Modulation of Single Nephron GFR in the db/db Mouse Model of Type 2 Diabetes Mellitus. II. Effects of renal mass reduction

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Running Head: Renal mass reduction and SNGFR in db/db mice

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ABSTRACT

This study examines for the first time the effects of uninephrectomy (Nx) on modulation of whole kidney GFR, single nephron GFR (SNGFR) and progression of diabetic nephropathy in the db/db mouse model of type 2 diabetes mellitus. To characterize SNGFR and tubuloglomerular feedback (TGF) responses to Nx and chronic neuronal nitric oxide synthase (nNOS) inhibition in the db/db mouse, we studied the effects of Nx on whole kidney GFR, SNGFR and TGF characteristics in both db/db and WT mice after Nx or sham Nx. We also documented progression of glomerular changes over a six month period. Whole kidney GFR and SNGFR in db/db Nx mice were significantly higher than in db/db sham mice, without change in proximal tubule reabsorptive rates. The TGF responses, determined as proximal-distal SNGFR differences, were brisk (WT sham, 12.1 ± 1.0 vs. 8.4 ± .6 nl/min, p<0.05; WT Nx 15.7 ± 1.0 vs. 12.0 ± 1.0 nl/min, p<0.05; db/db Nx, 17.8 ± 1.3 vs. 14.3 ± 1.0 nl/min, p<0.05). Chronic ingestion of the nNOS inhibitor, S-methylthiocitrulline, for 2-3 weeks post Nx had no effect on SNGFR or the TGF response. These studies show that after Nx, these hyperglycemic morbidly obese db/db mice have further elevations in whole kidney and single nephron GFR with an intact TGF system. In addition, in the db/db Nx mice, 4-6 months post Nx, there is an exacerbation of the lesions of diabetic nephropathy as quantified by a significant increase in the ratio of mesangial surface area to total glomerular surface area.
INTRODUCTION

The epidemic of diabetic nephropathy resulting from type 2 diabetes mellitus (DM2) is growing, and still remains the most common cause of kidney failure in patients on dialysis life-support (33). Many clinical studies have attempted to evaluate the relation of early elevations in GFR to the subsequent appearance of diabetic nephropathy (26). Rat models of diabetic hyperfiltration—typically the streptozotocin (STZ) type 1 diabetes (DM1) preparation—have provided evidence that the accompanying increase in glomerular capillary pressure contributes to the progression of diabetic nephropathy with specific glomerular damage (1, 7, 8, 15, 37).

For more than 25 years the effect of loss of renal mass has also attracted attention because of demonstrations of the acceleration of preexisting renal disease, and changes in the control of GFR in the remnant kidney (4, 15). Expectedly, the combined effects of reduction of renal mass and diabetic nephropathy on renal function and progression of diabetic nephropathy have aroused clinical (11) and experimental interest. Again, these investigations usually focused on the insulin treated rat streptozotocin model of type 1 diabetes (DM1) (23, 27).

With respect to whole kidney GFR, 30 years ago Gartner (12) documented hyperfiltration in the obese db/db (B6.Cg-m^+/+Lepr^db/J) model of DM2. Shortly thereafter, Bower et al (6) in a key study using the DM2 db/db mouse, documented exacerbation of diabetic nephropathy induced by uninephrectomy (Nx) and
suggested that alterations in glomerular hemodynamics subsequent to Nx influence the rate of development of diabetic glomerular lesions. Few studies have attempted to address in vivo single nephron effects of DM2 because appropriate rat models are not readily available, and in vivo micropuncture assessments in hyperglycemic DM2 mouse models with glycosuria can be technically overwhelming (36). We recently established that single nephron GFR (SNGFR) in the db/db mouse is significantly elevated compared to heterozygote and wild type controls (21) and reported associated changes in the tubuloglomerular feedback (TGF) system as well as effects related to ECV changes and acute nNOS inhibition.

Several intrarenal factors may contribute to the early increase in whole kidney GFR and SNGFR in experimental models of diabetes mellitus (17-19, 29). These factors include, separately or in combination, hypertrophy of glomerular and tubular structures, altered tubular transport, changes in TGF responses, and alterations in intrarenal NOS activity. The tubular hypothesis of glomerular hyperfiltration, supported by several studies in the STZ DM1 rat preparation, points to enhanced proximal tubule fluid reabsorption in early diabetes as a key determinant of hyperfiltration (31, 32).

