Progression of Glomerular Filtration Rate Reduction Determined in Conscious Dahl Salt-Sensitive Hypertensive Rats

Allen W. Cowley Jr, Robert P. Ryan, Terry Kurth, Meredith M. Skelton, Daniel Schock-Kusch, Norbert Gretz

Abstract—Sequential changes in glomerular filtration rate during development of hypertension in the conscious Dahl salt-sensitive rats were determined using a new method for measurement. Using a miniaturized device, disappearance curves of fluorescein isothiocyanate–sinistrin were measured by transcutaneous excitation and real-time detection of the emitted light through the skin. Rats with implanted femoral venous catheters (dye injection and sampling) and carotid catheters (mean arterial pressure by telemetry) were studied, while maintained on a 0.4% NaCl diet and on days 2, 5, 7, 14, and 21 after switching to 4.0% (high-salt [HS]) diet. A separate group of rats were maintained on 0.4% for 21 days as a time control. Mean arterial pressure rose progressively from the last day of 0.4% (130±2 mm Hg) reaching significance by day 5 of HS and averaged 162±7 mm Hg by day 21. Urine albumin excretion was significantly elevated (x3) by day 7 of HS in Dahl salt-sensitive rats. Glomerular filtration rate reduced on day 14 of HS falling from 1.53±0.06 mL/min per 100 g body weight to 1.27±0.04. By day 21, glomerular filtration rate had fallen 28% to 1.1±0.04 mL/min per 100 g (t1/2 28.4±1.1 minute.) No significant reductions of creatinine clearance were observed throughout the study in response to HS demonstrating the insensitivity of creatinine clearance measurements even with creatinine measured using mass spectrometry. We conclude that the observed reduction of glomerular filtration rate was a consequence and not a cause of the hypertension and that this noninvasive approach could be used in these conscious Dahl salt-sensitive rats for a longitudinal assessment of renal function. (Hypertension. 2013;62:00-00.) • Online Data Supplement

Key Words: creatinine clearance ▪ Dahl SS rat ▪ GFR ▪ salt-sensitive hypertension

The present study had 2 goals: (1) to evaluate the ability of a recently developed transcutaneous monitoring system to make sequential measurements of glomerular filtration rate (GFR) in conscious unrestrained rats for several weeks; and (2) to determine the progression of changes of GFR that occur during the first 21 days as hypertension develops in Dahl salt-sensitive (SS) rats. Despite the central role of the kidneys in diseases of hypertension, investigators have been severely hampered in obtaining such measurements for several technical reasons. To overcome the recognized limitations of making repetitive GFR measurements in the conscious state, we used a recently developed miniaturized device that enables the transcutaneous determination of the elimination kinetics of fluorescein isothiocyanate (FITC)–sinistrin, a fluorescently labeled inulin analog. The details and validation of this technique for determination of GFR in freely moving rats and mice have been reported by Schock-Kusch et al and Gretz et al. Sinistrin is a plant-derived fructose polymer-like inulin but has much greater water solubility than inulin. Excretion half-life (t1/2) of FITC-sinistrin, as determined using the single exponential excitation phase, was shown to provide a valid measurement of GFR as compared with sinistrin clearance measured enzymatically in 20 healthy awake Sprague Dawley rats. This approach enabled not only measurements in conscious rats but also repetitive determination of GFR on sequential days.

We applied this novel technique in this study to determine the relationship of GFR and mean arterial pressure (MAP) during the development of salt-sensitive hypertension in the conscious SS rat after a step change in salt intake from 0.4% to 4.0% NaCl. Because creatinine clearance (Cкр) has become the accepted experimental and clinical indicator of GFR and renal function, comparative measurements of Cкр were also determined, together with periodic measurements of urine albumin excretion. The results of these studies show the great utility and sensitivity of these new techniques and the significant limitations of creatinine as a useful marker of renal function.

