Ontogeny of rabbit proximal tubule urea permeability

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Received 2 November 2000; accepted in final form 20 February 2001

Ontogeny of rabbit proximal tubule urea permeability. Am J Physiol Regulatory Integrative Comp Physiol 280: R1713–R1718, 2001.—Urea transport in the proximal tubule is passive and is dependent on the epithelial permeability. The present study examined the maturation of urea permeability (Purea) in in vitro perfused proximal convoluted tubules (PCT) and basolateral membrane vesicles (BLMV) from rabbit renal cortex. Urea transport was lower in neonatal than adult PCT at both 37 and 25°C. The PCT Purea was also lower in the neonates than the adults (37°C: 45.4 ± 10.8 vs. 88.5 ± 15.2 × 10⁻⁶ cm/s; P < 0.05; 25°C: 28.5 ± 6.9 vs. 55.3 ± 10.4 × 10⁻⁶ cm/s; P < 0.05). The activation energy for PCT Purea was not different between the neonatal and adult groups. BLMV Purea was determined by measuring vesicle shrinkage, due to efflux of urea, using a stop-flow instrument. Neonatal BLMV Purea was not different from adult BLMV Purea at 37°C [1.14 ± 0.05 × 10⁻⁶ vs. 1.25 ± 0.05 × 10⁻⁶ cm/s; P = not significant (NS)] or 25°C (0.94 ± 0.06 vs. 1.05 ± 0.10 × 10⁻⁶ cm/s; P = NS). There was no effect of 250 μM phloretin, an inhibitor of the urea transporter, on Purea in either adult or neonatal BLMV. The activation energy for urea diffusion was also identical in the neonatal and adult BLMV. These findings in the BLMV are in contrast to the brush-border membrane vesicles (BBMV) where we have previously demonstrated that urea transport is lower in the neonate than the adult. Urea transport is lower in the neonatal proximal tubule than the adult. This is due to a lower rate of apical membrane urea transport, whereas basolateral urea transport is the same in neonates and adults. The lower Purea in neonatal proximal tubules may play a role in overall urea excretion and in developing and maintaining a high medullary urea concentration and thus in the ability to concentrate the urine during renal maturation.

THE FINAL NITROGEN WASTE PRODUCT from metabolism of amino acids in mammals is urea (8, 11). Glomerular filtration rate and tubular transport are the main determinants of urinary excretion of urea. Despite changes in hydration status and protein intake that alter the overall excretion of urea, the proximal tubule reabsorbs ~50% of the filtered load of urea. Most evidence indicates that urea transport in the proximal tubule occurs by passive diffusion through the lipid bilayer (8, 11). Solute permeabilities are influenced by the phospholipid composition of membranes and thus may directly affect proximal tubule transport of urea (6).

There are developmental changes in the lipid composition of both apical and basolateral membranes that may play a role in the maturation of transport processes (1, 2, 4, 20). We have recently reported that the renal brush-border membrane permeability for urea is lower in neonates than adults (16). The purpose of the present study was to examine directly the maturation of proximal convoluted tubule urea permeability to determine the overall transport rates for urea in the adult and neonatal tubules.

METHODS

In vitro perfusion of tubules. Juxtamedullary proximal convoluted tubules (PCT) from adult and neonatal (9–16 days old) New Zealand White rabbits were perfused in vitro as previously described (3, 13, 21). The tubule segment used was primarily S1 PCT with some portion of the S2 PCT. There was no portion of the proximal straight tubule included in these experiments. Briefly, PCTs were dissected in cooled (4°C) modified Hank’s solution containing (in mM) 137 NaCl, 5 KCl, 0.8 MgSO4, 0.33 Na2HPO4, 0.44 KH2PO4, 1 MgCl2, 10 Tris-HCl, 0.25 CaCl2, 2 glucose, and 2 l-lactate. This solution was bubbled with 100% O2 and had a pH of 7.4. Tubules were then transferred to a 1.2-ml thermostatically controlled (25 or 38°C) bathing chamber and perfused with concentric glass pipettes. The perfusion solution simulated late proximal tubule fluid and contained (in mM) 140 NaCl, 5 KCl, 0.8 MgSO4, 0.33 Na2HPO4, 0.44 KH2PO4, 1 MgCl2, 10 Tris-HCl, 0.25 CaCl2, 2 glucose, and 2 l-lactate. This solution was exchanged at a rate of 0.5 ml/min to keep the osmolality constant. The bathing solution was adjusted to 295 mosmol/kg H2O by the addition of water or NaCl. The bathing solution was exchanged at a rate of 0.5 ml/min to keep the osmolality and pH constant.