In the present investigations, we evaluated functional and structural changes following nephrectomy (Nx) in the gene targeted hyperglycemic obese db/db mouse model of DM2. We hypothesized that, 1) Nx of 3 weeks duration leads to an additional increase in whole kidney GFR and single nephron GFR (SNGFR) in the already hyperfiltering db/db mice; 2) this additional increase in
SNGFR is likely associated with a resetting of the TGF system, 3) chronic NOS1 inhibition attenuates the hyperfiltration; and 4) Nx in these type 2 diabetic mice eventually leads to exacerbation of the lesions of diabetic glomerulopathy. More specifically, to understand possible mechanisms underlying filtration changes in our db/db DM2 mouse model, in the context of the tubular hypothesis of glomerular hyperfiltration (32), we focused on three single nephron parameters before and after Nx, using WT and sham operated mice as controls, in the four groups of mice: distal tubule measured SNGFR (without modification of flow to the macula densa site), proximal SNGFR with zero distal flow, and proximal tubule fluid reabsorption rates to assess net fluid transport.

METHODS

For these experiments, as in our previous study (21), wild type (WT) and db/db C57 BL/6J mice (stock number: 000697) were again purchased from Jackson Laboratories. After receiving the mice from Jackson, a period of about 4 weeks of acclimatization followed. Then Nx, or sham Nx, surgery was carried out with a subsequent ~ 3 week recovery period before the stressful micropuncture experiments. Thus, in vivo studies were carried out in db/db or WT mice approximately 13-15 weeks of age, 3 weeks after Nx or the sham procedure. In addition to micropuncture and histopathology studies in 15 week old mice, histopathological changes were also assessed in mice at 30 and 40 weeks of age.

Overview of experimental groups.
Groups 1-4. To assess the effects of unilateral nephrectomy (Nx), micropuncture studies were carried out on 4 groups of mice 13-15 weeks of age, approximately 3 weeks after Nx or sham Nx. These were db/db Nx mice (Group 1), and WT Nx mice (Group 3) with corresponding db/db sham and WT sham mice, Groups 2, and 4, respectively.

Groups 5 and 6: To examine the possible effects of chronic nNOS inhibition on SNGFR and related parameters, commencing 2 days post Nx, SMTC in drinking water was ingested for 2-3 weeks by db/db Nx mice, in the amount of approximately 12 or 24 µg day (Groups 5 and 6, respectively). These ingested SMTC doses are similar on a per weight basis to that used in rats by Komers et al (16) who were able to modify the lesions of diabetic nephropathy after 12 weeks of ingestion.

All studies were approved by the Animal Care Committee of the University of Ottawa.

Micropuncture preparation. Mice were anaesthetized with 100 mg/kg i.p. thiobutabarbital sodium (Inactin, Sigma-Aldrich Co. St. Louis, MO, USA) and 100 mg/kg i.m. with Ketamine Hydrochloride (Bimeda-MTC, Cambridge, ON, Canada). After anaesthesia, the mouse was placed on a heated mouse operating table; a tracheostomy was performed with PE-90 tubing inserted. Using pulled PE-10 tubing, the left carotid artery was cannulated for continuous blood pressure measurement, blood sampling for glucose and inulin 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 excessive 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 Inc). 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. The mice were infused with a prime of 5-10 µCi of [H3] inulin and a sustaining infusion of an equal amount at 3.3ml/100 g BW/hr of 2.25% albumin in 0.9% saline for the duration of the experiment.

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 hematocrit, glucose and inulin. Urine was collected under oil for determination of inulin concentration and urine flow from both kidneys. The mice were infused with a prime of 5-10 µCi of [H3] inulin and a sustaining infusion of an equal amount per
hour in 0.9% saline. Again this protocol was designed to replicate the time, infusion, post operative days, age and blood sampling during micropuncture experiments.

**Single-nephron GFR (SNGFR) and tubuloglomerular feedback (TGF) responses.** SNGFR was determined at the late proximal and distal tubular sites by the usual technique of $[\text{H}^3]$ inulin infusion, sparse use of lissamine green dye and timed collections of quantitative tubular flow by careful oil block insertions. To assess TGF activity, we measured, in a paired fashion, proximal-distal SNGFR differences as described by Schnermann (22). This method is sensitive to SNGFR changes in the “normal” to low range, is more likely to detect TGF responses to very low flows than the use of the $P_{\text{SF}}$ technique, and may be more sensitive to changes in ECV (22, 31).

