A vasopressin-regulated urea transport process is normally expressed in the rat terminal inner medullary collecting duct (IMCD) (1). A urea transporter cDNA (rUT2), which is thought to be the vasopressin-regulated urea transporter (VRUT), has been cloned from the rat inner medulla (2-4). Recently, Nielsen et al. prepared a polyclonal antibody to rUT2 by immunizing rabbits with a synthetic polypeptide corresponding to the carboxyl-terminal 19 amino acids of the polypeptide sequence (5). The antibody prepared by Nielsen et al. recognizes a single protein band at 97 kD in rat inner medulla (5). The successful expression cloning of UT2 (6) and preparation of an antibody to this VRUT (5) provides molecular probes that can be used to investigate the regulation of urea transport in the IMCD.

Over 20 years ago, Knepper et al. showed that administering glucocorticoids (dexamethasone) to adrenalectomized rats for 3 days doubles their fractional excretion of urea (7). This result suggests that adrenalectomy can increase tubular transport of urea or that glucocorticoids can reduce it. The purpose of the study presented here was to test whether adrenalectomy or glucocorticoids can change urea transport in either the rat initial or terminal IMCD and to examine the mechanism of any change in urea transport. To accomplish the latter goal, we prepared a polyclonal antibody to rUT2 (3,4) and used it, along with our rat UT2 cDNA (2), to measure changes in immunoreactive protein and/or mRNA abundance, respectively.

Materials and Methods

Animal Preparation

Pathogen-free male Sprague-Dawley rats (National Cancer Institute, Frederick, MD) weighing 60 to 70 g were kept in filter-top cages with autoclaved bedding and received free access to an 18% protein diet (NIH-31; Ziegler Brothers, Gardner, PA) and 0.9% saline for 7 days after adrenalectomy or sham operation. A third group of rats underwent adrenalectomy and were treated twice a day with subcutaneous dexamethasone for 7 days at a dose (5 μg/100 g body wt) designed to approximate the glucocorticoid levels of rats under stress (8,9).

On the sixth day after adrenalectomy or sham operation, rats were weighed (Table 1) and placed into individual metabolic cages for collection of a 24-h urine sample, after which blood was collected and the kidneys removed. Urine osmolality was measured using a vapor pressure osmometer (Model 5500; Wescor, Logan, UT). Plasma concentrations of urea, creatinine, glucose, sodium, albumin, and total protein, and urine concentrations of urea, creatinine, sodium, and...
Tissue Preparation for Tubule Microperfusion

Twenty minutes before each experiment, furosemide (5 mg ip) was administered to reduce medullary osmolality and prevent osmotic shock to the inner medulla after it was removed from the animal and placed into dissecting solution (described below) (10). Initial or terminal IMCD were dissected as described previously (1,11) in a dissecting solution gassed with 95% O2/5% CO2 and containing (in mM): NaCl, 118; NaHCO3, 25; CaCl2, 2; K2HPO4, 2.5; MgSO4, 1.2; glucose, 5.5; and creatinine, 4. Using standard techniques, the tubules were perfused in a 37°C bath, which was exchanged continuously and bubbled with 95% O2/5% CO2 gas (1,2,10,11).

Urea Permeability Measurement

To measure urea permeability, 5 mM murea was added to the bath solution, and 5 mM raffinose was added to the perfusate to create a 5 mM bath-to-lumen urea gradient without any osmotic gradient (1,2,10-12). Previous studies have shown that the same urea permeability value is obtained regardless of whether a bath-to-lumen or lumen-to-bath urea gradient is imposed (10). Bath and perfusate solutions were otherwise identical to the dissecting solution described above. First, basal urea permeability was measured. Next, 10 nM vasopressin (AVP; Sigma, St. Louis, MO) was added to the bath, and after 30 min, the response to vasopressin was measured (1). The urea concentration in perfusate, bath, and collected fluid was measured using a continuous-flow ultramicrofluorometer as previously described (2,10-12). Urea flux and urea permeability were calculated as previously described (1,2,10-12).