Volume absorption (Jv; in nl·min⁻¹·mm⁻¹) was measured as the difference between the perfusion and collection rates and normalized per millimeter of tubule length. The collection rate was determined by timed collections using a constant volume pipette. Exhaustively dialyzed methoxy-¹³H]inulin (New England Nuclear) was added to the perfusate at a concentration of 50 μCi/ml so that the perfusion rate could be calculated. Urea transport (Jurea) was
determined by adding $[^{14}C]urea$ to the perfusate at 15 $\mu$Ci/ml and was calculated by

$$J_{\text{urea}} = \frac{(V_c C_0^c - V_i C_0^i)}{L} \frac{C_0^c}{C_0^i}$$

and urea permeability was calculated by the following equation (12, 14)

$$P_{\text{urea}} = \frac{V_c C_0^c - V_i C_i^i}{L} \frac{C_0^c - C_i^i}{\ln \left( \frac{C_i^i}{C_0^c} \right)}$$

where $V_0$ and $V_i$ represent the perfusion and collection rates, respectively, $C_0$ and $C_i$ represent the $^{14}C$ counts in the perfusate and collected fluid, respectively, and $C_0$ represents the urea concentration in the perfusate. Urea permeability was normalized to the inner surface area of the tubule using the inner diameter. The tubule length and inner diameter were measured using an eyepiece micrometer.

Preparation of basolateral membrane vesicles. Basolateral membrane vesicles (BLMV) were isolated from rabbit renal cortex by a modification of the method described by Grassl and Aronson (7), as previously reported in our laboratory (17). Kidneys were removed and immediately placed in ice-cold PBS (in mM: 137 NaCl, 2.7 KCl, 10.1 Na$_2$HPO$_4$, 1.7 KH$_2$PO$_4$, pH 7.4). The kidneys were decapsulated, and the cortexes were removed and minced with a razor blade. Cortex (1.5–2 g) was placed in 15 ml of ice-cold isolation buffer (in mM: 250 sucrose, 2 EDTA, 10 HEPES, adjusted to pH 7.6 with tetramethylammonium hydroxide) and 0.1 mM phenylmethylsulfonyl fluoride. All subsequent steps were carried out on ice or in refrigerated centrifuges (4°C). Tissue was homogenized by 30 strokes of a Teflon-glass homogenizer. The homogenate was centrifuged at 1,100 g for 10 min, and the resulting supernatant was decanted and kept on ice. The pellet was resuspended in 15 ml of isolation buffer, homogenized (20 strokes), and centrifuged at 1,100 g for 10 min. The two supernatants were combined and homogenized with an additional 10 strokes and centrifuged at 48,000 g for 10 min. The white, fluffy, upper layer of the resulting pellet was resuspended in 15 ml of isolation buffer, homogenized once again (10 strokes), and centrifuged at 48,000 g for 30 min. At this point, the fluffy upper layers from two adult rabbits or from one or two neonatal litter(s) were combined and resuspended in 15 ml of isolation buffer. Percoll was added (final concentration of 12%), and the resuspension was homogenized (10 strokes) and centrifuged at 40,000 g for 66 min. The resulting Percoll gradient was aspirated from the top with a Haake-Bucher Auto Densi-Flow apparatus (LABCONCO, Kansas City, MO). The basolateral membrane formed the upper band in the Percoll gradient. Fractions (40 drops/tube) were collected using an ISCO Foxy Jr. fraction collector (ISCO, Lincoln, NE). Fractions 3–5 were pooled and centrifuged at 200,000 g for 60 min. The membranous material on top of the Percoll was resuspended in ice-cold isolation buffer with 23- and 25-gauge needles and then centrifuged at 200,000 g for 40 min. The resulting pellet was resuspended in an 80 mosmol/kg H$_2$O mannitol solution (55 mM mannitol, 10 mM HEPES, adjusted to pH 7.4 with Trizma base) at a concentration of 10–15 mg protein/ml.

Osmolalities were determined by freezing-point depression using an Advanced Osmometer model 3D3 (Advanced Instruments, Norwood, MA). Protein was measured using bicinchoninic acid assay (Pierce Chemical, Rockford, IL). Na$^+$-K$^+$-ATPase activities were measured in the crude homogenate and the BLMV preparation to assess enrichment, as previously described by our laboratory (1). Na$^+$-K$^+$-ATPase enrichment was not different between the neonatal and adult BLMV (8.5 ± 3.0 vs. 12.5 ± 5.2-fold increase, respectively). BLMV size was previously determined by transmission electron microscopy in our laboratory by measuring diameters of vesicles from a randomly selected sample of greater than 100 vesicles for both adult and neonates.