Materials and Methods

Experimental Animals

Male Dahl SS rats (SS/JrHsdMcw) were obtained as weanlings maintained at the Medical College of Wisconsin since 1991. From weaning, rats were fed a purified AIN-76A rodent food (Dyets,
Bethlehem, PA) containing 0.4% NaCl with ad libitum water. The high-salt (HS) diet used was the same formulation and source but with 4% NaCl (HS). All experimental protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Phenotyping Protocol
At 8 to 9 weeks of age, rats were surgically prepared (see online-only Data Supplement) for measurement of MAP by telemetry and injection of indicator (FITC-sinistrin; ELS-525–892 EPIGAP Optoelektronik GmbH, Berlin, Germany). After 5 to 7 days of surgical recovery and 2 to 3 days of baseline control measurements of MAP and GFR, a venous blood sample (0.2 mL) was obtained to determine serum creatinine concentration and rats were placed in metabolic cages for an overnight (18 hours) urine collection for the determination of urine volume (by weight), urine creatinine, protein, and albumin. The rats were then switched from the 0.4% salt diet to HS, and the measurements of MAP and GFR were determined on days 2, 5, 7, 14, and 21 of HS with urine and blood samples collected on a control day and days 7, 14, and 21 of HS for measurement of urine albumin, protein, creatinine, and plasma creatinine (Methods in the online-only Data Supplement).

Measurement GFR in Conscious Rats
For the measurement of GFR, a miniaturized device (NIC-Kidney, Mannheim Pharma & Diagnostics, Mannheim, Germany) was used that was composed of 2 light-emitting diodes that could transcutaneously excite FITC-sinistrin at 480 nm and a photodiode to detect the emitted light signal at 521 nm as described. The device (batteries, diodes, and microprocessor) was contained within a rodent jacket (Lomir Biomedical, Malone, NY) and the optical components affixed on a depilated region of the back using a double-sided sticky patch (Lohmann GmbH KG, 56567, Neuwied, Germany) during a brief (<5 minutes) light anesthesia with 2% isoflurane. The rat was then placed in a polystyrene box residing on the receiver for the implanted transmitter, collecting data from the carotid artery for the measurement of arterial blood pressure. The rat could move freely about this area as desired, and measurements were initiated 30 to 40 minutes after affixing the device. The transcutaneous device contained a microprocessor for the amplification and digitization (10 bit) of the signal, and the data were transferred by radio telemetry to a remote computer for storage and analysis. After a resting baseline period of 10 minutes, a bolus of FITC-sinistrin (15 mg/100 g body weight, dissolved in 0.5 mL sterile isotonic saline) was injected through the femoral venous catheter. The excretion kinetics of FITC-sinistrin were determined using a sampling rate of 60 measurements/min with an excitation time of 10 ms/measurement. Excretion kinetics were determined after each injection for >120 minutes, which resulted in an excitation time of 10 ms/measurement.

Statistical Analysis
Data are presented as mean±SEM. A 2-way ANOVA for repeated measures was used followed by a Holm–Sidak test for multiple comparisons to determine differences across and between groups over the time course of the study. A P value <0.05 was considered significant.

Results
FITC-Sinistrin Excretion Kinetics
Figure 1 illustrates the kinetics of FITC-sinistrin elimination determined in 2 SS rats; one from the HS group (top) and one from the 0.4% time control group (bottom). The prolonged rate of disappearance of the fluorescent signal is evident in the excretion kinetics (eg, reduced slope) after 21 days of the HS. This is in marked contrast to the SS rat receiving the diet of 0.4% salt for the duration of the study. In this time control SS rat, the curves from the first time point and the last time point are superimposable, indicating that the excretion kinetics remained constant during the 3-week period of study.

GFR, Elimination $t_{1/2}$, MAP, and Albumin Excretion
Figure 2 summarizes the GFR, $t_{1/2}$, MAP, and urinary albumin excretion in the 2 groups of SS rats studied. In each group, baseline measurements were obtained, while the animals were on 0.4% salt diet. After the measurements on the last day of baseline, the diet was switched in one group of SS rats to HS (4.0% NaCl; n=9), whereas the other group was maintained on the 0.4% salt diet for the duration of the study as a time control (n=6). Measurements were obtained on days 2, 5, 7, 14, and 21 after switching to the 4.0% salt diet and on the same days for the time control group maintained on 0.4% salt. Because urine could not be collected simultaneously with the hemodynamic data, time points for urine albumin correspond to the next morning after the experimental period of control day 1 and HS days 7, 14, and 21.