Preparation of a Polyclonal Antibody to the Rat Vasopressin-Regulated Urea Transporter

Nielsen et al. (5) prepared a polyclonal antibody to the rat VRUT by immunizing rabbits against an HPLC-purified synthetic polypeptide corresponding to the carboxyl-terminal 19 amino acids of rUT2 (3,4). We conjugated the same synthetic polypeptide (synthesized by the Emory University Microchemical Facility) to keyhole limpet hemocyanin (KLH; kit #77107, Pierce, Rockford, IL) and injected it, dissolved in Freund's complete adjuvant, into three rabbits (A, B, and C). The rabbits were reinjected periodically with the KLH-linked peptide dissolved in Freund's incomplete adjuvant. The immunizing polypeptide was linked to a Sulfo-Link column (Pierce) and used to affinity-purify the antisemur from rabbit "C." Although the three antisera yielded similar results, the studies reported here were obtained with the antisemur from rabbit "C."

Western Blot Analysis

Rat kidney inner medullae were dissected into two regions, (1) base and (2) tip, and rapidly frozen in liquid N2 and stored at -80°C (2). Total RNA from the base or tip region of four inner medullae (two rats) was isolated using TRI-REAGENT®. After separation in 1.2% agarose/2.2 M formaldehyde gels, RNA was immobilized onto a nylon membrane (Hybond-N®; Amersham), hybridized with 32P-labeled probes under high stringency conditions (50% formamide at 42°C overnight), and washed in 2× standard saline citrate (SSC), 0.5% SDS at 42°C, then twice in 0.5× SSC, 0.5% SDS at 65°C. The probes used in this study were: (1) a rat UT2 cDNA obtained by polymerase chain reaction PCR as described previously (2); and (2) a rat GAPDH cDNA (15). GAPDH is considered to be a “housekeeping” gene and was used to assess loading of the gels (15,16). These cDNA were labeled with 32P-dCTP using the random primer technique (DNA Random Primed Labeling Kit; Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

Statistical Analyses

Data are presented as mean ± SE (N), where N indicates the number of rats studied. If tissue from two rats was pooled, the sample was counted as an N of 1. For Northern analysis experiments, statistical analysis was performed on data expressed as a ratio of the autoradiographic band density of rUT2 mRNA to that of GAPDH mRNA (17). For the tubule experiments, data from three to four collections were averaged to obtain a single value from each experimental phase in each tubule. To test for statistically significant differences, an analysis of variance was used, followed by a multiple comparison, protected t test (17).

Results

Blood and Urine Values

There were no significant differences in weight or in plasma levels of creatinine, urea nitrogen, total protein, sodium, or...
glucose between sham-operated rats, adrenalectomized rats, and adrenalectomized rats receiving dexamethasone (Table 1). Adrenalectomized rats had a significantly higher urine osmolality and significantly lower urine volume, sodium, potassium, Na/K ratio, and urea than did sham-operated rats or adrenalectomized rats receiving dexamethasone (Table 1). The plasma albumin values were significantly higher in adrenalectomized rats receiving dexamethasone than in untreated adrenalectomized rats or sham-operated rats (Table 1).

Urea Permeability
In initial IMCD, basal urea permeability was unchanged by adrenalectomy or dexamethasone replacement (Figure 1, left panel, \( N = 4 \)). Vasopressin did not change urea permeability in initial IMCD from any group of rats (\( N = 4 \), data not shown).

In contrast, in terminal IMCD (Figure 1, right panel), basal urea permeability was increased 54% in adrenalectomized rats, compared with sham-operated rats (\( P < 0.01, N = 6 \)) and decreased 44% in adrenalectomized rats receiving dexamethasone, compared with sham-operated rats (\( P < 0.01, N = 6 \)). Vasopressin (10 nM) significantly increased urea permeability in terminal IMCD from all three groups of rats (Figure 2).