Rapid kinetics for urea permeability measurement. Experiments were performed using a stop-flow instrument to ensure rapid mixing of vesicles and extravesicular buffer as previously described in our lab (16, 18). Vesicles were loaded with a solution containing (in mM) 500 urea, 80 mannitol, and 16 HEPES-Tris (pH 7.4). The extravesicular solution was 580 mM mannitol and 16 mM HEPES-Tris (pH 7.4). Intravesicular and extravesicular solutions were adjusted to 580 mosmol/kg H$_2$O. The stop-flow apparatus (SFM-3, Biologic, dead time ~7.5 ms) was set to mix 100 $\mu$l of vesicles 1:1 (final concentration of 0.3 mg protein/ml) with extravesicular buffer so that the final urea gradient was 250 mM. The reaction cuvette was temperature controlled with a circulating water bath. Excitation was from a 75-W xenon arc lamp via a monochrometer set at 400 nm, and emission was measured using a photomultiplier tube attached to the cuvette (Biologic). Data were collected at a rate of 10 points/s for 20 s using BIKINE software (Molecular Kinetics, Pullman, WA). For each experiment, five raw tracings were collected and averaged for subsequent analysis.

The data were fit to a double exponential curve, and the urea permeability was calculated from the following equation (16, 25)

$$P_{\text{urea}} = k/(S/V_0)$$

where $k$ is the weighted average rate constant from the double exponential fit of the data and $S$ is the surface area of the vesicle. This approach has been used by us and others (17, 24).

Activation energy (Ea) was calculated from the Arrhenius equation

$$\ln \left( \frac{P_1}{P_2} \right) = -\frac{Ea}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right)$$

where P, is the permeability, T is the temperature in degrees Kelvin, and R is the gas constant.

All data are expressed as means ± SE. Data from the micropерfusion experiments represent the mean of three or four collections in each period for each experiment. Comparisons between adult and neonatal groups were made by unpaired t-tests, whereas effects of temperature or phloretin were assessed using paired analysis. Significance was determined by a P value <0.05.

RESULTS

PCT urea transport. The tubule lengths were 1.1 ± 0.1 mm for both the adult and neonatal groups. The transport of urea in the PCT was lower in the neonates than the adults at both 37°C (17.8 ± 3.9 vs. 38.0 ± 4.7 pmol·mm$^{-1}$·min$^{-1}$, P < 0.01, n = 14 for both groups) and 25°C (14.5 ± 3.3 vs. 27.1 ± 4.4 pmol·mm$^{-1}$·min$^{-1}$, P < 0.05, n = 10 for neonates and 11 for adults). The transport in both groups was significantly greater at 37 than 25°C (P < 0.01 for the neonates and the adults). These data are shown in Fig. 1.
PCT urea permeability. As shown in Fig. 2, the urea permeability of the neonatal PCT was significantly lower than that of adult PCT at 37°C (45.4 ± 10.8 vs. 88.5 ± 15.2 × 10⁻⁶ cm/s, P < 0.05, n = 10 for neonates and 11 for adults) and at 25°C (28.5 ± 6.9 vs. 55.3 ± 10.4 × 10⁻⁶ cm/s, P < 0.05, n = 14 for both groups). The urea permeability was higher at 37°C in both neonatal and adult PCT (P < 0.01 for the neonates and P < 0.05 for the adults). The activation energy was 4.82 ± 1.13 kcal·degree⁻¹·mol⁻¹ in neonatal PCT and 6.97 ± 2.00 kcal·degree⁻¹·mol⁻¹ in the adult PCT [P = not significant (NS)]. Thus transepithelial permeability was lower in the neonates.

BLMV urea permeability. Figure 3 shows a typical tracing of neonatal and adult BLMV shrinkage. The vesicles were initially loaded with 500 mM urea so that after mixing with the mannitol solution, there was a 250-mM outwardly directed urea gradient. As urea diffused out of the vesicles, water exited the vesicles and the resulting shrinkage caused an increase in light scattering. The rate constants of the neonatal and adult BLMV were not different (0.27 ± 0.02 vs. 0.31 ± 0.03, P = NS, n = 6 for both groups). Thus, with identical initial vesicle size and rate constants, the urea permeability was found to be identical in neonatal and adult brush-border membrane vesicles at 25°C (0.94 ± 0.06 vs. 1.05 ± 0.10 × 10⁻⁶ cm/s, respectively, P = NS, n = 6 for both groups). These results are shown in Fig. 4.

