Collecting duct-specific knockout of endothelin-1 alters vasopressin regulation of urine osmolality

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IN VITRO STUDIES SUGGEST THAT collecting duct (CD)-derived endothelin-1 (ET-1) may be an autocrine inhibitor of vasopressin (AVP)-stimulated water reabsorption in the kidney. Within the kidney, the CD is the major renal site of ET-1 synthesis (6, 18, 31, 41, 42) and possibly binding (7, 35, 36). In vitro, exogenous ET-1, through activation of the ETB receptor (ETRB), reduces AVP-stimulated water permeability in cortical CD (35, 40) and inhibits AVP-stimulated cAMP accumulation (10, 21, 39) and osmotic water permeability (27, 29) in inner medullary CD (IMCD). Blockade of endogenous ET-1 action increases AVP-stimulated cAMP levels in cultured IMCD cells (19), providing the most direct evidence for a potential autocrine role of the peptide.

There is less convincing, and no direct, evidence demonstrating an autocrine role for ET-1 in regulating CD water transport in vivo. Part of the problem stems from the inability to discriminate between direct ET-1 effects on the nephron as opposed to the vasculature. Administration of ET agonists or antagonists usually alters renal plasma flow and glomerular filtration rate, effects that can impact urinary water excretion. Studies have shown that (17) systemically administered ET, at doses with minimal detectable effects on renal hemodynamics, increases water excretion (14, 16, 32). Additionally, chronic water loading in rats and humans is associated with elevated ET-1 excretion (although the source of urinary ET-1 could not be fully ascertained) (20, 24, 46), whereas water deprivation decreases urinary ET-1 excretion (20, 25). Thus circumstantial evidence suggests that ET-1 may inhibit renal water reabsorption. However, the physiological role that the peptide plays in regulating renal water transport, particularly through autocrine regulation of CD function, remains unknown.

Alternative methods for examining the physiological role of CD-derived ET-1 in regulating renal water excretion have not yet been employed. Traditional gene knockout methodologies have proven problematic in that every mouse homozygous for knockout of a component of the ET system [ET-1, ET-2, ET-3, ET receptor A (ETRA), ETRB] has a lethal phenotype (2, 15, 22, 23, 30). Renal function has been assessed on a limited basis in spotted lethal rats, which carry a natural mutation in the ETRB gene resulting in toxic megacolon (13). Spotted lethal rats that have been “rescued” with a transgene expressing ETRB under control of the dopamine β-hydroxylase promoter exhibit hypertension; however, renal water excretion has not been reported in this model. Furthermore, these animals lack ETRB expression in the kidney [almost every cell in the kidney expresses ETRB (17)], while circulating ET-1 levels are likely to be markedly elevated (3). Consequently, this model was not employed. We recently reported the development of mice with CD-specific knockout of ET-1. These mice express Cre recombinase under control of the aquaporin-2 (AQP2) promoter and are homozygous for loxp-flanked exon 2 of the ET-1 gene. We now report that these mice have altered renal water handling, thereby providing direct evidence for a physiological role of CD-derived ET-1 in regulating renal water excretion.

METHODS

Transgenic mice lines. Mice with CD-specific disruption of the ET-1 gene were generated in a manner similar to that previously described (1). Briefly, mice containing the loxp-flanked (floxed) ET-1
gene (obtained from Dr. M. Yanagisawa at the Howard Hughes Institute at University of Texas Southwestern Medical Center) were mated with AQP2-Cre mice. The floxed mice contain exon 2 of the ET-1 gene flanked by loxP sites [exon 2 is critical to ET-1 gene functional expression (23)]. AQP2-Cre mice contain a transgene with 11 kb of the mouse AQP2 gene 5'-flanking region driving expression of Cre recombinase. These mice are phenotypically identical to those previously described by our group in which 14 kb of the human AQP2 promoter were used to drive Cre expression (28). An SV40 nuclear localization signal is located on the NH2 terminus of Cre and an 11-amino acid epitope tag, derived from Herpes simplex virus glycoprotein D, is located on the COOH terminus of Cre. Female AQP2-Cre mice were mated with male floxed ET-1 mice; female offspring heterozygous for both AQP2-Cre and floxed ET-1 were bred with males homozygous for floxed ET-1. Animals homozygous for floxed ET-1 and heterozygous for AQP2-Cre (CD ET-1 KO) were used in all studies. Sex-matched littersmates that were homozygous for the floxed ET-1 gene, but without Cre, were used as controls in all studies.

