Erythrocyte Water Permeability and Renal Function in Double Knockout Mice Lacking Aquaporin-1 and Aquaporin-3*

Received for publication, September 21, 2000, and in revised form, October 3, 2000 Published, JBC Papers in Press, October 16, 2000, DOI 10.1074/jbc.M008664200

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Aquaporin (AQP) water channel AQP3 has been proposed to be the major glycero- and non-AQP1 water transporter in erythrocytes. AQP1 and AQP3 are also expressed in the kidney where their deletion in mice produces distinct forms of nephrogenic diabetes insipidus. Here AQP1/AQP3 double knockout mice were generated and analyzed to investigate the functional role of AQP3 in erythrocytes and kidneys. 53 double knockout mice were born out of 756 pups from breeding double heterozygous mice. The double knockout mice had reduced survival and impaired growth compared with the single knockout mice. Erythrocyte water permeability was 7-fold reduced by AQP1 deletion but not further reduced in AQP1/AQP3 null mice. AQP3 deletion did not affect erythrocyte glycero permeability or its inhibition by phloretin. Daily urine output in AQP1/AQP3 double knockout mice (15 ml) was 9-fold greater than in wild-type mice, and urine osmolality (194 mosM) was 8.4-fold reduced. The mice remained polyuric after DDAVP administration or water deprivation. The renal medulla in most AQP1/AQP3 null mice by age 4 weeks was atrophic and fluid-filled due to the severe polyuria and hydronephrosis. Our data provide direct evidence that AQP3 is not functionally important in erythrocyte water or glycero permeability. The renal function studies indicate independent roles of AQP1 and AQP3 in countercurrent exchange and collecting duct osmotic equilibration, respectively.

The route for water movement across the erythrocyte plasma membrane has been a subject of longstanding interest. Erythrocyte osmotic water permeability is inhibited by ~90% by mercurial sulfhydryl compounds and has biophysical properties of a pore pathway including a low Arrhenius activation energy and a high ratio of osmotic to diffusional water permeability (1). The water-selective transporter AQP1 is the major erythrocyte water transporter as proven by the reduced water permeability in erythrocytes from Colton erythrocyte water transporter as proven by the reduced water exchange and collecting duct osmotic equilibration, independent roles of AQP1 and AQP3 in countercurrent exchange and collecting duct osmotic equilibration, respectively.

* This work was supported by National Institutes of Health Grants DK35124, HL59198, HL60288 and DK43840 and Research Development Grant R613 from the National Cystic Fibrosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: AQP, aquaporin; PBS, phosphate-buffered saline.

**AQP3** provides the major non-AQP1 pathway for water transport across the erythrocyte plasma membrane as well as the transport pathway for glycero (4). Erythrocyte glycero permeability is substantially greater than that across lipid bilayers and is inhibited by phloretin (5); however, the molecular identity of the putative glycero transporter has not been established. The principal goal of this study was to determine the functional role of AQP3 in erythrocyte water and glycero permeability. Transport measurements were done on erythrocytes lacking AQP1 and AQP3 individually and AQP1/AQP3 together. We reasoned that the very low water permeability of AQP1-deficient erythrocytes would permit the detection of even small amounts of functional AQP3 as a further decrease in permeability in AQP1/AQP3-deficient erythrocytes.

A secondary goal of this study was to investigate the roles of AQP1 and AQP3 in the urinary-concentrating mechanism. AQP1 and AQP3 are expressed in the kidney: AQP1 in proximal tubule, thin descending limb of Henle, and medullary vasa recta (6, 7) and AQP3 at the basolateral membrane of collecting duct principal cells (8, 9). Wild-type mice have base-line urine osmolalities of 1000–1500 mosM that increase to >3,000 mosM after water deprivation. Mice lacking AQP1 are polyuric and have urine osmolalities of 500–700 mosM that do not increase after water deprivation or DDAVP administration (3). In vivo micropuncture revealed defective proximal tubule fluid absorption in AQP1 null mice (10), and isolated tubule microperfusion showed remarkably reduced osmotic water permeability in thin descending limb of Henle (11) and outer medullary descending vasa recta (12). Mice lacking AQP3 also manifest nephrogenic diabetes insipidus but with a very different pattern (13). AQP3 null mice are remarkably polyuric with base-line urine osmolalities of <280 mosM but are able to concentrate their urine to 1000–1400 mosM after water deprivation or DDAVP administration.

