

Chapter 2

Assessment of Diabetic Nephropathy in the Akita Mouse

Jae-Hyung Chang and Susan B. Gurley

Abstract

Akita mice have type 1 diabetes mellitus caused by a spontaneous point mutation in the *Ins2* gene which leads to misfolding of insulin, resulting in pancreatic β -cell failure. Akita mice develop pronounced and sustained hyperglycemia, high levels of albuminuria, and consistent histopathological changes, suggesting that these mice may be suitable as an experimental platform for modeling diabetic nephropathy. One key feature of diabetic kidney disease in Akita mice is that the severity of renal injury is significantly influenced by genetic background. In this chapter, we describe the Akita model and present some of the experimental studies utilizing Akita mice as a model of type 1 diabetes. For example, deficiency in bradykinin receptors, endothelial nitric oxide synthase, or angiotensin-converting enzyme 2 leads to development of functionally and structurally more advanced diabetic nephropathy in these mice, while ketogenic diet has been shown to reverse kidney injury associated with diabetes. This chapter also describes the application of 24-h urine collections from mice for careful measurement of urinary albumin excretion.

Key words: Mouse model of type I diabetes, Diabetic nephropathy, Genetic susceptibility, Albuminuria, Glomerular filtration rate, Bradykinin receptor, Endothelial nitric oxide synthase, Angiotensin-converting enzyme 2, Ketogenic diet, Mouse metabolic cage, 24-h urine collection

1. Introduction: The Akita Mouse as an Experimental Model of Diabetic Nephropathy

Diabetes mellitus is a serious public health problem with incidence reaching epidemic proportions in developed countries (1, 2). Among its complications, diabetic nephropathy (DN) is a leading cause of chronic kidney failure and end-stage renal disease (ESRD) worldwide (1–3). DN is characterized by microalbuminuria as the earliest detectable sign of glomerular injury followed by the development of macroalbuminuria and a progressive decline in glomerular filtration rate (GFR) (4). Histopathological hallmarks of the disease are glomerular mesangial matrix expansion, nodular glomerulosclerosis, and tubulointerstitial fibrosis (5). Consequently, much effort has been devoted to understanding disease mechanisms

in DN with the goal of developing new treatment strategies for this devastating disorder. In this regard, mouse models of diabetes mellitus are useful for studying both pathogenesis and treatment of kidney disease, in particular because of the potential for genetic manipulation of the mouse genome (6). However, while significant progress has been made in the past few years, current mouse models still do not display the full spectrum of functional and pathological characteristics of human DN (6, 7).

The Akita mouse, which is a recently described genetic model of type 1 diabetes mellitus, shows promise in this regard (6, 8, 9). Akita mice harbor an autosomal dominant, spontaneous point mutation which was originally identified in a colony of C57BL/6 mice in Akita, Japan (10). This mutation introduces a Cys to Tyr substitution at the seventh amino acid in the A chain of mature insulin (*Ins2^{C96Y}*) and leads to a disruption of an intramolecular disulfide bond (11). This causes improper folding of the insulin protein resulting in selective proteotoxicity to the pancreatic β -cell (6, 11). Subsequently, islets from Akita mice are depleted of β -cells, and those remaining β -cells release very little mature insulin (11). While homozygosity for the mutation leads to perinatal lethality, mice heterozygous for the Akita mutation are viable and fertile, and develop spontaneous hyperglycemia around 3–4 weeks of age.

One clear finding from studies of Akita mice is that the renal phenotype of these mice is significantly influenced by their genetic background (6, 8, 9). The heterogeneity of disease severity across different strains suggests that genetic determinants might modulate susceptibility to kidney injury from diabetes in Akita mice as it has been previously shown to affect susceptibility to DN in humans (12). Thus far, a number of Akita mice on different inbred genetic background have been created and characterized, and studies using these mice have been performed to better understand pathogenic mechanisms of DN as well as to evaluate novel treatment strategies (8, 9). Table 1 provides a comparison of the DN-related features exhibited by these mouse models. In this chapter we present the key features of several experimental studies in DN that have been conducted using Akita mice as a model of kidney injury associated with type 1 diabetes mellitus and describe in detail our current protocol for 24-h urine collection in mice for urine albumin measurements, an important component of the renal phenotyping process.