**Unilateral nephrectomy and sham nephrectomy.** Male WT and 8-10 week old db/db mice with hyperglycemia, polydipsia and polyuria were selected for unilateral nephrectomy (Nx) by surgical technicians of the University of Ottawa’s Animal Care Veterinarian Services (ACVS.) in a sterile, climate controlled facility. The db/db mice weighed between 40-50 grams while the WT mice weighed between 25 and 30 grams. The mice were placed in a restrainer without anesthesia and one hind leg was extended. For blood glucose determination, the lower surface of the hind leg was shaved so the saphenous vein could be visualized and a needled. One hour prior to surgery, mice were given 0.05 mg/kg subcutaneously of buprenorphine (R.B. Pharmaceuticals Inc) and a baseline body weight was determined. Anesthesia was induced with isofluorane with the flow
varying between 0.5%-3%. The isofluorane was mixed with oxygen at a rate of 1L/min and prior to the first incision, the animal was given 1 ml sc of normal saline and a BNP ophthalmic ointment (Vetcom) was applied. For kidney access, the dorsal area was shaved and prepped, and a small cm incision was made dorsally to pull out and isolate the right kidney. The fat at the lower pole of the kidney was grasped and blunt dissected (sparing the kidney’s adrenal gland) and three, 4-0 silk ligatures were passed under the ureter, renal artery and renal vein, tying one distally and two proximally. A cut was made between the ligatures and the proximal ties were shortened while the distal tie was removed, and then the kidney was removed. After carefully checking for bleeding, the peritoneum was closed with 6-0 prolene suture using a simple interrupted pattern and the skin was closed with autoclips. The right kidney was promptly weighed after the fat was removed. The mice were kept in an incubator until conscious and moving. Post operative care included 1 ml sc of sterile saline for dehydration and 0.05 mg/kg sc of buprenorphine over 3 days, as per ACVS guidelines.

Sham mice underwent the same procedure except the kidneys were only touched by the instruments, without incision. All Nx and sham mice were allowed about 3 weeks to recover prior to subsequent micropuncture experiments.

Histopathology. For histopathological assessment, whole left kidneys from usually 2-3 mice per group were bivalved, fixed in buffered formalin, embedded in paraffin, and sectioned at 3 µm. Each kidney was stained with hematoxylin and eosin, Jones silver methenamine, periodic acid-Schiff, Masson trichrome, and Sirius red. One full half-section was assessed on hematoxylin and eosin stains for
glomerular diameter. All glomeruli in that section were measured using an ocular grid micrometer at X 400 magnification. Approximately 60-100 glomeruli were examined per section. Mesangial matrix expansion was assessed using the Sirius red stained sections. High resolution digital photographs of 50 glomeruli sectioned through, or close to the hilum, were prepared from the Sirius red stained sections from each animal. These photographs were subsequently quantified by assessing the ratio of surface area of mesangium to total glomerular surface area with color assisted image analysis using Image Pro software (version 5.1).

Statistical Analyses. For 2-group comparisons 2-tailed unpaired t tests were used. For 4-group comparisons ANOVA was used to test the hypothesis that a given parameter was equal in the 4 groups (e.g. db/db Nx, db/db sham, WT Nx, WT sham). If the hypothesis of equality between groups was rejected, then an unpaired 2-tailed t test or Tukey’s post test was used to determine group differences. In these tests, data involving repeated measures were averaged for each mouse. Paired t tests were used to compare parameters such as SNGFR within a single group. For a single group analysis of 1 parameter, where each mouse served as its own control, the paired t test data included each repeated measure as a separate observation. The significance level to reject the null hypothesis was set at .05 for each test.

Statistical analysis for histopathological assessment was done using SPSS 15.0. Glomerular diameters of db/db mice, with and without Nx, were assessed using planned comparisons between Nx and sham Nx mice at each of 15 weeks, 30 weeks, and 40 weeks of age (two-tailed t-tests). Also planned comparisons of
glomerular diameters of WT Nx and db/db Nx mice at the 30 weeks and 40 weeks were done. A one-way ANOVA was done to compare glomerular diameters of 15 weeks, 30 weeks and 40 weeks db/db Nx mice. Post hoc analyses included polynomial contrasts and pair wise comparisons (Tukey HSD). Similarly, glomerular mesangial ratios of db/db sham and db/db Nx mice were each compared at 15 weeks and 30 weeks (two-tailed t test). Glomerular mesangial ratios in 30 week WT Nx and WT sham mice were also compared (two-tailed t test). A comparison of glomerular mesangial ratios was also done between WT Nx and db/db Nx mice at 30 and 40 weeks (One Way ANOVA).

RESULTS

Whole Animal Data. Table 1 summarizes Groups 1-4 whole animal data from db/db Nx, db/db sham, WT Nx, WT sham mice infused with a solution of 2.25% albumin in 0.9% saline at 3.3 ml·100 g body wt⁻¹·h⁻¹ for the duration of the experiment. In our previous db/db mouse micropuncture investigation (21) we noted whole kidney GFR tended to decline during the experiment. In the present study, using a constant albumin infusion, there was no significant GFR difference when considering the first period or the mean of 2 or 3 periods, indicating albumin has a stabilizing effect. In detailed preliminary experiments we also assessed possible effects of this infusion on ECF volume, SNGFR and TGF responsiveness of this sustaining infusion. We studied an additional group of db/db Nx mice infused with the same albumin solution but at a 40% lower rate. Single nephron responses were virtually identical those obtained from Group 1, as given below.
The left kidney weight in Group 4 WT sham mice grew by 40% after Nx. The db/db Nx (Group 1) kidney weight showed more modest but still significant hypertrophy when compared with sham db/db controls. The increase in GFR in both db/db and WT mice (Groups 1 and 3) was significant after Nx only when expressed in absolute terms (not corrected for kidney weight). When corrected for kidney weight, the increase in GFR was not significant in either db/db or WT groups.

