A Sensitized Screen of N-ethyl-N-nitrosourea–Mutagenized Mice Identifies Dominant Mutants Predisposed to Diabetic Nephropathy

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Diabetic nephropathy (DN) is a late diabetic complication that comprises progressively increasing albuminuria, declining GFR, and increased cardiovascular risk. Only a minority of patients with diabetes (25 to 40%) develop nephropathy, and there is evidence that heritable genetic factors predispose these “at-risk” individuals to DN. Comparing variability among inbred mouse strains with respect to a specific phenotype can model interhuman variability, and each strain represents a genetically homogeneous system with a defined risk for nephropathy. C57BL/6 mice, which are relatively resistant to DN, were mutagenized using N-ethyl-N-nitrosourea and screened for mutants that developed excess albuminuria on a sensitizing type 1 diabetic background contributed by the dominant Akita mutation in insulin-2 gene (Ins2Akita). Two of 375 diabetic G1 founders were found to exhibit albumin excretion rates persistently 10-fold greater than albumin excretion rates in non-mutagenized Ins2Akita controls. This albuminuria trait was heritable and transmitted to approximately 50% of Ins2Akita G2 and G3 progeny, consistent with a simple, dominantly inherited trait, but was never observed in nondiabetic offspring. During the course of 1 yr, albuminuric Ins2Akita G2 and G3 progeny developed reduced inulin clearance with elevated blood urea nitrogen and plasma creatinine. Glomerular histology revealed mesangial expansion, and glomerular basement membrane thickening as determined by electron microscopy was enhanced in diabetic mutant kidneys. Hereditary albuminuric N-ethyl-N-nitrosourea–induced mutants were redesignated as Nphrp1 (nephropathy1) and Nphrp2 (nephropathy2) mice for two generated lines. These novel mutants provide new, robust mouse models of DN and should help to elucidate the underlying genetic basis of predisposition to DN.


Diabetic nephropathy (DN) is the single greatest cause of kidney failure in the United States, accounting for >40% of patients with ESRD (1). DN is a clinical syndrome that is manifested by glomerular sclerosis, progressively worsening albuminuria, declining GFR, and an associated increase in cardiovascular disease (2,3). Importantly, DN is a late complication, occurring after approximately 20 yr of diabetes in only 20 to 40% of all patients with diabetes (4). Although the risk for developing ESRD depends partially on glucose control, even when glucose control is poor (e.g., glycosylated hemoglobin [HbA1c] >10%), only a minority of patients with diabetes develop nephropathy, suggesting that other factors are important (4). Sibling studies show a strong familial component for the risk for developing persistent proteinuria, suggesting a genetic basis for DN risk (5–7). The identity and the mode of inheritance of these genetic modifiers in human DN remain uncertain (6) and are the subject of two large, ongoing, clinical genetic studies: Family Investigation of Nephropathy and Diabetes (FIND) and Genetics of Kidney in Diabetes (GoKinD) (8,9).

As in humans, most inbred strains of diabetic mice do not develop nephropathy, as judged by only mild albuminuria and no fall in normal GFR (10,11). Recent studies in mice indicate that genetic background also may predispose to the development of nephropathy. Whereas C57BL/6 mice are relatively resistant to diabetic kidney disease, other strains, including DBA/2 and KK/HI, seem to exhibit greater diabetic albuminuria (10,11), suggesting the existence of specific modifier loci that confer sensitivity to DN among different strains. Although

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several studies have examined the impact of deletion of candidate genes (e.g., bradykinin B2 receptor, apolipoprotein E) on the progression of DN in mice (12,13), unbiased genome-wide approaches to identify genes that predispose to DN in mice have not been undertaken.

The use of phenotype-driven whole-genome mutagenesis to recover new N-ethyl-N-nitrosourea (ENU)-induced (14) heritable mutations in mice, together with the extensive homology between the mouse and human genomes, makes mutagenesis an attractive approach to discover new genes that predispose to DN. Mutagenesis offers significant advantages for the analysis of the complex traits (15). ENU is a supermutagen of spermatozoa. Mutagenesis offers significant advantages for the analysis of the complex traits (15). ENU is a supermutagen of spermatozoa.

In this study, we performed a sensitized screen of G1 progeny for mutants that exhibit renal dysfunction only in the presence of diabetes as the sensitizing condition. ENU-induced mutations in the nephropathy-resistant C57BL/6 strain were bred into a diabetic environment by crossing mutagenized male mice with diabetic C57BL/6 Ins2Akita (hereafter referred to as Ins2Akita) female mice (19), a model of type 1 diabetes. We report the identification and phenotypic characterization of two heritable mutations that result in dominantly inherited nephropathy that is evident only in a diabetic background.

