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Maturation Changes in Rabbit Renal Basolateral Membrane Vesicle Osmotic Water Permeability

R. Quigley¹, N. Gupta¹, A. Lisec¹, and M. Baum^{1,2}

¹Departments of Pediatrics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9063, USA

²Departments of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9063, USA

Abstract

We have recently demonstrated that while the osmotic water permeability (P_f) of neonatal proximal tubules is higher than that of adult tubules, the P_f of brush-border membrane vesicles from neonatal rabbits is lower than that of adults. The present study examined developmental changes in the water transport characteristics of proximal tubule basolateral membranes by determining aquaporin 1 (AQP1) protein abundance and the P_f in neonatal (10–14 days old) and adult rabbit renal basolateral membrane vesicles (BLMV). At 25°C the P_f of neonatal BLMV was significantly lower than the adult BLMV at osmotic gradients ranging from 40 to 160 mOsm/kg water. The activation energies for osmotic water movement were identical in the neonatal and adult BLMV (8.65 ± 0.47 vs. 8.86 ± 1.35 kcal · deg⁻¹ · mol⁻¹). Reflection coefficients for sodium chloride and sodium bicarbonate were identical in both the neonatal and adult BLMV and were not different from one. Mercury chloride (0.5 mM) reduced osmotic water movement by $31.3 \pm 5.5\%$ in the adult BLMV, but by only $4.0 \pm 4.0\%$ in neonatal vesicles ($P < 0.01$). Adult BLMV AQP1 abundance was higher than that in the neonate. These data demonstrate that neonatal BLMV have a lower P_f and AQP1 protein abundance than adults and that a significantly greater fraction of water traverses the basolateral membrane lipid bilayer and not water channels in neonates compared to adults. The lower P_f of the neonatal BLMV indicates that the basolateral membrane is not responsible for the higher transepithelial P_f in the neonatal proximal tubule.

Introduction

The proximal tubule reabsorbs the bulk of the glomerular ultrafiltrate (Schafer, 1990). This process is nearly isoosmotic because of the high osmotic water permeability (P_f) of this segment. The high proximal tubule P_f is due, in part, to the presence of AQP1 water channels (Sabolic et al., 1992). There is a developmental increase in the expression of AQP1 in the rat proximal tubule with minimal staining for AQP1 prior to birth and intense staining by six days of age (Bondy et al., 1993). We have recently demonstrated that there is also a developmental increase in rabbit renal brush border membrane vesicle AQP1 protein

abundance and a developmental increase in osmotic water permeability (Quigley et al., 1998). Despite these maturational changes in the apical membrane, the neonatal rabbit proximal tubule has a higher transepithelial P_f than the adult segment (Quigley & Baum, 1996). Thus, the difference in the epithelial P_f between the adult and neonatal proximal tubule must be due to developmental changes in other components of the route for water transport.

There are differences between the proximal tubule apical and basolateral membranes. The basolateral membrane has a lower P_f than the apical membrane (Meyer & Verkman, 1987; van Heeswijk & van Os, 1986). The lipid composition of the basolateral membrane is different from that of the apical membrane and the basolateral membrane fluidity is higher than that of the apical membrane (Le Grimmelée et al., 1982). Thus developmental changes in the P_f of the basolateral membrane may account for the developmental changes in proximal tubule P_f .

The purpose of the present study was to determine the characteristics of neonatal basolateral membrane water transport. To this end, we determined the osmotic water permeability and AQP1 protein abundance of adult and immature rabbit proximal tubule basolateral membranes. The temperature dependence and mercury sensitivity of water transport were measured to compare the routes for water transport in neonatal and adult BLMV. Solute reflection coefficients were also studied to examine the interactions of solute and water in the BLMV.

