Dissecting the genetic basis of kidney tubule response to hyperoxaluria using chromosome substitution strains

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Whether genetics may play a role in the pathophysiologic response of kidney tubules to oxalate exposure remains unexplored despite that as many as 15% of the U.S. population annually will experience a kidney stone composed of calcium oxalate. To explore this issue, we utilized a panel of chromosome substitution strains in which one chromosome at a time was transferred from the Brown Norway (BN) rat onto the Dahl salt-sensitive (SS) genetic background. Hyperoxaluria was induced by adding hydroxyproline (HP) to the drinking water. A dose-response (0–2% HP) study found that both SS and BN exhibited the same level of oxalate excretion as HP concentration increased, but only the BN exhibited changes in urothelial pathology and demonstrated crystal deposition at sites of urothelial injury as a function of dose (at 1.5–2.0%). The consomic panel was treated with 2.0% HP and evaluated for hyperoxaluria, renal injury, and crystal deposition. Tubular injury (% Area) and crystal deposition (% Area) were similar between the resistant SS and SS-4, -6, -7, -8, -9, -11, -16, and -20BN consomic rats. However, tubular injury was significantly increased in SS-2BN compared with the SS parental (9.8 ± 1.56 and 4.2 ± 1.09%, respectively). Crystal deposition was observed in SS-2BN and SS-18BN (4.7 ± 0.70 and 3.5 ± 1.3%, respectively) to the same extent as seen in the susceptible BN (3.2 ± 0.44%). The fact that crystal deposition was observed in SS-18BN without extensive overall tubule injury, compared with the more severe widespread tubular injury seen in SS-2BN, suggests that the underlying mechanism of each locus is different. In conclusion, these studies establish that BN rats demonstrate oxalate-associated pathology and they retain calcium oxalate crystals coincident with urothelial injury but SS rats do not. These observations establish that BN rat chromosome 2 and 18 harbor genes that contribute to these processes.

Dahl S; spontaneously hypertensive rat; congenic strains; kidney stones

KIDNEY STONE DISEASE is a substantial health problem associated with significant pain, suffering, and economic costs. By the age of 70, 5 to 15% of the population will have a symptomatic episode of a stone within the urinary tract (4, 24). Calcium oxalate stone disease represents the majority of stone events and stone recurrence is often a significant problem. Kidney stones are caused by a wide array of etiologic factors. For calcium-containing stone formers, these may include the urinary conditions hypercalciuria, hyperoxaluria, and hypocitraturia (24). Calcium oxalate stone disease also runs in families, suggesting a genetic contribution to the disease (8, 25). Diet, as well as other environmental conditions, has been reported as influencing calcium oxalate stone occurrence (6, 7).

In some stone patients, hyperoxaluria, regardless of its etiology, is often prominent and is frequently associated with crystalluria (21). However, not all episodes of crystalluria result in stone formation even among patients with the same metabolic disorder (9). We hypothesize that genetic factors may play an important role in an individual’s susceptibility or resistance to hyperoxaluria-associated injury. While some human-based studies have been performed to investigate the genetics of kidney stone disease, progress has been limited (24, 32), partly due to the inherent difficulties associated with genetic analysis in humans (23, 28). On the other hand, the rat provides a particularly fertile tool to investigate the genetic basis of complex disease because it overcomes some of the limitations associated with using human subjects. Typically, whole genome linkage analysis is used to identify chromosome regions linked to strain-specific differences in a given trait. Subsequently, chromosome substitution strains (CSS) or “consomic” strains are developed to isolate and study the genetic contribution of each locus (usually) from the resistant strain on the genetic background of susceptible strain (5, 10). Significant differences in phenotype (e.g., renal injury, crystal retention, or kidney stones) detected between the consomic and the control strain indicate that a gene(s) is present on that particular chromosome that influences the phenotype of interest. This strategy has proven useful in understanding the genetic basis of a number of complex diseases (1, 10). However, the genetics of susceptibility to injury induced by hyperoxaluria and its contribution to calcium oxalate crystal deposition in the tubule, or to calcium oxalate stone disease, have not been systematically studied using this methodology.

