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Antidiuretic hormone resistance in the neonatal cortical collecting tubule is mediated in part by elevated phosphodiesterase activity

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Abstract

Neonates cannot concentrate their urine to the same degree as adults. One of the key factors in concentrating the urine is the renal collecting duct osmotic water permeability (P_f) response to antidiuretic hormone (ADH). Neonatal cortical collecting ducts have a blunted P_f response to ADH compared with adult tubules (P_f : 119.0 ± 12.5 vs. 260.1 ± 29.5 $\mu\text{m/s}$, $P < 0.05$). We found that the phosphodiesterase activity in the neonatal collecting ducts was higher than that in the adult collecting ducts ($3,970 \pm 510$ vs. $2,440 \pm 220$ $\text{cpm} \cdot \mu\text{g tubular protein}^{-1} \cdot 20 \text{ min}^{-1}$, $P < 0.05$). After pretreatment of in vitro microperfused tubules with the nonspecific phosphodiesterase inhibitor IBMX (10^{-6} M in the bath), the P_f response to ADH in neonatal collecting ducts was 271.4 ± 51.7 $\mu\text{m/s}$, which was identical to that of the adult collecting duct [315.3 ± 31.3 $\mu\text{m/s}$, $P = \text{not significant (NS)}$]. Rolipram, a specific type IV phosphodiesterase inhibitor, lowered the elevated phosphodiesterase activity in the neonatal tubules to that in the adult tubules ($2,460 \pm 210$ vs. $2,160 \pm 230$ $\text{cpm} \cdot \mu\text{g tubular protein}^{-1} \cdot 20 \text{ min}^{-1}$, $P = \text{NS}$). Neonatal tubules pretreated with rolipram (10^{-5} M) in the bath also had a P_f response to ADH that was comparable to that of the adult tubules (258.2 ± 17.0 vs. 271.4 ± 32.6 $\mu\text{m/s}$, $P = \text{NS}$). Thus the elevated phosphodiesterase activity in the neonatal tubules appears to be due to an increase in type IV phosphodiesterase activity. Hence, one of the key factors in the decreased ability of neonates to concentrate their urine is overactivity of phosphodiesterase in the cortical collecting duct that blunts the neonatal collecting duct P_f response to ADH.

Keywords

in vitro microperfusion; water permeability; development; adenosine 3',5'-cyclic monophosphate

Neonates cannot concentrate their urine to the same degree as adults (11, 17, 25, 37, 40).

The factors that contribute to this inability to concentrate the urine in the neonate include a

lower medullary osmotic gradient compared with adult animals and a blunted water permeability response of the collecting duct to antidiuretic hormone (ADH) (12, 13, 19, 31, 36). The factors that play a role in the lower medullary osmotic gradient are the low rates of sodium chloride transport in the neonatal thick ascending limb compared with the adult and the low protein intake that limits the generation of urea (12, 13, 31). Both of these factors contribute to the limited accumulation of sodium chloride and urea in the medulla of the neonate.

The collecting duct water permeability response to ADH is a well-known independent factor in the neonate's inability to concentrate urine (19, 36). However, the factors that contribute to the blunted water permeability response of the neonatal collecting duct to ADH remain elusive. The expression of ADH receptors in the neonatal kidney is comparable to that in the adult kidney (27, 30). The synthesis and release of ADH in response to hypertonicity appear to be intact in the neonate (24, 32). Thus the blunted response of the neonatal collecting duct to ADH must be due to a factor that is downstream from ADH and its receptor.

After cAMP is generated by adenylate cyclase, it is degraded by phosphodiesterase. This provides a feedback mechanism to reverse the action of ADH. We hypothesized that a higher phosphodiesterase activity in the collecting duct would thus limit the response of the neonatal kidney to ADH by limiting the maximal production of cAMP in response to ADH. The purpose of the present study was to directly determine whether differences in phosphodiesterase activity between the neonatal and adult cortical collecting duct (CCD) could account for the decreased responsiveness of the neonatal collecting duct to ADH compared with the adult.

