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Glucocorticoids increase osmotic water permeability (P_f) of neonatal rabbit renal brush border membrane vesicles

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Abstract

Glucocorticoids increase osmotic water permeability (P_f) of neonatal rabbit renal brush border membrane vesicles. *Am J Physiol Regul Integr Comp Physiol* 288: R1417–R1421, 2005. First published January 20, 2005; doi:10.1152/ajpregu.00448.2004.— During postnatal maturation, there is an increase in renal brush border membrane vesicle (BBMV) osmotic water permeability and a parallel increase in aquaporin-1 (AQP1) protein abundance. The mechanisms responsible for these changes remain unknown. Because serum glucocorticoid levels rise postnatally and have previously been linked to other maturational changes in renal function, we examined the effects of glucocorticoids on osmotic (P_f) and diffusional (P_{DW}) water permeability and AQP1 protein abundance of renal BBMV. Neonatal rabbits were treated with dexamethasone (10 μ g/100 g) for three days and compared with control neonates and adults. P_f and P_{DW} were measured at 20°C with a stopped-flow apparatus using light-scattering and aminonaphthalene trisulfonic acid (ANTS) fluorescence, respectively. P_f was significantly higher in BBMV from dexamethasone-treated neonates compared with vehicle-treated neonates, but remained lower than in BBMV from adults ($P < 0.05$). P_{DW} in dexamethasone and vehicle-treated neonatal BBMV was lower than in adult BBMV. P_f/P_{DW} ratio increased from neonate (5.1 ± 0.3) to dexamethasone (7.0 ± 0.1) and adult BBMV (6.3 ± 0.1). AQP1 expression was increased by dexamethasone treatment to adult levels. Membrane fluidity, which is inversely related to generalized polarization (GP) of steady-state laurdan fluorescence, was significantly higher in neonatal BBMV than both dexamethasone and adult BBMV (GP: neonate 0.285 ± 0.002 , dexamethasone treatment 0.302 ± 0.006 , and adult 0.300 ± 0.005 ; $P < 0.05$). These combined results show that dexamethasone-treatment during *days 4–7* of life increases BBMV water permeability despite a decrease in membrane fluidity. This occurs by increasing channel-mediated water transport, as reflected in an increase in AQP1 protein abundance and a higher P_f/P_{DW} ratio. This mimics the maturational changes and suggests a physiological role for glucocorticoids in maturation of proximal tubule water transport.

Keywords

stop-flow kinetics; diffusional water permeability; development; aquaporin

The proximal tubule reabsorbs approximately three fourths of the glomerular ultrafiltrate (26). This occurs in a nearly isoosmotic manner due to the high water permeability of this nephron segment provided by the abundance of aquaporin-1 (AQP1), the water channel in the kidney proximal tubule (1, 18, 26). The functional aspects of water transport in the proximal tubule during development have recently been studied and reviewed (21–25). In vitro microperfusion revealed that the osmotic water permeability coefficient (P_f) of the proximal tubule is higher in the neonatal rabbit than that in the adult (21). Studies with isolated membrane vesicles, on the other hand, have indicated that P_f of both the apical (brush border) and basolateral membranes are lower in the neonatal tubule compared with the adult (23, 24). For both membranes, this was attributed primarily to a lower expression of AQP1. The neonatal brush border membrane also had a higher activation energy (E_a) for water permeability and a lower sensitivity to mercury inhibition (24). We have also demonstrated that the intracellular compartment of the tubule cells plays a role in the developmental changes in the water permeability that likely explained the discrepancy (22). Thus the maturational changes in water transport in the proximal tubule involve several factors.

Since the discovery and identification of AQP1, much has been learned about its developmental expression (5, 7, 29, 32, 34). In rats, renal expression of AQP1 mRNA increased greatly in the first week after birth to remain high throughout adult life (5, 29, 34). This increase in mRNA expression is also seen in sheep and humans, but in these species, it is initiated during fetal life (6, 7, 32). The regulation of the developmental changes in proximal tubule water transport remains to be elucidated. Several researchers have provided evidence that glucocorticoids play an important role in maturational changes in the kidney (2–4, 20). Serum glucocorticoid levels rise postnatally in rabbits and rats (9, 10). These have been linked to an increase in proximal tubular acidification (3, 4), Na^+/K^+ -ATPase gene transcription (31) and a decrease in phosphate transport during neonatal life (2, 20). Furthermore, glucocorticoids have been shown to induce AQP1-expression in membranes prepared from whole neonatal rat kidney (11) and in fetal sheep (32). Other factors that affect water permeability, for example, alterations in membrane lipid composition, may also be regulated by glucocorticoids (2, 27, 28).

