Collecting duct-specific knockout of adenylyl cyclase type VI causes a urinary concentration defect in mice

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Roos KP, Strait KA, Raphael KL, Blount MA, Kohan DE. Collecting duct-specific knockout of adenylyl cyclase type VI causes a urinary concentration defect in mice. Am J Physiol Renal Physiol 302: F78–F84, 2012. First published September 21, 2011; doi:10.1152/ajprenal.00397.2011.—Collecting duct (CD) adenylyl cyclase VI (AC6) has been implicated in arginine vasopressin (AVP)-stimulated renal water reabsorption. To evaluate the role of CD-derived AC6 in regulating water homeostasis, mice were generated with CD-specific knockout (KO) of AC6 using the Cre/loxP system. CD AC6 KO and controls were studied under normal water intake, chronically water loaded, or water deprived; all of these conditions were repeated in the presence of continuous administration of 1-desamino-8-D-arginine vasopressin (DDAVP). During normal water intake or after water deprivation, urine osmolality (U osmol) was reduced in CD AC6 KO animals vs. controls. Similarly, U osmol was decreased in CD AC6 KO mice vs. controls after water deprivation+DDAVP administration. Pair-fed (with controls) CD AC6 KO mice also had lower urine osmolality vs. controls. There were no detectable differences between KO and control animals in fluid intake or urine volume under any conditions. CD AC6 KO mice did not have altered plasma AVP levels vs. controls. AVP-stimulated cAMP accumulation was reduced in acutely isolated inner medullary CD (IMCD) from CD A6 KO vs. controls. Medullary aquaporin-2 (AQP2) protein expression was lower in CD AC6 KO mice vs. controls. There were no differences in urinary urea excretion or IMCD UT-A1 expression; however, IMCD UT-A3 expression was reduced in CD AC6 KO mice vs. controls. In summary, AC6 in the CD regulates renal water excretion, most likely through control of AVP-stimulated cAMP accumulation and AQP2.

MATERIALS AND METHODS

Animal study approval. All experiments were carried out in accordance with the guidelines of the animal care and use policy of the University of Utah Health Sciences Center.

Transgenic mouse lines. The adenylyl cyclase 6 gene (Adcy6; NM_007405.2) fragment was obtained by high-fidelity PCR (Stratagene Pfu Ultra II Fusion HS DNA Polymerase, La Jolla, CA) of 129x1/SVJ genomic DNA (Jackson Laboratory, Bar Harbor, ME) using primers AC6-F 5'-AAC TGG TGA GAT GGC TCC TCA G-3' and AC6-R 5'-GAG CCT CAG AAA ACA GAA GGG G-3', adding BamHI to the upstream end and NOTI to the downstream end. This product was cloned into the pStart vector (generous gift from Mario Capecchi) (25) that was digested with BamH1 and NOTI. Site-directed mutagenesis was performed using a Quick Change Mutagenesis Kit (Promega, Madison, WI) to insert the 3' loxP site (underlined sequence), primer 3'-CGA AAG CAG AGT GTT GCA GCC GGT GGC-3' and its complement, and the PML1 site (underlined sequence) upstream of exon 3, primer 5'-GAA CAC AGT GTT AAG CAC GTG GGT GCC CCA AAG GAA CTG-3' and its complement. A FRT-flanked neomycin resistance gene with a 3' loxP site (generously provided by Gail Martin) (14) was subcloned into the PML1 site. This generated the targeting vector shown in Fig. 1. This was swapped from the pStart vector into the PWS-TK2 vector using Red Recombinase. This was linearized and transfected into 129x1/SVJ ES cells by the transgenic core facility at the University of Utah.

This nephron segment. Our group confirmed this finding by showing that AC3 small interfering RNA (siRNA) inhibited AVP-stimulated cAMP accumulation in cultured mouse IMCD (23). This latter study also found that AC6 might also be of particular importance in controlling AVP action in the CD. Pharmacological manipulations suggested that a protein kinase C- and calcium-inhibitable AC isoform could substantially mediate AVP-regulated cAMP content in the CD; in addition, siRNA knockdown of AC6 in mouse IMCD cells decreased AVP-enhanced cAMP levels (23). While these studies suggested an important role for AC6 in mediating AVP action in the CD, they were based on in vitro analysis. More direct evidence for a role of AC6 in regulating AVP action comes from two recent in vivo studies. These investigators found that mice that were globally deficient in AC6 had decreased urine osmolality, increased urine output, and increased fluid intake (6, 19). However, these studies did not directly address the role of CD AC6 in modulating AVP effects on renal water transport. Consequently, in the current studies, we have generated a CD principal cell-specific knockout (KO) of AC6. We report that mice with relatively selective deficiency of AC6 in CD principal cells have a urinary concentration defect and decreased AVP-enhanced cAMP levels, thereby providing the most direct evidence to date that CD AC6 plays a role in mediating AVP-stimulated water reabsorption.

