Antidiuretic Hormone (ADH) Resistance in the Neonatal Cortical Collecting Tubule is Mediated in part by Elevated Phosphodiesterase Activity

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Running Title: Phosphodiesterase inhibits ADH response in neonatal CCD

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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 to adult tubules ($P_f : 119.0 \pm 12.5 \mu m/sec$ vs $260.1 \pm 29.5 \mu m/sec$, $p<0.05$). 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 $2,440 \pm 220$ cpm/µg tubular protein/20 minutes, $p<0.05$). After pretreatment of in vitro microperfused tubules with the nonspecific phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, $10^{-6}$ M in the bath), the $P_f$ response to ADH in neonatal collecting ducts was $271.4 \pm 51.7 \mu m/sec$ which was identical to that of the adult collecting duct ($315.3 \pm 31.3 \mu m/sec$, $p=NS$). Rolipram, a specific type IV phosphodiesterase inhibitor, lowered the elevated phosphodiesterase activity in the neonatal tubules to that of the adult tubules ($2,460 \pm 210$ vs $2,160 \pm 230$ cpm/µg tubular protein/20 minutes, $p=NS$). 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 ($258.2 \pm 17.0 \mu m/sec$ vs $271.4 \pm 32.6 \mu m/sec$, $p=NS$). Thus, the elevated phosphodiesterase activity in the neonatal tubules appears to be due to an increase in type IV phosphodiesterase activity. Thus, 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.
INTRODUCTION

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 when compared to adult animals and a blunted water permeability ($P_f$) response of the collecting duct to antidiuretic hormone (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 to 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 their 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 of the adult kidney (27; 30). The synthesis and release of ADH in response to hypertonicity appears 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 if differences in phosphodiesterase activity between the neonatal and adult cortical collecting duct could account for the decreased responsiveness of the neonatal collecting duct to ADH when compared to the adult.
METHODS

IN VITRO TUBULE PERFUSION:

Cortical collecting tubules from neonatal (8-12 day old) and adult (greater than 8 weeks 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 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 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 mOsm/kg water. 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 and also 6 gm/dl of albumin. The osmolality of the bathing solution was 300 mOsm/kg water. The perfusion and bathing solutions were bubbled with 95% O₂ and 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 (Advanced Instruments, Model 3D3; Norwood, Mass.) 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 (Jᵥ; in nl/min•mm) was measured as the difference between the perfusion and collection rates and normalized per mm of tubule length. The collection rate was determined by timed collections using a constant volume pipette. Exhaustively
dialyzed [methoxy-$^3$H] inulin (New England Nuclear) was added to the perfusate at a concentration of 50 µCi/ml so that the perfusion rate could be calculated. The perfusion rates were approximately 10 nl/min in both groups of tubules. The tubule length (L) was measured using an eyepiece micrometer. The tubule lengths were 0.9 ± 0.1 mm for the neonatal tubules and 1.2 ± 0.1 mm for the adult tubules.

The osmotic water permeability was calculated from the following equation (1):

\[
P_f = \frac{-V_0}{A/V_w} \left( \frac{C_0 - C_L}{C_b C_L} + \frac{1}{C_b^2} \ln \left( \frac{C_L - C_b}{C_b} \right) \right)
\]

where \( V_0 \) is the perfusion rate, \( C_o \), \( C_b \), 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 \( V_w \) is the molar volume of water. The collected fluid osmolality was calculated from the relationship:

\[
C_L = \frac{V_0 C_0}{V_L}.
\]

The transepithelial potential difference (PD) was measured using the perfusion pipet 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 M KCL/0.9 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, Inc., Cleveland Ohio).

Tubules were perfused for 45 minutes prior to control measurements of \( J_V \) and \( P_f \) to washout the endogenous ADH effect. After four control measurements of \( J_V \), \( P_f \) and PD,
ADH (200 pM) was added to the bathing solution. Four measurements were then performed 45 minutes 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 to 30 mm of tubules. The tubules were transferred from Hank’s 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 the phosphodiesterase SPA kit (Amersham). Tubules were dissected in ice cold Hank’s solution, their length measured and were then transferred in 10 µl of solution to an Ependorf 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 to 2.5 mm). Distilled water (60 µl) 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 30ºC in a water bath and 20 µl of assay buffer and 10 µl of 3H-cAMP tracer solution were added. This mixture was incubated at 30ºC for 20 minutes. At that time, 50 µl of SPA bead solution was added to stop the reaction. After 20 minutes 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 CPM/µg of tubule protein/20 minutes.

