Adaptation to Water Depletion in the Newborn Rat — Immunocytochemical Localization of Aquaporin-2 —

HIROKO MAESHIRO

Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume 830-0011, Japan

Summary: Little is known about the effect of prematurity on water reabsorption in the renal tubules by anti-diuretic hormone (vasopression: ADH). The purpose of this study was to assess the different mechanisms of maintaining water balance in newborn and in adult rats on ADH and aquaporin-2 (AQP2) axis. After the dehydration, the plasma ADH in newborn and adult rose 104.6% and 117.2%, respectively. In immunocytochemical study, AQP2 stained more intensively in dehydrated rats. The dehydrated adult rats apical membrane in the IMCD cells showed more intensive staining than in the control rats. Adult rats revealed more intensive staining than newborn after the dehydration in the IMCD cells. We conclude that low ADH secretion in response to dehydration might lead to inadequate production of AQP2 resulting in an increased tendency in newborn to become dehydrated.

Key words newborn, dehydration, anti-diuretic hormone, aquaporin-2, immunocytochemistry

INTRODUCTION

It is well known that newborns may experience severe dehydration due to prematurity of the renal tubular system.

The reduction in urinary concentrating capacity in newborns in comparison with older children and adults is due to a reduced glomerular filtration rate (GFR), tubular cell immaturity, reduced nephron length, reduced medullary solute gradient due to increased medullary blood flow and low urea production, and diminished tubular responsiveness to anti-diuretic hormone (vasopression: ADH) [1-5]. Among these factors, little is known about the effect of prematurity on water reabsorption in the renal tubules by the ADH. Vasopressin regulates water transport in the terminal portion of the collecting duct. This step depends both on the levels of expression of water channels located in the collecting duct cells, and the capacity of ADH to activate these channels. Recently, a vasopressin-sensitive water channel protein (aquaporin-2) and its DNA sequence were described [6]. As a mechanism to adapt to dehydration in mammalians, AQP2 protein is an important mediator of urinary concentration in the collecting duct [6-10].

In addition to the V2 receptor abnormality [11], renal diabetes insipidus with dehydration caused by AQP2 dysfunction has recently been reported [12].

The purpose of this study was to assess the different mechanisms of maintaining water balance in newborn and in adult by examining the cellular distribution of AQP2 in the IMCD (inner medullary collecting duct). We also studied the ADH and AQP2 axis, which is important in the homeostasis of water balance.

MATERIALS AND METHODS

Studies were performed on male Sprague-Dawley rats ages 7 days (newborn) and 40 days (adult). All rats were subdivided into three groups based on dehydrated patterns. The pups were kept with their dam until the day of the study.

Group I (dehydration group): The rats in this group were maintained more than 48 hours in a cage, followed by 24 hours of water deprivation and food restriction. Blood samples were then taken and left

Received for publication January 7, 1998
kidneys were used for histochemical analysis.

**Group II (inner control group: non-treated group):**
The non-dehydrated rats in this group were killed either at day 0, day 2 (24 hrs), day 4 (48 hrs after day 2) for histochemical analysis. In each rat, blood samples were taken and left kidneys were used for histochemical analysis.

**Group III (recovery group):** The rats in this group were subjected to water and food restriction for 24 hrs to induce dehydration. After dehydration, rats were given free access to chow and water ad libitum for 48 hrs to determine if the changes caused by dehydration were reversible. Blood samples were taken and left kidneys were used for histochemical analysis.

In each group, cardiac puncture was performed and blood samples were used for the measurement of hematocrit, plasma osmolality, and plasma ADH. Values were expressed as mean±SD, and p values <.05 were considered statistically significant. Statisticlal data were calculated by the Student t test. All values are two-sided.

**Immunocytochemistry**

For histochemical analysis of AQP2, rats in each group were killed. Using pentobarbital sodium (3 mg/kg) injected intraperitoneally, PE-50 or PE-90 tubing was immediately introduced into the descending aorta. The right renal artery and upper portion of a branch of the left renal artery were occluded. After perfusion of the left kidney with cold 1XPBS (4°C), the kidney was perfused with cold periodate-lysine-paraformaldehyde fixative (4°C) for 5 min. After removal, the left kidney was sliced with 1-mm cuts and immersion fixed with PLP fixative at 4°C for 120 min. The slices were then rinsed three times with 1X PBS and paraffin-embedded. For immunocytochemical staining, paraffin sections were cut at a thickness of 3 μm. The sections were deparaffinized with xylene and rehydrated with ethanol and distilled water.