Histopathology (Figure 1).

1. **Glomerular mesangial ratio differences and glomerular diameter in db/db Nx vs. WT Nx mice aged 30 and 40 weeks.** As expected, the db/db Nx mice showed a significant increase in mean diameter in comparison to the WT Nx mice at both 30 weeks (mean 76.3 ± 2 µm vs. 95.8 ± 8.8µm, F(1,800)=20.2, P< 0.01, partial eta squared = .20) and 40 weeks (95.6 ± 2 µm vs. 108.3 ± 8.5 µm, F(1,754) =76, P< 0.01, partial eta squared =.09). There was also a significant and marked difference in mean mesangial ratio between db/db Nx vs. WT Nx both at 30 weeks: 0.46 ± .17 vs. 0.12 ± .02, F(1,800)=64.3, P < 0.01, partial eta squared = .10 and at 40 weeks: 0.31 ± .15 vs. 0.17 ± .01, F(1,284) =14.4, P< 0.01, partial eta squared =.05.

2. **Effects of both time and Nx on glomerular diameter and mesangial ratio in db/db mice.**

**Glomerular diameter:** At the baseline 15 week measurement, there was no difference between the db/db Nx vs. db/db sham mice with respect to glomerular diameter (mean 65.8 ± 14.0 vs. 66.5 ± 14.6 µm, P> 0.1) Similarly, the glomerular diameter of db/db Nx vs. db/db sham mice at 30 weeks or at 40 weeks showed no
significant differences, consistent with the absence of a separate effect of the Nx status on db/db mouse glomerular diameter. However, within the db/db Nx group, glomerular diameter does significantly increase with time. There was a significant difference in the glomerular diameters at 15 weeks when compared to those at 30 or 40 weeks: F(2,1854)= 187.7, P< 0.001, partial eta squared = .17. Post hoc polynomial contrast showed a linear trend of increasing diameter with time, F(1, 1854)=347.6, P< 0.001 and post hoc analysis (Tukey HSD) shows a significant difference between mean diameter at 15 week and 30 week old as well as 30 week and 40 week old db/db Nx mice (P< 0.001).

With respect to glomerular mesangial ratio (representing the mesangial matrix surface area vs. total glomerular surface area), at 15 weeks, there was no difference between the db/db Nx and db/db sham mouse mesangial ratio (mean 0.08 ± .06 vs. 0.04 ± .08 µm, P> 0.1); however with time there was an increase of the mesangial matrix expansion when comparing db/db Nx versus ddb/db sham mice. Thus, in the 30 week db/db Nx vs. db/db sham mice the mesangial ratio was 0.46 ± .17 vs. 0.27 ± .02, P < 0.01, although glomerular diameters, as already stated, did not differ. In contrast the control mice showed no difference in the mesangial ratio at 30 weeks between WT Nx versus WT sham mice (0.12 ± .05 vs. 0.18 ± .08, P > 0.05).

3. Arteriolar hyalinization: Although arteriolar thickening and hyalinization also appeared to be more pronounced in the db/db Nx mice vs. the db sham mice (Figure 1), this has not been further quantified. As previously described by Bower et al (5), the PAS positive arteriolar hyaline type thickening does have the
appearance of flowing into the mesangial expansion. After comparison with the Sirius red stain, however, it seems that the mesangial expansion and arteriolar hyaline changes are separate, but geographically adjacent processes, with the same progression characteristics.

**Micropuncture data.** (Tables 2 and 3) Table 2 shows both paired data (proximal and distal measurements from the same nephron) combined with a small number of unpaired collections. Table 3 shows only paired data, with Figure 2 showing the individual paired proximal-distal SNGFR values—8-14 pairs per group corresponding to Table 3. Table 2 data, derived usually from 1-2 nephrons per mouse, were meaned for each mouse. For each within-group comparison of these means (i.e. db/db Nx proximal SNGFR vs. db/db Nx distal SNGFR) paired t testing was done. For comparisons between the 4 groups, ANOVA and Tukey post testing was carried out. Group 5, Nx db/db mice (4 mice) ingested 12 µg daily of SMTC (2-3 weeks) and Group 6, db/db Nx mice (5 mice), ingested 24 µg daily (2-3 weeks) of SMTC. The data from these two db/db Nx + SMTC groups were virtually identical, not significantly different in any parameter. Therefore, they were combined as a single group in Table 2, and only compared (using unpaired t testing) with db/db Nx mice (Group 1) not receiving SMTC.