In SS rats switched from 0.4% to HS, MAP rose progressively from the last control day (130±2 mm Hg) reaching

**Figure 1.** Excretion kinetics for fluorescein isothiocyanate–sinistrin in a representative Dahl salt-sensitive (SS) rat from each group studied. **Top,** The measurement in an SS rat obtained on the last control day on 0.4% (blue line) and the measurement in the same SS rat on day 21 (d21) of 4.0% salt diet (high salt; red line). **Bottom,** The measurement in a SS rat from the time control group on the same days but with the animal maintained on 0.4% salt for the duration of the study.
By day 5 of HS and averaged 162±7 mmHg by day 21. Although there tended to be a small reduction of GFR by the second day of HS, significant changes from control were not observed until day 14 when GFR was reduced from 1.53±0.06 mL/min per 100 g body weight (t1/2 =20.8±0.9 minutes) to 1.27±0.04 (t1/2 =24.7±0.7). By day 21, GFR had fallen by 28% to an average of 1.1±0.04 mL/min per 100 g body weight (t1/2 =28.4±1.1 minute.). Subsequent histological analysis of the kidneys removed on day 21 quantified glomerular injury as we have described and found significantly greater injury in the SS rats receiving the 4.0% salt diet than the time control SS rats fed with 0.4% diet (data not shown; Table S1 in the online-only Data Supplement).

Urinary excretion of albumin, however, changed significantly from a control of 20±4 to 65±11 by HS day 7. This change clearly preceded any measurable reductions of GFR and was significantly different from the time control group by HS day 14 with a 7-fold increase above control values by HS day 21.

In contrast to rats fed the HS diet, SS rats maintained on 0.4% salt diet throughout the study (time control) exhibited no significant changes in MAP, t1/2, GFR, or urinary excretion of albumin throughout the study. Although SS rats eventually develop hypertension even when maintained on low-salt diets, MAP remained constant through the entire period of experimental monitoring (131±2 mmHg on the first day of measurement and 131±2 on the last day of measurement). Importantly, this time control group of rats maintained on 0.4% salt diet provide the first assessment of the reproducibility of the transcutaneous FITC-sinistrin GFR and t1/2 measurements obtained at 7 different time points during a total period of ≈25 days (including control measurements). Specifically, the average coefficient of variation of GFR during these 25 days within the rats in this group averaged 5.3%.

Creatinine and Urine Indices of Renal Injury

The Table summarizes the creatinine measurements and the Ccre values, as well as urinary protein excretion. The goal was to use the sensitive and reliable mass spectrometry method of measurement of creatinine, then compare the commonly used index of Ccre with the transcutaneous method. Urine and plasma collections were matched to 4 of the 7 days of measurements of MAP and GFR. In contrast to the reductions of GFR detected by FITC-sinistrin excretion kinetics, no significant decrease of Ccre from the control measurement was observed in response to HS. There was not even a tendency for a decrease of Ccre by HS day 14 although GFR, determined by FITC-sinistrin, had significantly fallen by this time (Figure 2). The increases of total urine protein excretion in SS rats fed HS mirrored that of albumin, rising significantly from control values of 53±18 to levels of 130±18 by day 7, whereas no significant changes were observed in SS rats maintained on 0.4% salt diet. Body weight used for the calculation of GFR, was not different between the 2 groups of SS rats but increased progressively during the 3 to 4 weeks of study, appropriate for the growth pattern for this young age.

Discussion

There are several novel aspects of the present study. First, sequential changes of GFR have not been previously measured in unanesthetized rodents during the development of any form of hypertension. Second, the progressive changes of GFR were uniquely quantified during the critical period of hypertension development in the most commonly used model of salt-sensitive hypertension, the Dahl S rat. Third, increases of albumin excretion preceded any observed reductions of GFR and represented the most sensitive index of renal injury measured in this study followed by GFR measurements using FITC-sinistrin. The least sensitive index was Ccre which did not detect relatively large reductions of GFR (eg, 28%) even when using mass spectrometry to measure creatinine.

The results show that MAP rose progressively after SS rats were switched from 0.4% salt diet to HS reaching significance by day 5 of HS. Lagging by nearly 10 days, a significant decrease of GFR was observed by day 14 of HS with an average decrease of 28% by day 21. Given the delay in the reduction of GFR relative to the increase in blood pressure, it is likely that this reduction was a consequence of the glomeruli being subjected to injurious levels of elevated renal perfusion pressures. The concept of pressure-induced injury is...
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clinical assessment of GFR. The noninvasive techniques used in the present study clearly demonstrate the feasibility of performing such measurements in conscious, unrestrained rats for a period of many weeks.