METHODS

In vitro tubule perfusion

Cortical collecting tubules from neonatal (8 to 12 day old) and adult (>8 wk of age) New Zealand White rabbits were perfused in vitro as previously described (7, 28). Briefly, cortical collecting tubules were dissected in cooled (4°C) modified Hanks' 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 glutamine, 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 (38°C) bathing chamber and perfused with concentric glass pipettes. The perfusion solution contained (in mM) 30 NaCl, 25 NaHCO₃, 2.3 Na₂HPO₄, 10 Na acetate, 1.8 CaCl₂, 1 MgSO₄, 5 KCl, 8.3 glucose, and 5 alanine and had an osmolality of 150 mosmol/kgH₂O. The bathing solution was designed to simulate plasma and contained (in mM) 115 NaCl, 25 NaHCO₃, 2.3 Na₂HPO₄, 10 Na acetate, 1.8 CaCl₂, 1 MgSO₄, 5 KCl, 8.3 glucose, and 5 alanine as well as 6 gm/dl of albumin. The osmolality of the bathing solution was 300 mosmol/kgH₂O. The perfusion and bathing solutions were bubbled with 95% O₂-5% CO₂ at 37°C and had a pH of 7.4. The osmolalities of the perfusion and bathing solutions were measured with a Wide Range Osmometer (model 3D3, Advanced Instruments, Norwood, MA) and adjusted to the desired osmolality 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 (J_V ; in $\text{nl} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$) 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- ^3H]inulin (New England Nuclear) was added to the perfusate at a concentration of $50 \mu\text{Ci/ml}$ so that the perfusion rate could be calculated. The perfusion rates were $\sim 10 \text{ nl/min}$ in both groups of tubules. The tubule length (L) was measured using an eyepiece micrometer. The tubule lengths were $0.9 \pm 0.1 \text{ mm}$ for the neonatal tubules and $1.2 \pm 0.1 \text{ mm}$ for the adult tubules.

The osmotic water permeability was calculated from the following

$$P_f = - \frac{\dot{V}_0 C_0}{A \bar{V}_w} \left[\frac{C_0 - C_L}{C_0 C_b C_L} + \frac{1}{(C_b)^2} \ln \frac{(C_L - C_b) C_0}{(C_0 - C_b) C_L} \right] \quad \text{equation (1)}$$

where $\dot{V}_0$ is the perfusion rate; C_0 , C_b , and C_L represent the osmolality of the perfusate, bath, and collected fluid, respectively; A is the surface area calculated from the inner radius; and $\bar{V}_w$ is the molar volume of water. The collected fluid osmolality was calculated from the relationship

$$C_L = \dot{V}_0 C_0 / \dot{V}_L$$

The transepithelial potential difference was measured using the perfusion pipette as the bridge into the tubular lumen. The recording and reference calomel half-cells were connected to the perfusion and bathing solutions via agarose bridges containing $3.6 \text{ M KCl}/0.9 \text{ M KNO}_3$. This arrangement avoided direct contact between the solution bathing the tubule and the KCl/KNO_3 -agarose bridge. The recording and reference calomel half-cells were then connected to the high- and low-impedance sides, respectively, of an electrometer (model 601, Keithley Instruments, Cleveland, OH).

The tubules were perfused for 45 min before control measurements of J_V and water permeability to wash out the endogenous ADH effect. After four control measurements of J_V , water permeability, and potential difference, ADH (200 pM) was added to the bathing solution. Four measurements were then performed 45 min after the addition of ADH.

Tubule protein content

Because the neonatal tubules were much smaller in diameter than the adult tubules, we measured the tubular protein content so that the enzyme activities could be factored by protein. There were three samples of adult and neonatal tubules that were measured, each sample consisting of 20–30 mm of tubules. The tubules were transferred from Hanks' solution to RIPA buffer using glass beads. Tubular protein was estimated using the BCA reaction (Pierce).

Phosphodiesterase activity

Phosphodiesterase activity was measured directly in the neonatal and adult CCD with a phosphodiesterase SPA kit (Amersham). Tubules were dissected in ice-cold Hanks'

solution, their length was measured, and they were then transferred in 10 μ l of solution to an Eppendorf tube. Assays were performed on individual adult tubules (length ranging from 1.3 to 2.5 mm) and on pairs of neonatal tubules (total length for each assay of 1.5–2.5 mm). Distilled water (60 μ l) was then added for hypotonic shock, and the tubule underwent three cycles of freeze and thaw using liquid nitrogen to permeabilize the cells. The tubules were then warmed to 30°C in a water bath, and 20 μ l of assay buffer and 10 μ l of [3 H]cAMP tracer solution were added. This mixture was incubated at 30°C for 20 min. At that time, 50 μ l of SPA bead solution were added to stop the reaction. After 20 min of incubation, the tubes were placed in a liquid scintillation counter. The SPA beads serve as the scintillant in this assay. Results are expressed as counts per minute per microgram of tubule protein per 20 min.