Materials and Methods

Preparation Of Basolateral Membrane Vesicles

Basolateral membrane vesicles were isolated from rabbit renal cortex by modification of the methods described by Grassl and Aronson (1986). Kidneys were removed and immediately placed in ice-cold PBS (in mM: 137 NaCl, 2.7 KCl, 10.1 Na₂HPO₄, 1.7 KH₂PO₄, pH 7.4) prior to dissection of the cortex. Kidneys were decapsulated, then the cortices were dissected and minced. Minced tissue was divided into 1.5 to 2 g portions which were placed in 15 mL of ice-cold isolation buffer (250 mM sucrose, 2 mM EDTA, 10 mM HEPES, adjusted to pH 7.6 with tetramethylammonium (TMA) hydroxide; 0.1 mM phenylmethylsulfonyl fluoride was added immediately before use) prior to homogenization. All subsequent steps were carried out on ice or in refrigerated centrifuges (4°C). Tissues were homogenized 30 strokes using a Teflon-glass homogenizer. The homogenate was centrifuged at $1,100 \times g$ for 10 min in a Beckman JA-20 rotor, and the resulting supernatant was decanted off and kept on ice. The pellet was resuspended in 15 ml of resuspension buffer (250 mM sucrose, 10 mM HEPES, adjusted to pH 7.6 using TMA hydroxide), homogenized 20 strokes as before, and centrifuged at $1,100 \times g$ for 10 min. The two supernatants were combined and homogenized with 10 strokes, and centrifuged at $48,000 \times g$ for 10 min. The white, fluffy, upper layer of the resulting pellet was resuspended in 15 mL of the buffered sucrose medium described above, homogenized once again (10 strokes) and centrifuged at $48,000 \times g$ for 30 min. At this point, the fluffy upper layers from two adults or from a neonatal litter(s) were combined and resuspended in 15 mL of buffered sucrose medium. Percoll was added (final concentration of 12%), and the resuspension was homogenized 10 strokes and centrifuged at

40,000 $\times g$ for 66 min. The resulting Percoll gradient was aspirated from the top with a Haake-Buchler 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 (usually tubes 3–5, depending on amount of starting material) were pooled and centrifuged at 200,000 $\times g$ for 60 min. The membranous material on top of the hard Percoll pellet was resuspended in ice-cold buffered sucrose medium with 23- and 25-gauge needles then centrifuged at 200,000 $\times g$ for 40 min. The resulting pellet was resuspended in an 80 mOsm/kg water 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 after which total protein concentration was determined by bicinchoninic acid assay (BCA, Pierce Chemical, Rockford, IL). Osmolalities were determined by freezing point depression using an Advanced™ OSMOM-ETER model 3D3 (ADVANCED INSTRUMENTS, Norwood, MA). Na-K-ATPase activities were measured in the crude homogenate and the BLMV preparation to assess enrichment, as previously described in our laboratory (Arar, Levi, & Baum, 1994). Na-K-ATPase enrichment was not different between the neonatal and adult BLMV (7.5 ± 0.8 – vs. 11.5 ± 3.4 -fold increase). Initial vesicle size was determined by transmission electron microscopy. Diameters were measured on vesicles from a randomly selected sample of greater than 100 vesicles for each adult and neonate.

Immunoblotting For AQP1

Immunoblots for AQP1 were performed by loading 20 μ g total protein of neonatal and adult BLMV in final volume of 30 μ l of loading buffer (1.7 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 1% 2-mercaptoethanol, pinch bromphenol blue) in each sample lane, with a separate lane containing 20 μ l Prestained Molecular Weight Standards (#SDS-7B, Sigma). Proteins were transferred onto PVDF (Immobilon) overnight at 4°C using Bio-Rad Transblot electroblotting apparatus.

Anti-AQP1 (Alomone Labs, Jerusalem, Israel) and anti- β -actin (Sigma) were used at 1:1,000 and 1:5,000 dilutions, respectively. All antibodies were diluted in Blotto (5% nonfat dried milk, 0.05% Tween-20 in autoclaved Phosphate Buffered Saline (PBS): 150 mM NaCl, 10 mM sodium phosphate, 2.6 mM KCl and 1 mM KH_2P_4 ; pH 7.4). Blots were wetted briefly in methanol, rinsed in H_2O and PBS-Tween (0.05% Tween-20), preincubated in Blotto for 1 hr and incubated with the primary antibody for 2 hr at room temperature. Blots were then rinsed 3 times over 30 min in PBS-Tween (1% Tween-20), incubated with HRP-conjugated anti-rabbit monoclonal Ab for 1 hr. The blot was washed with Blotto and enhanced chemiluminescence was used to detect antibody (Amersham ECL kit).

Rapid Kinetics For Water Permeability (P_f) Measurement

Stop-Flow Kinetics—Because of the speed of water movement across the basolateral membrane vesicles, these experiments required rapid mixing with a stop-flow device. The stopped flow apparatus (SFM-3, BioLogic, dead time ~7.5 msec) was set to mix 100 μ l of vesicles 1:1 (final concentration of 0.3 mg protein/ml) with resuspension buffer of the desired osmolality (as above, adjusting the osmolality with D-mannitol). Excitation was from a 75-watt xenon arc lamp via a monochromator set at 400 nm and emission was measured

via Biologic photomultiplier tube. The fluorometer was interfaced to the mixing device via randomly wound, fused-silica fiber optic cables. Data were collected in photon counting mode at 250 points/second for 2 sec using Biologic software. For each experiment, 5 raw tracings were collected and averaged for subsequent analysis.