The purpose of this study was to examine the role of glucocorticoids in the maturational changes in water permeability of the brush border membrane of the proximal tubule. We determined P_f and the diffusional water permeability (P_{DW}) using stop-flow kinetics in brush border membrane vesicles (BBMV) from control and dexamethasone-treated neonates and adult rabbits. The physiological significance of measuring P_f and P_{DW} in the same preparation in this study is that it enables us to calculate the P_f/P_{DW} ratio. In membranes that lack water channels, this ratio is ~ 1 , but in channel-containing membranes it is greater than 1 (8). It is, therefore, an informative characteristic regarding the predominant pathway for

water to move across membranes. In addition, we measured the protein expression of AQP1 and membrane fluidity using the generalized polarization (GP) of laurdan.

METHODS

Animals

New Zealand white rabbits were housed at the University of Texas Southwestern Medical Center at Dallas. Pregnant does were received at least 9 days before delivery to care for their neonates. They were fed a standard laboratory chow. Neonates received daily, subcutaneous injections with dexamethasone (10 µg/100 g body weight) or vehicle (in mM): 150 NaCl, 2 K₂HPO₄, pH 7.5 for 3 days, including 2 h before death. Neonates were studied at 7 days of age. Adult rabbits ("adult" hereafter) were >9 wk of age. The protocols for this study were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

Materials

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and Fluka BioChemika (Buchs, Germany). 8-Aminonaphthalene- 1,3,6-trisulfonic acid (ANTS) and 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan) were obtained from Molecular Probes (Eugene, OR). Solution osmolalities were measured by freezing point depression (Advanced Instruments, Norwood, MA; osmometer model 3D3). Osmolalities of solutions prepared with D₂O could not be determined in this manner, but small osmotic gradients were previously shown not to affect measurements of P_{DW} (35).

Brush border membrane vesicle preparation

BBMV were prepared as described previously (24). Animals were killed by decapitation after sedation. Kidneys from 3–7 neonates were pooled, while adults were studied individually. Kidneys were removed and immediately placed in ice-cold PBS in mM: 137 NaCl, 2.7 KCl, 10.1 Na₂HPO₄, 1.7 KH₂PO₄, with pH 7.4). The capsule was removed, and the cortex was dissected. After mincing, the cortex was put in 15 ml of isolation buffer (in mM) 300 D-mannitol, 16 HEPES, 5 EGTA, adjusted to pH 7.5 with Tris-HCl; containing the protease inhibitors aprotinin (2 µg/ml), leupeptin (2 µg/ml), and phenylmethylsulfonyl fluoride (175 µg/ml). Tissue was homogenized by 15 strokes with a Potter homogenizer, after which precipitation was induced using 230 µl of 1.0 M MgCl₂. The homogenate was shaken vigorously for 10 s every 5 min for 20 min. Subsequently, the homogenate was centrifuged at 2,500 g for 15 min at 4°C. 230 µl 1.0 M MgCl₂ was added to the supernatant and shaken vigorously for 10 s every 5 min for 20 min. After this, it was centrifuged for 15 min at 2,500 g at 4°C. The supernatant was then centrifuged at 48,400 g for 30 min at 4°C. The pellets were resuspended in ice-cold 1.5 mL resuspension buffer (5.0 mM HEPES, pH 7.4; osmolality adjusted to 80 mosmol/kgH₂O with D-mannitol) using 22- and 25-gauge needles. The protein content was determined in the crude homogenate and BBMV using bicinchoninic acid protein assay (Pierce, Rockford, IL). Alkaline phosphatase activity was determined to assess enrichment of brush border membrane (BBM), as was previously described (2, 24). This was not different between control neonates, dexamethasone-treated neonates, and adults (fold increase: 7.6 ± 0.5, 8.4 ± 0.5, and 6.6 ± 0.7, respectively; *P* = NS).