cAMP generation

As a measure of adenylate cyclase activity in neonatal and adult CCDs, cAMP generation was also measured. Tubules were dissected, placed in an Eppendorf tube with 155 μ l of Hanks' solution, and incubated for 30 min. At that time, ADH (200 pM) and IBMX (0.1 mM) were added and the tubule was incubated for 30 more min. Lysis buffer (20 μ l; provided in the cAMP ELISA kit, Amersham) was added, and the tube was vortexed and allowed to stand at room temperature for 10 min to complete the cell lysis. The supernatant was then assayed (per the manufacturer's protocol) for cAMP and expressed as femtomoles per microgram protein per 30 min.

All data are expressed as means $\pm$ SE. Comparisons between groups were made by ANOVA or unpaired *t*-test as appropriate. Significance was determined by a *P* value <0.05.

RESULTS

Figure 1 shows the response to ADH in neonatal and adult CCDs. As can be seen, the baseline water permeability is low in both the neonatal and adult tubules. After addition of ADH (200 pM), there was a prompt increase in the water permeability that reached a plateau after 35–40 min. As shown in Fig. 2, the adult tubules had a larger increase in water permeability in response to ADH than did the neonatal tubules (260.1 ± 29.5 vs. 119.0 ± 12.5 μ m/s; *P* < 0.05, *n* = 6). These results are comparable to previously reported findings (5, 19, 36).

The phosphodiesterase activity in the neonatal and adult CCDs is shown in Fig. 3. The activity is normalized to the protein content of the tubules. We found that the neonatal tubules had a significantly smaller protein content per millimeter of tubule length than did the adult tubules (0.189 ± 0.006 vs. 0.303 ± 0.013 μ g/mm, *P* = 0.001, *n* = 3). This is consistent with other investigators' findings (4) as well as tubular volume estimates from the inner and outer diameters measured during perfusion. As can be seen, the neonatal collecting duct had a significantly higher phosphodiesterase activity than the adult tubules ($3,970 \pm 510$ vs. $2,440 \pm 220$ cpm· μ g tubular protein $^{-1}$ ·20 min $^{-1}$, *P* < 0.05). In both the neonatal and adult tubules, IBMX significantly lowered phosphodiesterase activity, at both 0.1 and 1.0 mM concentrations (Fig. 4, A and B). In the neonatal tubules, rolipram, a specific type IV phosphodiesterase inhibitor, significantly inhibited phosphodiesterase activity to a level that

was not different from the adult control value (Fig. 4, A and B). In the adult tubules, however, rolipram had no effect on phosphodiesterase activity. These data suggest that the neonatal CCDs have higher phosphodiesterase activity that appears to be due to an elevation of type IV phosphodiesterase.

We next examined the response of the collecting duct water permeability to ADH after pretreatment with the phosphodiesterase inhibitors IBMX (0.1 mM) or rolipram (10 μ M). Neither inhibitor was able to augment the ADH effect in the adult tubules; however, both IBMX and rolipram augmented the ADH effect in the neonatal tubules (Fig. 5). More importantly, after addition of ADH, the water permeability in the neonatal tubules pretreated with the phosphodiesterase inhibitors was not different from that of the adult tubules. Thus after inhibition of the elevated phosphodiesterase activity, the neonatal tubules had a water permeability response to ADH that was not different from the adult tubules.

To assess the response of adenylate cyclase to generate cAMP, neonatal and adult tubules were incubated in the presence of ADH (200 pM) and IBMX (0.1 mM). The cellular cAMP content after treatment with ADH and IBMX was not different between the neonatal and adult tubules ($6,669 \pm 797$ vs. $5,433 \pm 516$ fmol/ μ g protein, $P = \text{NS}$; $n = 5/\text{group}$). Thus adenylate cyclase activity in neonatal tubules does not appear to be a limiting factor in the ADH response.