**Albumin Excretion**

The key measurements generally used to define chronic kidney disease are a reduced GFR and increased albuminuria, both of which can coexist with hypertension and other recognized cardiovascular risk factors. In the present study, a 3-fold increase of urinary albumin excretion was observed as early as 6 days after the start of the HS diet and one week before significant reductions of GFR. It was indeed a sensitive predictor of the reductions of GFR that followed. This observation is consistent with recent epidemiological evidence from the multicenter ONTARGET/TRANSCEND studies of 23,480 patients, which found changes in albuminuria were significantly associated with a useful predictor of risk of cardiovascular death and renal disease.

**Creatinine Clearance**

Determination of endogenous $c_{\text{cre}}$ has been the most commonly used indicator of GFR in many experimental and clinical studies. Although it is generally conceded that serum creatinine and $c_{\text{cre}}$ measurements lack sufficient sensitivity to meaningfully assess moderate reduction of renal function, eGFR, as extrapolated from serum creatinine measurements, is most often used as an estimate of GFR and to classify the contribution of renal function to population risk of cardiovascular events and mortality. Although lower eGFR has been found to predict the composite cardiovascular end point and stroke, this index of renal function seems to be a weaker predictor of outcome than 24-hour systolic blood pressure. Several studies in various species indicate that from 30% to 60% of total $c_{\text{cre}}$ is attributable to tubular secretion, which therefore overestimates the real GFR. In addition, the Jaffe colorimetric method to determine plasma creatinine concentrations is not accurate at low concentration ranges and even when accurately measured by using high-performance liquid chromatography or by mass spectrometry, the GFR is influenced by the serum creatinine dependency on muscular tissue mass and dietary habits. The results in the present study comparing GFR measured by FITC-sinistrin kinetics with $c_{\text{cre}}$ measurements confirm limitations described by others and demonstrate that creatinine is an unreliable surrogate marker of GFR. It has been estimated by others to reflect reductions of GFR follows the development of hypertension ≈ 6 days after the start of the HS diet and one week after the development of the hypertension following an increase of sodium intake. MAP was significantly elevated above control levels by day 5 of the HS diet, whereas significant reductions of GFR or the $t_{1/2}$ were not observed until day 14. This sequence of events is consistent with what seems to be a gradual impairment of renal blood flow myogenic autoregulation in SS rats, a response which follows only after the appearance of morphological changes. No evidence of reduced tubuloglomerular feedback has been found in SS rats fed a HS diet. It should be recognized that the sequence of events observed in SS rats in the present study does not imply that the same sequence occurs in other forms of hypertension.

**Perspectives**

The use of the novel noninvasive method with FITC-sinistrin has enabled repetitive tracking of GFR in freely moving rats during disease progression. We have shown that this method is far more sensitive than standard measurements of $c_{\text{cre}}$. Importantly, it has enabled us to determine that the reduction of GFR in SS rats follows the development of hypertension by ≈ 2 weeks and is therefore a consequence of the hypertension and not the cause. Given the ease of such measurements in even small rodent model systems, it would seem logical to apply this noninvasive approach to assess renal function in human subjects for routine clinical evaluations of patients at risk and to refine risk stratification assessments.

**Acknowledgments**

We thank Jennifer Phillips and Camille Taylor for the measurement of albumin and protein.

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**Disclosures**

Own patents for the production of fluorescein isothiocyanate (FITC)-sinistrin and the transcutaneous device. D. Schock-Kusch is cofounder of Mannheim Pharma & Diagnostics GmbH, supplier of FITC-Sinistrin and the NIC-Kidney devices. The other authors report no conflicts.

**References**


22. Novelties and Significance
What Is New?

- There are several novel aspects of the present study: (1) sequential changes of glomerular filtration rate (GFR) have never been previously measured in unanesthetized rodents during the development of any form of hypertension; (2) GFR was uniquely quantified during the critical period of hypertension development in the most commonly used model of salt-sensitive hypertension, the Dahl salt-sensitive rat.

What Is Relevant?