Thus, in the present study we took advantage of a consomic panel of rats in which chromosomes from the Brown Norway (BN) rat were introgressed onto the Dahl salt-sensitive (SS) genetic background, a well-studied model of cardiovascular disease (27). The SS and BN rats were originally selected to derive CSS because of the significant and divergent differences in cardiovascular-related phenotypes (hypertension, cardiac hypertrophy, renal, etc.) with the goal of providing a genetic resource to better study cardiovascular disease (22). The availability of these strains provides an opportunity to dissect the genetics of other complex diseases. In particular, it provided an opportunity to forego linkage analysis and to directly examine the role of each chromosome on hyperoxaluria-induced tubule injury and on possible crystal deposition. Initial studies sought to characterize phenotypic differences in hyperoxaluria-associated renal injury and on crystal deposition between the SS and BN using a model of hydroxyproline (HP)-induced hyperoxaluria. Subsequently, a standardized phenotyping protocol

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was performed on each consomic strain, as well as on the SS and BN parental rats to evaluate the phenotypes associated with these endpoints.

**MATERIALS AND METHODS**

**Animals.** All animal protocols were reviewed and approved by the Clement J. Zablocki, Veterans Administration Animal Care Committee. Experiments were performed on inbred Dahl SS (SS/JHsdMcwi), BN (BN/NHsdMcwi), and a panel of CSS was developed at the Medical College of Wisconsin (MCW). Only male rats were used in this study. The complete CSS panel consists of 22 strains in which each of the 20 autosomes as well as the X and Y chromosomes from the BN rat were transferred onto the SS genetic background (22, 27). Due to the availability of consomic rats, the present study used a subset of the consomic panel, including: SS-2BN, SS-4BN, SS-6BN, SS-7BN, SS-8BN, SS-11BN, SS-16BN, SS-18BN, and SS-20BN. The strains are formally designated as SS-nBN/Mcwi, where n designates the substituted chromosome (e.g., SS-1BN). The CSS were developed by MCW using the marker-assisted selection approach as previously described (22) and were obtained through Physiogenix (Wauwatosa, WI). Rats were maintained on a 0.4% NaCl diet (Dyets, Bethlehem, PA).

**Experimental protocol.** Development of hyperoxaluria, oxalate-induced kidney injury, and retention of calcium oxalate crystals to urothelium was studied by inducing hyperoxaluria with the inclusion of trans-4-hydroxy-L-proline (HP; ICN, Aurora, OH) in the drinking water. HP is a precursor of oxalate and has been successfully used in rodents to increase urine oxalate levels (2, 16). Fresh HP was prepared and provided twice a week. The rats were studied using two different protocols. In **protocol 1**, 8- to 10-wk-old SS and BN rats were studied for response to various doses of HP (0, 0.1, 0.5, 1.0, 1.5, or 2.0%, HP in the drinking water) for a period of 5 wk. Preliminary work using X-ray diffraction or FTIR found that papillary calculi and/or encrustations in the BN parental were calcium oxalate in origin. The number of rats used per dose ranged from n = 3–10 per strain. A total of 66 animals (SS = 32 and BN = 34) were used to establish appropriate dose of HP. In **protocol 2**, consomic animals as well as parental SS and BN rats received 2.0% HP in the drinking water. Rats were started on 2.0% HP at ~9 wk of age and continued for a period of 5 wk. The number of rats ranged from n = 4–6 per strain. A set of untreated control animals for each strain (n = 3) was also followed for a period of 5 wk. A total of 88 animals were studied under **protocol 2** (HP treated = 55 and control = 33).