1.1. *Ins2-Akita Mutation Versus Streptozotocin-Induced Diabetes in Mice*

STZ has been used widely to induce type 1 diabetes in rodents (6, 9, 13). Streptozotocin (STZ) causes diabetes in animals secondary to pancreatic β -cell failure, however, it may be toxic to a variety of other tissues which complicates interpretation of results (6) and also requires administration of a drug to animals. To investigate potential differences in the character of kidney injury between chemical and genetic models of type 1 diabetes, we compared C57BL/6 mice treated with STZ with C57BL/6 Akita mice (9).

Table 1
Summary of selected Akita mouse models used in diabetic nephropathy research

Mouse strains	Blood pressure	Blood glucose	Albuminuria ^a	Glomerular filtration rate	Renal structure	Primary references
C57BL/6	↑	↑	1+	↑	Mesangial expansion (mild)	(8, 9)
129/SvEv	↑	↑	3+	↑	Mesangial expansion (mild)	(8)
DBA/2	=	↑	7+	Not reported	Unchanged	(8)
FVB/NJ	↑	↑	10+	↑	Mesangial expansion (mild-to-moderate)	(Chang J. H. et al. unpublished)
F1(D2:B6)	↑	↑	5+	↑	Mesangial expansion (mild)	(8)

^aAlbuminuria scale 1 + ≈50 µg/day

Throughout the study period, male Akita mice developed hyperglycemia that was more pronounced and durable than that observed in the STZ model (518 ± 25 vs. 388 ± 50 mg/dL at 6 months of age). Male C57BL/6 Akita mice also developed mild hypertension and decrease in heart rates compared to their nondiabetic controls while C57BL/6 mice treated with STZ tended to have reduced BPs. Moreover, male Akita mice on C57BL/6 background developed more severe albuminuria, mesangial expansion, and renal and glomerular hypertrophy than the male mice with STZ-induced diabetes. These studies comparing both models on the same genetic background suggest that the Akita model offers advantages over chemical models of diabetes and thus, it may provide a better experimental platform for DN model development.

1.2. Impact of Genetic Background on Diabetic Kidney Injury in Akita Mice

In humans with diabetes, it is well established that there is a clear-cut genetic susceptibility to the development of kidney injury (12). Genetic background may also influence the development of diabetic renal complications in mice (8, 9, 13). In this regard, studies in mice with STZ-induced diabetes provided evidence for differential susceptibility to kidney injury among genetically distinct mouse lines (9, 13). To further test this hypothesis using the Akita model, we recently characterized renal manifestations of diabetes in several inbred strains of Akita mice (C57BL/6-*Ins2*^{C96Y/+}, DBA/2-*Ins2*^{C96Y/+}, and 129SvEv-*Ins2*^{C96Y/+}) (8). Male Akita mice on all three backgrounds developed marked and equivalent hyperglycemia compared with their respective wild-type controls. The marked levels of hyperglycemia were sustained through the 6-month study period. However, despite similar levels of hyperglycemia, there were significant differences in the magnitude of albuminuria

among the three Akita strains at 6 months of age. While the absolute level of urinary albumin excretion was highest in the DBA/2-*Ins2^{C96Y/+}* group (345 ± 110 $\mu\text{g/day}$), it was lowest in the C57BL/6-*Ins2^{C96Y/+}* mice (40 ± 3 $\mu\text{g/day}$), and intermediate in the 129SvEv-*Ins2^{C96Y/+}* group (151 ± 77 $\mu\text{g/day}$). Compared with their respective nondiabetic controls, there was a fivefold increase in urinary albumin excretion in the 129SvEv-*Ins2^{C96Y/+}* animals (151 ± 77 $\mu\text{g/day}$ (Akita) vs. 33 ± 20 $\mu\text{g/day}$ (control); $P=0.03$), threefold increase in the DBA/2-*Ins2^{C96Y/+}* group (345 ± 110 $\mu\text{g/day}$ (Akita) vs. 116 ± 27 $\mu\text{g/day}$ (control); $P=0.048$), and 2.8-fold increase in the C57BL/6-*Ins2^{C96Y/+}* mice (40 ± 3 $\mu\text{g/day}$ (Akita) vs. 14 ± 2 $\mu\text{g/day}$ (control); $P<0.0001$). Similarly, estimated GFR was significantly higher in the 129SvEv-*Ins2^{C96Y/+}* mice compared to the C57BL/6-*Ins2^{C96Y/+}* group at 6 months of age (24.5 ± 1.9 $\mu\text{L/min/g}$ body weight vs. 16.0 ± 1.3 $\mu\text{L/min/g}$ body weight; $P=0.0004$). At this point in the disease process, increased GFR represents hyperfiltration. Examination of the kidneys by light microscopy revealed modest mesangial matrix expansion of similar severity in diabetic 129SvEv and C57BL/6 mice compared to their respective nondiabetic controls (2- to 2.5-fold increase in mesangial score) with preservation of normal morphology in the tubular and interstitial regions.