P_f measurements

Fresh BBMVs were brought to a concentration of 0.6 mg protein/ml. BBMVs (100 μ l) were rapidly mixed with 240 mosmol/kgH₂O resuspension buffer (100 μ l) using a stopped-flow apparatus (SFM-3, Biologic, France). The cuvet was illuminated using a 75-W xenon arc lamp and a monochromator, which was set at 400 nm. Light scattering was measured using a photomultiplier tube (Biologic, France) oriented at a 90° angle with regard to the illumination axis. Data were collected at 10-ms intervals for 2–3 s using Bio-kin software (Biologic, France). Five raw tracings were averaged and analyzed subsequently. P_f was calculated as previously described (23).

P_{DW}-measurements

Recently, we successfully used a combination of stop-flow kinetics and aminonaphthalene-trisulfonic acid (ANTS) fluorescence to measure P_{DW} in rabbit renal BBMVs (16). This methodology takes advantage of the fact that the fluorescence of ANTS changes with the D₂O:H₂O ratio. In a separate set of experiments, fresh BBMVs suspensions were brought to a concentration of 10 mg protein/ml and loaded with ANTS (10 mM) overnight (12–16 h), in the dark at 4°C. After loading, BBMVs were washed five times with ice-cold 80 mosmol/kgH₂O-resuspension buffer and ultracentrifugation (368,000 g) for 10 min at 4°C. After the fifth spin, pellets were resuspended using 22- and 25-gauge needles, and vesicles were brought to a final concentration of 1.0 mg protein/ml. This suspension (100 μ l) was rapidly mixed with two volumes of isoosmotic resuspension buffer (2 $\times$ 100 μ l) made with D₂O as solvent using the previously described experimental setup. As the D₂O diffuses through the membrane, the D₂O:H₂O ratio will change, which results in an increase in the fluorescence from the ANTS. ANTS was excited by the 75-W xenon arc lamp with the monochromator set at 380 nm. The photomultiplier tube was equipped with a 500-nm cut-on filter to monitor the intensity of emitted light. Data were collected at 100- μ s intervals for 50 ms using the Bio-kin software. Five raw tracings were averaged, normalized to initial fluorescence, and fitted to a single-exponential curve using Biokine Software (Molecular Kinetics, Pullman, WA). P_{DW} was calculated using the following relation: $P_{DW} = 1/[\tau_{ex}(S/V)]$, where τ_{ex} is the exchange time (1/k), and S/V is the surface area to volume ratio of BBMVs (12). In our calculations, the diameter of BBMVs was assumed to be 200 nm, as previously measured in our laboratory in neonates and adults (24).

Immunoblotting for AQP1

Immunoblots were performed as described previously (23, 24). Briefly, BBMVs samples (50- μ g total protein) were brought to a 40- μ l volume using isolation buffer. After addition of 10 μ l of 5 $\times$ loading buffer (final SDS concentration of 10 mg/ml), samples were heated to 95°C for 5 min, chilled on ice, and loaded on a 10% SDS gel. Samples were transferred overnight onto PVDF membrane at 4°C.

Anti-AQP1 (Alomone Labs, Israel) was used at 1:1,000 and anti- β -actin (Sigma) at 1:5,000 dilutions. After preincubation with Blotto (5% nonfat milk and 0.05% Tween-20 in PBS), membranes were incubated with the primary antibody at room temperature for 2 h, washed four times over 40 min, and incubated with the secondary antibody at room temperature for 1 h. Detection was performed using an ECL kit (Amersham).

Membrane lipid fluidity

Membrane fluidity was assessed with a fluorescence technique using laurdan (13). BBMVs (100 µg protein in 100 µL buffer) were incubated with 0.3 nmol laurdan at 37°C for >20 min, after prolonged mixing by vortex. Steady-state emissions at 440 and 490 nm were measured at an excitation wavelength of 340 nm using a spectrofluorometer (PC1, ISS, Urbana-Champaign, IL). The generalized polarization (GP) was calculated using ISSPC software using the following relation: $GP_{\text{laurdan}} = [I_{440} - I_{490}] / [I_{440} + I_{490}]$, where I_{440} and I_{490} are the emission intensities at 440 and 490 nm, respectively. GP is inversely related to membrane fluidity, as laurdan will produce in a polar environment a higher-intensity emission at 440 nm; whereas in a crystalline phase, it will produce an even higher-intensity emission at 490 nm (19).

Statistical analysis

Data were analyzed using SigmaStat statistical software (Jandel, San Rafael, CA). Data were presented as means $\pm$ SE, unless otherwise noted. Comparisons were performed using *t*-tests or ANOVA (with Student-Newman-Keuls post hoc tests), as appropriate.