In this study, mice lacking AQP1 and AQP3 were generated and characterized. A comparison of water and glycero permeabilities of erythrocytes from the single and double knockout mice permitted a direct assay of the functional role of AQP3. A comparison of urinary-concentrating ability in the single and double knockout mice tested whether the different patterns of nephrogenic diabetes insipidus result from distinct defects in countercurrent exchange and collecting duct function.

**EXPERIMENTAL PROCEDURES**

Generation of AQP1/AQP3 Double Knockout Mice—Because the AQP1 and AQP3 genes are localized on different chromosomes in the mouse genome, AQP1/AQP3 double knockout mice were generated by intercross of the single knockouts. The breeding of F2 generation double heterozygous mice yielded 53 AQP1/AQP3 knockout mice out of 756 pups.

**Immunofluorescence**—Red blood cells and bone marrow aspirates were smeared onto glass slides. Samples were fixed in acetone/methanol (1:1) and incubated for 30 min with PBS containing 1% bovine

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serum albumin and then incubated with AQP1 or AQP3 antiserum (1:1000) for 1 h at 23 °C in PBS containing 1% bovine serum albumin. Slides were rinsed with 2.7% NaCl and then with PBS, and they were incubated with a secondary Cy3-conjugated sheep anti-rabbit F(ab)2 fragment (1:200) for visualization by fluorescence microscopy.

**Water and Glycerol Permeability Measurements**—Fresh erythrocytes obtained by tail bleeding (100–200 μl/bleed) were washed three times in PBS to remove serum and the cellular buffy coat. Stopped-flow measurements were carried out on a Hi-Tech SF-51 instrument (Wiltshire, United Kingdom). For measurement of osmotic water permeability, suspensions of erythrocytes (∼0.5% hematocrit) in PBS were subjected to a 100 mM inwardly directed gradient of sucrose. The kinetics of decreasing cell volume was measured from the time course of 90° scattered light intensity at 530 nm wavelength. Osmotic water permeability coefficients (P) were computed from the light-scattering time course as described previously (14). For measurement of glycerol permeability, the erythrocyte suspension was subjected to a 100 mM inwardly directed gradient of glycerol. In some experiments, 0.3 mM HgCl2 (four mice of each genotype, mean ± S.E.).

**Renal Morphology—**Mice were anesthetized by intraperitoneal pentobarbital (30 mg/g body weight). Kidneys were fixed in situ by perfusion with 4% paraformaldehyde in PBS. Fixed tissues were processed by routine histological methods, and 6-μm paraffin sections were stained with hematoxylin.

## RESULTS

AQP1/AQP3 double knockout mice were generated by intercross of AQP1 and AQP3 null mice. The mice were grossly phenotypically normal when given free access to food and water except for obvious polyuria. Over the first 10 weeks of life, the double knockout mice were 20–25% smaller by body weight than wild-type littermates. At 6 weeks, the mean body weights were 27.8 ± 2.4 g (wild-type) and 21.7 ± 3.2 g (AQP1/AQP3 knockout). The AQP1 null mice were 15–20% smaller than wild-type mice, whereas the AQP3 null mice were not impaired in their growth. The survival of AQP1/AQP3 double knockout mice was also impaired compared with the single knockout mice. Although >90% of living AQP1 and AQP3 null mice that were genotyped at 5 days remained alive at 8 weeks, only 50% of the double knockout mice were alive at 8 weeks.