Osmotic Water Permeability—The osmotic water permeability was calculated from the stop-flow light scattering data as described by Van Heeswijk and van Os, 1986). Briefly, the scattered light intensity was normalized to an initial value of 1, then fit to a double exponential curve:

$$I(t) = b - c_1 e^{-k_1 t} - c_2 e^{-k_2 t}$$

where $I(t)$ is the light intensity at time t , b is the asymptote, c_i are the coefficients and k_i are the rate constants. The mean rate constant was determined, by the following equation:

$$\bar{k} = \frac{c_1 k_1 + c_2 k_2}{c_1 + c_2}$$

P_f was then calculated from the following equation:

$$\bar{k} = \bar{V}_w P_f (A/V_0) C_m,$$

where $\bar{V}_w$ is the molar water volume, A/V_0 is the surface area to initial vesicle volume ratio and C_m is the osmolality of the extravesicular solution. The initial surface area to volume ratio was calculated assuming that the vesicles were spheres.

All data are mean $\pm$ SEM. Comparisons were made using unpaired analysis (t-test and linear regression where appropriate) and significance was taken to be $P < 0.05$. Calculations were made using SigmaStat software (Jandell Scientific).

Results

Immunoblot For AQP1

Figure 1 shows the immunoblot of AQP1 and β -actin in both the neonatal and adult BLMV. As can be seen, AQP1 abundance is greater in the adult vesicles than the neonatal vesicles. The ratio of AQP1/ β -actin densitometry readings were compared using the Mann-Whitney test. The median ratio for the adults was 0.14 (25–75% range: 0.12–0.33) and for the neonates was 0.05 (25–75% range: 0.03–0.06; $P < 0.02$). Thus, there is a maturational increase in the expression of AQP1 in the rabbit proximal tubule basolateral membrane.

Initial Vesicle Size

Initial vesicular size was measured using transmission electron microscopy. There was no significant difference observed between adult ($n = 109$) and neonatal ($n = 172$) initial vesicle diameters (adult: 204.5 ± 5.6 nm vs. neonate: 211.6 ± 6.9 nm; $P = \text{NS}$). Thus, any difference

in the calculated P_f is not due to a difference in the initial vesicle size. These values were also similar to our previously reported values for BBMVs (Quigley et al., 1998).

Osmotic Water Permeability

Typical tracings of adult and neonatal BLMV preparations are shown in Fig. 2. As can be seen, the adult BLMV curve shows a faster rate of shrinkage. The P_f of the adult preparations were greater than that of the neonatal BLMV at all osmotic gradients examined (Fig. 3). Thus, osmotic water transport through the basolateral membrane is slower in the neonate and cannot be the cause of the higher transepithelial P_f found in the in vitro perfused tubule (Quigley & Baum, 1996).

Activation Energy

To determine the activation energy for osmotic water permeability, adult and neonatal BLMV were exposed to an 80 mOsm/kg water osmotic gradient with mannitol at temperatures from 20° to 40°C. The data were fit to a double exponential curve and an Arrhenius plot was constructed using the natural logarithm of the mean rate constants (Fig. 4). The activation energies (calculated from the slopes of the lines) were $8.65 \pm 0.47 \text{ kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$ for the neonates and $8.86 \pm 1.35 \text{ kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$ for the adults ($P = \text{NS}$). This is in contrast to the BBMVs where the neonatal membranes had a higher activation energy than the adult preparation (Quigley et al., 1998).

Inhibition With Mercury Chloride

To determine the degree to which mercury inhibits water transport, BLMVs were incubated with 0.5 mM HgCl_2 and subjected to an 80 mOsm/kg water osmotic gradient at 25°C. The size of the vesicles was followed with light scattering. The percent inhibition was greater in the adult BLMV than the neonatal BLMV ($31.3 \pm 5.5\%$ vs. $4.0 \pm 4.0\%$, $P < 0.01$ (Fig. 5)). This is consistent with less channel mediated water transport in the neonatal than the adult BLMV. To measure the effect of mercury on the activation energy, vesicles were examined at 40°C. There was no significant change in the activation energy for either the adult or neonatal vesicles with the addition of mercury.

Reflection Coefficients

Reflection coefficients for sodium chloride and sodium bicarbonate were determined as previously described (Quigley, Flynn, & Baum, 1999). Briefly, BLMVs were exposed to identical osmotic gradients of mannitol, sodium chloride and sodium bicarbonate. The refractive index of the salt solutions were adjusted to the mannitol solution by the addition of polyvinyl pyrrolidone. The initial slope of the curve for the solute being tested was then divided by the initial slope of the mannitol curve for that preparation.