ARGinine VATopressin (AVP) plays a key role in the regulation of renal salt and water excretion (20, 21, 24). A major target of AVP in renal tissue is the collecting duct (CD) principal cell. AVP effects in CD principal cells are mediated in large part by adenylyl cyclase (AC)-stimulated production of cAMP (18). To date, 10 AC isoforms have been described (2). Previous studies suggest that the isoforms that predominate in the CD are AC3, AC4, and AC6 (23). There is ongoing debate as to the exact isoforms that are responsible for AVP-stimulated accumulation of cAMP. Hoffert et al. (9) found that inhibition of calmodulin decreased the cAMP response to AVP in the rat inner medullary collecting duct (IMCD). Since AC3 was thought to be the only known calmodulin-regulated AC expressed in rat IMCD, these investigators concluded that AC3 is a key modulator of AVP-stimulated cAMP accumulation in this nephron segment. Our group confirmed this finding by showing that AC3 small interfering RNA (siRNA) inhibited AVP-stimulated cAMP accumulation in cultured mouse IMCD (23). This latter study also found that AC6 might also be of particular importance in controlling AVP action in the CD. Pharmacological manipulations suggested that a protein kinase C- and calcium-inhibitable AC isoform could substantially mediate AVP-regulated cAMP content in the CD; in addition, siRNA knockdown of AC6 in mouse IMCD cells decreased AVP-enhanced cAMP levels (23). While these studies suggested an important role for AC6 in mediating AVP action in the CD, they were based on in vitro analysis. More direct evidence for a role of AC6 in regulating AVP action comes from two recent in vivo studies. These investigators found that mice that were globally deficient in AC6 had decreased urine osmolality, increased urine output, and increased fluid intake (6, 19). However, these studies did not directly address the role of CD AC6 in modulating AVP effects on renal water transport. Consequently, in the current studies, we have generated a CD principal cell-specific knockout (KO) of AC6. We report that mice with relatively selective deficiency of AC6 in CD principal cells have a urinary concentration defect and decreased AVP-enhanced cAMP levels, thereby providing the most direct evidence to date that CD AC6 plays a role in mediating AVP-stimulated water reabsorption.

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Fig. 1. Constructs used in generating collecting duct (CD)-specific knockout sequence (NLS), a carboxy-terminal HSV glycoprotein D epitope tag, Cre Adcy6 gene with exons 3–12 flanked by loxP sites as well as a FRT-flanked neomycin resistance gene.

Fig. 2. Adcy6 recombination and AC6 mRNA expression in CD AC6 KO mice. A: representative PCR (n = 6) of target organ recombination of Adcy6 gene. PCR was performed with primers spanning exons 2–13 of the mouse Adcy6 gene. The 0.5-kb band represents recombination of the target Adcy6 gene. B: relative mRNA expression of AC6/GAPDH in brain and renal papilla. Real-time PCR was performed on cDNA using a Taqman gene expression assay; n = 6 for each data point. *P < 0.05 vs. control.

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mg/ml DNase (Sigma) and incubated at 37°C for 30–45 min. IMCD fragments were washed and incubated with HBSS. IMCD were incubated for 30 min with 1 mM IBMX (Sigma) before addition of AVP (10−9 M, Sigma) for 10 min. Cells were extracted with ethanol, and cAMP levels were measured by ELISA (Enzo Life Sciences, Plymouth Meeting, PA). Total cell protein was measured by a Bradford assay (Bio-Rad, Hercules, CA).