VRUT Protein
Our antibody recognized a 97-kD protein in the membrane fraction (17,000 \( \times g \) pellet) from the tip of a rat inner medulla

### Table 1. Blood and urine values

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>Adx</th>
<th>Dex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat weight, final (g)</td>
<td>126 ± 3</td>
<td>112 ± 11</td>
<td>137 ± 3</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dl)</td>
<td>0.4 ± 0.03</td>
<td>0.4 ± 0.0</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Plasma urea nitrogen (mg/dl)</td>
<td>14 ± 1</td>
<td>13 ± 1</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Plasma albumin (g/dl)</td>
<td>2.4 ± 0.0</td>
<td>2.1 ± 0.2</td>
<td>3.1 ± 0.2b</td>
</tr>
<tr>
<td>Plasma total protein (g/dl)</td>
<td>4.0 ± 0.1</td>
<td>3.6 ± 0.3</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>Plasma sodium (mEq/l)</td>
<td>133 ± 6</td>
<td>131 ± 2</td>
<td>130 ± 3</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>159 ± 8</td>
<td>157 ± 12</td>
<td>164 ± 9</td>
</tr>
<tr>
<td>Urine osmolality (mosmol/kg H₂O)</td>
<td>561 ± 51</td>
<td>746 ± 18b</td>
<td>592 ± 27</td>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>49 ± 7</td>
<td>16 ± 3b</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>Urine sodium (mEq/day)</td>
<td>8.8 ± 1.0</td>
<td>2.6 ± 0.6b</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Urine potassium (mEq/day)</td>
<td>2.9 ± 0.1b</td>
<td>1.5 ± 0.3b</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Urine Na/K ratio</td>
<td>3.0 ± 0.3</td>
<td>1.7 ± 0.1b</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Urine urea (mg/day)</td>
<td>9.1 ± 0.4</td>
<td>6.0 ± 1.3b</td>
<td>10.9 ± 0.4</td>
</tr>
</tbody>
</table>

* Data are shown as mean ± SE (\( N = 4 \) rats/group). Sham, sham-operated rats; Adx, adrenalectomized rats; Dex, adrenalectomized rats receiving dexamethasone (5 \( \mu g/100 \) g body wt, twice daily, sc) for 7 days. All rats received 0.9% saline and an 18% protein diet ad libitum.

b \( P < 0.05 \) versus Sham (by one-way analysis of variance).

Figure 1. In terminal IMCD (\( N = 6 \), right panel), basal urea permeability (no vasopressin added) is significantly different between rats undergoing sham operation (Sham), adrenalectomy (Adx), or Adx plus replacement with glucocorticoid (Dex). In contrast, in initial IMCD (\( N = 4 \), left panel), basal urea permeability was similar in all three groups of rats. * \( P < 0.01 \) versus Sham rats.

Figure 2. Vasopressin (10 nM AVP added to the bath) significantly increased urea permeability in terminal IMCD from rats undergoing sham operation (Sham), adrenalectomy (Adx), or Adx plus replacement with glucocorticoid (Dex). * \( P < 0.01 \) versus Basal value; +, \( P < 0.01 \) versus Sham rats.
Figure 3. Immunoblots of membrane fraction (17,000 × g pellet) proteins from a rat inner medullary tip and probed with (left-to-right): our three antibodies to the VRUT and their respective pre-immune sera (lanes A, pre-A, B, pre-B, C, and pre-C refer to antiserum or pre-immune serum from rabbits A, B, and C), and affinity-purified antibody C, which was preabsorbed with the immunizing peptide (lanes 0, 0.1 and 1 μg/ml peptide). There is a band at 97 kD detected by all three antibodies (A, B, and C) that is not present when probed with pre-immune serum or after preabsorption with the immunizing peptide.