To assess the heritability of strain-specific modifiers affecting susceptibility to kidney injury associated with diabetes in Akita mice, we extended these studies to include a cohort of F1 intercross mice between the two strains, C57BL/6 and DBA/2 (8), with C57BL/6 being a strain resistant to developing DN and DBA/2 as a susceptible strain. These F1(DBA/2 $\times$ C57BL/6) *Ins2^{C96Y/+}* animals developed robust and sustained hyperglycemia similar to the other strains examined in our study. Interestingly, however, the extent of albuminuria in the F1-*Ins2^{C96Y/+}* mice was equivalent to the parental DBA/2-*Ins2^{C96Y/+}* line (240 ± 54 $\mu\text{g/day}$ vs. 345 ± 110 $\mu\text{g/day}$; $P=\text{NS}$) while that of mesangial pathology was very similar to that seen in the parental C57BL/6-*Ins2^{C96Y/+}* line. These studies suggest that there is a dominant pattern of susceptibility alleles for albuminuria from the DBA/2 strain. In a separate set of experiments, we determined the functional and structural characteristics of kidney disease in FVB/NJ Akita mice. In this study, male FVB/NJ Akita mice developed sustained hyperglycemia and albuminuria by 4 and 8 weeks of age, respectively. By 20 weeks of age, diabetic animals developed a tenfold increase in albuminuria (480 ± 73 $\mu\text{g/day}$ (Akita) vs. 40 ± 7 $\mu\text{g/day}$ (control); $P<0.0001$) as well as renal and glomerular hypertrophy. Levels of albuminuria in FVB/NJ Akita mice suggest a moderate to severe susceptibility to renal injury resulting from type 1 diabetes, with 24-h urinary albumin excretion values being greater than the strains reported by Gurley and coworkers (8, 9). Mild-to-moderate glomerular mesangial expansion was also observed in FVB/NJ Akita mice at 30 weeks of age (Chang J.H. et al., unpublished

observation). Therefore, FVB/NJ Akita mice develop significant renal injury that may make this strain suitable for disease modeling.

Together, these findings provide evidence for strong genetic modifiers influencing kidney phenotypes associated with diabetes, such as albuminuria and mesangial pathology. Identification of these naturally occurring genetic modifiers among different genetic backgrounds may allow mapping of specific gene variants which can accelerate kidney injury associated with diabetes. Moreover, they are important considerations when selecting a model of type 1 diabetes.

1.3. Akita Mice as Type 1 Diabetes Models

Below are some examples of studies where the *Ins2*^{C96T/+} mutation is used to induce type 1 diabetes in mice, illustrating the utility for disease modeling with Akita mice.