Osmotic water permeability was measured in erythrocytes from wild-type mice and mice lacking AQP1 and AQP3 individually and AQP1/AQP3 together. Fig. 1A shows the time course of osmotic cell shrinking in response to a 100 mM inwardly directed osmotic gradient of sucrose. The data are plotted using three contiguous time scales to show the full time course of decreasing cell volume. Erythrocyte water permeability was remarkably reduced by AQP1 deletion but not further reduced by AQP3 deletion. Fig. 1B shows the inhibition of water transport by the mercurial HgCl2. Water transport was strongly inhibited in erythrocytes from wild-type and AQP3 null mice and was inhibited to a lesser extent in erythrocytes from AQP1 null mice and AQP1/AQP3 double knockout mice.

Fig. 2 summarizes osmotic water permeability coefficients (P) determined in four mice of each genotype. AQP3 deletion did not reduce P in wild-type or AQP1 null mice nor did it affect the inhibitory potency of HgCl2. Temperature dependence measurements were performed to determine the Arrhenius activation energy for erythrocyte water transport. Computed activation energies (10–37 °C) were <4 kcal/mol for wild-type and AQP3 null mice and >8 kcal/mol for AQP1 null mice and the double knockout mice.

Erythrocyte glycerol permeability was measured from the time course of cell swelling in response to a 100 mM inwardly directed gradient of glycerol. As shown in Fig. 3A, the glycerol gradient produced an initial rapid decrease in cell volume due to osmotically induced water efflux followed by slower cell swelling that was due to glycerol and secondary water influx. At 20 °C, glycerol equilibrated across erythrocytes from wild-type mice with a half-time of ~20 s, giving a permeability coefficient (Pgly) of 2.63 × 10^-6 cm/s (top curve). Glycerol permeability was inhibited by 64% by 0.5 mM phloretin (second curve) and was strongly temperature-sensitive (bottom curves), increasing 2.8-fold from 10 to 30 °C that was consistent with a facilitated transport pathway.

Fig. 3B shows glycerol permeability in erythrocytes from AQP3 null mice in the absence and presence of phloretin. Results were similar to those in erythrocytes from wild-type mice. Data from a series of mice showed that AQP3 deletion did not reduce erythrocyte glycerol permeability or its inhibition by phloretin (Fig. 3C). Together the permeability measurements indicate that AQP3 does not contribute measurably to erythrocyte water or glycerol permeability.

Immunocytochemistry was done to look for AQP3 protein in erythrocytes from AQP3 null mice. Fig. 4A shows little AQP3 antibody labeling of permeabilized erythrocyte smears from humans (left) and wild-type mice (middle). Similar low levels of labeling were detected in erythrocytes from AQP3 null mice (right). In contrast, AQP3 was readily detected in the kidney-collecting duct in wild-type mice (Fig. 4B, left) but not in AQP3 null mice (right).

Urinary-concentrating function was compared in the wild-type, single knockout, and double knockout mice. Fig. 5A shows
daily fluid consumption and urinary output in the mice. Polydipsia and polyuria were greater in AQP3 than in AQP1 null mice and further increased in the AQP1/AQP3 double knockout mice. The difference in fluid intake and urinary output, primarily representing insensible respiratory losses, was similar in all groups. Fig. 5 summarizes urine osmolalities in mice given free access to food and water and in mice deprived of food and water for 24 h. Base-line urine osmolality was high (>1500 mosm) in wild-type mice and nearly doubled after water deprivation. Base-line urine osmolality was much lower in the AQP1 null mice and changed little after water deprivation. Urine osmolality was lower in AQP3 null mice but increased 2.4-fold after water deprivation. Interestingly, the deletion of AQP1 and AQP3 together resulted in an even lower base-line urine osmolality, which unlike in AQP1 null mice increased 2.9-fold after water deprivation (see “Discussion”).