**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 Ependorf tube with 155 µl of Hank’s solution and incubated for 30 minutes. At that time, ADH (200 pM) and IBMX (0.1 mM) were added and the tubule was incubated for 30 more minutes. Lysis buffer (20 µl, provided in the kit; Amersham cAMP Elisa kit) was added and the tube was vortexed and allowed to stand at room temperature for 10 minutes to complete the cell lysis. The supernatant was then assayed (per manufacturer’s protocol) for cAMP and expressed as fmol/ µg protein/ 30 minutes.

All data are expressed as mean±SEM. Comparisons between groups were made by ANOVA or unpaired t-test as appropriate. Significance was determined by a p value less than 0.05.
RESULTS

Figure 1 shows the response to ADH in neonatal and adult cortical collecting ducts. 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 minutes. As shown in Figure 2, the adult tubules had a larger increase in water permeability in response to ADH than did the neonatal tubules (260.1±29.5 µm/sec vs 119.0±12.5 µm/sec; p<0.05, n=6). These results are comparable to previously reported findings (5; 19; 36).

The phosphodiesterase activity in the neonatal and adult CCD is shown in Figure 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 mm of tubule length than the adult tubules (0.189 ± 0.006 µg/mm vs 0.303 ± 0.013 µg/mm, p=0.001, n=3). This is consistent with other investigator’s 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 (3970 ± 510 cpm/µg tubular protein/ 20 minutes vs 2440 ± 220 cpm/µg tubular protein/ 20 minutes, p<0.05). In both the neonatal and adult tubules, IBMX significantly lowered the phosphodiesterase activity, at both the 0.1 mM and 1.0 mM concentrations (Figures 4A and 4B). In the neonatal tubules, rolipram, a specific type 4 phosphodiesterase inhibitor, significantly inhibited the phosphodiesterase activity to a level that was not different from the adult control value (Figures 4A and 4B). In the adult tubules, however, rolipram had no effect on phosphodiesterase activity. These data
suggest that the neonatal cortical collecting ducts have a higher phosphodiesterase activity that appears to be due to an elevation of type 4 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 µM). Neither inhibitor was able to augment the ADH effect in the adult tubule, however both IBMX and rolipram augmented the ADH effect in the neonatal tubules (Figure 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 (6669±797 fmoles/ µg protein vs 5433±516 fmoles/ µg protein, p=NS; n= 5 in each group). Thus, the adenylate cyclase activity in the 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 cortical collecting ducts were found to have a higher phosphodiesterase activity than adult tubules. When the 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 the adult tubules. Thus, one of the primary defects in the neonatal cortical collecting duct that limits its water permeability response to ADH is an elevated activity of phosphodiesterase.

When ADH binds to its receptor on the basolateral membrane of the cortical collecting duct, a series of events occurs that culminate in the insertion of 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 have remained elusive.

The expression of ADH receptors in the neonatal kidney is comparable to that of the adult, 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 stimulated directly 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 the neonatal tubules compared to the 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 or not phosphodiesterase inhibitors were present. In a more recent study that employed IBMX to inhibit phosphodiesterase, the 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 the control neonatal tubules and remained much lower than that of the 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 the 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 the adult CCD. The intracellular resistance to water flow in the neonatal tubules is probably less than that of the adult tubules that 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 if the abundance of AQP2 could be a limiting factor in the 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 to 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. Since the renal cortex is comprised 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 post-cAMP signaling defects (5; 36). The cAMP analogs, 8-Br-cAMP and 8-pCPT-cAMP, 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 (NDI mice) (10; 39). Analysis of these mice revealed that the cause of their NDI was overactivity of type 4 phosphodiesterase (18). This would limit the amount of cAMP generated when ADH binds to the tubule and blunt the water permeability response to ADH. When these mice were treated with a phosphodiesterase inhibitor, their urine osmolality increased (8). Another condition that is associated with increased phosphodiesterase activity is glucocorticoid deficiency (15; 35). When rabbits were adrenalecomized, they developed a concentrating defect. This was found to be due to an increased phosphodiesterase activity. This could be corrected by short term treatment with phosphodiesterase inhibitors or by treating 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 protein kinase C 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 the neonatal tubules was significantly higher than that of the adult tubules. The phosphodiesterase activity in both sets of tubules was inhibited with IBMX. However, only the phosphodiesterase activity of the neonatal tubules was inhibited with rolipram, a specific inhibitor of type 4 phosphodiesterase. The phosphodiesterase activity of the neonatal tubules after treatment with rolipram was not different from that of the adult tubules. Thus, the augmented phosphodiesterase activity appears to be due to the presence of type 4 phosphodiesterase. When the neonatal tubules were perfused in vitro with either rolipram or IBMX, the water permeability response to ADH was not different from that of the 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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FIGURE LEGENDS