For either **protocol 1** or **2**, rats were weighed and 24-h urine samples were collected twice a week for 5 wk. Samples were collected in tubes containing 100 μl 6 N HCl as a preservative. Urine was analyzed for oxalate and creatinine levels. Oxalate was measured using an oxalate oxidase assay (3). Urine creatinine was determined by the Jaffe method (Teco Diagnostics, Anaheim, CA). Urine oxalate levels are expressed in units of concentration (mmol/l). Similar results were found when the data were normalized to creatinine because no significant difference in creatinine was observed between the strains and HP concentration in the water increased from 0 to 2%. In general, urine oxalate levels gradually increased from week 1 to 3 and plateaued from week 4 to 5, regardless of the treatment (data not shown). A semiquantitative evaluation of tubular injury and crystal deposition in response to increasing HP doses is shown in Fig. 2, A and B, respectively. The tubular injury score was normalized to control rats (0% HP) to assess the degree of tubular damage caused specifically from oxalate. As shown in Fig. 2A, the SS rats had tubular injury at all levels of HP tested, ranging from 0.27 ± 0.24 to 1.6 ± 0.0. No tubular injury was observed in the BN rats below 1.5% HP. Tubular injury in the proximal convoluted tubule was graded semiquantitatively, using computer imaging, to provide a better measure of injury compared with the initial study (**protocol 1**). Stained sections were examined under ×10 light microscopy and 10 randomly selected areas (from cortex and outer medulla) from each slide (both experimental and control groups) were evaluated for degree of tubule dilation. In addition, 10 randomly selected areas were digitally captured at ×20 power using polarized light for the detection of the birefringent calcium oxalate crystal deposits. Images were captured using an Olympus-BX51 microscope with Olympus-DP70 microscope digital camera (Olympus, Melville, NY) and analyzed using MetaMorph image analysis software (Downingtontown, PA). For calculation of tubular area, the open space within all tubules (per field) was evaluated by applying a threshold (MetaMorph) to measure the amount of white area vs. total image area. Percent tubular area was normalized to control animals from each group to both subtract normal tubule area and damage normally associated with the Dahl SS rat, giving a measure of injury resulting directly from exposure to oxalate. Similarly, a threshold was applied to each image to distinguish between the crystals and the background area. Tubule injury or crystal deposition is expressed as the percent of total area of each captured image.

**Statistical analysis.** Data are expressed as means ± SE. Strains were compared using one-way ANOVA followed by post hoc multiple comparisons using Tukey’s test (**protocol 1**) or using Dunnett’s (**protocol 2**).

**RESULTS**

Figure 1 illustrates the dose-response relationship of increasing amounts of HP in the drinking water on urine oxalate levels in the parental SS and BN rats. No significant difference in urine oxalate levels was observed between the strains as HP concentration in the water increased from 0 to 2%. In general, urine oxalate levels gradually increased from week 1 to 3 and plateaued from week 4 to 5, regardless of the treatment (data not shown). A semiquantitative evaluation of tubular injury and crystal deposition in response to increasing HP doses is shown in Fig. 2, A and B, respectively. The tubular injury score was normalized to control rats (0% HP) to assess the degree of tubular damage caused specifically from oxalate. As shown in Fig. 2A, the SS rats had tubular injury at all levels of HP tested, ranging from 0.27 ± 0.24 to 1.6 ± 0.0. No tubular injury was observed in the BN rats below 1.5% HP. Tubular injury in the proximal convoluted tubule was graded semiquantitatively, using computer imaging, to provide a better measure of injury compared with the initial study (**protocol 1**). Stained sections were examined under ×10 light microscopy and 10 randomly selected areas (from cortex and outer medulla) from each slide (both experimental and control groups) were evaluated for degree of tubule dilation. In addition, 10 randomly selected areas were digitally captured at ×20 power using polarized light for the detection of the birefringent calcium oxalate crystal deposits. Images were captured using an Olympus-BX51 microscope with Olympus-DP70 microscope digital camera (Olympus, Melville, NY) and analyzed using MetaMorph image analysis software (Downingtontown, PA). For calculation of tubular area, the open space within all tubules (per field) was evaluated by applying a threshold (MetaMorph) to measure the amount of white area vs. total image area. Percent tubular area was normalized to control animals from each group to both subtract normal tubule area and damage normally associated with the Dahl SS rat, giving a measure of injury resulting directly from exposure to oxalate. Similarly, a threshold was applied to each image to distinguish between the crystals and the background area. Tubule injury or crystal deposition is expressed as the percent of total area of each captured image.

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observed in the BN rat exposed to 1.5 and 2.0% HP in their drinking water (1.7 ± 0.33 and 2.2 ± 0.40, respectively). Based on these finding, 2.0% HP treatment was selected as the appropriate dose to evaluate the susceptibility to tubular injury and crystal deposition in the CSS derived from the SS and BN rat.