Materials and Methods

Experimental Animals

All experimental procedures were in compliance with the Vanderbilt University Guide for Care and Use of Laboratory Animals. Mice were housed in a pathogen-free veterinary facility that is accredited by the American Association for the Accreditation of Laboratory Animal Care. Mice were maintained under a controlled 12-h light/dark cycle at a constant temperature of 21 ± 2°C and humidity of 55 ± 10%. Male C57BL/6 and C57BL/6 Ins2Akita female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Carriers of the Akita mutation were housed at the mouse metabolic phenotyping centers (http://www.mmpc.org) that have developed a standard protocol that includes a fast between 7 a.m. and 1 p.m. and blood drawing at 1 p.m. This protocol has been adopted by the Animal Models of Diabetic Complications Consortium (http://www.amdccc.org). Blood glucose was determined using 4 μL of whole blood, freshly collected from saphenous vein (21), using B-glucose analyzer (HemoCueAB, Angelholm, Sweden) after a 6-h fast.

Urine Protein Assays

For detection of albumin/creatinine ratio (ACR; expressed as μg/mg), a 20- to 200-μL volume of spot urine was collected from each mouse that was transferred to the urine collection station that was designed as a corral for individually caged animals. Urinary albumin was detected using Albuwell M kit, and urinary creatinine was measured using the Creatinine Companion murine ELISA kit (Exocell, Philadelphia, PA). For determination of 24-h urinary albumin excretion rate, urine was collected in metabolic cages, and 24 h urinary excretion levels in urine, expressed as μg/24 h, also were assayed with Albuwell M assay kit.

Inulin Clearance

Renal inulin clearance was measured using previously described methods (22,23). Briefly, mice were anesthetized by isoflurane (Baxter Pharmaceutical Products, Deerfield, IL) for 60 s, and sterile FITC-inulin (Sigma) solution was injected retro-orbitally (3.74 μL/g body wt). Plasma (approximately 10 μL) was obtained from blood that was collected via the saphenous vein at 3, 7, 10, 15, 35, and 75 min after bolus FITC-inulin injection. FITC concentration was determined by fluorescence of titrated plasma samples that were loaded onto a 96-well plate using a Fluoroscan Ascent FL (FIN-00811; Labsystems, Helsinki, Finland). GFR was calculated using two-compartment clearance analysis (23).

Plasma Creatinine and Blood Urea Nitrogen

Blood urea nitrogen (BUN) was measured by an iSTAT analyzer (Heska Corp., Waukesha, WI) in 75 μL of whole mouse blood. Plasma creatinine was measured as described previously (24). Briefly, plasma was obtained from whole blood, and plasma proteins were precipitated with cold acetonitrile acidified with glacial acetic acid. After evaporation of acetonitrile and any of the residual aqueous phase in a SpeedVac (Farmington, NY), the residue that contained creatinine was resuspended in 25 μL of 5 mM sodium acetate (pH 4.2). Samples were centrifuged at 3000 rpm for 5 min (microcentrifuge 5415D; Eppendorf, Hamburg, Germany), and supernatants were loaded into the autosampler of an HPLC system (Perkin-Elmer, Wellesley, MA). Creatinine peak elution was detected at 225 nm at 3.65 ± 0.02 min. The concentration of creatinine was determined from a weighted regression formula that was created using an external standard regression line (Perkin-Elmer).

Renal Histopathology and Electron Microscopy

Kidneys were perfused at 140 mmHg with PBS (pH 7.0) followed by 4% paraformaldehyde solution as described previously (10), dissected, and embedded in paraffin, and cross-sections of 4 μm were cut and stained with periodic acid-Schiff. The mesangial expansion score was determined as described previously (25). All glomeruli on single cross-section were examined in each kidney from three animals per group. Mesangial expansion was scored from 0 to 4 according to the proportion of glomerular involvement: score 0, a normal glomerulus; score 1, increased mesangial matrix of up to 25% of glomerular tuft; score 2, mesangial expansion of 25 to 50% of glomerular tuft; score 3, mesangial

Fasting Blood Glucose Detection

The National Institutes of Health has established mouse metabolic phenotyping centers (http://www.mmpc.org) that have developed a protocol for determining fasting blood glucose levels in mice. In this study, mice were fasted for 12 h before blood glucose measurements were made. Blood glucose was determined using 4 μL of whole blood, freshly collected from the tail vein (21), using B-glucose analyzer (HemoCue AB, Angelholm, Sweden) after a 6-h fast.

ENU Mutagenesis

Sixty male C57BL/6 mice (G0) received an intraperitoneal injection of 85 mg/kg ENU (Sigma) each week for 3 wk beginning at 8 to 12 wk of age to produce high mutation rates in this strain (20). After recovery of fertility, G0 mice then were bred with diabetic C57BL/6 Ins2Akita female mice to generate G1 offspring for primary renal phenotype screening.
expansion of 50 to 75%; and score 4, mesangial expansion of >75% of
glomerular tuft. Average tuft score then was obtained for each animal.

Portions of cortex were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), processed, and embedded in Spurr resin. Thin
sections were examined using a FEI/Phillips CM12 transmission electron microscope. Glomerular basement membrane (GBM) was mea-
sured in areas of the cross-section of the GBM as evidenced by endo-
thelial cell appearance. At least four measures were made in each
glomerulus, and an average was calculated. Foot process effacement
was assessed semiquantitatively by estimation of proportion of capil-
lar loops with overlying effacement. All morphologic assessments
were done without knowledge of the animal group.

