Tubular Resistance to Furosemide Contributes to the Attenuated Diuretic Response in Nephrotic Rats¹,²

K.A. Kirchner, J.R. Voelker, and D.C. Brater

K.A. Kirchner, Department of Medicine, University of Mississippi Medical Center, Jackson, MS
J.R. Voelker, D.C. Brater, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN

ABSTRACT
A blunted response to loop diuretics frequently occurs in nephrotic syndrome (NS). Observations that nephrotic humans have reduced sodium excretion at normal rates of diuretic excretion have suggested that tubular resistance to the drug may contribute to diuretic resistance. To determine if tubular resistance to furosemide exists in NS, late proximal and early distal tubular micropuncture was performed in rats with puromycin aminonucleoside-induced NS and in control rats after an i.v. bolus dose of furosemide of 1 mg/kg body wt. Absolute and fractional urinary sodium excretions were less ($P < 0.05$) in NS rats than in control rats after furosemide. Inulin clearance and total urinary furosemide excretion, however, were not different between groups. Thus, similar to reports in humans, the urinary sodium-to-furosemide excretion ratio was less ($P < 0.05$) in NS than in control rats. Single-nephron GFR and chloride delivery to late proximal sites were not different between groups after furosemide. In contrast, absolute and fractional chloride deliveries to early distal sites were less ($P < 0.05$) in NS rats after furosemide. Calculated loop chloride reabsorption after furosemide was greater ($P < 0.05$) in NS than in control rats when expressed either as percentage of filtered load ($39.4 ± 3.1$ versus $28.2 ± 2.0\%$) or delivered load ($67.9 ± 4.7$ versus $48.3 ± 3.0\%$). Loop fluid reabsorption was not different between groups. Thus, loop chloride reabsorption is inhibited to a lesser extent by i.v. furosemide in NS than in normal rats. Furthermore, as total urinary furosemide excretion is equivalent in both groups, the decrease in loop chloride reabsorption appears to result from resistance by that segment to the diuretic.

Nephrotic syndrome (NS) is frequently associated with a reduced response to loop diuretics. The mechanism for the resistance is unknown. A pharmacokinetic cause seems unlikely because nephrotic individuals with a well-maintained GFR deliver normal amounts of diuretic into the tubule lumen yet still demonstrate a reduced response (1,2). Thus, urinary sodium excretion is lower in nephrotic than normal individuals at equivalent urinary furosemide excretion rates (1,2). The reduced urinary sodium-to-furosemide excretion ratio that occurs in NS has led to the hypothesis that the thick ascending limb of Henle (TALH), the site of action for loop diuretics, is resistant to the effects of the drugs. This supposition has not been confirmed in humans because the nephron segments in humans are inaccessible for study.

Interestingly, few studies have examined diuretic response in animal models of NS, and determination of the response by individual tubule segments to loop diuretics in this condition has not been reported. Micropuncture studies of segmental sodium reabsorption in experimental model of NS during euvoemia suggest that reabsorption in the superficial loop segment is either unchanged or slightly decreased compared with that in normal animals (3–5). Thus, at least under basal conditions, solute reabsorption by the TALH does not appear to be intrinsically altered in NS. This study was designed to compare in vivo the effects of furosemide on solute transport by the whole organ and by specific nephron segments in nephrotic and normal rats. Our goals are to determine if diuretic resistance occurs in the puromycin aminonucleoside (PAN) model of NS and to examine the contribution of loop segment tubular resistance to this effect.