(Figure 3). Antibodies from all three rabbits recognized the 97-kD protein. This protein was not recognized by pre-immune serum. The 97-kD protein band was not detected when antibody from rabbit “C” was preabsorbed against the immunizing peptide (0.1 to 1 μg/ml). Densitometry showed a linear increase in immunoreactive VRUT protein with increased protein loading (2, 4, 6, and 8 μg) of the gel (r = 0.992).

In the inner medullary tip, adrenalectomized rats had a 37% higher level of VRUT protein (N = 8, P < 0.01) than sham-operated rats, whereas adrenalectomized rats receiving dexamethasone had levels that were not significantly different than sham-operated rats (Figure 4). Adrenalectomized rats receiving dexamethasone had levels that were not significantly different than those of sham-operated rats. In the inner medullary base, there were no significant differences among the three groups of rats (data not shown).

In inner medullary tip proteins from the vesicle fraction (200,000 × g pellet), adrenalectomized rats had a 211% higher level of VRUT protein (N = 8, P < 0.01) than sham-operated rats, whereas adrenalectomized rats receiving dexamethasone had levels that were not significantly different than sham-operated rats (Figure 5). In the inner medullary base, there were no significant differences among the three groups of rats (data not shown).

Northern Blot Analysis
In the inner medullary tip, our rat UT2 cDNA probe (2) hybridized almost exclusively to a 4.0-kilobase (kb) mRNA band, but a faint 2.9-kb mRNA band could be seen in 50% of the dexamethasone-treated rats (Figure 6A, left panel). Densitometry of the 4.0-kb mRNA band showed no significant difference between sham-operated rats, adrenalectomized rats, and adrenalectomized rats receiving dexamethasone (Figure 6B, left panel).

Figure 4. (A) Representative immunoblot of membrane fraction (17,000 × g pellet) from inner medullary tip from rats undergoing sham operation (Sham), adrenalectomy (Adx), or Adx plus replacement with glucocorticoid (Dex) and probed with our VRUT antibody “C.” Each lane shows protein from an individual rat. The band at 97 kD is significantly increased in Adx versus Dex or Sham rats. (B) Summary of laser densitometric analysis. VRUT protein in the inner medullary tip from Adx rats is significantly increased, compared with Sham or Dex rats. * P < 0.01.
Glucocorticoids Regulate IMCD Urea Transport

Figure 5. (A) Immunoblot of vesicle fraction (200,000 × g pellet) from inner medullary tip from rats undergoing sham operation (Sham), adrenalectomy (Adx), or Adx plus replacement with glucocorticoid (Dex) and probed with our VRUT antibody “C.” Each lane shows protein from an individual rat. The band at 97 kD is significantly increased in Adx versus Dex or Sham rats. (B) Summary of laser densitometric analysis. VRUT protein in the inner medullary tip from Adx rats is significantly increased, compared with Sham or Dex rats. * P < 0.01.

In the inner medullary base, our rat UT2 cDNA probe hybridized to 4.0- and 2.9-kb mRNA bands (Figure 6A, right panel). There were no significant differences in either mRNA band among sham-operated rats, adrenalectomized rats, and adrenalectomized rats receiving dexamethasone (Figure 6B, right panel).

Discussion

Our main conclusion is that glucocorticoids downregulate urea transport in the rat terminal IMCD and decrease the immunoreactive VRUT protein content of the inner medullary tip. The mRNA abundance of rUT2 is not changed by adrenalectomy or dexamethasone replacement, suggesting that glucocorticoids regulate urea transport by a post-translational mechanism. However, we cannot exclude the possibility that rUT2 mRNA increased transiently and then returned to control values by the time we harvested inner medullary tissue.

Urea Transport

This study is the first to demonstrate that glucocorticoids can change urea transport in a mammalian collecting duct segment. However, the studies presented here cannot determine whether the change in terminal IMCD urea transport and VRUT protein is a direct effect of glucocorticoids or whether it is secondary to other physiologic changes that occur in rats after adrenalectomy and glucocorticoid replacement.

