal SNGFR differences as described by Schnermann (18). This method is sensitive to SNGFR changes in the normal-to-low range, and is more likely to detect TGF responses to very low flows than the use of the Pₛ₊ technique and may be more sensitive to reductions in ECV (18, 28). The proximal SNGFR exceeded the distal SNGFR by about 11% in db/m and WT mice, while the difference was 24% in the db/db mice with the lower sustaining infusion, which did not account for urine losses. With a higher infusion rate in group 4 mice, designed to render these mice euvolemic (increasing 3.3 to 4.0 ml·100 g body wt⁻¹·h⁻¹), SNGFR increased significantly, compared with the db/m and WT mice, with a loss of the TGF response, despite the increased flow rate past the macula densa site (see RESULTS and Table 2). However, to confirm there is a right shift in the TGF curve over a wide range, even higher delivery rates to the macula densa site would have to be undertaken by closed loop perfusion while...
tracking changes in early proximal flow rates or P_{SF}. Still our data are consistent with the reported decrease in efficiency of the TGF mechanism in the rat STZ DM1 model (26), as well as with the effect of hyperglycemia, per se, to suppress TGF (1, 30).

As shown in Fig. 3 and Table 2, the proximal-distal SNGFR difference in the WT mice were not only small, but barely met the P < 0.05 significance level. This is in contrast to the clear effects in the db/m mice (P < 0.02). These WT mice from the Jackson colony (see METHODS), although originally based on the C57BL/6J strain, have been inbred for at least 80 generations since last backcrossed to noncolony C57BL/6J mice (per Jackson Web site). It is possible, therefore, that TGF responses have been downregulated as a result of this inbreeding, but we note, small free-flow proximal distal SNGFR differences have already been reported in WT mice (11, 28).

Effects of different infusion rates. It is appropriate to reiterate that in our preliminary experiments to establish a viable preparation in our db/db mice, we were unable to successfully use the more conventional infusion rate of 0.3–1.5 ml·100 g body wt·h⁻¹. Accordingly, our db/m and WT mice, with lower than usually reported proximal fractional reabsorptive rates, may be considered to have an element of ECV expansion, but, as noted below, TGF responses have been maintained. When the infusion rate was increased from 3.3 to 4.0 ml·100 g body wt·h⁻¹, SNGFR (distal) increased almost 30% (P < 0.05) in the db/db mice, and the proximal-distal SNGFR difference was abolished. In contrast, and as noted in METHODS and RESULTS, even with much higher infusion rates (8.0 ml·100 g body wt⁻¹·h⁻¹), SNGFR in db/m mice did not increase, and the TGF response was present. The most reasonable explanation for these findings is that at the lower infusion rate, the db/db mice were somewhat volume contracted, and despite the assumption that the efficiency of the TGF response is reduced in diabetes based on STZ DM1 rat data (26), the reduction associated with a decrease in SNGFR. In this para-

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