**Systolic BP Measurements in Conscious Mice**

Systolic BP was determined simultaneously in conscious mice by
using a computerized tail-cuff system (IITC, Life Science, Woodland
Hills, CA) at the Vanderbilt Mouse Metabolic Phenotyping Center.

**Statistical Analyses**

All data are expressed as mean ± SEM. Chance differences proba-
bilites (P) were calculated using ANOVA test. Statistical analysis for
expected inheritance of Ins2Akita mutation in ENU-induced G1 progeny
was performed by χ² test. P < 0.05 was considered to be statistically
different.

**Results**

**Generation of a Population of Diabetic Progeny Carrying ENU Mutations**

Sixty male C57BL/6 G0 mice were treated with three intra-
peritoneal injections of 85 mg/kg ENU at weekly intervals. The
dosage resulted in the expected sterility (20) in all injected male
C57BL/6 mice. Approximately 70% of ENU-injected mice re-
gained their fertility by week 15 after the final ENU injection. ENU-mutagenized male mice were mated with C57BL/6
Ins2Akita heterozygous female mice to produce 429 first-gener-
ation mice (G1 progeny) during a 2-yr period (Figure 1A). A
number of G1 progeny exhibited visible defects such as mi-
crophthalmia, as well as craniofacial and skeletal dysmorphol-
ogy and died in the perinatal period. The remaining 375 G1
survivors were serially screened over 1 yr for a renal pheno-
type.

**Inheritance of Ins2Akita Mutation in G1 Progeny**

PCR genotyping showed that 55% of offspring of non–ENU-
treated parents carried the Ins2Akita mutation, as expected for
inheritance of a dominant trait. In contrast, genotyping of G1
progeny from ENU-mutagenized mice (Figure 1B) demon-
strated that the inheritance of the Ins2Akita mutation signifi-
cantly deviated from the expected 50% (P < 0.0001, χ² test).
Only 29% of G1 progeny (9% female and 20% male) were
Ins2Akita mutation carriers, whereas 71% of mice (32% female
and 39% male) carried wild-type Ins2+/− allele. This decreased
survival of Akita mice was observed only in G1 progeny and
was not observed in subsequent generations of Ins2Akita G2 and
G3 progeny. This is consistent with a decreased survival of
several ENU mutants in the context of the diabetic Ins2Akita
mutation. At 8 wk of age, Ins2Akita G1 progeny exhibited hy-
perglycemia with fasting blood glucose of 415 ± 144 mg/dl in
female mice (n = 34) and 615 ± 116 mg/dl in male mice (n =
74). Fasting blood glucose in age-matched, nonmutagenized
Ins2Akita heterozygous female mice was 293 ± 78 mg/dl (n = 9)
versus 725 ± 85 (n = 7) in male mice. As expected, Ins2+/−
(Akita negative) G1 progeny exhibited lower fasting blood
glucose levels of 189 ± 28 mg/dl in female mice (n = 120) and
200 ± 24 mg/dl in male mice (n = 146).

**Identification of Putative Primary Mutants with Albuminuria**

Starting at 8 wk of age, all Ins2Akita G1 mice were screened for
microalbuminuria every 2 wk for at least 2 mo. Frequency
histograms of these averaged urine ACR values for each animal
were generated (Figure 2; three or more determinations were
averaged). ACR values were transformed to Log10 to “normal-
ize” the ratiometric data. The average ACR of multiple spot
urine samples that were obtained from 23 control diabetic
Ins2Akita male and female mice was 22 ± 10 μg/mg, corre-
sponding to a median Log10ACR value of 1.30. In contrast, the
median for the Log10ACR for mutagenized Ins2Akita G1 progeny
was 1.64. Ten G1 outliers that exhibited an ACR ≥100 μg/mg
(Log10ACR ≥2) were identified, exceeding this empiric upper
limit for ACR values in nonmutagenized Ins2Akita mice.