Within a single group, there were always significant proximal-distal SNGFR differences. For between group comparisons, as shown in Table 2, proximal SNGFR was significantly elevated in db/db Nx vs. db/db sham mice, and distal
SNGFR was significantly higher in WT Nx mice vs. WT sham mice. The combined SMTC group was compared only with Group1 db/db Nx mice, and shows virtually identical proximal and distal SNGFR values.

With reference to the tubular hypothesis of diabetic hyperfiltration (see Discussion), there were no significant changes in proximal fluid reabsorption between groups. Similarly, distal flow rates were unchanged except for the significant difference between WT sham and db/db sham, where a significant increase occurs (5.2 ± 0.05 vs. 3.2 ± 0.03 nl/min, P < 0.05).

DISCUSSION

We demonstrate that 3 weeks after unilateral nephrectomy, type 2 diabetic db/db mice show further increases in both kidney growth and whole kidney GFR, increases in single nephron GFR (SNGFR) measured proximally, and, four to six months later, exacerbation of the lesions of diabetic nephropathy as demonstrated by a marked increase in the ratio of the mesangial matrix surface to the total glomerular surface area. These functional and morphological observations derive from comprehensive comparisons using db/db and WT mice subjected to both Nx and sham Nx procedures. The basis for the SNGFR changes are likely related to the effects of a reset and possibly more responsive TGF system, without change in proximal tubule fluid reabsorptive rates.

Single nephron GFR and proximal tubule fluid reabsorption. In Group 1 db/db Nx mice, mean proximal and distal SNGFR were about 3 nl higher when compare with the db/db sham group, but only the proximal SNGFR difference reached statistical significance using 4-group post ANOVA analysis. However,
distal SNGFR in the WT sham mice was strikingly less than that measured in the WT Nx mice or db/db sham mice, suggesting an upward resetting of steady-state SNGFR (28).

With respect to control mechanisms possibly operative in our preparations, it is appropriate to consider whether the hyperfiltration we demonstrate is associated with changes in proximal tubule reabsorption or distal flow rates as suggested by Thomson et al (32). The distal SNGFR in WT sham (Group 4) mice is significantly lower when compared to distal SNGFR in db/db sham mice (Group 2), as shown in Table 2. Accordingly, in comparing Group 2 vs. Group 4—in a sense evaluating the effect of DM2 on the response to the sham procedure—we document an increase in steady-state distal SNGFR without change in proximal tubular fluid reabsorption. In addition, the distal flow rate in Group 4 WT sham mice is significantly lower than that measured in Group 2 db/db sham controls, although proximal SNGFR is similar in both groups. Thus, under the free-flow conditions of our studies we do not show an association of the rate of proximal fluid reabsorption or distal flow rates with SNGFR. Of course, to more comprehensively address the validity of the tubulocentric proposal (32) for the db/db mouse, specific studies are needed to evaluate the effects of the concurrent hyperglycemia, a reported suppressor of the Na\(^+\)-glucose cotransporter (13) and down regulator of the TGF response in a rat model of DM1 (5). If feasible, measurement of P\(_{sf}\) responses to higher than normal flow rates could possibly induce greater changes in proximal reabsorption and measured distal Cl\(^-\) delivery. It would be also be desirable to eventually describe 1) the detailed morphology of
db/db mouse proximal tubular epithelium, 2) its trans apical membrane transport characteristics—i.e. \( \text{Na}^+ \)-glucose cotransport—with and without hyperglycemia, and 3) whether ornithine decarboxylase plays a role as described in the STZ DM1 rat (31). Until other data emerge, it appears reasonable to suggest that hyperfiltration in the db/db diabetic mouse is likely sustained by a resetting of the TGF system at least in range of zero to normal distal flow rates.

It is of course possible that hemodynamic or myogenic effects, rather than macula densa signalling \textit{per se}, account for some of the changes we observed. A primary change in afferent arteriolar tone may occur in diabetic preparations, and may account for a right shift in the TGF curve as described by Vallon et al (34). Importantly, using direct videoscopic measurements, Hayashi et al (14) demonstrated blunted myogenic responses to pressure by afferent arterioles from STZ DM1 rats. Such changes may be related to alterations in voltage dependent calcium channels, and \( \text{K}^+ \) channels (2).