1.3.1. Bradykinin B1 and B2 Receptor Deficiency in Akita Mice

Accumulating evidence suggests that kallikrein-kinin system (KKS) plays a key role in the pathogenesis of diabetic kidney disease (14–16). In this regard, the deletion (D) allele of the common insertion/deletion (I/D) polymorphism of the angiotensin-converting enzyme (ACE) gene in humans is associated with higher serum levels of ACE (17) with resultant-enhanced inactivation of bradykinin (14) and increased risk for type 1 DN. Additionally, ACE inhibitors have well-documented beneficial effects on diabetic kidney injury that are beyond those attributable to changes in BP (18, 19). One possibility to explain these findings is that bradykinin may partly mediate the renoprotective effects of ACE inhibitors (14, 16). In mammals, two receptors for bradykinin have been identified: B1R and B2R (14). While B2R is constitutively expressed in most tissues, B1R is expressed minimally under normal circumstances, but is induced by lipopolysaccharide (LPS) in kidney (14), diabetes in heart (20), inflammation in endothelial cells, fibroblasts, urothelial cells and sensory neurons (21–23), and in the absence of B2R in kidney and heart (24). In their studies, Kakoki and coworkers investigated the role of both bradykinin receptors in the development of DN using Akita mice on C57BL/6 background (15, 16). By 6 months of age, diabetic Akita mice lacking the B2R developed more severe kidney pathology than their diabetic littermates expressing B2R. B2R knockout-Akita mice exhibited a fourfold increase in albuminuria, severe renal hypertrophy and mesangial sclerosis that closely resembles glomerular changes seen in humans with diabetic glomerulosclerosis. At 12 months of age, all of the renal diabetic phenotypes observed in Akita mice, including albuminuria, glomerulosclerosis, interstitial fibrosis, glomerular basement membrane (GBM) thickening, mitochondrial DNA deletions, and renal expression of fibrogenic genes (transforming growth factor (TGF)- β 1, connective tissue growth factor, endothelin-1) were enhanced by lack of both B1R and B2R. Interestingly, diabetic Akita mice lacking both bradykinin receptors had more severe kidney injury than Akita mice lacking only B2R

suggesting that expression of B1R may have beneficial effects in DN in the absence of constitutively expressed B2R. These results demonstrate the critical role of the bradykinin system in protecting kidney from damage caused by diabetes mellitus, and provide a clear example in how the Akita model can be used effectively to study the pathomechanisms of diabetic kidney injury in mice.

1.3.2. Endothelial Nitric Oxide Synthase Deficiency in Akita Mice

Endothelial dysfunction and damage are prevalent in patients with diabetes mellitus and contribute to the development of DN (25). Nitric oxide (NO) is a vasodilator that can modulate permeability in the vasculature (26), and besides its potency to control vascular tone and therefore BP, NO also inhibits platelet aggregation and smooth muscle cell proliferation. There are three different isoforms of nitric oxide synthase (NOS): endothelial, neuronal, and inducible (27). In endothelial cells, endothelial nitric oxide synthase (eNOS) is expressed constitutively and produces endothelial cell-derived NO (27). In humans, vascular eNOS activity can be altered in diabetes, and three functionally significant polymorphisms in the eNOS gene, *NOS3*, have been shown to lead to a decreased production of NO and are associated with the development of advanced DN in both patients with type 1 and type 2 diabetes (28–31). To better understand the role of eNOS-derived NO in the pathogenesis of DN, Wang and coworkers generated C57BL/6 *eNOS*^{-/-} *Ins2*^{C96Y/+} as a mouse model of type 1 diabetes with deficient eNOS activity (31). These diabetic mice, however, died by the age of 5 months before development of DN. Therefore, *eNOS*^{-/-} *Ins2*^{C96Y/+} and *eNOS*^{+/-} *Ins2*^{C96Y/+} mice that are F1 between C57BL/6 and 129S6/SvEvTac were created, which were as genetically uniform as inbred mice but lacked the detrimental recessive mutations that affected the survival of their parents. Compared with Akita mice having fully intact *eNOS* expression, *eNOS*^{-/-} Akita and *eNOS*^{+/-} Akita mice developed 2.5-fold higher urinary albumin excretion at both 3 and 7 months of age. The GFR in diabetic mice was increased by both reduction (*eNOS*^{+/-}) and absence (*eNOS*^{-/-}) of eNOS at 3 months of age. Morphometric analysis revealed that mesangial expansion, glomerulosclerosis, and GBM thickening were progressively worse in diabetic Akita mice in the order: *eNOS*^{+/+} Akita < *eNOS*^{+/-} Akita < *eNOS*^{-/-} Akita, and these changes were independent from BP. Finally, along with exacerbation of DN, loss of eNOS in diabetic Akita mice increased renal fibrin deposition, and expression of inflammatory and fibrinogenic factors including tumor necrosis factor (TNF), interleukin (IL)-6, monocyte chemoattractant protein 1 (MCP1), intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), and TGF-β. In summary, eNOS deficiency in Akita mice accelerated the renal injury associated with diabetes and provided insight into molecular pathways involved in the development of DN.