**Urinary Albumin Excretion in ENU-Induced G1 Phenotyping Variants**

The progression of albuminuria in the outliers was assessed by
sequential monitoring every 2 wk during the first 4 mo of
life and then monthly for up to 1 yr to confirm the phenotype.
ACR in urine from the remainder of the G1 mice (i.e., nonout-
liers) were examined at least six times in a 12-mo period.
Among the 10 outliers initially identified, albuminuria per-
stained only in six Ins2Akita G1 phenotypic variants that were
derived from different ENU-mutagenized C57BL/6 male
mice. These phenotypic variants, identified as ENU10, ENU18,
ENU20, ENU57, ENU76, and ENU161, showed significantly
that unlinked to the dominant mutation that is responsible for diabetic albuminuria. Ants were bred with C57BL/6 female mice. For a simple G2 P/H11021 ENU161) transmitted the albuminuric trait to diabetic four G1 phenotypic variants (ENU10, ENU20, ENU76, and produce any albuminuric offspring in G2 progeny. However, Two of G1 phenotypic variants (ENU18 and ENU57) failed to six pups, and of the four diabetic G2 offspring. The ENU10 founder produced only one litter of G2 offspring. The ENU10 founder produced only one litter of G1 phenotypic variants (ENU10, ENU20, ENU76, and notypes were heritable, these six G1 progeny and five (45.5%) of 11 Ins2Akita mice from both mutant lines to generate G3 progeny confirmed the transmission of albuminuria in 46.7% of ENU76 (seven of 15 Ins2Akita mice) and 47.6% ENU20 (10 of 21 Ins2Akita mice) diabetic offspring, respectively. Importantly, none of the nondiabetic Ins2+/+ G3 progeny of the ENU76 line (20 of 35 mice) or the ENU20 line (22 of 43 mice) exhibited albuminuria. In contrast, approximately one third of nondiabetic Ins2+/+ G3 mice in the ENU161 line exhibited albuminuria, indicating that the ENU-induced renal phenotype in ENU161 is distinct from that in ENU20 and ENU76 and not dependent on diabetes as a sensitizing condition. Hereditary albuminuric ENU-induced mutants were redesignated as Nphrp1 (nephropathy1 for ENU20 line) and Nphrp2 (nephropathy2 for ENU76 line) mice. Progeny of Nphrp1 and Nphrp2 mutants exhibited significantly increased ACR in diabetic Ins2Akita mice but not in nondiabetic Ins2+/+ mice. Half of the diabetic Ins2Akita mice from both Nphrp1 and Nphrp2 mutant lines exhibited albuminuria (averaging 153 ± 93 and 140 ± 106 μg/mg, respectively) versus 22 ± 10 μg/mg ACR in control Ins2Akita mice, again consistent with the presence of dominant mutations that cause diabetic albuminuria. Albuminuria was confirmed by measurement of 24-h urinary albumin excretion rates (AER) in progeny from Nphrp1 and Nphrp2 founders. AER was significantly greater in Nphrp1/Ins2Akita (227 ± 139 μg/24 h; n = 10) and Nphrp2/Ins2Akita mice (116 ± 12 μg/24 h; n = 4) versus control Ins2Akita mice (36 ± 14 μg/24 h; n = 8; Figure 3B) at the average age of 9 mo. Progressively increasing albuminuria was observed in both mutant lines during a period of 19 mo (Figure 4). By 3 mo of age, mutant mice from both lines exhibited elevated ACR (93 ± 19 μg/mg for Nphrp1, n = 26) and 81 ± 14 μg/mg for Nphrp2 (n = 30). ACR continued to

Figure 2. Identification of albumin/creatinine ratio (ACR) outliers in diabetic G1 progeny. C57BL/6 (Akita−) and control Ins2Akita mice (Akita+; top) and G1 Ins2+/+ mice (ENU+ Akita−; bottom left) follow a normal distribution in LogACR values. This is in contrast to the bimodal distribution that was observed in G1 Ins2Akita heterozygotes that inherited ENU mutation (ENU+ Akita+; bottom right). Among 10 identified outliers during primary screening (circled bars), six phenotypic variants showed persistent ACR (see Figure 3) during 1 yr.

greater urinary ACR than control Ins2Akita mice from non-mutagenized sires (223 ± 141, 199 ± 150, 177 ± 136, 214 ± 134, 302 ± 144, and 107 ± 129, respectively, versus 18 ± 15 μg/mg; P < 0.005; Figure 3A). Importantly, urine ACR in Ins2+/+ (Akita negative) progeny from mutagenized G0 sires was not different from control Ins2Akita mice, and increased ACR was observed in ENU-induced phenotyping variants in only the setting of diabetes.

Inheritance of ENU-Induced Mutations in G2 and G3 Progeny from G1 Founders

To confirm the genetic transmission of ENU-induced mutations and determine whether these diabetic albuminuria phenotypes were heritable, these six G1 Ins2Akita phenotypic variants were bred with C57BL/6 female mice. For a simple dominant mutation that is responsible for diabetic albuminuria that is linked to the Ins2Akita mutation, approximately 50% of G2 Ins2Akita diabetic heterozygotes should exhibit albuminuria. Two of G1 phenotypic variants (ENU18 and ENU57) failed to produce any albuminuric offspring in G2 progeny. However, four G1 phenotypic variants (ENU10, ENU20, ENU76, and ENU161) transmitted the albuminuric trait to diabetic Ins2Akita G2 offspring. The ENU10 founder produced only one litter of six pups, and of the four diabetic Ins2Akita G2 offspring, two were albuminuric and sterile and exhibited low sperm count (sperm was collected for cryopreservation). ENU20 and ENU76 founders produced five litters each, with an average of approximately four and six pups per litter, respectively. As was expected for a dominant trait, six (46.1%) 13 Ins2Akita ENU76 G2 progeny and five (45.5%) of 11 Ins2Akita ENU20 G2 progeny increased albuminuria. Breeding of albuminuric G2 mice from both mutant lines to generate G3 progeny confirmed the transmission of albuminuria in 46.7% of ENU76 (seven of 15 Ins2Akita mice) and 47.6% ENU20 (10 of 21 Ins2Akita mice) diabetic offspring, respectively. Importantly, none of the nondiabetic Ins2+/+ G3 progeny of the ENU76 line (20 of 35 mice) or the ENU20 line (22 of 43 mice) exhibited albuminuria. In contrast, approximately one third of nondiabetic Ins2+/+ G3 mice in the ENU161 line exhibited albuminuria, indicating that the ENU-induced renal phenotype in ENU161 is distinct from that in ENU20 and ENU76 and not dependent on diabetes as a sensitizing condition.