1. The response of neonatal and adult CCD water permeability to ADH. The baseline $P_f$ was not different between the neonatal and adult tubules. After addition of ADH (200pM) there was an increase in the $P_f$. The response in the neonatal tubules was significantly less than that in the adult tubules.

2. Water permeability of neonatal and adult CCDs after treatment with 200 pM ADH. The $P_f$ in the adult CCDs was significantly higher than that of the neonatal CCDs ($p<0.05$).

3. Phosphodiesterase activity in the neonatal and adult CCDs. Phosphodiesterase activity was measured directly in microdissected tubules. $^3$H-cAMP was added to the permeabilized tubules and the formation of 5′-AMP was assayed. The activity in the neonatal tubules was significantly higher than that of the adult tubules.

4. A. Effect of IBMX and rolipram on the phosphodiesterase activity in the neonatal CCD. Rolipram (10 $\mu$M) significantly inhibited the phosphodiesterase activity ($p<0.05$ compared to control). IBMX at 0.1 and 1 mM also significantly inhibited phosphodiesterase activity ($p<0.05$).

4. B. Effect of IBMX and rolipram on the phosphodiesterase activity in the adult CCD. IBMX at 0.1 and 1 mM significantly inhibited phosphodiesterase activity ($p<0.05$). In contrast to the neonatal tubules, rolipram (10 $\mu$M) had no effect on the phosphodiesterase activity in the adult CCD.

5. Effect of IBMX (0.1 mM) and rolipram (10 $\mu$M) on the ADH stimulated water permeability in neonatal and adult CCDs. As can be seen, there was no difference
in the P_f between the neonatal and adult tubules after treatment with the phosphodiesterase inhibitors. Both phosphodiesterase inhibitors significantly increased the neonatal tubule response to ADH but had no effect on the ADH response in the adult tubules.
Figure 1

CCD Water Permeability

- Adult
- Neonate
Figure 2

![Graph comparing P_f (µm/sec) for Neonatal CCD and Adult CCD. The graph shows that Neonatal CCD has a significantly lower P_f (µm/sec) compared to Adult CCD, with p<0.05.](image)
Figure 3.

![Bar graph showing PDE activity (cpm/µg protein/20 min) for Neonatal CCD and Adult CCD.](image)

- **Neonatal CCD**
  - n=31
  - *p < 0.02*

- **Adult CCD**
  - n=25

The graph illustrates a significant difference in PDE activity between neonatal and adult CCD, with neonatal CCD having a higher activity level. The asterisk indicates a statistically significant difference at the p < 0.02 level.
Figure 4 A

Neonatal CCD

[Pie chart and bar graph showing PDE activity (cpm/µg protein/20 minutes) for Control, Rolipram, 0.1 mM IBMX, and 1 mM IBMX.

- Control: n=31
- Rolipram: n=27
- 0.1 mM IBMX: n=12
- 1 mM IBMX: n=17

*p<0.05 vs Control
**p<0.05 vs Rolipram
Figure 4 B

Adult CCD

PDE activity (cpm/µg protein/20 minutes)

Control
Rolipram
0.1 mM IBMX
1 mM IBMX

n=25
n=10
n=10
n=10

p<0.05 vs Control
Figure 5

![Bar chart showing the effect of different treatments on P (µm/sec).]

- Neo Control
- Neo Rolipram
- Neo IBMX
- Adult Control
- Adult Rolipram
- Adult IBMX

*p<0.05 vs all other groups*

n=6 for all groups.