RESULTS

Osmotic water permeability (P_f)

Typical tracings of osmotically induced vesicle shrinkage for neonatal, dexamethasone, and adult BBMVs are shown in Fig. 1. Both maturation and dexamethasone-treatment affected P_f (Figs. 1 and 2). P_f was significantly different between all three groups ($P < 0.05$). Comparisons between the groups were made using ANOVA with Student-Newman-Keuls post hoc tests. P_f was lower in control neonates than in dexamethasone-treated neonates or adults. Dexamethasone treatment led to an increase in P_f , which did not reach adult levels. Dexamethasone treatment partially mimicked maturational changes.

These results indicate that during maturation, P_f increases, which is consistent with our earlier findings of a higher P_f in adults (24). Dexamethasone accelerates the maturational increase of P_f .

Diffusional water permeability (P_{DW})

Figure 3 shows the results of P_{DW} measurements for the three groups. P_{DW} was significantly lower in both neonatal groups than in adults ($P < 0.05$). This is consistent with our recent findings of P_{DW} in adults and 11-day-old neonates (16).

We also compared the P_f to P_{DW} ratio between the three groups. The ratio was lower in the control neonatal groups compared with the dexamethasone-treated neonates and the adult BBMVs (5.1 ± 0.3 , 7.0 ± 0.1 and 6.3 ± 0.1 , respectively; $P < 0.001$).

Immunoblot

To determine the effect of glucocorticoids on the expression of AQP1 in the proximal tubule apical membrane, we performed Western blots on the BBMVs from the three groups, as previously described from our laboratory (24). As seen in Fig. 4, the expression of AQP1 in

the neonatal BBMV was significantly less than that in the adult BBMV. Treatment of the neonates with dexamethasone for 3 days increased the amount of AQP1 expression to a value that was between that of the neonatal and adult BBMV. Densitometry of the expression of AQP1 factored for β -actin expression was neonates: 407 ± 299 ; dexamethasone: $4,435 \pm 1,183$; adults: $11,201 \pm 424$; $P < 0.05$ for differences between all three groups. Thus glucocorticoids have an effect on the expression of AQP1.

Membrane fluidity

Characteristics of water transport in BBMV are not only determined by the expression of AQP1. Changes in the membrane composition may have occurred and contributed to our findings of increased water permeability in dexamethasone and adult BBMV compared with neonatal BBMV. To examine possible changes in membrane fluidity, we measured the steady-state laurdan fluorescence. Generalized polarization of laurdan (GP_{laurdan}), which is inversely related to membrane fluidity, was found to be significantly lower in neonatal BBMV than both dexamethasone and adult BBMV (Fig. 5). In the latter two, GP was not different. Therefore, neonatal BBMVs were found to be more fluid than the other two groups. This is consistent with the finding that P_{DW} of neonatal BBMV, with lower AQP1 protein expression, is not different from dexamethasone and adult BBMV at higher temperatures, since increased membrane fluidity has been linked directly to higher water permeability (33).

DISCUSSION

The present study examined the role of glucocorticoids in the maturation of renal BBM water transport. Glucocorticoids were shown to accelerate maturational changes of BBM water transport in rabbit neonates. Treatment with dexamethasone on *days 4–7* of life resulted in an increase of P_f , P_{DW} , and P_f/P_{DW} ratio. This was accompanied by an increase in AQP1 protein expression. This increased expression of AQP1 was paralleled by a decrease in membrane fluidity, indicating that glucocorticoids had multiple effects on water transport.

The osmotic water permeability of the proximal tubule apical membrane was clearly affected by the administration of dexamethasone. The P_f of the BBMV from the dexamethasone-treated neonates was greater than that of BBMV from the control neonates and less than the adult. We have previously shown similar results in the maturational changes in brush border membrane water permeability characteristics (16, 24). In our previous study of osmotic water permeability, we used fluorescence quenching of an entrapped fluorophore to measure the shrinkage of the brush border membrane vesicles (24). We had found that maturational changes in P_f included an increase in P_f and AQP1 expression, a decreased E_a , and an increase in mercury sensitivity (24). The present study also demonstrates a maturational increase in P_f and AQP1 expression.

The diffusional water permeability of the proximal tubule apical membrane was not affected by the administration of dexamethasone. The maturational changes in the diffusional water permeability that we had previously found were also similar to those seen in the present study (16). Both of these studies used the same techniques for measuring the P_{DW} and found

that the adult membranes had a higher P_{DW} (16). The P_f/P_{DW} ratio was calculated from the values for P_f and P_{DW} from corresponding preparations. The results indicate that there is a significant increase in the ratio that occurs with development and that dexamethasone accelerates the maturational changes. This corresponds to an increase in the fraction of water that moves through water channels instead of through the lipid bilayer.