Sections were incubated with 1X PBS containing 1% bovine serum albumin for 20 min prior to application of the primary antibody, diluted 1:300, for 1 hr at room temperature. Sections were washed three times for 5 min in 1X PBS, and the secondary antibody was applied for 1 hr at room temperature. The labeling was visualized using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (DAKO Japan, Kyoto, Japan) for 30 min. Peroxidase-rabbit anti-peroxidase complex (1:100 dilution) (DAKO Japan) was applied for 30 min and the peroxidase reaction product was developed with diaminobenzidine. Controls done at the light microscopic level revealed no non-specific labeling. These controls used (1) preimmune sera, (2) antiserum absorbed with excess synthetic peptides, and (3) omission of primary or secondary antibody. A polyclonal antibody against a synthetic portion of the C-terminus of rat AQP2 raised in rabbits [13] (gifted by Dr. Sei Sasaki) was used for immunocytochemical analysis.

**RESULTS**

Data were collected for the control group, the 24-hours dehydration group, and the recovery group of newborn and adult rats.

The changes in body weight are shown in Fig. 1. After 24 hrs of dehydration, the mean (±SD) body weight in newborn rats showed a 12.3% reduction (10.6±0.9 g to 9.3±0.6 g) (p=0.001). However, 48 hrs after termination of water restriction, body weights returned to 10.2±1.0 g, similar to the control levels. In adult rats, after 24 hrs dehydration, the mean body weight showed a 4.7% reduction (212.5±4.3 g to 202.8±4.3 g) (p=0.0004). However, 48 hrs after termination of water restriction, body weight returned to 206.0±6.3 g, similar to control levels.

The changes in plasma osmolality are shown in Fig. 2. After 24 hrs of dehydration, the mean (±SD) plasma osmolality in newborn rats rose 8.3% (299.6±2.9 to 324.4±4.7 mOsm/kgH₂O) (p=0.0000). However, 48 hrs after termination of water restriction, plasma osmolality returned to 300.7±3.1 mOsm/kgH₂O which is similar to the control levels. In adult rats, after 24 hrs of dehydration, the mean plasma osmolality rose 7.4% (314.0±9.4 to 337.3±8.3 mOsm/kgH₂O) (p=0.0001). However, 48 hrs after termination of water restriction, osmolality returned to 314.0±1.0 mOsm/kgH₂O similar to control levels.

The changes in plasma ADH are shown in Fig. 3. After 24 hrs of dehydration, the mean plasma ADH in newborn rat rose 104.6% (15.3±5.3 to 31.3±7.9 pg/mL) (p=0.0001). However, 48 hrs after termination of water restriction, plasma ADH levels recovered to 18.1±6.3 pg/ml which is similar to the control levels. In adult rats, after 24 hrs of dehydration, the mean plasma ADH rose 117.2% (29.9±3.3 to 51.9±18.7 pg/mL) (p=0.013). However, 48 hrs after the termination of water restriction, ADH levels rose to 26.9±5.5 pg/mL, similar to control levels. The immunocytochemistry of AQP2 in the renal col-
Fig. 1. Body weights of control, after dehydration, and 48 hrs after dehydration groups in newborn and adult rats.

**Newborn**

![Bar chart showing body weights](chart1)

- Control (n=7)
- Dehydrated (n=7)
- Post (48hr) (n=7)

**Adult**

![Bar chart showing body weights](chart2)

- Control (n=7)
- Dehydrated (n=7)
- Post (48hr) (n=7)

*** P=0.001

*** P=0.0004

Fig. 2. Plasma osmolalities of control, after dehydration, and 48 hrs after dehydration groups in newborn and adult rats.

**Newborn**

![Bar chart showing plasma osmolalities](chart3)

- Control (n=7)
- Dehydrated (n=7)
- Post (48hr) (n=7)

**Adult**

![Bar chart showing plasma osmolalities](chart4)

- Control (n=7)
- Dehydrated (n=7)
- Post (48hr) (n=7)

*** P=0.0000

*** P=0.001
Fig. 3. Plasma ADH levels of control, after dehydration, and 48 hrs after dehydration groups in newborn and adult rats.