Key Words: Micropuncture, loop segment, puromycin aminonucleoside
METHODS

Under light pentobarbital anesthesia (50 mg/kg body wt i.p.), male Sprague-Dawley rats weighing between 200 and 300 g were injected i.v. with a PAN dose of 7.5 mg/100 g body wt. Control rats were injected with an equal volume of the vehicle for PAN (0.15 M NaCl). The animals were allowed to waken, were placed in individual metabolic cages, and were fed standard rodent chow and tap water until study. Rats were initially studied at either 4 days or 15 to 18 days after the PAN or vehicle injection, because preliminary studies demonstrated that PAN-treated rats had significant proteinuria but well-maintained GFR at these intervals. On the day of study, animals were anesthetized with an i.p. injection of 80-mg/kg body wt 5 sec-butyl-5-ethyl-2-thiobarbituric acid (Inactin; Promonta, Hamburg, Germany) and were placed on a heated animal table. Rectal temperatures were maintained between 37 and 38°C by using a servoactivated controller (Yellow Springs Instrument Co., Yellow Springs, Ohf). After tracheostomy, a PE-50 polyethylene catheter was introduced into the right jugular vein for infusion of isotonic Ringer's bicarbonate solution containing 5% polyfructosan (Inustest; Laevosan Gesellschaft, Linz, Austria) at a rate of 1.2 mL/h. A PE-50 polyethylene catheter was placed into the left jugular vein for volume expansion and administration of furosemide. A PE-50 polyethylene catheter was placed in the right femoral artery for blood sampling and continuous blood pressure monitoring. This catheter was connected to a pressure transducer (Model 4-327-I; Trans-America Devel, Pasadena, CA), and arterial pressure was recorded continuously on a polygraph (Lafayette Instrument Co., Lafayette, IN). A flanged PE-50 polyethylene catheter was placed in the bladder through a suprapubic incision for urine collection from the right kidney. The left kidney was exposed through a subcostal incision and was gently separated from the adrenal gland and perirenal fat. The kidney was placed in a Lucite cup, and the upper one-third segment of the ureter was cannulated with PE-50 polyethylene tubing. Agar was then placed around the kidney to form a well on the surface, and the kidney was bathed with mineral oil warmed to 37°C. Cortical surface convolutions of four to five latest proximal and four to five earliest distal tubule segments were identified by observing the passage of 0.05 mL of 7.5% FD&C green dye. Localization of puncture sites by dye transit time has been shown to correlate well with puncture sites identified by microdissection. After the specific tubular segments were identified, rats received a volume of 0.15 M NaCl equivalent to 3% of their body weight over a 60-min interval. After volume expansion, the 0.15 M NaCl infusion was reduced to 2 mL/h and rats received a 1-mg/kg i.v. bolus of furosemide (Hoechst-Roussel Pharma-

Analytical Techniques

Urine flow rate was estimated from the change in weight of collection vials. Sodium concentrations in serum and urine were analyzed by flame photometry (Model 943; Instrumentation Laboratories, Lexington, MA). Inulin concentrations in urine and plasma were determined by the diphenylamine method of Walser et al. (7). Urinary protein concentration was determined by the Coomassie blue technique (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, CA). Furosemide concentration in urine was measured by HPLC by a modification of a previously described procedure (8). Briefly, 200 μL of metolazone internal standard and 200 μL of acetonitrile were added to 200 μL of urine. The sample was vortexed and then centrifuged. The supernatant was removed and placed in a autoinjection vial. Forty-five microliters of the sample was injected onto a 5-μm C18 column by using a Waters WISP 712 autosampler and was eluted with a mobile phase consisting of 67% 50 mM sodium acetate (adjusted to pH 3.6 with glacial acetic acid) and 33% acetonitrile at a flow rate of 1 mL/min (Spectra-Physics Model SP 8810 Isocratic Pump; Spectra-Physics, San Jose, CA). Drugs were detected with a Perkin-Elmer 650-15 fluorescent spectrophotometer (The Perkin-Elmer Corp., Norwalk, CT) employing excitation and emission wavelengths of 344 and 410 nM, respectively. Sample concentration was determined by comparing the peak-height ratio of the sample: internal standard to a standard curve.