Effect of temperature on BLMV urea permeability. To determine the temperature dependence of urea transport in neonatal and adult BLMV, the experiments were repeated at 37°C. The rate constants of the neonatal and adult BLMV were also not different at 37°C (0.32 ± 0.01 vs. 0.37 ± 0.02, P = NS, n = 6 for both groups). Although there was a significant increase in

![Fig. 1. Urea transport (J_{urea}) in neonatal and adult juxtamedullary proximal convoluted tubules (PCT). At both 25 and 37°C, J_{urea} was lower in the neonatal tubules than the adult tubules.](http://ajpregu.physiology.org/)

![Fig. 2. Urea permeability (P_{urea}) in neonatal and adult PCT. At 25 and 37°C, P_{urea} was lower in the neonatal PCT.](http://ajpregu.physiology.org/)

![Fig. 3. Representative tracings from experiments with adult basolateral membrane vesicles (BLMV; top curve) and neonatal BLMV (bottom curve). The data were fit with a double exponential, and P_{urea} was calculated from the average rate constant.](http://ajpregu.physiology.org/)

![Fig. 4. P_{urea} for neonatal and adult BLMV at 25 and 37°C. The adult and neonatal BLMV P_{urea} were not different at either 25 or 37°C. The P_{urea} was higher in both neonatal and adult BLMV at 37°C than at 25°C.](http://ajpregu.physiology.org/)
urea permeability for both neonatal and adult BLMV, the urea permeability for the neonatal BLMV remained comparable to adult BLMV at 37°C (1.14 ± 0.05 vs. 1.25 ± 0.05 × 10⁻⁶ cm/s, respectively; Z = NS; n = 6 for both groups). These results are shown in Fig. 4. The activation energy for urea transport, calculated from the Arrhenius relationship, was the same in the neonatal and adult BLMV (3.14 ± 0.52 vs. 3.00 ± 1.03 kcal-degrees⁻¹·mol⁻¹; Z = NS). Thus the temperature dependence of urea transport in the neonatal and adult BLMV is the same.

Effect of phloretin on BLMV urea permeability. Phloretin is a known inhibitor of the red blood cell and renal urea transporter (8, 11). To determine the phloretin sensitivity of urea transport in the proximal tubule basolateral membrane, the experiments were performed in the presence of 250 μM phloretin. Phloretin had no effect on urea permeability in either the neonatal or the adult BLMV (Fig. 5) at 25°C. These data are consistent with diffusion of urea through the lipid bilayer of the proximal tubule cell membrane and not through a specific, phloretin-sensitive transporter.

**DISCUSSION**

The present study examined the maturation of rabbit PCT and renal BLMV urea permeability. Urea transport and permeability in the proximal tubule was lower in neonatal tubules than in the adults at both 37 and 25°C. In contrast to the brush-border membrane vesicles, there was no difference between the neonatal and adult BLMV urea permeability. There was no effect of phloretin on the transport of urea in the BLMV, which is consistent with diffusion of urea through the lipid bilayer and not through a phloretin-sensitive urea transporter. The urea permeability of the basolateral membrane in both the neonates and adults was higher than the respective apical membranes. Thus the developmental increase in urea transport in the proximal tubule is due to an increase in apical membrane permeability.

Urea transport across cell membranes can occur through specific urea transporters or by diffusion through the lipid bilayer (8, 11). Specific transporters for urea have been identified in red blood cells (HUT11) and kidneys (UT2) (8). Other members of the urea transporter family have been identified and are currently designated UT-A for renal urea transporters and UT-B for the red cell urea transporter (9, 19, 23). These transporters have a high degree of homology and both are reversibly inhibited by phloretin. UT-A is located in the medulla of the kidney and is regulated by protein intake and hydration state of the animal (8, 19). There are several lines of evidence against a urea transporter in the proximal tubule. First, there was no expression of UT2 found in the proximal tubule (8).

Second, the diffusional permeability of urea in the proximal tubule is not higher than that found in lipid bilayers, indicating that urea transport in the proximal tubule could be explained by simple diffusion across the lipid bilayer with no need for facilitated diffusion (11). Last, phloretin had no effect on urea transport in either the neonatal or adult BLMV or brush-border membrane vesicle (16). This is consistent with the hypothesis that urea diffuses through the lipid bilayer of the basolateral membrane and not through a urea transporter. However, it remains possible that urea transport could occur via a phloretin-insensitive transporter.