For each CSS strain, one chromosome from the BN rat (in this case, susceptible to oxalate-associated tubular injury and to oxalate crystal attachment) was substituted onto the genetic background of the SS rat. It is therefore expected that a consomic animal would be more susceptible to oxalate-induced tubular injury and to crystal deposition than the parental SS if the transferred chromosome is linked to these traits. However, the chromosome or genes associated with oxalate-induced injury may or may not be the same as those associated with crystal deposition. However, it is clear that the mechanistic events of injury are a requirement for effective crystal deposition. Figure 3 shows the relative urine oxalate levels achieved in the consomic strains. Most strains demonstrated similar urine oxalate levels, but SS-2BN, -7BN, and -18BN had urine oxalate levels significantly higher than the SS. Semiquantitative evaluation of tubular injury across the CSS panel, based on the percent area of tubule dilation, is shown in Fig. 4A. The

BN rat treated with 1.5 or 2.0% HP was not significantly different (3.0 ± 0.58 and 3.2 ± 0.47, respectively), but was more severe than that observed in SS rats. Figure 2B shows the relative crystal deposition score in parental SS and BN rats at each dose of HP. No crystals were observed in SS rats at any dose of HP. However, considerable crystal deposition was

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**Fig. 2.** Semiquantitative histological evaluation of kidney sections. The grading criteria: 0, absent; 2, slight; 3, moderate; 4, severe. A: tubular injury. B: crystal deposition. Results were normalized to 0% HP controls. Numbers of animals used are the same as denoted in Fig. 1. ND, not detected. Error bars represent ± SE.

**Fig. 3.** Urine oxalate levels across the panel of consomic SSnBN rats in response to 2.0% HP in the drinking water. *Significantly higher than SS (P < 0.05). Numbers of animals used for experiments are denoted above the bar graphs. Error bars represent ± SE.

**Fig. 4.** Semiquantitative histological evaluation of kidney sections from a panel of consomic SSnBN rats in response to 2.0% HP in the drinking water. A: tubular injury. B: crystal deposition. Results were normalized to 0% HP controls for each strain (n = 3). Numbers of animals used are the same as denoted in Fig. 3. *Significantly higher than SS or †significantly lower than SS. Error bars represent ± SE.
SS-2BN strain (9.8 ± 1.56%) had significantly more tubule injury compared with the SS rat (4.2 ± 1.09%, P < 0.05). The degree of injury in this consomic strain was similar to that seen in the BN rat (11.8 ± 1.09%). There were several strains (SS-4BN, SS-6BN, SS-8BN, SS-16BN, and SS-20BN) that had significantly less tubular injury compared with the SS (Fig. 4A).

The semiquantitative evaluation of crystal deposition (% Area) across the CSS panel is shown in Fig. 4B. Crystals were only observed in SS-2BN, SS-18BN, and the parental BN. Similar to the finding of tubular injury, the same degree of crystal deposition was also found between SS-2BN and BN (4.7 ± 0.70 and 3.2 ± 0.44%, respectively). Interestingly, SS-18BN had crystal deposition (3.5 ± 1.3%) comparable to that found in the SS-2BN and in the BN even though no significant tubule injury was observed (Fig. 4A).

Figure 5 shows representative histology images of kidneys from both parental strains and SS-2BN, the strain most susceptible to tubule injury and crystal deposition. Kidneys from SS-2BN and SS were visually larger than the BN (Fig. 5A). However, no significant difference in kidney weight normalized to body weight was observed between the SS-2BN (2.15 ± 0.34 g/100 g body wt) and BN (1.92 ± 0.33 g/100 g body wt). Kidney weights in both strains, however, were significantly greater than that seen in the SS (1.14 ± 0.33, P < 0.05). Severe tubular dilation was observed throughout the entire kidney in both the SS-2BN and BN, but not in the SS. Additionally, a high degree of immune cell infiltration was observed in the interstitial regions surrounding injured tubules (Fig. 5B). Crystal deposition in the lumen of the tubules and most often in tubules where significant injury is observed can be clearly seen under polarized light in SS-2BN and BN rats (Fig. 5C).

**DISCUSSION**

To explore the question of whether genetics may play a role in susceptibility to kidney urothelial injury associated with oxalate exposure and crystal deposition, we evaluated a unique panel of consomic animals developed at the MCW as a genomic resource to better understand complex diseases (i.e., hypertension, kidney disease, diabetes, etc.) (27). These consomic strains were derived from the Dahl SS and the BN rat. The SS rat has been extensively used as a model to study the genetics of SS hypertension (1, 12, 22), renal injury (11, 13, 29), and cardiovascular disease (1, 17, 30). In contrast, the BN rat is resistant to these complex diseases, although the strain...
does exhibit mild hydronephrosis and some abnormal arterial phenotypes (e.g., IEL rupture, PDA, aortic elastin deficit) (18, 19). Due to the genetic predisposition of the SS rat to develop hypertension and renal injury, it might be expected that the SS rat would be more susceptible to develop kidney stones. In fact, clinical studies found that kidney stones affect hypertensive patients disproportionately compared with normotensive individuals (20, 26). However, our initial experiments indicated that the BN, not the SS, rat was more susceptible to hyperoxaluria-induced tubular injury and crystal deposition, despite both strains attaining similar urine oxalate levels. This finding suggests that a genetic susceptibility to hypertension and renal injury alone is not sufficient to promote kidney stone disease and that other genetic factors (or environmental stimuli) are necessary.