We found no evidence for malnutrition (based upon total protein and albumin levels and rat weight) or renal dysfunction (based upon creatinine and blood urea nitrogen levels) in the three groups (sham-operated, adrenalectomized, and adrenalectomized rats receiving dexamethasone) (Table 1). However, 24-h urinary urea values were significantly lower in adrenalectomized rats than in either sham-operated rats or adrenalectomized rats receiving glucocorticoid replacement (Table 1).

Knepper et al. (7) reported an increase in the fractional excretion of urea in adrenalectomized rats when they are given dexamethasone, suggesting that glucocorticoids act to decrease tubular reabsorption of urea. This is consistent with the findings that the terminal IMCD has glucocorticoid receptor mRNA (18) and is the primary site for facilitated urea reabsorption in the kidney (1,10). The effect of reducing urea permeability, as we observed in the dexamethasone-replaced rats, would be to reduce urea reabsorption. This is a possible explanation for the increase we observed in urine urea (Table 1) and the increase Knepper et al. found in the fractional excretion of urea (7) in adrenalectomized rats given glucocorticoid replacement. Another possibility is that glucocorticoids decrease the sensitivity of urea transporter to stimulation by vasopressin. However, we found that vasopressin stimulates urea permeability comparably in all three groups (sham-operated, adrenalectomized, and adrenalectomized rats receiving dexamethasone) of rats. Thus, these data are consistent with a regulatory role for glucocorticoids on the tubular transport of urea.

We also tested for a change in urea transport in the initial IMCD because feeding rats a low-protein diet for 2 wk increases vasopressin-regulated urea transport in this segment (2,11,12). However, we found no effect of adrenalectomy or glucocorticoids in the initial IMCD.

Molecular Regulation of the Renal Vasopressin-Regulated Urea Transporter

Northern blot analysis of rat and rabbit inner medullary mRNA with a UT2 cDNA shows mRNA bands at 4.0 and 2.9 kb (2,3,6). These two mRNA bands are thought to be splice-variants of a single gene with identical 3' ends (4). The two transcripts differ at their 5' ends, where splicing of the 4.0-kb rUT2 mRNA produces an additional 1590 bp of coding region at the 5' end (4). In Xenopus oocytes, cAMP increases urea flux after injection of the 4.0-kb UT2 mRNA, suggesting that only the 4.0-kb UT2 mRNA encodes a vasopressin-regulated urea transporter (4). We found the appearance of a faint 2.9-kb mRNA band in the inner medullary
tip of 50% of the adrenalectomized rats given glucocorticoid replacement. However, we do not know the physiologic significance of this finding.

Nielsen et al. prepared a polyclonal antibody to rUT2 by immunizing rabbits with a synthetic polypeptide corresponding to the carboxyl-terminal 19 amino acids of the polypeptide sequence (5). Although this amino acid sequence is common to both rUT2 mRNA transcripts, the Nielsen et al. immunoblots show a single protein band at 97 kD in the rat inner medulla (5). The antibody used in this study was prepared by immunizing rabbits with the identical synthetic polypeptide and also identified a single protein band at 97 kD in rat inner medulla. This 97-kD protein band is specific because it is not present when blots were probed with pre-immune serum or when our antibody was preabsorbed against the immunizing peptide.

By Western analysis, we determined that immunoreactive VRUT protein was increased in membrane fraction (17,000 × g pellet) proteins from the inner medullary tip of adrenalectomized rats, suggesting that the increase in urea transport in the terminal IMCD may be explained, at least in part, by the increase in VRUT protein in the tip of the inner medulla.

Adrenalectomy also increased immunoreactive VRUT protein in vesicle fraction (200,000 × g pellet) proteins from the inner medullary tip. Nielsen et al. proposed that regulation of vasopressin-stimulated urea transport may involve “shuttling” of urea transporter-containing vesicles between the cytoplasm and the apical membrane (5). Our finding of VRUT protein in both membrane and vesicle fraction proteins is similar to the pattern of immunolocalization shown by Nielsen et al. (5).

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