DISCUSSION

The present study examined the role of phosphodiesterase in the blunted water permeability response of the neonatal CCD to ADH. Neonatal CCDs were found to have higher phosphodiesterase activity than adult tubules. When neonatal tubules were perfused in vitro in the presence of a phosphodiesterase inhibitor, the water permeability response to ADH was not different from that of adult tubules. Thus one of the primary defects in the neonatal CCD that limits its water permeability response to ADH is elevated activity of phosphodiesterase. When ADH binds to its receptor on the basolateral membrane of the CCD, a series of events occurs that culminates in the insertion of aquaporin-2 (AQP2) water channels into the apical membrane of the tubule, which increases the tubule's water permeability (21–23, 26). The mechanism for this process includes activation of adenylate cyclase, increased cAMP concentrations, activation of protein kinase A, and then phosphorylation of target proteins. While it has been known for some time that neonatal collecting ducts have a limited water permeability response to the addition of ADH, the factor(s) responsible for this blunted response has remained elusive.

The expression of ADH receptors in the neonatal kidney is comparable to that of the adult, and thus the defect in the neonatal tubule is thought to be downstream from the receptor (27, 30). The activity of adenylate cyclase has been studied in the neonatal and adult collecting duct. When isolated membranes from the kidneys of neonatal and adult rats and rabbits were directly stimulated with fluoride, the activity of the neonatal adenylate cyclase was comparable to that of the adult (34). Thus it appears that in the isolated membranes of neonatal kidneys, adenylate cyclase activity is present. However, when studied in individual tubules, the response of adenylate cyclase to ADH was blunted in neonatal tubules

compared with adult tubules (20, 34). These studies measured the generation of cAMP in response to the hormone treatment. It was not clear in these early studies whether phosphodiesterase inhibitors were present. In a more recent study that employed IBMX to inhibit phosphodiesterase, neonatal tubules formed less cAMP in response to ADH than adult tubules (4). In our study, we examined the ability of the neonatal tubule to generate cAMP after stimulation with ADH. In the presence of IBMX, we demonstrated that the neonatal tubule has the ability to generate cAMP to the same degree as the adult tubule. This discrepancy between our findings and the recently published findings is not entirely clear. However, adenylate cyclase activity does not appear to be a limiting factor in the neonatal collecting duct response to ADH.

Prostaglandins have been implicated as the downstream factor in the blunted ADH response of neonatal collecting ducts (4). Pretreatment of adult tubules with PGE₂ inhibits the water permeability response to ADH (16). Receptors for PGE₂ are located in the collecting duct and may modulate the adenylate cyclase response to ADH by activation of G proteins (6). Incubation of the neonatal tubules with indomethacin augmented the accumulation of cAMP after the addition of ADH (4). Thus it was thought that PGE₂ might be mediating the blunted response of the neonatal tubule to ADH. However, when neonatal tubules were perfused in the presence of indomethacin, the water permeability response to ADH was not different from that in control neonatal tubules and remained much lower than that of adult tubules (5). Thus while PGE₂ might modulate the adenylate cyclase response to ADH, this does not account for the blunted ADH response in the neonatal collecting duct.

The expression of AQP2 in the neonatal kidney is lower than that of the adult kidney (2, 3, 9, 41). Thus it is possible that a decreased abundance of water channels might limit the tubular response to ADH. However, recent studies indicate that when neonatal rats were thirsted, the abundance of AQP2 quickly increased to that of the adult kidney (3). Also, the ability of AQP2 to traffic from the cellular compartment to the apical membrane was intact (3). In addition, the cell height in the neonatal CCD is much smaller than that in the adult CCD. The intracellular resistance to water flow in neonatal tubules is probably less than that in adult tubules, which would require fewer water channels. This is similar to the findings in our studies with the proximal tubule, where we found a high water permeability in the neonatal proximal tubule despite having a lower abundance of water channels (29). Thus it is not clear whether the abundance of AQP2 could be a limiting factor in ADH-stimulated water permeability.

One factor that has not been investigated in the blunted response to ADH of the neonatal collecting duct is the activity of phosphodiesterase. After cAMP is generated by adenylate cyclase, phosphodiesterase is responsible for its degradation, which limits the hormonal response. Phosphodiesterase activity in the developing rat kidney was shown to be elevated compared with adult kidneys and thus could be an important mediator of the blunted ADH response in the neonate (14). However, the study examined phosphodiesterase activity in the renal cortex. Because the renal cortex is composed of mostly proximal tubules, the developmental changes of phosphodiesterase in the developing CCD remain unknown. Previous studies examining the water permeability response of the developing CCD employed cAMP analogs to examine postcAMP signaling defects (5, 36). The cAMP

analogs 8-BrcAMP and 8-pCPTcAMP that were used were thought to be resistant to hydrolysis by phosphodiesterase. Recent studies examining the specificity of cAMP analogs showed that while these analogs were somewhat resistant to phosphodiesterase, they did undergo hydrolysis (33). Thus the role of phosphodiesterase in the blunted ADH water permeability response of the neonatal CCD remained unclear.