1.3.3. Angiotensin- Converting Enzyme 2 Deficiency in Akita Mice

Activation of renin–angiotensin system (RAS) with subsequent generation of angiotensin II (ANGII) holds an important role in the pathogenesis of kidney injury associated with diabetes (18). Blockade of the RAS has been shown to ameliorate the development of DN (18, 19). Angiotensin-converting enzyme 2 (ACE2) is a homologue of classical ACE that is also membrane-bound, but is a monocarboxypeptidase that generates ANG from the decapeptide ANGI and from the octapeptide ANGI (32, 33). As such, ACE2 represents an endogenous negative regulator of the RAS by degrading the vasoconstrictor ANGI while producing the vasodilator ANG-(1–7). In this regards, early increases in ACE2 mRNA levels, protein expression (34), and ACE2 activity have been reported in animal models of diabetes (33), whereas ACE2 mRNA and protein levels were decreased in older experimentally induced diabetic rats (32). In humans with type 2 diabetes, glomerular and tubular ACE2 expressions are reduced while ACE expression is increased (35, 36). Together, these data suggest a protective role for ACE2 in the early stage of DN. To test this hypothesis, Wong and coworkers recently studied the effect of deletion of the *Ace2* gene (*Ace2*^{-/-}) on DN in Akita mice (37). They generated *Ace2*^{-/-}*Ins2*^{C96Y/+} by crossing ACE2 knockout mice with Akita mice. At 3 months of age, the absence of ACE2 led to a twofold increase in urinary albumin excretion in *Ace2*^{-/-}*Ins2*^{C96Y/+} mice compared with Akita mice with *Ace2* gene intact despite similar blood glucose levels. Histomorphometric analysis of the kidneys of mice at 3 months of age revealed increased glomerular volume, mesangial matrix expansion, and GBM thickening accompanied by increased glomerular expression of fibronectin and α -smooth muscle actin as markers of glomerular injury. Although ANGI level was not increased in kidneys of diabetic *Ace2*^{-/-}*Ins2*^{C96Y/+} mice, treatment with an angiotensin type 1 (AT1) receptor blocker (ARB) reduced urinary albumin excretion rate in these diabetic animals. In a study by Oudit and coworkers, treatment of Akita mice for 4 weeks with human recombinant ACE2 (hrACE2) increased plasma ACE2 activity, reduced urinary albumin excretion, and decreased glomerular volume, mesangial matrix expansion, and GBM thickness (38). Treatment with hrACE2 also lowered ANGI levels in renal cortex, and reduced ANGI-induced oxidative stress and NADPH oxidase activity (38). Taken together, these findings in diabetic Akita mice suggest that ACE2 plays a protective role in the diabetic kidney as a negative regulator of RAS and again demonstrate the utility of the Akita model.

1.3.4. Reversal of Kidney Injury by a Ketogenic Diet in Akita Mice

Increasing evidence suggests that the ketogenic diet may prevent and even reverse kidney pathologies associated with diabetes (39–41). In this regard, chronic caloric restriction and ketogenic diet reroute cellular metabolism away from glucose utilization and toward the use of alternative fuels, including the ketone

3-beta-hydroxybutyric acid (3-OHB) (39, 42). Prolonged elevation of 3-OHB, on the other hand, has been recently shown to reduce molecular response to glucose metabolism (43) and may ultimately lead to the reversal of diabetic kidney pathology (39). To address this hypothesis, Poplawski and coworkers examined if a ketogenic diet can reverse DN in C57BL/6 Akita mice (39). After the development of DN had been confirmed in the Akita mice at 20 weeks of age by the presence of tenfold increase in albuminuria, half of the Akita mice and half of the nondiabetic controls were placed on a ketogenic diet while the remaining animals were maintained on a standard high-carbohydrate diet. Ketogenic diet increased blood 3-OHB level, and after 2 months on the diet, both blood glucose level as well as urinary albumin excretion was completely normalized in diabetic Akita mice without insulin treatment. Ketogenic diet also normalized expression of kidney genes induced by oxidative and other forms of stress associated with diabetes in Akita mice. Interestingly, however, histological pathology such as glomerular sclerosis was only partially reversed by the dietary intervention in diabetic animals suggesting that reversal of functional and molecular markers of DN occur more rapidly than reversal of histological aspects of DN. These studies demonstrate that DN can potentially be altered in Akita mice by therapies such as prolonged maintenance on a ketogenic diet. The mechanism by which the simple dietary intervention reverses kidney injury associated with this model of type 1 diabetes, however, remains to be determined.