Most adult AQP1/AQP3 double knockout mice showed marked tumor-like swelling of the flanks bilaterally (Fig. 6A, left), which was never seen in wild-type mice. The flank swelling was caused by kidney enlargement (Fig. 6A, middle and right). Examination of the morphology in kidneys from wild-type mice showed well demarcated cortex and papilla (Fig. 6B, top panels). In contrast, kidneys from AQP3 null mice (Fig. 6B, bottom panels) and AQP1/AQP3 double knockout mice (not shown) showed medullary atrophy and cortical thinning. At 4 weeks of age, >50% of kidneys from AQP3 null mice and >90% of kidneys from AQP1/AQP3 double knockout mice showed these changes. Many adult mice showed hydrenephrosis. The kidneys with severe hydrenephrosis were markedly enlarged and transparent enough to reveal dilated renal blood vessels (Fig. 6A, right panel). Similar changes in renal morphology have been seen in polyuria causing increased intrarenal pressures (15). The medullary atrophy appeared to be an age-dependent phenomenon that was infrequently seen in mice under the age of 2 weeks but found in ~50% of mice at age 4 weeks (Fig. 6B). The mice with flank swelling and renal enlargement had serum azotemia (blood urea nitrogen 78 ± 27 mg/dl, normal <15 mg/dl) and generally did not survive beyond 10–12 weeks.

**DISCUSSION**

The results here provide direct evidence against a role for AQP3 in erythrocyte water and glycerol permeabilities. The low osmotic water permeability in AQP1-deficient erythrocytes was not further reduced by AQP3 deletion. Erythrocyte glycerol permeability was not affected by AQP3 deletion nor was the inhibition of glycerol permeability affected by phloretin. The conclusion of the prior study suggesting a role for AQP3 in erythrocyte water and glycerol transport was based primarily on indirect immunocytochemical evidence (4). Our AQP3 antibodies were unable to detect AQP3 in human or mouse erythrocytes by immunostaining, whereas AQP3 was readily detected in the kidney-collecting duct. Although small amounts of erythrocyte AQP3 expression cannot be ruled out given the limitations of available antibodies, our data provide functional evidence against an important contribution of AQP3 to erythrocyte water and glycerol permeability.

The molecular mechanisms of erythrocyte glycerol transport and non-AQP-mediated water transport remain unknown. The high and phloretin-inhibitable glycerol permeability suggests the existence of an as yet unidentified glycerol transport protein. Interestingly, the residual water permeability after AQP1 deletion was partially inhibited by mercurials, suggesting the expression of another water-transporting protein. Erythrocytes contain UT-B (originally called UT3 in rat and mouse and UT11 in human) (for review see Refs. 16 and 17), a urea-transporting protein that also transports water when expressed in Xenopus oocytes (18); however, quantitative analysis suggests that UT-B does not contribute significantly to water permeability in native erythrocytes (19). Measurement of water permeability in the erythrocytes lacking UT-B should be informative and when available, transport measurements in erythrocytes lacking UT-B and AQP1 together. Alternately, the non-AQP1 erythrocyte water transport pathway might involve a different aquaporin or non-aquaporin water transporter, such as a water-permeable glucose carrier (20) or Na+-coupled solute cotransporter (21). As in erythrocytes, water permeability in vesicles derived from the renal proximal tubule is inhibited by mercurials (3). However, in contrast to the data presented here on erythrocytes, water permeability in proximal tubule vesicles from AQP1 null mice is not inhibited by mercurials and probably involves a lipid pathway.