Hereditary albuminuric ENU-induced mutants were redesigned as Nphrp1 (nephropathy1 for ENU20 line) and Nphrp2 (nephropathy2 for ENU76 line) mice. Progeny of Nphrp1 and Nphrp2 mutants exhibited significantly increased ACR in diabetic Ins2Akita mice but not in nondiabetic Ins2+/+ mice. Half of the diabetic Ins2Akita mice from both Nphrp1 and Nphrp2 mutant lines exhibited albuminuria (averaging 153 ± 93 and 140 ± 106 μg/mg, respectively) versus 22 ± 10 μg/mg ACR in control Ins2Akita mice, again consistent with the presence of dominant mutations that cause diabetic albuminuria. Albuminuria was confirmed by measurement of 24-h urinary albumin excretion rates (AER) in progeny from Nphrp1 and Nphrp2 founders. AER was significantly greater in Nphrp1/Ins2Akita (227 ± 139 μg/24 h; n = 10) and Nphrp2/Ins2Akita mice (116 ± 12 μg/24 h; n = 4) versus control Ins2Akita mice (36 ± 14 μg/24 h; n = 8; Figure 3B) at the average age of 9 mo. Progressively increasing albuminuria was observed in both mutant lines during a period of 19 mo (Figure 4). By 3 mo of age, mutant mice from both lines exhibited elevated ACR (93 ± 19 μg/mg for Nphrp1, n = 26) and 81 ± 14 μg/mg for Nphrp2 (n = 30). ACR continued to

Figure 3. (A) ACR in ENU-induced albuminuric variants. Each column represents the mean ± SEM for at least six measurements during 15 mo for each phenotypic variant. *P < 0.005 versus control mice. (B) Twenty-four-hour albumin excretion rates in G2 and G3 progeny from two fertile ENU-induced albuminuric founders. □, control Ins2Akita mice (n = 8); ■, Nphrp1/Ins2Akita mice (ENU20 diabetic progeny; n = 10); △, Nphrp2/Ins2Akita mice (ENU76 diabetic progeny; n = 4). **P < 0.05 for ENU founders’ Ins2Akita progeny versus control Ins2Akita mice. Data are means ± SEM.
increase with age, with ACR of 187 ± 47 and 376 ± 115 μg/mg in \textit{Ins2Akita} (n = 8; \( P < 0.05 \)) and \textit{Nphrp2} (n = 7; \( P < 0.05 \)) mice, respectively. These values were four- and eight-fold higher than control \textit{Ins2Akita} mice (45 ± 4 μg/mg; \( n = 6 \)) at 19 mo of age.

Reproductive Histopathology in Diabetic \textit{Nphrp1}/\textit{Ins2Akita} and \textit{Nphrp2}/\textit{Ins2Akita} Mutants

Reproductive histopathology was examined in albuminuric \textit{Ins2Akita} G3 progeny of both \textit{Nphrp1} and \textit{Nphrp2} mutant lines after 9 to 12 mo of sustained hyperglycemia (Figure 5, A through C). \textit{Ins2Akita} G3 progeny from \textit{Nphrp1} and \textit{Nphrp2} founders exhibited significantly greater mesangial expansion with scores of 1.49 ± 0.38 (n = 4) and 1.47 ± 0.28 (n = 3), respectively, with increased mesangial periodic acid-Schiff–positive staining versus only mild mesangial expansion in glomeruli from nonmutagenized \textit{Ins2Akita} C57BL/6 mice (score 0.71 ± 0.16; \( n = 4 \); Figure 5G). Electron microscopy (Figure 5, D through F) confirmed increased mesangial matrix and also demonstrated greater GBM thickening of 406 ± 32 (P < 0.05) and 358 ± 19 nm (P < 0.05) in \textit{Nphrp1}/\textit{Ins2Akita} and \textit{Nphrp2}/\textit{Ins2Akita} mutants versus 244 ± 25 nm in control diabetic \textit{Ins2Akita} mice (Figure 5H) despite that HbA1c values were not different among these three groups. In addition, foot process effacement was greater (5 to 30%) in both mutant lines. These morphologic changes are consistent with DN in the ENU-induced mutants.