**TABLE 1.**
Summary of immunocytochemical study on AQP2 mobilization in rat innermedullary collecting duct cells from control and dehydrated Sprague-Dawley rats

<table>
<thead>
<tr>
<th>distribution</th>
<th>control apical and subapical membrane</th>
<th>cytoplasm</th>
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<tbody>
<tr>
<td></td>
<td>apical and subapical membrane</td>
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<tr>
<td>newborn</td>
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<td>adult</td>
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<td>dehydration</td>
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IMCD cells stained with anti-AQP2 in newborn rats (Figs 6-A, 6-B) and adult rats (Figs 7-A, 7-B) after 24 hrs dehydration are also shown. In newborn IMCD cells, compared to controls (4-A, 4-B) AQP2 stained more intensively in the dehydrated rat (6-A, 6-B). Similarly, the dehydrated adult rat apical membrane in the IMCD cells (7-A, 7-B) showed more intensive staining than in the control rats (5-A, 5-B). Adult rats revealed more intensive staining (7-A, 7-B) than newborn rat (6-A, 6-B) after 24 hrs of
Fig. 4. Localization of AQP2 by peroxidase staining on 3 μm thin sections of rat IMCD from a control newborn Sprague-Dawley rat.

Fig. 5. Localization of AQP2 by peroxidase staining on 3 μm thin sections of rat IMCD from a control adult Sprague-Dawley rat.

Fig. 6. Localization of AQP2 by peroxidase staining on 3 μm thin sections of rat IMCD from a dehydrated newborn Sprague-Dawley rat.

Fig. 7. Localization of AQP2 by peroxidase staining on 3 μm thin sections of rat IMCD from a dehydrated adult Sprague-Dawley rat.
dehydration. Furthermore, AQP2 staining in the cytoplasm in IMCD cells was more pronounced after 24 hrs of dehydration in both newborn and adult rats (6-B, 7-B). However, the cytoplasmic AQP2 staining in the adult rats (7-B) was much more intense than in newborns (6-B). We summarized the immunocytochemical study on AQP2 mobilization before and after dehydration in newborn and adult rats in Table 1.

Staining patterns in newborn and adult rat of recovery group were consistent with those in control group (data is not shown). This result enables us to understand that 24-hours water restriction is reversible change in newborn and adult rats.

**DISCUSSION**

Infants consume much larger amounts of water per unit of body weight compared with adults. The daily consumption of fluid by healthy infants is equivalent to 10 to 15% of their body weight, compared with 2 to 4% in adults. At birth and for several months thereafter, tubular functional capabilities are less than adult levels. The minimal response noted in infants to administration of vasopressin has led many observers [1-5] to conclude that, due to immaturity, the renal tubules are relatively insensitive to antidiuretic hormone (ADH). However, the exact mechanism is not interpreted as simply a resistance to the hormone since vasopressin-regulated water channel protein has not been evaluated. It has been recognized in humans that infants tend to be more likely to become dehydrated than adults. We have confirmed that 24 hrs of water restriction results in significant body weight loss in newborn rats compared to adult rats. Consistent with the changes in body weight in newborn and adult rats, plasma osmolality in newborn rats rose much more than in adult rats after water restriction. The magnitude of elevation in plasma osmolality reflects the severity of dehydration, increasing in parallel with degree of weight loss after dehydration. These data demonstrate that newborns can easily lose body water during water restriction.

Interestingly, in adult rats, plasma ADH levels rose 117.2% after water restriction, compared to only 104.6% in newborn rats. With regard to dehydration or elevations of plasma osmolality, newborns were more severely affected than adults, whereas ADH secretion in response to elevations of plasma osmolality in newborns was less than that in the adult rats. In a previous report, it had been shown that in terms of ADH secretion, infants were less responsive than adults, which is consistent with our observations [14]. Our data suggest that newborn infants can not inhibit the progression of severe dehydration, because newborns can not secrete enough endogenous ADH to reabsorb the water necessary to maintain homeostasis.