\textit{Intrarenal nitric oxide modulation of SNGFR.} We and others have reviewed the literature relating to 1) changes in intrarenal NO metabolism in DM1 preparations, 2) how NO may attenuate intrarenal vasoconstrictor influences in diabetes, and 3) whether macula dense cells, containing nNOS, may be a paracrine source of NO vasodilator influences e.g. (17, 18, 29). Thus, the TGF constrictor effect might be attenuated by higher levels of ambient NO in diabetes, thereby contributing to hyperfiltration. Indeed, intrarenal nitric oxide metabolism has been of interest in rodent models examining early and late effects of reduction in renal mass e.g. (30) and in the type 2 diabetic obese Zucker rat (10). In the
remnant kidney, our real time, direct tubular fluid [NO] measurements (20) shows a four-fold increase in tubular fluid [NO]. Also, long term effects of SMTC ingestion in the STZ DM1 rat with and without Nx have been described (16). We have previously reported that in both db/db and db/m mice, an acute systemic infusion of SMTC which did not alter systemic BP, was associated with suppression of SNGFR (21). In the present study, we examined the possible effects of SMTC ingestion on SNGFR adaptation to Nx when given for the 2-3 week period after the nephrectomy procedure. Two doses of SMTC, similar to that used in the rat on a per weight basis (16) in db/db Nx mice showed no effect on SNGFR or the TGF response (Results). It is possible that the effects of SMTC, although not apparent after 3 weeks of ingestion, might have been evident if examined earlier. In rats, Olerstam et al (25) examined the effect of chronic selective inhibition of nNOS with 7-nitro indazole (7-NI) on the TGF system and found greater sensitivity at 1 week but no change after 4 weeks. There are also other uncertainties regarding the disposition of NOS inhibitors in vivo (9). Finally, the intrarenal metabolism and paracrine concentrations of nitric oxide may well be different in different rodent strains of DM1 and DM2. We have shown in the db/db mouse model, tubular fluid concentrations of NO are vastly different than either in the STZ-induced diabetic mouse, or in the conventional STZ rat preparation (19).

The Preparation. This study has limitations which are, in part, inseparable from its unique goal to evaluate for the first time the effects of nephrectomy on single nephron function in this fragile db/db DM2 mouse model. In these db/db mice, hyperglycemia and hyperinsulinemia are present, factors already suggested
to influence TGF responsiveness. It is also possible that the GFR responses we have measured after reduction in renal mass may have been suppressed in the more fragile hyperglycemic diabetic mice when compared with WT mice. These and other issues have been reviewed by Bak et al who have monitored the sequence of renal hypertrophy and hyperfiltration in the STZ diabetic rat (3).

Another variable relates to our attempt to meticulously control for the effects of Nx. To better compare data with non-diabetic WT mice, we introduced sham controls, which entailed major surgical interventions as described in Methods. This procedure, *per se*, may have modified GFR or TGF responsiveness, and it is possible that other means of inducing nephron loss may differently modify GFR and TGF responses. As already noted, we did not directly measure distal tubular Cl' concentrations, nor did we examine progressive $P_{sf}$ and TGF responses in the range of zero to higher than normal flow. Rather, we restricted our assessment to within the normal flow range using distal SNGFR to assess so-called steady-state GFR, and we used proximal-distal SNGFR differences to assess the ambient restraining effect of the macula densa/TGF mechanism. Schnermann (28), and Vallon et al (35) have described difficulties, in mice, of determining by microperfusion effects on $P_{sf}$ of changes in very low loop flow rates, and have concluded proximal-distal SNGFR differences reasonably assess TGF responses in the ambient flow range. Finally, it is appropriate to note that the present study, as in all *in vivo* micropuncture studies, we were restricted to evaluation of accessible superficial nephrons. In fact, juxtamedullary nephrons, with an
intrinsically higher SNGFR, and reportedly even further elevations in SNGFR in early diabetes (24), cannot be assessed in this experimental in vivo setting.

**Perspectives and Significance**

The worldwide epidemic of type 2 diabetes is the major cause of kidney failure requiring dialysis life-support. The present study, in gene targeted db/db obese mice with type 2 diabetes, is the first to simultaneously assess progressive kidney damage along with single nephron changes in early diabetic hyperfiltration with reduction in kidney mass by uninephrectomy (Nx). Our results show further hyperfiltration and kidney hypertrophy after Nx, and evidence for an upward resetting of the control system regulating single nephron filtration. We could not demonstrate changes in single nephron filtration after 3 week ingestion of an inhibitor of nitric oxide synthase. Four to six months after Nx, there was a marked increase in mesangial matrix expansion. Future in vivo investigations in these db/db mice should focus on single nephron functional changes as GFR deteriorates, the effects of hyperglycemia, and factors regulating afferent arteriolar tone.
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Prevention of diabetic glomerulopathy by pharmacological amelioration of
Figure Legends