Renal Function in \textit{Ins2Akita} G2 and G3 Progeny from \textit{Nphrp1} and \textit{Nphrp2} Founders

Renal function was assessed in diabetic \textit{Nphrp1}/\textit{Ins2Akita} (n = 11) and \textit{Nphrp2}/\textit{Ins2Akita} (n = 6) mice and compared with age-matched control \textit{Ins2Akita} mice (n = 9). Both the \textit{Nphrp1} and \textit{Nphrp2} lines exhibited renal enlargement with increased absolute kidney weight and kidney:body weight ratios of 10.92 ± 0.72 and 11.73 ± 0.84 versus 7.83 ± 1.67 mg/g, than in control \textit{Ins2Akita} mice (P < 0.005 for both mutant lines; Table 1). Importantly, \textit{Nphrp1}/\textit{Ins2Akita} and \textit{Nphrp2}/\textit{Ins2Akita} mice also exhibited significantly increased BUN and plasma creatinine at 30 to 40 wk of age compared with diabetic control \textit{Ins2Akita} mice of the same age (Table 1). Furthermore, FITC-inulin clearance was reduced in progeny from both \textit{Nphrp1}/\textit{Ins2Akita} (330 ± 90 μl/min per mouse; \( P < 0.05 \)) and \textit{Nphrp2}/\textit{Ins2Akita} mutants (320 ± 50 μl/min per mouse; \( P = 0.005 \)) compared with control \textit{Ins2Akita} mice (520 ± 120 μl/min per mouse; Figure 6), confirming decreased renal function in these mice.

Although blood glucose and HbA1c levels were not different between mutants and control \textit{Ins2Akita} mice, plasma lipids (triglycerides and cholesterol) were increased significantly in both mutant lines compared with diabetic control \textit{Ins2Akita} mice (Table 1). No significant differences in the white blood cell (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) or platelet count, mean corpuscular volume, and mean corpuscular hemoglobin were observed in the complete blood count in the same age (Table 1).
GFR was measured by FITC-inulin clearance in conscious mice.

Table 1. Anatomic and metabolic profiles of ENU-induced mutant mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Ins2Akita (n = 9)</th>
<th>Nphrp1/Ins2Akita (n = 11)</th>
<th>Nphrp2/Ins2Akita (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>26.40 ± 2.28</td>
<td>23.56 ± 1.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.38 ± 2.30&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Kidney weight (left; mg)</td>
<td>197.86 ± 30.65</td>
<td>247.86 ± 19.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>249.33 ± 20.89&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney weight:body weight (mg/g)</td>
<td>7.83 ± 1.67</td>
<td>10.92 ± 0.72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.73 ± 0.84&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>30.14 ± 2.16</td>
<td>40.30 ± 4.68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39.10 ± 1.80&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dl)</td>
<td>0.089 ± 0.003</td>
<td>0.110 ± 0.029&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.134 ± 0.030&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>688 ± 17</td>
<td>697 ± 44</td>
<td>684 ± 22</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>46.11 ± 2.25</td>
<td>47.7 ± 3.89</td>
<td>48.67 ± 2.22</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>15.6 ± 0.8</td>
<td>16.0 ± 1.3</td>
<td>16.4 ± 0.7</td>
</tr>
<tr>
<td>Hba1c (%)</td>
<td>12.4 ± 0.2</td>
<td>12.13 ± 0.17</td>
<td>12.0 ± 1.28</td>
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<td>Triglycerides (mg/dl)</td>
<td>63.33 ± 13.20</td>
<td>102.71 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>143.25 ± 36.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>70.10 ± 1.03</td>
<td>107.00 ± 12.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>144.25 ± 25.97&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>116 ± 9</td>
<td>119 ± 12</td>
<td>128 ± 2</td>
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</tbody>
</table>

<sup>a</sup>Data are means ± SEM. Plasma creatinine was measured by HPLC. BUN, blood urea nitrogen; ENU, N-ethyl-N-nitrosourea; HbA1c, glycosylated hemoglobin.
<sup>b</sup>P < 0.05, <sup>c</sup>P < 0.005 versus control group.

Figure 6. GFR in progeny from Nphrp1 and Nphrp2 founders. GFR was measured by FITC-inulin clearance in conscious mice. □, control Ins2Akita mice (n = 8); ■, Nphrp1/Ins2Akita mutants (n = 10); ▭, Nphrp2/Ins2Akita mutants (n = 11). *P < 0.05, **P = 0.005 for Nphrp1/Ins2Akita and Nphrp2/Ins2Akita mice versus control Ins2Akita mice. Data are mean ± SEM.

Discussion

Strong familial factors that predispose to DN were reported previously (26,27). For example, there is a striking difference between the incidence of nephropathy in diabetic siblings of patients with DN versus diabetic siblings of patients without DN. When an index patient with type 1 diabetes had persistent proteinuria, the risk for proteinuria in the patient’s sibling with diabetes was 71 versus only 25% risk when the index patient was not proteinuric (6,27). Likewise, the role of dominant modifiers that predispose to DN was supported by studies of diabetic Pima Native Americans (28). Most recent studies have focused on associations between certain candidate genes and DN, including the carnosinase gene (29), RANTES receptor gene in immunocompetent cell (30), and engulfment and cell motility gene 1 (31). In more diverse, less homogeneous populations, both recessive and dominant modifiers are likely to play important roles (6). Elucidation of the pathogenesis of DN will be critical to the development of therapeutic interventions that aim to normalize renal function in these patients.