Urea can move through the proximal tubule by either a transcellular route or the paracellular pathway. The transcellular route would include the apical and basolateral membranes and the intracellular compartment. If the intracellular compartment provided resistance to the diffusion of urea, the epithelium could be modeled as two membranes in series, and the transepithelial permeability should be equivalent to that of the two membranes as shown in Fig. 6. If the intracellular compartment contributed significant resistance to the transcellular movement of urea, then the measured permeability should be less than the permeability calculated from the apical and basolateral membrane measurements. This model offers some insight into the development of urea transport.

For this analysis, the apical membrane urea permeability was taken from our previous measurements of the adult and neonatal rabbit brush-border membrane vesicle (16). The apical and basolateral membrane surface areas were taken from Evan et al. (5). For comparison of the calculated to the measured transepithelial permeability, all data were normalized to 1-mm tubule length. This removes the problem of which diameter to use to normalize the tubular data to surface area and is also the approach used by Kokko (10). The results of this comparison are shown in Table 1. As can be seen, both the neonatal and adult urea permeabilities calculated from the individual membranes are comparable although less than the measured urea permeability of the PCT. Thus the intracellular compartment offers no resistance to the movement of urea through the cell. The movement of urea through the paracellular pathway could account for the somewhat higher measured permeability than the calculated permeability. However, the contribution of urea move-
that of the adult, but the surface area is also about one-half of the adult (1.47 vs. 2.21 × 10^6 μm²/mm). Thus, although the basolateral membrane of the neonate and adult proximal tubule may have equal urea permeability per unit area, the total surface area per millimeter of tubule length is less in the neonate. Thus the developmental increase in the PCT urea permeability is due primarily to the increase in the apical membrane urea permeability and secondarily to the increase in surface area of both the apical and basolateral membranes.

The present study demonstrates that neonatal and adult BLMV have identical urea permeability. Urea transport in both neonate and adult membranes is not affected by phloretin, which is consistent with a simple diffusion model of urea transport in the proximal tubule apical membrane. The apical membrane appears to be the limiting step to urea transport during development. The lower urea transport in neonatal tubules may directly affect urinary concentrating ability.

**Perspectives**

It is unclear how the lower urea transport in neonatal proximal tubules would impact urea handling in the kidney. The neonatal kidney has a limited ability to concentrate the urine (22). Many factors affect the concentrating ability of the kidney, including distal delivery of urea. The proximal tubule in adult kidneys reabsorbs approximately one-half of the filtered load of urea, which appears to be an important factor in urea recycling throughout the cortex (11). The intricate handling of urea in the adult nephron beyond the proximal tubule will then depend on how much is delivered to the remaining segments and will ultimately determine the medullary hypertonicity and urinary concentrating ability. The lower proximal tubule transport rates in neonates may be an important factor in maintaining the delivery of urea to the medulla, which may then affect the medullary interstitial concentration of urea. This may be a factor contributing to the development of neonates to concentrate their urine (22).

The authors thank J. McQuinn for able secretarial assistance. This work was supported by National Institutes of Diabetes and Digestive and Kidney Diseases Grants K08-DK-02232 (to R. Quigley) and DK-41612 (to M. Baum) and The National Kidney Foundation of Texas.

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Table 1. Comparison of calculated to measured transepithelial permeability data

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<tr>
<th></th>
<th>BBMV P_u</th>
<th>Apical membrane surface area</th>
<th>BLMV P_u</th>
<th>Basolateral membrane surface area</th>
<th>Calculated P_total</th>
<th>Measured P_total</th>
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<tr>
<td>25°C</td>
<td>0.34 ± 0.04</td>
<td>2.7 ± 0.3</td>
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<td>1.47 ± 0.10</td>
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<tr>
<td>37°C</td>
<td>0.45 ± 0.04</td>
<td>3.1 ± 0.3</td>
<td>1.14 ± 0.05</td>
<td>1.84 ± 0.12</td>
<td>0.45</td>
<td>0.79</td>
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<td><strong>Adult</strong></td>
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<tr>
<td>25°C</td>
<td>0.56 ± 0.03</td>
<td>4.5 ± 0.5</td>
<td>1.05 ± 0.10</td>
<td>2.21 ± 0.11</td>
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<td>37°C</td>
<td>0.66 ± 0.03</td>
<td>5.3 ± 0.6</td>
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Values are means ± SE. P, permeability; PCT, proximal convoluted tubules; BBMV, brush-border membrane vesicles; BLMV, basolateral membrane vesicles.
REFERENCES