**Figure 1.** Histopathology. A. Glomerulus from a db/db sham mouse (30 weeks) showing segmentally accentuated mesangial expansion (Sirius red stain, 600 x). B. Glomerulus from a db/db Nx mouse (15 weeks) showing no mesangial matrix expansion (Sirius red, 600 x). C. WT Nx mouse (30 weeks) with no thickening or hyaline changes of arteriole (PAS, 400 x). D. Thickening of arterioles in a 30 week db/db Nx mouse (PAS, 400 x). * Arteriolar wall

**Figure 2.** Paired late proximal (Prox)-tubule and distal (Dist)-tubule SNGFR determinations in db/db and WT mice after unilateral nephrectomy (Nx) and control (sham) surgery (Groups1-4). Each line connects one SNGFR value determined at the late-proximal tubule site with a previously determined SNGFR from the distal tubule of the same nephron. A-D: (Group 1 mice) db/db Nx, (Group 2 mice) db/db sham, (Group 3 mice) WT Nx, (Group 4 mice) WT sham. The TGF response was significant in all groups (A-D, P<0.05).
Table 1. Whole body GFR in Nx and sham Nx db/db and WT mice.

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<td>Days, post Nx</td>
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<td>25 ± 2</td>
<td>21 ± 1</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Blood Glucose, mM/L</td>
<td>13.3 ± 0.9</td>
<td>15.8 ± 1.9</td>
<td>7.6 ± 0.5 (\alpha)</td>
<td>8.4 ± 0.4 (\alpha)</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>49 ± 1</td>
<td>51 ± 1</td>
<td>30 ± 1 (\alpha)</td>
<td>29 ± 1 (\alpha)</td>
</tr>
<tr>
<td>Left kidney weight, g</td>
<td>0.25 ± 0.01</td>
<td>0.21 ± 0.01 (\alpha)</td>
<td>0.25 ± 0.01</td>
<td>0.18 ± 0.01 (\alpha) (\alpha)</td>
</tr>
<tr>
<td>Syst. BP, mm Hg</td>
<td>104 ± 5</td>
<td>104 ± 3</td>
<td>101 ± 2</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>LK GFR, µl/min</td>
<td>295 ±15</td>
<td>220 ± 16 (\alpha)</td>
<td>269 ± 14</td>
<td>177 ± 14 (\alpha) (\alpha)</td>
</tr>
<tr>
<td>LK GFR, µl/min·g LKWt</td>
<td>1233 ± 84</td>
<td>1069 ± 90</td>
<td>1090 ± 88</td>
<td>942 ± 78</td>
</tr>
<tr>
<td>Number of Mice</td>
<td>9</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are means ± SE; db/db Nx, diabetic mice + unilateral nephrectomy; db/db Sham, diabetic control mice; WT Nx, wild-type mice + unilateral nephrectomy; WT Sham, control wild-type mice; GFR, glomerular filtration rate; LKWt, left kidney weight, \(\alpha\) ANOVA, Tukey post test vs. db/db Nx, \(P<0.05\); \(\alpha\) ANOVA, Tukey post test WT Nx, \(P<0.05\); ANOVA, Tukey post test vs. db/db sham, \(P<0.05\).
### Table 2. Micropuncture results of unpaired fluid collections from proximal and distal tubules in Nx and sham Nx db/db and WT mice