In addition to the physiological effects of glucocorticoids on water transport characteristics, we measured their effect on AQP1 expression. We found that 3 days of glucocorticoid treatment increased the expression of AQP1 in the BBMV to a level that was not different from that of the adult BBMV. Other investigators have studied glucocorticoids and their potential role in the developmental expression of AQP1 in the kidney (11, 32). These studies report a stimulating effect of glucocorticoids on AQP1 expression at both mRNA and protein levels, but there have been no reports of functional studies on isolated renal membrane vesicles. Glucocorticoids also induced the expression of AQP1 in lung endothelium (11) and AQP3 in airway epithelial cells (30). The functional effect of the latter was confirmed in stopped-flow experiments, in which P_f of the cell membrane was shown to increase (30).

The genetic mechanisms underlying the induction of AQP1 expression by dexamethasone have been studied in a mouse erythroleukemia cell line (15). This effect was shown to be mediated by two glucocorticoid response elements 0.5 kilo-bases upstream from the transcription initiation site. Although they suspected that this may play a central role in maturational changes, the authors remain cautious in their conclusions, because the induction by other compounds, including dimethyl sulfoxide, was found to be much greater (15). These compounds may mimic the effects of factors present in vivo that are involved in the maturational changes.

The laurdan measurements of membrane fluidity showed that both adult and dexamethasone BBMV were less fluid than neonatal BBMV. This is consistent with reported effects of aging (14) and dexamethasone (2, 13, 17) on renal and intestinal BBM fluidity. It is interesting that 3-day dexamethasone treatment increases AQP1 protein expression to adult levels and has an effect on fluidity, indicating that dexamethasone has differential effects on factors that determine BBMV water transport.

Although GP of laurdan fluorescence was not different between dexamethasone and adult BBMV, it cannot be concluded that the membrane composition of dexamethasone BBMV and adult BBMV are identical. Dexamethasone and normal maturation may affect the actual lipid and protein composition differently. Therefore, dexamethasone BBMV cannot simply be thought of as adult BBMV with lower AQP1 content.

In conclusion, dexamethasone increases the protein expression of AQP1 in neonatal BBMV, resulting in a higher P_f despite a decrease in membrane fluidity. This indicates that glucocorticoids accelerate maturation of water transport across the BBM and suggests a physiological role of glucocorticoids in the developmental changes. These results indicate that glucocorticoids have multiple effects on the maturation of the proximal tubule cell membranes.

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Fig. 1.

Typical tracings for measurement of P_f in neonatal, dexamethasone, and adult brush border membrane vesicle (BBMV) at 20°C. BBMV were prepared with 80 mosmol/kgH₂O resuspension buffer and exposed to a 80 mosmol/kgH₂O inward osmotic gradient. Light scattering is increased as a result of vesicle shrinkage. Averaged tracings were normalized to initial fluorescence and fitted to a double-exponential curve. The dotted line indicates the asymptote for the adult tracing.

Fig. 2.

P_f for neonatal ($n = 7$), dexamethasone treatment ($n = 7$) and adult ($n = 6$) groups. Error bars denote means $\pm$ SE.

Fig. 3.

P_{DW} for neonatal ($n = 7$), dexamethasone treatment ($n = 7$) and adult ($n = 6$) groups. Error bars denote means $\pm$ SE.

Fig. 4.

A: immunoblot of aquaporin 1 (AQP1) protein expression in BBMVs. Lanes were loaded with 50 μ g of protein. Expression was compared using densitometry. **B:** densitometry results. AQP1: β -actin ratios: neonates: 407 ± 299 ; dexamethasone treatment: $4,435 \pm 1,183$; adults: $11,201 \pm 424$. $P < 0.05$ for differences between all three groups, and there were 3 samples for each group.

Fig. 5.

Generalized polarization (GP) of steady-state laurdan fluorescence. BBMV (100 μ g protein) were incubated with 0.33 nmol laurdan at 37°C for 20 min before measurements. Excitation wavelength was 340 nm. GP was calculated from the emission intensities at 440 and 490 nm. The lower GP of neonatal BBMV corresponds to a higher membrane fluidity.