Genotyping. Tail DNA was prepared by standard methods and PCR amplified for the AQP2 Cre transgene using oligonucleotide primers mAQP2 F 5'- CCT CTC GAG GAA CTG CTG G3' and CreTag R 5’-GGC AAC ATC TTC AGG TTC TGC GG-3’, which amplify the 671-bp junction between the mouse AQP2 promoter and the Cre gene. Genotyping the ET-1 gene involved three primer sets. The first set used primers ET-1CF (5’- CGC CAA AGA TTC TGA ATT C-3’) and ET-1BR (5’-GAT GTG CTC GTG GCA GAA G3’), which amplify 800 bp of the endogenous ET-1 allele. The second set used primers ET-1AF (5’- CCC AAA GAT TCT GAA TGG ATA ACT TCG-3’) and ET-1BR (5’-GAT GTG CTC GTG GCA GAA G3’), which amplify the same 800-bp region; however, the forward primer overlaps loxP and hence only recognizes the floxed ET-1 allele. The third set used primers ET-1AF and ET-1DR (5’-AAC CTC CCA GTG GAT CAC GTA C-3’), which amplify the region in the targeted allele spanning the loxP sites. The PCR product of the nonrecombined allele is 2 kb, whereas the recombined allele yields a 300-bp product. PCR products were visualized after electrophoresis through 1.5% agarose.

Metabolic balance studies. All mice were studied at 3–5 mo of age. All mice were acclimated for 1 wk to Nalgene metabolic cages and a gel normal water diet for 1 wk. They were then placed into small metabolic cages that contained no food or water and given 2 ml of water ip. Subsequently, urine was collected under oil on an hourly basis for the next 6 hrs. Urine was analyzed for volume and osmolality. Urine was then pooled for analysis of ET-1 excretion. In some studies, mice were killed at different time points after acute water loading, and the kidneys were removed for determination of IMCD ET-1 mRNA levels.

CAMP studies. IMCDs were acutely isolated in a manner similar to that previously described (21). Briefly, inner medullas were minced in Kreb’s buffer with 1 mg/ml collagenase (In Vitrogen, Carlsbad, CA) and 0.1 mg/ml DNase (Sigma) and incubated at 37°C for 30 min. IMCD fragments were washed and all subsequent incubations were done in Kreb’s buffer. IMCDs were incubated in 1 mM isobutylmethylxanthine (Sigma) for 15 min before addition of varying concentrations of AVP or 1 μM forskolin for 10 min. Cells were extracted with ethanol and cAMP was measured by ELISA (R&D Systems, Minneapolis, MN) using a SpectraMax Plus Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Total cell protein was measured by the Bradford assay (Bio-Rad, Hercules, CA).

mRNA expression. Total RNA was prepared from kidneys using acid phenol. Twenty micrograms of RNA were electrophoresed on a 0.9% formaldehyde gel, transferred to a nylon membrane, and prehybridized for 3 h at 60°C in 50% formamide, 5× standard saline citrate, 5× Denhardt’s solution, 1% SDS, and 100 μg/ml salmon sperm DNA. Fresh solution was added for hybridization along with radioactively labeled probe. For probes, cDNA was made from mouse IMCD cell total RNA using oligo dT mRNA primer and SuperScript II reverse transcriptase (Invitrogen). The cDNA was then used as a template for PCR amplification using specific primers as follows: AQP2 (GenBank accession no. NM-000669), forward primer 5’-CTG GCT GCC CAG CTG GCA GAA G3’ and reverse primer 5’-ATC ACT AGT GTC ATC CTC ACG G-3’, which yields a product size of 596 bp. Products were purified, sequenced, and cloned into a pGEM-T cloning vector (Promega). The inserts were sequenced again to ensure cloning fidelity and to confirm orientation. Probes were digested with an appropriate restriction enzyme to give the antisense strand, and riboprobes were made by [32P]UTP incorporation with either T7 or SP6 RNA polymerase (Invitrogen). The radioactive probes were purified over a G-50 column, and specific activity was calculated. The probe was added to hybridization solution at 10 ng/ml with a specific activity 108 dpm/g and incubated overnight at 60°C. Blots were washed in decreasing concentrations of standard saline citrate and increasing temperature until background was removed. Labeled blots were subjected to autoradiography and densitometry. V2 RNA is 1.8 kb and AQP2 mRNA is 1.4 kb in size. All blots were stripped and reprobed for 1 small nuclear ribonucleoprotein (SNRNP) mRNA (mRNA size is 0.75 kb) using a probe provided by Dr. A. Thorburn at the University of Utah (37).

For determination of IMCD ET-1 mRNA, IMCD fragments were obtained as described above. IMCDs were placed in GITC solution for determination, and kidneys were removed for protein and mRNA measurements.