1.4. Potential for Akita Mice as a Model of DN

Significant progress has been made during the past decade toward development of better mouse models of DN, which has helped to advance our understanding of the pathogenesis of diabetic kidney injury in humans (6, 7). In this regard, the Akita model appears to be quite promising. These mice develop sustained and robust hyperglycemia as a result of a spontaneous mutation in the *Ins2* gene accompanied by enhanced levels of albuminuria and consistent mesangial pathology (6, 8, 9). Similar to humans, these phenotypic features are significantly influenced by genetic background (8). Moreover, Akita mice also develop hypertension, cardiac hypertrophy, and echocardiographic evidence of heart failure which are complications commonly associated with diabetes mellitus in humans (44). Finally, Akita mice are commercially available, and the *Ins2*^{+/C96T} mutation can be easily intercrossed on other transgenic mouse lines to facilitate model development. As described in this chapter, these features of the Akita model may provide an ideal experimental platform for studying the mechanisms of diabetic kidney injury that exist in humans, and ultimately advance our progress toward a treatment for DN.

2. Materials

2.1. 24-Hour Urine Collection for Mice (see Fig. 1)

1. Cage lid: Holds disposable air filter pad. Keeps animal securely in cage, but can be removed for easy access to the animal.
2. Mouse cage: Consists of an upper and a lower chamber and is designed to house individual mice. Constructed of transparent, gas sterilizable acryl. Easy to clean with warm soapy water or detergent solution.
3. Food chamber: Constructed of transparent acryl. Located outside cage. This tunnel allows easy access to the food drawer, while its small size discourages rodents from nesting or sleeping inside. It prevents spilled food from entering the cage and contaminating the urine collection.
4. Food drawer: Constructed of transparent acryl. Slides out for easy filling with slurries, liquids or powders, without disturbing the animal.
5. Water bottle holder: Constructed of transparent acryl. Located outside cage.
6. Water bottle: Constructed of acryl.



Fig. 1. Individual mouse metabolic cage (Courtesy of R. Goodman, Hatteras Instruments, NC, USA).

7. Stainless steel mesh screen floor for the cage: Lets excreta pass through the widely spaced bars.
8. Stainless steel funnel and separating cone: Urine flows along the inside surface of the collection funnel and is directed into the urine collection tube. Separates urine from feces.
9. Urine collection tube: Removable and disposable.
10. Digital scale.
11. Centrifuge capable of generating a force of $800\times g$.
12. Pipettes (200–1,000 μL).
13. Sterile microfuge tubes (1.5 mL).

3. Methods

The intent of a metabolic cage is to collect urine and separate it from feces. There are numerous commercial rodent metabolic cages available to collect urine. Here we describe 24-h urine collection using metabolic cages purchased from Hatteras Instruments (MMC100 Metabolic Cage, Hatteras Instruments, Cary, NC, USA).

1. Determine body weight of each mouse prior to study by using a digital scale.
2. Determine weight of each urine collection tube prior to study by using a digital scale as this will be used to determine volume of urine collected (see Note 1).
3. Fill feeder drawer with rodent diet (e.g., rodent chow) (see Note 2). Slide the filled drawer cautiously into the food chamber. Food consumption can be monitored by weighing food drawer and the food inside before and after the study.
4. Fill water bottle with distilled water and attach it to the holder (see Note 3). Water consumption can be monitored by weighing the water-filled bottles before and after the study (see Note 4).
5. Place urine collection tube in the lower chamber of the cage.
6. Put stainless steel funnel in the lower chamber of the cage with the spout of the funnel positioned directly above the urine collection tube to collect the urine (see Note 5).
7. Place stainless steel mesh screen floor above the funnel (see Note 5).
8. Attach the upper chamber to the lower chamber above the stainless mesh screen (see Note 6).
9. Put single mouse into the upper chamber on the stainless steel mesh.

10. Close the upper chamber of the mouse cage with the cage lid.
11. The start time of the study is recorded.
12. After 24 h mice are removed from the cages and the body weight and the amount of water consumed and urine produced recorded (see Notes 4, 7, and 8).
13. The collected urine is centrifuged at $800 \times g$ for 15 min to remove solid contaminants and then the supernatant is aliquotted into 1.5 mL tubes (see Note 9). Store samples at -70°C .
14. Avoid repeated freeze–thaw cycles (see Note 10).