Tubule fluid volume was measured in constant bore glass tubing with a microslide comparator (Gaertner Scientific, Chicago, IL). Tubule fluid inulin concentration was determined by the method of Vurek and Pegram (9). Tubule fluid chloride concentration was determined by electrometric titration according to the second method of Ramsay et al. (10).
Analysis of Data

The average urinary protein excretion rate was determined from urine collected over the 24-h interval beginning the day before the study while rats were housed in individual metabolic cages. The concentrations of inulin, chloride, and sodium in blood and urine and the urinary flow rate permitted calculation of whole-kidney GFR and urinary excretion rates of sodium and chloride according to standard expressions (6). Total urinary furosemide excretion during the study interval was calculated as the product of urinary furosemide concentration and total urine volume. Measurements of flow rate and inulin and chloride concentrations in tubule fluid samples allow determination of single-nephron GFR (SNGFR) and of fractional delivery rates of chloride and fluid to puncture sites as previously described (6). The fraction of filtered chloride reabsorbed between proximal (A) and distal (B) punctured nephron segments A and B was calculated according to the following expression:

\[ \frac{(TF/P)_{\text{cub}} - (TF/P)_{\text{u}}}{(TF/P)_{\text{u}}} \times 100 \]

where \((TF/P)_{\text{cub}}\) is the ratio of a tubule fluid to plasma concentration of chloride (Cl) and inulin (IN). This calculation was performed for each animal from the mean values obtained in that individual animal. Absolute chloride reabsorption was then calculated for each animal from mean values for early distal SNGFR, nephron filtered load, and appropriate values of fractional chloride reabsorption determined for that animal. In all statistical computations, \(N\) equals the number of animals and not tubules. The unpaired \(t\) test was used to determine statistical significance between the groups. Statistical significance was set at the \(P < 0.05\) level. Because no difference in the furosemide-induced urinary sodium excretion rate was noted between rats studied at 4 days and at 15 to 18 days after PAN administration, these results were combined and the data were presented collectively.

RESULTS

Whole Animal Data

Urinary protein excretion rates determined the day before micropuncture study were 13-fold greater in rats treated with PAN than in rats treated with PAN vehicle (Table 1). After preparation for micropuncture, mean arterial pressure was greater in nephrotic rats than in normal rats. Whole-kidney inulin clearance was not different between groups. Despite virtually identical amounts of furosemide excreted into the urine of normal and nephrotic rats, absolute and fractional urinary excretion rates of sodium and chloride were significantly greater in normal animals. As
a result, urinary sodium-to-furosemide excretion ratio was significantly less in nephrotic rats compared with normal rats (Figure 1).

**Micropuncture Data**

After i.v. furosemide, SNGFR determined from either late proximal or early distal tubular puncture sites was slightly but not statistically lower in nephrotic rats compared with normal rats (Table 2). Neither absolute nor fractional chloride delivery to the latest accessible segment of the proximal convoluted tubule was significantly different between rats with and without NS (Table 2; Figure 2, left panel).

In contrast to the similarities noted for the amount of filtered chloride delivered from the proximal collection site, absolute and fractional chloride deliveries to the early distal puncture site were significantly lower in nephrotic rats compared with normal rats (Table 2; Figure 2, right panel). Consistent with greater chloride reabsorption by the nephrotic rats was the finding that their distal tubule TF/Pc ratio was less than that of normal animals (Table 2).

This difference in chloride uptake by the loop segment was magnified when loop segment reabsorption was expressed as the percentage of the chloride load that was delivered beyond the proximal puncture site (Figure 3).

Fractional fluid reabsorption either in the proximal convoluted tubule or in the loop segment was not different between normal and nephrotic rats (Figure 4). Thus, the reduced TF/Pc measured at the distal site of nephrotic animals (Table 2) is consistent with this group having greater than normal loop segment chloride reabsorption in the presence of furosemide.

**DISCUSSION**

This study demonstrates that, compared with normal rats, rats with PAN nephrosis have a greater than 50% reduction in the chloruretic and natriuretic responses to a 1-mg/kg i.v. furosemide bolus. This blunted effect occurred despite similar GFR between groups and a greater mean arterial pressure in nephrotic rats. In as much as equivalent amounts of diuretic reached the final urine in both groups, these observations indicate that the nephrotic rat kidney is unable to respond normally to the loop diuretic.