The dramatic difference in renal injury and crystal deposition between the SS and BN provided a clear basis for evaluation of the consomic panel and a good likelihood that loci (chromosomes) would be found that influence these traits. An evaluation of nine consomic strains demonstrated that oxalate-induced tubule injury and crystal deposition in BN rats are genetically determined by at least two loci (on chromosome 2 and 18). The SS-2BN rats were the only strain that sustained kidney tubule injury and crystal deposition comparable to the BN rats, indicating that a major locus controlling oxalate-induced injury and crystal formation resides on rat chromosome 2. The deposition of crystals in SS-18BN rats appears to occur without extensive overall tubule injury. This is an interesting point given that it is generally thought that tubule injury is required for crystal attachment to the tubule; however, whether crystals attach to previously damaged cell surfaces or whether the crystals injure tubule cells is not completely understood (31). In the present study, we found that SS-18BN exhibited the same degree of crystal deposition as SS-2BN rats, but much less tubular injury. These results indicate that factors other than tubular injury are likely required to promote crystal retention. Genes on chromosomes 4, 6, 8, 16, and 20 appear to provide protection from tubule injury compared with parental SS rats even though they all had nearly equivalent urine oxalate levels. This is supported by the previous finding that consomic strains for most of these chromosomes demonstrated significantly reduced proteinuria (a marker of renal injury) compared with the SS parental (22, 27).

The current study is the first to our knowledge to examine the genetic factors that contribute to the susceptibility of renal tissue to oxalate-associated injury with hyperoxaluria. There has been work on the genetics of hypercalciuria, another important risk factor for stone disease, using a novel selectively inbred rat strain, the genetically hypercalciuric stone-forming rat (GHS) (2). The GHS rat exhibits significant hypercalciuria and also develops crystals that are attached to kidney tissues. Linkage analysis using a segregating population derived from the GHS and Wistar-Kyoto rat identified a locus on chromosome 1 linked to hypercalciuria (15). The linkage data were later confirmed by congenic strain analysis, but it was not demonstrated whether this locus contributes to or is associated with actual crystal formation under conditions of hypercalciuria (14).

One possible limitation of the current study relates to how hyperoxaluria was induced because hydroxyproline must be metabolized (through several intermediate compounds) to form oxalate. It was observed that both the SS-2BN and SS-18BN consomic exhibited significantly higher urine oxalate levels compared with the SS rat and subsequently the kidneys from these animals were found to contain crystal deposits. The logical question to ask is, “Are differences in crystal deposition between these strains simply a consequence of HP being metabolized more efficiently?” In other words, if higher urine oxalate levels were also attained in the SS, would the SS rat also develop crystal deposits? Arguing against this possibility is that the SS-7BN strain attained the same urine oxalate levels as SS-2BN and SS-18BN, but didn’t exhibit significant tubule damage or crystal deposits. Moreover, subsequent experiments performed on SS and BN rats treated with a diet containing oxalate (as opposed to HP in drinking water) showed marked crystal deposition in the BN and not in the SS (data not shown).

This suggests that hyperoxaluria in the absence of other renal susceptibility genes (such as the case on chromosome 2 and 18) is not sufficient to promote kidney stones.

In conclusion, the CSS used in this study provide useful information on the genetic involvement in the protection (or promotion) of oxalate-induced tubule tubule injury and crystal deposition. The data indicated that genes on chromosome 2 enhance susceptibility to oxalate-induced tubule injury and genes on chromosome 2 and 18 enhance retention of crystals in the tubules. There appears to be BN genes on other chromosomes that provide protection from oxalate-induced injury as well as crystal deposition. Further studies, including fine-mapping and expression profiling, will be needed to identify the specific genes and mechanisms involved in promoting oxalate-induced tubular injury and stone formation in this model.

REFERENCES