The reflection coefficients are shown in the Table. Although the neonatal NaCl reflection coefficient tended to be lower than the adult, there were no statistically significant differences found between the adult and neonatal BLMV and the coefficients were not different from one. These results are similar to our findings in the BBMVs and demonstrate that there is no evidence of solvent drag through the proximal tubule cells (Quigley et al., 1999).

Discussion

The present study examined the maturation of rabbit renal basolateral membrane vesicle osmotic water permeability. The P_f and abundance of AQP1 of BLMV from neonatal rabbits was lower than that of adult BLMV. The activation energy for P_f in the neonatal BLMV was found to be identical to that of the adult BLMV. The amount of water transport in the neonatal BLMV that was inhibitable by mercury was significantly less than that in the adult BLMV. This is consistent with more water transport in the neonatal membranes occurring through the lipid bilayer. Thus, the higher transepithelial P_f for neonatal proximal tubules previously found using in vitro microperfusion (Quigley & Baum, 1996) cannot be explained by a higher P_f in the basolateral membrane.

Because of its high transepithelial osmotic water permeability, the proximal tubule reabsorbs the bulk of the glomerular ultrafiltrate by a process that is nearly isoosmotic (Schafer, 1990). The driving force for this fluid movement is thought to be hypotonicity of the luminal fluid due to active transport of solute from the lumen (Schafer, 1990). In adult proximal tubules, the route for transepithelial water transport is thought to be transcellular, although this remains controversial (Preisig & Berry, 1985; Schafer, 1990). While the transepithelial pathway for water movement has not been directly addressed in neonatal tubules, solute reflection coefficients for sodium chloride and sodium bicarbonate have been measured (Quigley & Baum, 1996). The reflection co-efficients in both adult and neonatal proximal tubules perfused in vitro were found to be identical and not significantly different from one. This suggests that water movement through the paracellular pathway is minimal, although this remains an open issue (Quigley & Baum, 1996). Since the transcellular route for water movement consists of the apical and basolateral membranes and the intracellular compartment, any differences in transepithelial water permeability must be due to differences in one or more of these components.

Previous studies of water transport through the basolateral membrane in adult proximal tubules indicated that the P_f was slightly lower than that of the apical membrane (Meyer & Verkman, 1987; van Heeswijk & van Os, 1986). The activation energy for water movement was higher in the BLMV than the BBMV (Meyer & Verkman, 1987). This is consistent with the finding that the membrane fluidity was higher in BLMV than BBMV (Le Grimellec et al., 1982). The present study also demonstrated that the P_f of the BLMV is lower than that found in the BBMV (at 25°C BLMV: 102.6 ± 3.5 vs. BBMV: 173.4 ± 20.3 $\mu\text{m}/\text{sec}$) while the activation energy is higher (BLMV: 8.86 ± 1.35 vs. BBMV: 5.09 ± 0.57 $\text{kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$) (Quigley et al., 1998). These differences could be due to differences in lipid composition between the apical and basolateral membranes.

Although the P_f of neonatal BLMV has not been directly measured, it has been estimated indirectly (Lin-shaw, Welling, & Bauman, 1986). The rates of neonatal and adult rabbit proximal tubule swelling were compared in isolated nonperfused tubules. The authors concluded that the hydraulic conductivity of the basolateral membrane in adult and neonatal tubules were identical (Lin-shaw et al., 1986). The present study clearly demonstrated that the P_f of neonatal BLMV was less than that of adult BLMV. The difference found may be due to the time course of the experiments. The swelling in the tubule experiments was

induced by treating the tubules with ouabain to inhibit active transport and not by quickly inducing an osmotic gradient (Linshaw et al., 1986). Thus, the rate of swelling was limited by the entry of solute into the tubule cells.

Water movement through the cell membranes can occur via diffusion through the lipid bilayer or through specialized water channels. Water transport through channels is characterized by a low activation energy ($<6 \text{ kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$) and inhibition by mercury while water movement through the lipid bilayer has a high activation energy ($>10 \text{ kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$) and is not inhibited by mercury (Verkman et al., 1996). In the present study, the activation energy for the neonatal BLMV water transport was found to be the same as the adult ($8.65 \pm 0.47 \text{ kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$). This is comparable to the activation energy found in the neonatal BBMV (Quigley et al., 1998). The result for the adult BLMV ($8.86 \pm 1.35 \text{ kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$) is higher than that found for the BBMV value (Quigley et al., 1998), suggesting that there may be a higher fraction of water diffusing through the lipid bilayer in the basolateral membrane. Previous studies in adult dogs indicate that the BLMV have a higher fluidity than the BBMV (Le Grimellec et al., 1982). This may result in a greater temperature dependence of water transport as reflected in the higher activation energy.