Functional analysis of double knockout mice lacking two aquaporins has been useful in analyzing the role of aquaporins in organs that express more than one aquaporin. A small con-
Distribution of airway water channel AQP4 to airspace-capillary water permeability was demonstrated by comparative measurements in lungs from AQP1 null mice, which already have low water permeability, with lungs from AQP1/AQP4 double knockout mice (22). Similarly, the role of high basolateral membrane water permeability in the kidney-collecting duct was quantified in functional studies on mice lacking AQP3 versus AQP3/AQP4 together (13). AQP3 is expressed mainly in the cortical and outer medullary-collecting duct, whereas AQP4 is expressed mainly in the inner medullary-collecting duct. Measurements in double knockout mice provided information about whether the partial urinary-concentrating ability of AQP3 null mice in response to water deprivation results from residual AQP4-mediated water permeability in the inner medullary-collecting duct. The roles of AQP1 (microvascular water channel) and AQP5 (alveolar epithelial water channel) in lung physiology were investigated using AQP1/AQP5 null mice (23). Although airspace-capillary osmotic water permeability was reduced by more than 30-fold by AQP1/AQP5 deletion, active near-isosmolar alveolar fluid absorption was not affected, providing strong evidence against a role of aquaporins in lung physiology (24). We note that the AQP1/AQP3 double knockout mice. Although the reason(s) for the relatively poor growth and survival of AQP1/AQP3 null mice cannot be established from the data presented here, we speculate that defective gastrointestinal function might be involved. AQP1 null mice manifest dietary fat misprocessing (25), and AQP3 appears to be expressed strongly throughout the gastrointestinal tract (26, 27) so that their double deletion might further compromise intestinal nutrient absorption.

Urinary-concentrating ability in mice lacking AQP1 and AQP3 together was impaired to a greater extent than that in mice lacking AQP1 or AQP3 individually. AQP1 deletion results in defective isosmolar fluid absorption in proximal tubule as well as in defective countercurrent exchange, which produces a decrease in osmolality of the medullary interstitium. Although the relative importance of decreased proximal tubular absorption and defective thin descending limb of Henle and vasa recta function (for countercurrent exchange) was not determined directly, the vasa recta seemed more important based on the inability of DDAVP to increase urine osmolality (3) and the compensatory decrease in glomerular filtration rate in AQP1 null mice (10). The polyuria in AQP1 null mice and their inability to produce concentrated urine probably results from a maximal medullary osmolality of 600–700 mosm. If AQP3 deletion affects renal cortical function (collecting duct water permeability, for review see Ref. 13) and the countercurrent sys-

**FIG. 5. Renal function in aquaporin null mice.** A, daily fluid consumption (open bar) and urine output (black bar) in mice of indicated genotype (four mice in each group, mean ± S.E.). B, urine osmolality in mice of indicated genotype measured during free access to water (open bar) and after a 24-h water deprivation (black bar) (four mice in each group, mean ± S.E.).

**FIG. 6. Renal morphology in aquaporin null mice.** A, AQP3 null mouse with hydronephrosis (left) and hydronephrotic kidneys (middle and right). B, age-dependent progression of medullary atrophy in AQP3 null mice. Paraffin sections of kidneys from mice of indicated genotypes at different ages.
tem, it is predicted that urine osmolality in well hydrated mice should be very low and increase slightly in response to DDAVP administration or water deprivation. We found that compared with the results in well hydrated AQP3 null mice, urine osmolality was mildly reduced, and urine output was increased in the AQP1/AQP3 double knockout mice. The combined effects of low medullary interstitial osmolality and reduced collecting duct water permeability would account for this observation. Interestingly, urine osmolality in water-deprived AQP1/AQP3 null mice increased substantially, albeit to a level just lower than that in water-deprived AQP1 null mice. The submaximal increase in transepithelial water permeability in the AQP3-deficient cortical collecting duct in water-deprived AQP1/AQP3 double knockout mice permitted osmotic extraction of water in the collecting duct lumen to produce a urine with osmolality >500 mosmol. Together these results indicate distinct renal defects involving medullary countercurrent exchange (AQP1) and cortical collecting duct water permeability (AQP3). These data should be very useful in testing mathematical models of the urinary-concentrating mechanism.

Acknowledgment—We thank Liman Qain for mouse breeding and genotype analysis.

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