Our findings are consistent with reports from several investigative groups that have examined furosemide pharmacokinetics and dynamics in nephrotic humans. In these studies, nephrotic patients have been found to be less responsive than healthy individuals to equivalent amounts of diuretic drugs excreted by the kidney (1,2). On the basis of the absence

<table>
<thead>
<tr>
<th>SNGFR(nL/min)</th>
<th>Late Proximal</th>
<th>Early Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td>FDCl (%)</td>
<td>ADCl (pEq/min)</td>
</tr>
<tr>
<td>Distal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (N = 8)</td>
<td>33.5 ± 3.0</td>
<td>59.8 ± 2.6</td>
</tr>
<tr>
<td>Nephrotic (N = 8)</td>
<td>29.0 ± 2.8</td>
<td>57.9 ± 1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>FDCl (%)</th>
<th>ADCl (pEq/min)</th>
<th>TF/Pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (N = 8)</td>
<td>31.5 ± 2.1</td>
<td>1.294 ± 124</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td>Nephrotic (N = 8)</td>
<td>18.5 ± 2.5</td>
<td>564 ± 74°</td>
<td>0.72 ± 0.06°</td>
</tr>
</tbody>
</table>

*Values are means ± SE; N, number of animals studied; FDCl, fractional delivery of chloride to puncture site; ADCl, absolute chloride delivery to puncture site; TF/Pc, tubule fluid-to-plasma chloride ratio.

* P < 0.05 versus normal.
Figure 3. Fraction of delivered load reabsorbed after furosemide administration in the proximal convoluted tubule and loop segment in normal \((N = 8)\) and nephrotic rats \((N = 8)\). \(^*P < 0.05\) versus normal rats.

Figure 4. Fractional fluid reabsorption after furosemide administration in the proximal convoluted tubule and loop segment in normal \((N = 8)\) and nephrotic rats \((N = 8)\).

of a pharmacokinetic difference between subjects with and without NS, groups led by Smith and by Keller proposed that the diuretic resistance associated with NS is due to a reduced ability of the renal tubules to respond to furosemide \((1,2)\). This conclusion, however, has remained speculative because direct assessment of tubular responsiveness to furosemide in human or in animal models of NS has not been previously reported.

This study was designed to compare the response of the loop segment to a clinically relevant dose of furosemide in normal and nephrotic rats. Late proximal and early distal nephron locations were chosen for micropuncture because they allowed an assessment to be made of the action of furosemide in the TALH. As shown in Figures 2 and 3, the ability of furosemide to inhibit chloride reabsorption in the loop segment is 39% lower in nephrotic rats compared with normal rats. Fluid reabsorption in this segment was not different between normal and nephrotic rats after furosemide administration (Figure 4). Neither chloride nor fluid reabsorption in the proximal convoluted tubule was different between normal and nephrotic rats after furosemide administration (Figures 3 and 4). These observations imply that the attenuated furosemide response in this model of NS results from a direct reduction in the ability of furosemide to inhibit active chloride reabsorption in the water-impermeable TALH.

The mechanism for the blunted tubular response to furosemide in nephrotic rats is unknown, but prior work suggests the albuminuria that accompanies NS may be causal. In previous microperfusion studies in normal rats, we have shown that the presence of exogenously administered albumin in tubule fluid at concentrations representative of amounts measured in the proximal tubule of rats with PAN nephrosis attenuates the inhibitory effect of furosemide on loop segment chloride uptake \((11)\). Because the addition of competitive inhibitors of furosemide-albumin binding to the tubule perfusate restores furosemide potency, the impaired response appears to result in tubule fluid \((12)\). On the basis of these data, it is possible that inactivation of furosemide through binding to filtered albumin could account for the reduced activity of furosemide observed in nephrotic rats in this current study. Thus, despite finding equivalent cumulative amounts of furosemide in urine of the nephrotic and normal rats (Table 1), there may have been less free drug in the nephrotic groups. Although determination of free and total furosemide concentrations in early distal tubule fluid is necessary to confirm the above supposition, their measurement in tubule fluid has been technically difficult because of the small sample volume and lack of radio labeled furosemide of high specific activity. Until direct assessments of protein binding are made, it will be difficult to quantitate the importance of this phenomenon in causing diuretic resistance.