Murine models of DN theoretically offer significant advantages over human studies in the experimental identification of modifier genes. Recent studies indicate that, as in humans, most mice do not develop nephropathy. The genetic background of a mouse (e.g., different inbred mouse strains) determines the predisposition to the development of DN, with some strains being more prone to albuminuria than others (10,11). C57BL/6 mice show relative resistance to nephropathy either from low-dosage streptozotocin (STZ)-induced diabetes or in diabetes that is induced by the Ins2Akita mutation (10). In contrast, in the same studies, STZ-induced diabetic DBA/2 mice exhibited six-fold greater ACR than C57BL/6 mice, suggesting the presence of specific genes that confer relative protection in C57BL/6 mice. Differential susceptibility to DN in inbred mouse strains provides a possible approach for the genetic dissection of this diabetic complication.

To provide insight into the identity of genes that are important for the resistance of C57BL/6 to DN, we undertook a phenotype-driven screen of C57BL/6 diabetic Ins2Akita mice that also inherited a high load of paternally derived ENU mutations. The diabetic Ins2Akita mouse is a model of type 1 diabetes, carrying a heterozygous mutation in insulin-2, resulting in insulin misfolding and autosomal dominant diabetes (19). Homozygous C57BL6 Ins2Akita mice fail to thrive, and they die within 1 to 2 mo, but heterozygous mice are viable and fertile and exhibit hyperglycemia, hypoinsulinemia, polydipsia, and polyuria by 3 to 4 wk of age (19,32), making them an attractive tool for physiologically sensitizing the background so
that new diabetes-dependent renal dysfunction mutations might be identified.

When ENU mutagenized male mice were intercrossed with Ins2^Akita^ female mice, the first-generation offspring (G1) exhibited multiple abnormal phenotypes, including neonatal and juvenile lethality, microphthalmia, and craniofacial and skeletal dysmorphology. Survival of Ins2^Akita^ carriers also was diminished significantly in these G1 progeny. Adverse interaction between the Ins2^Akita^ mutation and ENU-induced mutations could contribute to lethality at embryonic or neonatal stages. However, we did not detect either major anatomic abnormalities or decreased survival in G2 and G3 mice that were generated from G1 founders.

We used albuminuria as the primary screen for nephropathy mutants. In humans, microalbuminuria (AER of 30 to 300 mg/24 h) has been used widely as a marker to identify patients who are at risk for DN (33). In human DN, the onset of microalbuminuria generally is followed several years later by overt proteinuria (AER >300 mg/24 h) and then subsequent renal failure (34–36). Similar thresholds for urine AER in mice that signify glomerular disease have not been established firmly and are likely to be variable among different mouse strains. Albuminuria is not a prominent feature in diabetic C57BL/6 Ins2^Akita^ mice (10,37). In our study, nonmutagenized C57BL/6 Ins2^Akita^ mice exhibited an average AER of 36 ± 14 µg/24 h, in agreement with a previous report (38). Among G1 progeny that were generated from mating between ENU-mutagenized male and female C57BL/6 Ins2^Akita^ heterozygotes, we identified six Ins2^Akita^ outliers that exhibited elevated ACR. True G1 genetic variants were confirmed by test-crosses of all six outliers with wild-type partners. Of these six diabetic founders, two confirmed genetic variants (ENU20 and ENU76) bred true and produced following generations of mice with renal phenotype. Importantly, albuminuria in the two generated lines (ENU20, or Nphpr1, and ENU76, or Nphpr2) was transmitted only to half of diabetic progeny but not to nondiabetic progeny. This is consistent with dominantly heritable mutations that segregate independent of the Ins2^Akita^ mutation and that result in renal disease only in the setting of diabetes. In contrast, increased albuminuria in 50% of all G3 progeny from ENU161 founder was evident in the absence of Ins2^Akita^ mutation. The cause of albuminuria in ENU161 progeny therefore is unlikely to be related to DN, as opposed to the results that were obtained for Nphpr1 and Nphpr2 lines.

The nephropathy that characterizes these two ENU-induced mutant lines was of comparable severity to the reported low-dosage STZ-treated DBA/2, db/db C57BLKS/6, and FVB OVE26 inbred strains that seem to be prone to DN and significantly more severe than in the relatively resistant STZ-treated wild-type C57BL/6 mice (Table 2). Daily AER in Nphpr1/Ins2^Akita^ and Nphpr2/Ins2^Akita^ mice were similar to diabetic DBA/2 and db/db C57BLKS mice at the age of approximately 9 mo (39,40) (Table 2). The AER in FVB/OVE26 mice (41) reportedly exceeded 15,000 µg/24 h, but they also exhibited hydronephrosis, an element that was not observed in our ENU-induced mutants or in STZ-treated FVB mice (10).