- The data show that reduction of GFR was a consequence and not a cause of the hypertension. The results also demonstrate the inability of creatinine to track even relatively large reductions of GFR (eg, 28%), showing how misleading creatinine clearance measurements can be even when accurately measured using mass spectrometry.

The use of the novel noninvasive method with fluorescein isothiocyanate–sinistrin has enabled repetitive tracking of GFR in freely moving rats during disease progression. We have shown that reduction of GFR in Dahl salt-sensitive rats follows the development of hypertension by ≈2 weeks and is therefore a consequence of the hypertension and not the cause. Given the ease of such measurements in even small rodent model systems, it would seem logical to apply this noninvasive approach to assess renal function in human subjects for routine clinical evaluations of patients at risk and to refine risk stratification assessments.
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TITLE: Progression of GFR reduction determined in conscious Dahl S hypertensive rats

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SHORT TITLE: GFR reduction in conscious Dahl S rats

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Phenotyping protocol

At 8-9 weeks of age, rats were anesthetized with a mixture of ketamine (28 mg/kg), xylazine (1.6 mg/kg), and acepromazine (0.4 mg/kg). A gel-filled catheter attached to a blood pressure telemetry transmitter (Data Sciences International, Minneapolis, MN) was implanted into the right carotid artery for measurement of blood pressure\(^1\) and a femoral intravenous catheter was implanted for injection of FITC-sinistrin (ELS-525-892 EPIGAP Optoelektronik GmbH, Berlin, Germany) or collection of blood. After a six-day recovery period, mean arterial pressure (MAP) and GFR were measured between 9 and 12 AM on two control days. Following these measurements, a venous blood sample (0.2 ml) was obtained to determine serum creatinine concentration and rats were placed in metabolic cages for an overnight (18 hr.) urine collection for the determination of urine volume (by weight), urine creatinine, protein and albumin. The rats were then switched from the 0.4% salt diet to HS and the measurements of MAP and GFR determined on days 2, 5, 7, 14 and 21 of HS with urine and blood samples collected on a control day and days 7, 14 and 21. Urine albumin was quantified using an Albumin Blue 580 fluorescence assay (Fluka, Inc.) standardized with rat albumin. Urine protein was measured with Weichselbaums’ biuret reagent on an ACE autoanalyzer (Alfa Wassermann). Serum and urine creatinine samples were determined by underivitized, stable isotope dilution LC-MS/MS\(^2\) by the UAB-UCSD O’Brien Core Center. Creatinine clearance (\(C_{\text{cre}}\)) was determined using accurately timed urine specimens and plasma samples collected in the unanesthetized rats on the morning at the end of the urine collection period using the standard equation \(C_{\text{cre}} = U_{\text{cre}} V / P_{\text{cre}}\).

Calculation of GFR

Elimination half-life (\(t_{1/2}\)) determinations were calculated using an established one compartment model\(^3\). The \(t_{1/2}\) was converted into GFR using an empirically derived and validated conversion factor for rats as previously described\(^3,6\).

\[
GFR = \left( \frac{ml}{min \cdot 100g \cdot bwgt.} \right) = 31.26 \frac{[ml \ 100g \ bwgt.]}{c_{t/2}(FITC - sinistrin)[min]}
\]

On those days that urine collections were made, the animal was moved from the home cage on the receiver to a metabolic cage overnight (18 hrs.) then returned to the home cage on the receiver. Except during the period of the GFR measurement, the animal had access to both food and water.
REFERENCES:


**Table S1:** Glomerular injury and tubular necrosis evaluation in kidneys collected in SS rats on day 21 of 4% salt and in SS rats in the time control (TC) group fed 0.4% salt for the same period. Gomori trichrome stained sections were examined at 20X magnification and 60 superficial and 30 juxtamedullary glomeruli evaluated and scored from 0 (no injury) to 4 as we have described. Tubular necrosis was determined by a threshold method as we have described that quantified the % of the outer medullary area with positive stained protein cast indicating tubular necrosis.

<table>
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<tr>
<th>Group</th>
<th>Cortical Glomerular Injury</th>
<th>Juxtamedullary Glomerular Injury</th>
<th>% Cast Positive Region</th>
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<td>2.33±0.10*</td>
<td>5.8±1.0*</td>
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<tr>
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<td>1.18±0.07</td>
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