Protein expression in inner medulla. Mice on a normal water diet were euthanized, and their kidneys were removed. Tissue was dissected into inner papillary tip, inner medullary base, and outer medulla and prepared as detailed previously (3). Fresh kidney tissues were homogenized in a glass tissue grinder in ice-cold isolation buffer (10 mM triethanolamine, 250 mM sucrose, pH 7.6, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% β-mercaptoethanol, 0.02% bromophenol blue) and heated at 100°C for 2 min. Proteins (10–20 µg/lane) were size separated by SDS-PAGE and then electroblotted to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were separated by SDS-PAGE and then electroblotted to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were stained with Ponceau S and prepared as detailed previously (3). Fresh kidney tissues were homogenized in a glass tissue grinder in ice-cold isolation buffer (10 mM triethanolamine, 250 mM sucrose, pH 7.6, 1 mM EDTA, 10% β-mercaptoethanol, 0.02% bromophenol blue) and heated at 100°C for 2 min. Proteins (10–20 µg/lane) were size separated by SDS-PAGE and then electroblotted to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were probed with primary antibody overnight at 4°C. Antibodies derived and characterized in this laboratory (10, 11, 15, 17) including COOH-terminal urea transporter UT-A1 (exclusively detects UT-A1 in the inner medulla and UT-A2 in the outer medulla), NH2-terminal UT-A1 (detects UT-A1 and UT-A3 simultaneously), and AQP2 were used to determine the level of respective protein abundances. The secondary antibody used for detection was Alexa Fluor 680-linked anti-rabbit IgG (Invitrogen, Chicago, IL). Proteins were visualized using infrared detection with the LI-COR Odyssey protein analysis system (LI-COR, Lincoln, NE). Laser densitometry was used to quantify the intensity of the resulting bands. Densitometry values were normalized to β-actin loading controls.

Electrolyte and hormone analysis. Urine was analyzed for osmolality (Osmett II, Precision System, Natick, MA). Plasma AVP was extracted using acetone and petroleum ether (7) and analyzed by ELISA (Enzo Life Sciences, Plymouth Meeting, PA). Urine urea was measured using a quantitative colorimetric urea assay (Quantichrom, BioAssay Systems, Hayward, CA).

Statistical analysis. Data are presented as mean percentage of control ± SE. Data were compared using one-sided (in pair-feeding studies) and two-sided (all other studies) unpaired Student’s t-tests. The criterion for significance was P < 0.05.

RESULTS

Characterization of CD AC6 KO mice. CD AC6 KO mice were born at the expected frequency and developed normally until at least 1 yr of age. Gross and microscopic morphology were normal in these mice. PCR of DNA from heart, liver, large intestine, lung, brain, kidney, and testes revealed some

| Table 2. Effect of CD AC6 KO on water and osmolyte metabolism in the presence of exogenous AVP |
|-----------------------------------|-----------------|-----------------|-----------------|
|                                  | Control         | CD AC6 KO       | P Value         |
| Normal water + DDAVP at 25 ng/h  |                 |                 |                 |
| Urine volume, ml                | 1.4 ± 0.26      | 2.0 ± 0.27      | 0.12            |
| Fluid intake, ml                | 3.6 ± 0.8       | 4.61 ± 1.06     | 0.5             |
| Urine osmolality, mosmol/l      | 1,938 ± 189.3   | 1,428 ± 91.7    | 0.04            |
| Osmolyte excretion, mosmol/day  | 2,466 ± 447.4   | 2,773 ± 329.7   | 0.59            |
| Chronic water loading + DDAVP at 25 ng/h |             |                 |                 |
| Urine volume, ml                | 34.5 ± 4.33     | 27.3 ± 5.93     | 0.35            |
| Fluid intake, ml                | 33.1 ± 3.9      | 29.7 ± 6.74     | 0.67            |
| Urine osmolality, mosmol/l      | 230 ± 56.4      | 268 ± 94.4      | 0.73            |
| Osmolyte excretion, mosmol/day  | 6,581 ± 866.3   | 4,911 ± 1,731   | 0.4             |
| Water deprivation               |                 |                 |                 |
| Urine volume, ml                | 10.0 ± 0.09     | 1.1 ± 0.16      | 0.71            |
| Urine osmolality, mosmol/l      | 2,777 ± 108.4   | 2,271 ± 160     | 0.02            |
| Osmolyte excretion, mosmol/day  | 2,776 ± 249.5   | 2,378 ± 307.5   | 0.33            |

Values are means ± SE. Control and CD AC6 KO mice underwent implantation of osmotic pumps delivering 1-desamino-8-D-arginine vasopressin (DDAVP) at 25 ng/h, followed by normal, high, or low water intake (n = 8/group). Urine volume, fluid intake, and urine osmolality were analyzed.
degree of the Adcy6 gene recombination in brain, testes, and kidney of CD AC6 KO mice (Fig. 2A). Real-time PCR of brain and renal papilla mRNA revealed an insignificant decrease in AC6 message in brain (P = 0.45) in CD AC6 KO mice compared with control animals, whereas renal papillary mRNA content was significantly reduced in knockout animals (39.5 ± 2.14% reduction, P = 0.02) (Fig. 2B). Six animals were used for the PCR analysis and, as shown in Fig. 2, the data had very modest variability. Note that renal papilla contains multiple cell types whereas AC6 was targeted only in collecting duct principal cells.