Reduction in GFR is a critically important feature of DN that is missing from most models. In these studies, GFR was determined using FITC-inulin clearance (22,23) and was significantly lower in Nphpr1 and Nphpr2 mutant lines than Ins2^Akita^, a feature that is lacking in db/db C57BLKS (42) and STZ-treated DBA/2 and C57BL/6 mice (10) (Table 2). Renal function decline was confirmed by measurement of BUN and HPLC plasma creatinine in both ENU-induced mutant lines. In this study, the finding of renal functional impairment in diabetic mutant mice is novel and consistent with the identification of two new, robust, and heritable mouse models of DN.

In humans, renal histopathologic alterations that are associated with the development of overt diabetic proteinuria include GBM thickening and mesangial expansion. As albuminuria and renal insufficiency progress, glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial fibrosis develop (43,44). In mice, pathologic criteria that are predictive of renal insufficiency have not been established, largely because renal failure was not reported previously in mouse models of DN (37). In these studies, in addition to reduced GFR, we observed glomerular lesions consistent with DN in these two ENU-induced DN lines. Light microscopy and ultrastructural evaluation revealed diffuse mesangial matrix expansion, foot process effacement, and increased GBM thickening, consistent with the development of DN. Although we did not observe nodular glomerulosclerosis or arteriolar hyalinosis in ENU-induced mutants, mesangial matrix expansion in Nphpr1/Ins2^Akita^ and Nphpr2/Ins2^Akita^ mice was comparable to that in all analyzed DN mouse models (Table 2). The degree of basement membrane thickening also was greater in Nphpr1 and Nphpr2 than in STZ-treated C57BL/6 (10) and db/db C57BLKS/6 (45) mice and similar to STZ-treated DBA/2 (10) and FVB/OVE26 (46) (Table 2). It is notable that although fasting blood glucose levels and HbA1c values were indistinguishable in the Ins2^Akita^ mutants from Nphpr1 and Nphpr2 lines versus control Ins2^Akita^ mice, GBM thickening was significantly greater in the mutants, suggesting that these novel mutations per se, rather than hyperglycemia alone, contribute to increased GBM deposition.

In addition to albuminuria and renal function decline, hypertension and hyperlipidemia have been implicated in progression of DN. Hypertension may occur early in the course of DN, and BP rises further as GFR falls. The rate of decline in GFR does not seem to be related directly to BP, although antihypertensive treatment seems to retard the rate of decline (47). Nphpr2 offspring showed a trend for increased systolic BP; this did not achieve statistical significance as compared with control Ins2^Akita^ mice. Although mutant mice from both lines were phenotypically similar (with albuminuria, glomerular sclerosis, and renal function decline), it is unlikely that both mutant lines carry identical mutations. These lines derived from different ENU-induced G1 founders. Furthermore, plasma creatinine (P = 0.03), triglycerides (P = 0.048), and cholesterol (P = 0.004) were significantly higher in Nphpr2/Ins2^Akita^ mice than in Nphpr1/Ins2^Akita^ mutants. Dyslipidemia is a common feature of humans with DN (48,49); however, whether this is a consequence of renal injury remains uncertain. Similarly, whether Nphpr1 and Nphpr2 mutations result in primary dyslipidemia or this is a consequence of DN per se remains to be determined.
Table 2. Renal characteristics in mouse models of diabetic nephropathy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Nphrp1/Ins2Akita</th>
<th>Nphrp2/Ins2Akita</th>
<th>Low-dosage STZ C57BL/6</th>
<th>Low-dosage STZ DBA/2</th>
<th>Db/db C57BlKS</th>
<th>FVB OVE26</th>
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<tbody>
<tr>
<td>Control (µl/min)</td>
<td>120</td>
<td>150</td>
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<td>100</td>
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<td>GBM (nm)</td>
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<td>325</td>
<td>325</td>
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</tr>
<tr>
<td>AER (µg/24 h)</td>
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<td>0.16</td>
<td>0.16</td>
<td>0.16</td>
<td>0.16</td>
<td>0.16</td>
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</tr>
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References

AER, albumin excretion ratio; CM, control mice; DM, diabetic mice; GBM, glomerular basement membrane; STZ, streptozotocin.

Conclusion

These studies support the existence of dominant genetic modifiers contributing to the development of DN. This phenotype-driven screen identified two independent heritable dominant mutations that result in renal disease only in diabetic mice. These two new mutant lines (Nphrp1 and Nphrp2) exhibit several critical features of DN, including late onset of increased plasma creatinine, decreased inulin clearance, progressive albuminuria, and histopathologic changes that are characteristic of DN. Insight into phenotypic similarities and disparities and the genetic mechanisms of renal decline in Nphrp1 and Nphrp2 mutants should be provided by mapping and identification of the genes that are targeted by these two ENU-induced mutations. It is hoped that this information will accelerate both the understanding of the pathogenesis of DN and progress toward a treatment for this major devastating complication of diabetes.

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Disclosures

None.

References