4. Notes

1. Label both urine collection tube as well as its lid for identification. Use fade and water resistant ink. This is important since spilled urine can wash away the ink.
2. Clean the food drawer each time with warm soapy water or detergent solution. Fill the drawer with fresh diet prior to each study.
3. Clean the water bottle each time with warm soapy water or detergent solution. Fill the bottle with fresh distilled water prior to each study.
4. Water consumption over a 24-h period can be calculated by using the formula: Water consumption per 24 h = Filled water bottle^{pre-study} – Filled water bottle^{post-study}.
5. Make sure to dry the stainless steel funnel and the mesh screen floor completely after cleaning prior to each study. Remaining debris from cleaning can prevent flow of urine, and contaminate and dilute sample, causing false results.
6. Take care to attach both chambers together to avoid spilling of food and water from food chamber and water bottle, respectively.
7. Urine 24-h volume can be calculated by using the formula: Urine 24-h volume = Urine collection tube^{post-study} – Urine collection tube^{pre-study} (empty).
8. In diabetic animals, urine may overflow the collection tube's capacity and spill in the lower chamber of mouse cage. Larger containers can be used or the overflow urine should be collected carefully in an additional urine collection tube. Avoid transferring accidentally feces into the collection tube. Determine volume of overflow urine by using above formula for urine volume.

9. Avoid disrupting the pellet since this contains feces, hair, cells, and crystals.
10. Repeated freeze–thaw cycles will damage and potentially denature most proteins in urine including albumin and should be avoided.

References

1. Reutens AT, Atkins RC (2011) Epidemiology of diabetic nephropathy. *Contrib Nephrol* 170:1–7
2. de Boer IH et al (2011) Temporal trends in the prevalence of diabetic kidney disease in the United States. *JAMA* 305:2532–2539
3. (2003) USRDS: the United States Renal Data System. *Am J Kidney Dis* 42:1–230
4. Chavers BM et al (1989) Glomerular lesions and urinary albumin excretion in type I diabetes without overt proteinuria. *N Engl J Med* 320:966–970
5. Dalla VM et al (2000) Structural involvement in type 1 and type 2 diabetic nephropathy. *Diabetes Metab* 26(Suppl 4):8–14
6. Breyer MD et al (2005) Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 16: 27–45
7. Brosius FC 3rd et al (2009) Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 20: 2503–2512
8. Gurley SB et al (2010) Influence of genetic background on albuminuria and kidney injury in *Ins2(+)/C96Y* (Akita) mice. *Am J Physiol Renal Physiol* 298:F788–795
9. Gurley SB et al (2006) Impact of genetic background on nephropathy in diabetic mice. *Am J Physiol Renal Physiol* 290:F214–222
10. Yoshioka M et al (1997) A novel locus, *Mody4*, distal to *D7Mit189* on chromosome 7 determines early-onset NIDDM in nonobese *C57BL/6* (Akita) mutant mice. *Diabetes* 46: 887–894
11. Ron D (2002) Proteotoxicity in the endoplasmic reticulum: lessons from the Akita diabetic mouse. *J Clin Invest* 109:443–445
12. Cowie CC (1993) Diabetic renal disease: racial and ethnic differences from an epidemiologic perspective. *Transplant Proc* 25:2426–2430
13. Qi Z et al (2005) Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. *Diabetes* 54:2628–2637
14. Kakoki M, Smithies O (2009) The kallikrein-kinin system in health and in diseases of the kidney. *Kidney Int* 75:1019–1030
15. Kakoki M et al (2010) Lack of both bradykinin B1 and B2 receptors enhances nephropathy, neuropathy, and bone mineral loss in Akita diabetic mice. *Proc Natl Acad Sci U S A* 107:10190–10195
16. Kakoki M et al (2004) Diabetic nephropathy is markedly enhanced in mice lacking the bradykinin B2 receptor. *Proc Natl Acad Sci U S A* 101:13302–13305
17. Rigat B et al (1990) An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 86:1343–1346
18. Gurley SB, Coffman TM (2007) The renin-angiotensin system and diabetic nephropathy. *Semin Nephrol* 27:144–152
19. Lewis EJ et al (1993) The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N Engl J Med* 329:1456–1462
20. Spillmann F et al (2002) Regulation of cardiac bradykinin B1- and B2-receptor mRNA in experimental ischemic, diabetic, and pressure-overload-induced cardiomyopathy. *Int Immunopharmacol* 2:1823–1832
21. Schremmer-Danninger E et al (1998) B1 bradykinin receptors and carboxypeptidase M are both upregulated in the aorta of pigs after LPS infusion. *Biochem Biophys Res Commun* 243:246–252
22. Ahluwalia A, Perretti M (1999) B1 receptors as a new inflammatory target. Could this be the 1? *Trends Pharmacol Sci* 20:100–104
23. Chopra B et al (2005) Expression and function of bradykinin B1 and B2 receptors in normal and inflamed rat urinary bladder urothelium. *J Physiol* 562:859–871
24. Duka I et al (2001) Vasoactive potential of the b(1) bradykinin receptor in normotension and hypertension. *Circ Res* 88:275–281
25. Futrakul N et al (2006) Early detection of endothelial injury and dysfunction in conjunction with correction of hemodynamic maladjustment can effectively restore renal function