An alternative explanation for the blunted furosemide response in nephrotic rats is the presence of an intrinsic defect in loop segment response to the diuretic. Although several groups have reported that fractional loop segment reabsorption is not different between nephrotic and normal rats under euolemic conditions \((3,5)\), pathophysiological changes associated with the nephrotic state may limit the ability of the TALH to respond normally to furosemide. For example, autacoids such as renal prostaglandins could be affected by the NS. Several investigators have suggested that stimulation of prostaglandin production during diuretic administration determines the magnitude of the natriuretic and diuretic responses \((13,14)\). We have shown that inhibition of prostaglandin production with indomethacin or meclofenamate blunts the inhibitory effect of furosemide on loop segment chloride uptake \((15)\). It is, therefore, possible that the production or response to renal prostaglandins in the presence of furosemide is disrupted in NS and results in an impaired tubular response to the drug.

Another system that modulates renal sodium excretion and, therefore, could antagonize diuretic re-
Diuretic Resistance in Nephrosis

response to furosemide in NS is the sympathetic nervous system. Koepke and DiBona have shown that conscious rats with adriamycin-induced NS have a blunted natriuretic response to exogenous atrial natriuretic factor that is partially reversed by renal denervation (16). They propose that elevated renal sympathetic nerve activity in NS blunts diuresis and natriuresis through direct effects on tubular transport, including solute reabsorption by the loop segment (17). Increased plasma norepinephrine levels have been reported in NS in humans and may reflect an increase in sympathetic nervous system activity to the kidney (18). Thus, increased renal sympathetic nerve activity may contribute to the attenuated furosemide response observed in nephrotic rats.

Although this study provides evidence that loop segment response to furosemide is impaired in NS, this phenomenon may not be the sole cause of the diuretic resistance. The observation that differences in furosemide response in the loop segment are not as great as are those for the whole kidney is consistent with a contribution of additional nephron segments to the overall blunted furosemide response observed in NS. Previous studies in euvoletic and volume-expanded rats have proposed that nephron segments beyond the cortical distal convoluted tubule or deep nephrons are the primary site for renal sodium retention in NS (3,4). Thus, increased reabsorption in these locations might also contribute to the attenuated whole-kidney response to furosemide observed in this condition. Direct evaluation of these segments is difficult because of their limited accessibility by micropuncture techniques. Comparison of the fraction of filtered chloride delivered to the early distal micropuncture site (Table 2) with that excreted in the urine (Table 1) demonstrates that locations beyond the TALH and deep nephrons accounted for reabsorption of 21.0 ± 2.4% of the filtered load in normal rats and 13.0 ± 2.6% of the filtered load in nephrotic rats (P > 0.05; NS). Although these values are not consistent with significant net contribution of these locations to the attenuated furosemide response observed in nephrotic rats in this study, differences in solute delivery out of the distal convoluted tubule (Table 2) and potential differences in filtered chloride load to deep nephrons between normal and nephrotic rats make acceptance of these very indirect data as conclusive evidence excluding contribution of these locations hazardous. Further investigations will be necessary to evaluate the contribution of sites beyond the TALH and deep nephrons to the diuretic resistance observed in NS.

In summary, this study provides the first direct data demonstrating that the attenuated response to furosemide in NS can be due to a blunted effect of the diuretic on loop segment chloride reabsorption. This subnormal diuretic effect cannot be accounted for solely by differences in the total amount of furosemide delivered to the TALH active site. Our findings are consistent with a reduction in the unbound furosemide concentration due to the presence of albumin in the lumen of the nephron and/or a decrease in tubular responsiveness to the drug. Further studies are required in order to elucidate a precise mechanism for this tubular resistance.

ACKNOWLEDGMENTS

The authors thank Ms. Bridget Crosby and Mrs. Tamara Walton for excellent technical assistance and Ms. Tammy Blakenev for secretarial help.

REFERENCES