Water metabolism studies. Urine osmolality, fluid intake, and urine volume were measured over 24 h in mice in metabolic cages (Table 1). On a diet containing 0.3% Na and free access to drinking water, urine volume tended to be higher in KO mice. Urine osmolality was decreased by ~25% in the CD AC6 KO animals. Animals were then given 5% sucrose in water, resulting in an increase in water intake from between 1 and 2 ml/day on normal water intake to 27–35 ml/day on high water intake. There were no differences in urine volume and fluid intake between control and CD AC6 KO mice on high water intake. In contrast to a normal water intake, urine osmolality was not different between KO and control animals on high water intake (urine was markedly diluted to 200–300 mosmol/l). Animals were then water deprived for 18 h and given normal food. Urine osmolality was significantly reduced by about 20% in AC6 knockout mice vs. controls. Urine osmolyte excretion was not different between control and CD AC6 KO mice under any conditions.

Osmotic minipumps were implanted that delivered DDAVP at 25 ng/h, and mice were exposed to normal, high, and low water intake as described above (Table 2). During normal water intake, there were no differences between control and CD AC6 KO mice in urine volume, fluid intake, or urine osmolality. Similarly, during water loading, urine volume, fluid intake, and urine osmolality were not different between the two groups of mice. With water restriction, urine volume was markedly reduced in both groups of mice and to a similar degree; urine osmolality was slightly, albeit significantly, lower in CD AC6 KO mice compared with controls. Urine osmolyte excretion was not different between control and CD AC6 KO mice under any conditions during DDAVP administration.

CD AC6 KO mice did not have an apparently greater fluid intake than controls; however, measurements of water intake in mice are subject to considerable variability. To control more carefully for water intake, KO and control animals were pair-fed the same volumes by using a gelled diet that contains a fixed amount of food and water (Table 3). Urine osmolality was significantly decreased in CD AC6 KO animals. Urine osmolyte excretion was not different between the two groups of mice. Thus the reduced urine osmolality in KO mice was not due to differences in fluid intake.

**Urea excretion.** There was no statistically significant difference in 24-h urinary urea excretion between control and CD AC6 KO mice given free access to drinking water and standard mouse chow, although CD AC6 KO mice tended to have higher urinary urea excretion rates (85.3 ± 10.4 control vs. 137.8 ± 21.2 mg/day KO, P = 0.09, n = 8/group) (Fig. 3).

**Plasma AVP levels.** Plasma AVP was extracted and analyzed from animals given a 0.3% Na diet and free access to water (Fig. 4). There was no difference in plasma AVP levels between control and KO animals, suggesting that circulating AVP did not account for the differences in urine osmolality between the groups.

**Protein expression in the medulla.** Expression of AQP2 protein was evaluated in the inner medulla and outer medulla from animals fed a normal water diet (Fig. 5). There was a significant reduction in AQP2 protein expression in both the inner and outer medullar in CD AC6 KO compared with control mice. UT-A1 and UT-A3 expression were evaluated in the inner medulla (Fig. 6). There was a decrease in both UT-A1 and UT-A3 in the inner medulla of CD AC6 KO animals.

**AVP-stimulated cAMP accumulation.** cAMP levels were evaluated in acutely isolated IMCD from control and CD AC6 KO mice on a normal water diet. Cells were pretreated with IBMX, followed by stimulation with AVP (Fig. 7). AVP-enhanced cAMP accumulation was reduced by ~40% in CD AC6 KO compared with control mice.

**DISCUSSION**

This study demonstrates that mice with CD-specific KO of AC6 have reduced urine concentration. This was associated with 1) decreased AVP-stimulated cAMP accumulation in IMCD acutely isolated from KO animals; 2) lower AQP2 protein content in the renal medulla of KO mice; and 3) diminished expression of UT-A3 in inner medulla from KO animals.