<table>
<thead>
<tr>
<th></th>
<th>Group 1, db/db Nx</th>
<th>Group 2, db/db sham</th>
<th>Group 3, WT Nx</th>
<th>Group 4, WT sham</th>
<th>Groups 5 &amp; 6, db/db Nx + SMTC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prox</td>
<td>Dist</td>
<td>Prox</td>
<td>Dist</td>
<td>Prox</td>
</tr>
<tr>
<td><strong>Collected Flow, nl/min</strong></td>
<td>12.7 ± 0.8</td>
<td>5.4 ± 0.6*</td>
<td>9.2 ± 0.7*</td>
<td>5.2 ± 0.5*</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.9 ± 0.7</td>
<td>4.4 ± 0.4*</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.7 ± 0.6*</td>
<td>3.2 ± 0.3*</td>
<td>8.7 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14.5 ± 0.6</td>
<td>6.92 ± 0.4§</td>
<td>14.5 ± 0.6</td>
</tr>
<tr>
<td><strong>TF/P_{in}</strong></td>
<td>1.42 ± 0.05</td>
<td>2.88 ± 0.42*</td>
<td>1.53 ± 0.06</td>
<td>2.49 ± 0.22*</td>
<td>1.33 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.33 ± 0.06</td>
<td>2.93 ± 0.28*</td>
<td>1.42 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.42 ± 0.06</td>
<td>2.95 ± 0.40*</td>
<td>1.23 ± .03†</td>
</tr>
<tr>
<td><strong>SNGFR, nl/min</strong></td>
<td>17.9 ± 1.0</td>
<td>14.3 ± 0.8*</td>
<td>13.7 ± 0.8*</td>
<td>11.6 ± 0.9*</td>
<td>14.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14.5 ± 1.0</td>
<td>12.0 ± 0.6*</td>
<td>12.3 ± 0.9§</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17.9 ± 0.9</td>
<td>14.9 ± 0.6§</td>
<td>17.9 ± 0.9</td>
</tr>
<tr>
<td><strong>Abs. Reab., nl/min</strong></td>
<td>5.2 ± 0.5</td>
<td>9.0 ± 0.8*</td>
<td>4.5 ± 0.6</td>
<td>6.8 ± 0.7</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.5 ± 0.7</td>
<td>7.6 ± 0.4*</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.6 ± 0.6*</td>
<td>5.6 ± 0.6*</td>
<td>3.2 ± 0.5†</td>
</tr>
<tr>
<td><strong>Frac. Reab</strong></td>
<td>0.29 ± 0.02</td>
<td>0.63 ± 0.04*</td>
<td>0.36 ± 0.06</td>
<td>0.58 ± 0.04*</td>
<td>0.35 ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.35 ± 0.09</td>
<td>0.70 ± 0.05*</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.31 ± 0.05</td>
<td>0.61 ± 0.03*</td>
<td>0.18 ± .02†</td>
</tr>
<tr>
<td><strong>Number of Mice</strong></td>
<td>9</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>Tubules, proximal/distal</strong></td>
<td>9/7</td>
<td>6/6</td>
<td>8/7</td>
<td>8/7</td>
<td>8/7</td>
</tr>
<tr>
<td><strong>Days Post Nx</strong></td>
<td>17 ± 1</td>
<td>20 ± 3</td>
<td>19 ± 1</td>
<td>20 ± 1</td>
<td>20 ± 1</td>
</tr>
</tbody>
</table>

Values (means ± SE); Prox, proximal; Dist, distal; TF/P_{in}, tubular fluid to plasma (H^3) inulin concentration ratio; SNGFR, single-nephron GFR; Abs. Reab, absolute net fluid reabsorption to the point of collection; Frac. Reab., fractional reabsorption. *Paired t test, two tail, proximal vs. distal, P < 0.05; §ANOVA, Tukey post test vs. db/db Nx, P < 0.05; †ANOVA, Tukey post test vs. WT Nx, P < 0.05; ANOVA, Tukey post test vs. db/db sham, P < 0.05; Unpaired t test, two tail, proximal vs. distal, P < 0.05; Unpaired t test, two-tail vs. db/db Nx, P < 0.05. See also Results.
<table>
<thead>
<tr>
<th></th>
<th>Group 1, db/db Nx</th>
<th>Group 2, db/db sham</th>
<th>Group 3, WT Nx</th>
<th>Group 4, WT sham</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></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.6 ± 1.1</td>
<td>5.4 ± 0.9*</td>
<td>9.4 ± 0.4</td>
<td>5.0 ± 0.5*</td>
</tr>
<tr>
<td>TF/P$_{in}$</td>
<td>1.43 ± 0.05</td>
<td>2.93 ± 0.38*</td>
<td>1.46 ± 0.08</td>
<td>2.36 ± 0.17*</td>
</tr>
<tr>
<td>SNGFR, nl/min</td>
<td>17.8 ± 1.3</td>
<td>14.3 ± 1.0*</td>
<td>13.6 ± 0.7</td>
<td>11.5 ± 1.0*</td>
</tr>
<tr>
<td>Abs. Reab., nl/min</td>
<td>5.2 ± 0.5</td>
<td>8.8 ± 0.7*</td>
<td>4.2 ± 0.7</td>
<td>6.5 ± 0.7*</td>
</tr>
<tr>
<td>Frac. Reab.</td>
<td>0.30 ± 0.02</td>
<td>0.63 ± 0.04*</td>
<td>0.34 ± 0.06</td>
<td>0.56 ± 0.03*</td>
</tr>
<tr>
<td>Prox-dist SNGFR, nl/min</td>
<td>3.6 ± 1.0</td>
<td></td>
<td>2.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Dist/Prox SNGFR (%)</td>
<td>82 ± 6</td>
<td></td>
<td>84 ± 5</td>
<td></td>
</tr>
<tr>
<td>Number of mice/pairs</td>
<td>7/8</td>
<td></td>
<td>6/10</td>
<td></td>
</tr>
<tr>
<td>Days Post Nx</td>
<td>19 ± 1</td>
<td></td>
<td>20 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Values (means ± SE); Prox, proximal; Dist, distal; TF/P$_{in}$, tubular fluid to plasma (H$_3$) inulin concentration ratio; SNGFR, single-nephron GFR; Abs. Reab, absolute net fluid reabsorption to the point of collection. *Paired t test, two tail P < 0.05.
Figure 1
Figure 2

db/db mice

WT mice

Figure 2