In the present study, we also performed immunocytochemical studies to characterize the developmental differences of vasopressin and AQP2 between the newborn and adult rat kidney. The mechanisms of the water reabsorption in response to ADH in the renal collecting duct is known, but the details have not been fully elucidated. Recently, an AQP2: vasopressin-regulated water channel protein was cloned from a rat cDNA library, and immunohistochemical studies and RT-PCR along the nephron clearly showed that its expression was limited to the collecting duct cells [6]. In terms of mechanisms of dehydration in infants, the roles of ADH and AQP2 remain unknown. In the present study, after 24 hrs of water restriction, AQP2 distribution in the kidney was histochemically analyzed in kidney sections. The anti AQP2 COOH-terminal antibody used in this study was localized to vasopressin-sensitive components in kidney collecting duct principal cells [15].

We also tried to characterize the long-term regulation of AQP2 and histologic distribution before and after water restriction in newborn and adult kidneys. Immunolabeling was increased by water deprivation, and distributional changes in AQP2 by endogenous vasopressin levels were quantitatively confirmed. In the water-deprived newborn and adult rat, both of which are presumed to have high levels of plasma vasopressin, immunostaining of AQP2 was highly intensified in both the apical membrane and subapical region, especially the former. These observations support the hypothesis that water channel-containing vesicles are mobilized and fuse to the apical membrane during hydration. Increases in the number and size of cytoplasmic vesicles and the development of large vacuoles have been observed in collecting duct cells stimulated with ADH [16,17]. It has been demonstrated by Lankford et al. that the intramembrane particle clusters in the terminal CD cells were markedly increased in dehydrated rats, and that this change was dominant in the terminal part of the IMCD compared with its proximal part [18]. In the present study, for both newborn and adult rats, AQP2 protein appeared to move into the apical membrane from the cytoplasm, suggesting AQP2
protein shuffling from the cytoplasm into the apical membrane by water deprivation.

It is widely recognized that vasopressin alters the osmotic water permeability of the apical membrane by trafficking water channel-containing vesicles between the endosomal reservoir and the apical membrane via an endo- and exocytotic mechanism [18-20]. This subcellular location of AQP2 closely resembles the predicted site of ADH-regulated water channels [21,22]. Ultrafine structural changes by vasopressin have been observed in toad bladders; it was shown that vasopressin and cyclic AMP induced membrane particle clustering in the apical plasma membrane of granular cells in the toad bladder with or without an osmotic gradient [23]. In mammalian collecting ducts, in vivo treatment of Brattleboro rats with vasopressin also induced clustered particles in the luminal membrane [24].

Our observation in part suggests that AQP2 is abundantly stored in the cytoplasmic vesicles as a reservoir of the ADH-regulated water channel, and then a small fraction thereof is normally expressed on the apical membrane as predicted in the shuttle hypothesis [25]. The mechanism of this adaptive change has not been clarified, and our present study suggests a partial explanation for this phenomenon. Yamamoto et al. have demonstrated an increase in AQP2 protein at the apical side of collecting duct cells in the first 4 weeks after birth, which is presumed to contribute to the maturation of urinary concentrating capacity during kidney development [26]. In our control group, the AQP2 staining in the apical membrane of adult rats was much more intense than in newborns. This result indicates that newborn rats not only secreted less ADH under basal conditions, but also contain less AQP2 protein than adults. Interestingly, after water restriction, despite more severe water loss in newborn rats than in adults, reactive ADH release was less in the newborns than in the adults. Protein expression after water restriction in newborns revealed less intensive staining with AQP2 than in adults, reflecting the smaller elevation of plasma ADH levels. Apical staining with AQP2 after water restriction in adults was much more intense than in newborns. Immunocytochemical localization of AQP2 in adult rats obtained from the present study was consistent with a previous study by Yamamoto et al. [27,28]. Yasui et al. have demonstrated that AQP2 protein and mRNA expression in homogenized kidney tissue in 10-day adult rats were significantly lower than in 40 day-old rats [7]. These data are supportive of the immunocytochemical difference we observed between newborn and adult rat kidneys stained with AQP2. The reason why newborns tend to become severely dehydrated may be in part due to the inadequate production of AQP2 release of endogeneous ADH in response to dehydration. As for the regulation of AQP2 in vivo, we conclude that low basal ADH levels and low ADH secretion in response to dehydration might lead to inadequate production of AQP2 resulting in an increased tendency in newborns to become dehydrate.

ACKNOWLEDGMENTS: The author is grateful to Dr. Tohru Matsumoto and Dr. Hirohisa Kato for their valuable comments. This work was supported in part by a Grant-in-Aid (to E.H.9770590) for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

12. Deen PMT, Verdijk MAJ, Knoers NNAM et al.