A key question is whether the decrease in urine concentration was due to a primary defect in renal concentrating ability or was caused by enhanced fluid intake. Our studies suggest...
that AC6 deficiency in the CD causes impaired water reabsorption. First, the study was designed to specifically target the CD. While this was largely achieved, there was evidence for some recombination of the floxed Adcy6 allele in the brain, albeit total brain AC6 mRNA levels were not reduced. While such AQP2-Cre-mediated recombination in the brain has not previously been reported, it is possible that the chromosomal location of the floxed AC6 allele renders it unusually highly susceptible to Cre, even if Cre is expressed at very low levels. Second, no changes in fluid intake were detected. Given that fluid intake in mice is very difficult to accurately measure, it was possible that small differences in fluid intake failed to be detected. However, reduced urine concentration in KO mice vs. controls was noted in pair-fed animals with matched fluid intake. Taken together, these findings indicate that CD-derived AC6 regulates renal water reabsorption. Notably, our studies contrast with two previous studies involving whole animal AC6 KO (6, 19). These previous investigations reported polydipsia in mice with global AC6 deficiency, whereas we failed to observe altered fluid intake in CD-specific KO animals. While the cause for the differing results is speculative, a likely explanation is that AC6 may affect water metabolism in cells outside the collecting duct, including other nephron segments or nonrenal tissues. Finally, there was a consistent trend toward increased urine volume in CD AC6 KO mice. Taken together, the reduced urine osmolality, consistent tendency toward increased fluid excretion, and unaltered total osmolyte excretion indicate that CD deficiency of AC6 causes a mild nephrogenic diabetes insipidus.

The mechanism(s) by which CD KO of AC6 impairs renal water excretion was not investigated in detail; however, the current study does point to possible mechanisms. Our data suggest that AVP actions are reduced by CD-specific knockout of AC6. This includes decreased AVP-stimulated cAMP content in isolated IMCD and lower AQP2 protein levels in the renal medulla of KO compared with control animals. In addition, reduced urine concentration was still evident during “clamping” of circulating AVP levels using endogenously delivered supraphysiological doses of AVP during water deprivation (when AVP levels would be maximized). With regard to this latter observation, it is notable that previous studies examining whole animal AC6 knockout either did not administer DDAVP in vivo (6) or only examined the response to acute administration of DDAVP in the setting of acute water loading (19).

The present studies also evaluated the effect of CD-specific AC6 KO on urea metabolism, albeit to a limited extent. CD AC6 KO mice manifested decreased inner medullary UT-A3 expression. However, this was not associated with statistically significant alterations in urinary urea excretion, although urea excretion tended to be increased in CD AC6 KO mice. In addition, inner medullary UT-A1 levels were unaffected. Since the primary goal of this study was to examine the effect of CD AC6 deficiency on water metabolism, additional studies clarifying the role of AC6 in modulating renal urea handling were not conducted, but are planned for future analyses.
CD-specific KO of AC6 caused only a modest defect in urine concentration. It is possible that this relates to incomplete KO of CD-derived AC6. However, it is likely that AC6 is not the only AC isoform involved in mediating renal water reabsorption. As mentioned earlier, studies by Hoffert et al. (9) suggested that AC3 was the major AVP-regulated AC isoform in the CD, while in vitro studies by our group suggested that both AC3 and AC6 modulate AVP-stimulated cAMP accumulation in the CD (23). In addition, a third isoform, AC4, is also present in the CD (23); very little is known about the biological role of AC4 in the CD. It is possible, therefore, that AC3 and AC4 could compensate for loss of AC6, either by simply being present in the CD or through possible upregulated expression that is induced by AC6 KO. We did not specifically examine how or whether AC6 KO affected other AC isoform expression in the CD. While this would be of interest, the ultimate question is what role each AC isoform has in modulating water handling by the CD. To best address this question, further studies are planned that will use CD-specific KO of different AC isoforms.

AVP, via modulation of cAMP, regulates CD sodium and chloride transport in addition to controlling urea and water reabsorption (20–22, 24). AVP increases CD epithelial sodium channel (ENaC) activity (13, 21) and augments CD chloride secretion (13, 24). The effect of AVP on ENaC has been shown to be mediated, at least in part, by cAMP (5, 8, 21). Similarly, chloride secretion has been linked to cAMP in IMCD via an increase in CFTR activity (4, 13). Consequently, it will be interesting to examine the effect of CD-specific AC isoform KO on renal salt excretion (and ultimately how this affects arterial pressure).

In summary, CD-specific KO of AC6 causes a urinary concentration defect associated with reduced AQP2 expression and lower AVP-stimulated cAMP accumulation. Thus CD AC6 KO causes a milder nephrogenic diabetes insipidus. These data suggest that CD-derived AC6 is involved in modulating AVP-regulated water reabsorption. Additional studies, using CD-specific KO of other AC isoforms (primarily AC3 and AC4), will help clarify the relative importance of the individual AC isoforms in modulating AVP actions in the CD.

**GRANTS**

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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