The mercury sensitivity of water transport through AQP1 is due to a cysteine residue at position 189 (Moon et al., 1993; Zhang et al., 1993). In the present study, osmotic water transport was inhibited to a smaller degree in the neonatal BLMV than the adult was. This is consistent with a larger portion of the water movement being through the lipid bilayer in the neonatal membranes than the adult and is similar to the findings in the brush border membrane vesicle (Quigley et al., 1998). The activation energy for water transport in the presence of mercury, calculated by measuring the P_f at 25° and 40°C, was not different from the activation energy in the control vesicles. This could be due to several factors. First, the degree of inhibition that we found with mercury in the adult vesicles was only 31%. Thus, the water channels may not have been totally blocked and allowed some water transport to occur. Second, there is accumulating data that other transporters (e.g., the glucose transporter) may transport water and would therefore allow for water transport in the presence of mercury (Fischbarg et al., 1990; Zeidel et al., 1992; Fischbarg & Vera, 1995). Third, the lipid content of the membrane is known to be different from the brush border membrane and may alter its interaction with the water channel and water transport (Le Grimellec et al., 1982). These factors may affect the activation energy in the presence of mercury.

The remaining component of transcellular water movement is the intracellular compartment. The cytoplasmic compartment is a complex unstirred layer (Berry & Verkman, 1988) and may account for over 50% of the transcellular resistance to water movement (Berry, 1985). Thus, small changes in the cellular compartment may significantly affect transepithelial water permeability. The cell height in neonatal proximal tubules is significantly lower than adult proximal tubules (Evan, Gattone, & Schwartz, 1983; Baum, 1990). This suggests that the path length for water movement through the cell may be much shorter in the neonatal proximal tubule than the adult. Thus, the higher transepithelial P_f in neonatal proximal tubules may be due to developmental changes in the intracellular compartment.

The present study examined maturation of the basolateral membrane P_f from rabbit renal cortex. There were significant differences found in the water transport pathway between the neonatal and adult vesicles. While these differences could be obtained because of technical considerations in the preparation of the vesicles, this is unlikely since they have identical diameters and Na-K-ATPase activities. There was a developmental increase in AQP1 expression. The P_f in neonatal BLMV was lower than the adult BLMV and there was less inhibition with mercury chloride. Thus, the movement of water in the neonatal proximal tubule basolateral membrane is more lipid mediated and less channel mediated than the adult membrane. The reason for the higher transepithelial P_f in the neonatal tubules is probably due to differences in the intracellular compartment, although differences in the paracellular pathway may also play a role.

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Fig. 1. Immunoblot of AQP1 abundance in neonatal and adult BLMV. Each lane was loaded with 20 μ g of total protein and probed with anti-AQP1 and anti- β actin antibody.

Fig. 2. Relative light intensity of neonatal (lower curve) and adult (upper curve) BLMV. Vesicles were loaded with resuspension buffer (80 mOsm/kg water) and exposed to an inwardly directed osmotic gradient (250 mOsm/kg water). Vesicle shrinkage results in increased light scattering. Relative light intensity was fit to a double exponential.

Fig. 3.

P_f of adult and neonatal BLMV. P_f was determined from a double exponential curve. At all osmotic gradients tested, the P_f of neonatal BLMV ($n = 4$) was significantly lower than adult BLMV ($n = 5$). * $P < 0.001$.

Fig. 4.

Arrhenius plots of temperature dependence of mean rate constants from the neonatal and adult BLMV. Temperature was varied from 20° to 40°C. Activation energy, calculated from the slopes of the plots, was $8.65 \pm 0.47 \text{ kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$ for the neonatal BLMV (open circles; $n = 4$) and $8.86 \pm 1.35 \text{ kcal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$ for the adult BLMV (closed circles; $n = 5$) ($P = \text{NS}$).

Fig. 5.
Effect of 0.5 mM HgCl₂ on osmotic water permeability in neonatal and adult BLMV at 25°C.
**P* < 0.01.

Table

Adult and neonatal BLMV solute reflection coefficients

Solute	Reflection coefficient			
	Adult	<i>n</i>	Neonate	<i>n</i>
NaCl	1.14 ± 0.29	5	0.73 ± 0.13	6
NaHCO ₃	0.96 ± 0.09	5	1.23 ± 0.48	5