- in type 2 diabetic nephropathy. *Clin Hemorheol Microcirc* 34:373–381
26. Ignarro LJ et al (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 84:9265–9269
 27. Bogdan C (2001) Nitric oxide and the immune response. *Nat Immunol* 2:907–916
 28. Zanchi A et al (2000) Risk of advanced diabetic nephropathy in type 1 diabetes is associated with endothelial nitric oxide synthase gene polymorphism. *Kidney Int* 57:405–413
 29. Ksiazek P et al (2003) Endothelial nitric oxide synthase gene intron 4 polymorphism in type 2 diabetes mellitus. *Mol Diagn* 7:119–123
 30. Ezzidi I et al (2008) Association of endothelial nitric oxide synthase Glu298Asp, 4b/a, and -786 T>C gene variants with diabetic nephropathy. *J Diabetes Complications* 22: 331–338
 31. Wang CH et al (2011) A modest decrease in endothelial NOS in mice comparable to that associated with human NOS3 variants exacerbates diabetic nephropathy. *Proc Natl Acad Sci U S A* 108:2070–2075
 32. Tikellis C et al (2003) Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. *Hypertension* 41:392–397
 33. Wysocki J et al (2006) ACE and ACE2 activity in diabetic mice. *Diabetes* 55:2132–2139
 34. Ye M et al (2004) Increased ACE 2 and decreased ACE protein in renal tubules from diabetic mice: a renoprotective combination? *Hypertension* 43:1120–1125
 35. Mizuiri S et al (2008) Expression of ACE and ACE2 in individuals with diabetic kidney disease and healthy controls. *Am J Kidney Dis* 51:613–623
 36. Reich HN et al (2008) Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease. *Kidney Int* 74:1610–1616
 37. Wong DW et al (2007) Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. *Am J Pathol* 171:438–451
 38. Oudit GY et al (2010) Human recombinant ACE2 reduces the progression of diabetic nephropathy. *Diabetes* 59:529–538
 39. Poplawski MM et al (2011) Reversal of diabetic nephropathy by a ketogenic diet. *PLoS One* 6:e18604
 40. Al-Khalifa A et al (2009) Therapeutic role of low-carbohydrate ketogenic diet in diabetes. *Nutrition* 25:1177–1185
 41. Badman MK et al (2009) A very low carbohydrate ketogenic diet improves glucose tolerance in ob/ob mice independent of weight loss. *Am J Physiol Endocrinol Metab* 297(5): E1197–204
 42. Chmiel-Perzynska I et al (2011) Novel aspect of ketone action: beta-hydroxybutyrate increases brain synthesis of kynurenic acid in vitro. *Neurotox Res* 20:40–50
 43. Ma W, Berg J, Yellen G (2007) Ketogenic diet metabolites reduce firing in central neurons by opening K(ATP) channels. *J Neurosci* 27:3618–3625
 44. Hong EG et al (2007) Nonobese, insulin-deficient Ins2Akita mice develop type 2 diabetes phenotypes including insulin resistance and cardiac remodeling. *Am J Physiol Endocrinol Metab* 293:E1687–1696

Animal Models in Diabetes Research

Joost, H.-G.; Al-Hasani, H.; Schürmann, A. (Eds.)

2012, XI, 325 p., Hardcover

ISBN: 978-1-62703-067-0

A product of Humana Press