The importance of the activity of phosphodiesterase in the regulation of the ADH water permeability response is demonstrated in two animal models of elevated PDE activity. Recently, a strain of mice was found to have nephrogenic diabetes insipidus (10, 39). Analysis of these mice revealed that the cause of their nephrogenic diabetes insipidus was overactivity of type IV phosphodiesterase (18). This would limit the amount of cAMP generated when ADH binds to the tubule and blunts the water permeability response to ADH. When these mice were treated with a phosphodiesterase inhibitor, their urinary osmolality increased (8). Another condition that is associated with increased phosphodiesterase activity is glucocorticoid deficiency (15, 35). When rabbits were adrenalectomized, they developed a concentrating defect. This was found to be due to increased phosphodiesterase activity. This could be corrected by short-term treatment with phosphodiesterase inhibitors or by treatment of the in vitro microperfused tubule with glucocorticoid (35). The significance of the elevated phosphodiesterase activity in the neonatal CCD remains unclear. There is recent evidence that PKC activation might stimulate phosphodiesterase activity (38). Thus activation of PKC in developing tissue such as the neonatal CCD might be a cause of the elevated phosphodiesterase activity.

In the present study, we demonstrate directly that the phosphodiesterase activity in neonatal tubules was significantly higher than that in adult tubules. The phosphodiesterase activity in both sets of tubules was inhibited with IBMX. However, only the phosphodiesterase activity of neonatal tubules was inhibited with rolipram, a specific inhibitor of type IV phosphodiesterase. The phosphodiesterase activity of neonatal tubules after treatment with rolipram was not different from that of adult tubules. Thus the augmented phosphodiesterase activity appears to be due to the presence of type IV phosphodiesterase. When neonatal tubules were perfused in vitro with either rolipram or IBMX, the water permeability response to ADH was not different from that of adult tubules. Thus we have shown for the first time that a key factor in the blunted response of the neonatal collecting duct to ADH is an elevated phosphodiesterase activity.

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Fig. 1.

Response of neonatal and adult cortical collecting duct (CCD) water permeability (P_f) to antidiuretic hormone (ADH). The baseline P_f was not different between the neonatal and adult tubules. After addition of ADH (200 pM), there was an increase in P_f . The response in neonatal tubules was significantly less than that in adult tubules.

Fig. 2.

P_f of neonatal and adult CCDs after treatment with 200 pM ADH. Values are means $\pm$ SE.

n , No. of tubules. The P_f in adult CCDs was significantly higher than that in neonatal CCDs ($P < 0.05$).

Fig. 3. Phosphodiesterase (PDE) activity in neonatal and adult CCDs. PDE activity was measured directly in microdissected tubules. Values are means $\pm$ SE. *n*, No. of tubules. [^3H]cAMP was added to the permeabilized tubules, and the formation of 5'-AMP was assayed. Activity in neonatal tubules was significantly higher than that in adult tubules.

Fig. 4.

A: effect of IBMX and rolipram on the PDE activity in the neonatal CCD. Rolipram (10 μ M) significantly inhibited PDE activity ($P < 0.05$ compared with control). IBMX at 0.1 and 1 mM also significantly inhibited PDE activity ($P < 0.05$). **B:** effect of IBMX and rolipram on PDE activity in the adult CCD. IBMX at 0.1 and 1 mM significantly inhibited PDE ($P < 0.05$). In contrast to neonatal tubules, rolipram (10 μ M) had no effect on PDE activity in the adult CCD. Values are means $\pm$ SE. *n*, No. of tubules.

Fig. 5.

Effect of IBMX (0.1 mM) and rolipram (10 μM) on ADH-stimulated P_f in neonatal and adult CCDs. As can be seen, there was no difference in the P_f between neonatal and adult tubules after treatment with both PDE inhibitors. Both PDE inhibitors significantly increased neonatal tubule response to ADH but had no effect on the ADH response in adult tubules. Values are means $\pm$ SE. *n*, No. of tubules.