Acute water loading. Mice were acclimatized to metabolic cages and a gel normal water diet for 1 wk. They were then placed into small metabolic cages that contained no food or water and given 2 ml of water ip. Subsequently, urine was collected under oil on an hourly basis for the next 6 h. Urine was analyzed for volume and osmolality. Urine was then pooled for analysis of ET-1 excretion. In some studies, mice were killed at different time points after acute water loading, and the kidneys were removed for determination of IMCD ET-1 mRNA levels.
F914  COLLECTING DUCT ET-1 KNOCKOUT ALTERS WATER METABOLISM

5′-CCT TCA TTG ACC TCA ACT ACA TGG-3′ and GAPDH-R
5′-GCA GTG ATG GCA TGG ACT GTG GTG-3′, which yielded a
442-bp product. All PCR reactions were carried out with and without
reverse transcription to check for genomic DNA contamination. PCR
products were run on a 1.5% agarose gel, and products were quanti-
fied by densitometry.  

Protein expression. Kidneys were minced and homogenized in 250
mM sucrose, 10 mM triethanolamine, and a 1:1,000 dilution of
protease inhibitor cocktail set III (Calbiochem, La Jolla, CA). The
sample was spun at 4,000 g for 15 min to remove nuclei, mitochon-
dria, and larger cellular fragments. The supernatant containing the
crude membrane fraction was centrifuged at 17,000 g for 30 min. Protein
centration was determined using the Bradford assay. Samples
were solubilized at various concentrations in Laemmli buffer
containing 0.5% lithium dodecyl sulfate and run on a denaturing
NUPAGE-4–12% Bis-Tris minigel using the MOPS buffer system
(Invitrogen). Proteins were transferred to PVDF plus nylon mem-
brandes by electroelution. The blots were blocked with 5% nonfat dry
milk + TBST (10 mM Tris base, 150 mM NaCl, 0.1% Tween 20, pH
7.5) and then washed in TBST. Blots were incubated overnight at 4°C
with the appropriate primary antibody, washed, and incubated with a
horseradish peroxidase-conjugated (HRP) secondary antibody for 1 h.
After final washes, antibody binding was visualized using the en-
chanced chemiluminescent system (Amersharm International). Blots
were stripped using 200 mM glycine, pH 2.8, washed with TBST, and
reblotted before further probing. Antibodies used for total AQP2
were 1:500 goat anti-human AQP2 (Santa Cruz Biotechnology, Santa
Cruz, CA) and 1:5,000 HRP-rabbit anti-goat IgG (Santa Cruz Bio-
technology). Antibodies for phosphorylated AQP2 (P-AQP2) detec-
tion were 1:2,000 rabbit anti-rat P-AQP2 [AN-244-PP-AP (generously
provided by S. Nielsen, University of Aarhus, Denmark, which
recognizes Ser-256 phosphorylated AQP2) (8)] and 1:2,000 HRP-
donkey anti-rabbit IgG (Amersharm International). Antibodies against
β-actin were 1:10,000 rabbit anti-human β-actin (Abcam, Cambridge,
MA) and 1:2,000 HRP-donkey anti-rabbit IgG. All blots were re-
probed for β-actin.  

Electrolyte and hormone analysis. Plasma from all animals was
analyzed for Na concentration (EasyVet analyzer, Medica, Bedford,
MA) and osmolality (Osmett II, Precision Systems, Natick, MA).
Plasma from guillotined mice was also analyzed for AVP. For AVP
determination, heparinized samples (100 μl) were mixed with 100 μl
of water and 2 ml of ice-cold acetone, centrifuged for 20 min at 3,500
rpm, the supernatant was mixed with 2 ml of petroleum ether, and the
bottom layer was lyophilized. AVP was assayed using a radioimmu-
noassay kit (Peninsula Laboratories, San Carlos, CA).  

Urine was measured for osmolality and ET-1. Urine was applied to
a 200-mg Bond Elut C8 column (Varian, Palo Alto, CA), equilibrated,
equiluted as previously described (34), and ET-1 was determined by
ELISA (QuantiGlo, R&D Systems) using a Dynex Technologies
MLX luminometer (Chantilly, VA).  

Statistics and ethics. Comparisons between floxed ET-1 and CD
ET-1 KO mice were analyzed by the unpaired Student’s t-test.
Comparisons of urine volume after acute water loading, AVP-stimu-
lated cAMP accumulation, protein levels, mRNA levels, and daily
weight change between floxed and CD ET-1 KO mice were made
using one-way analysis of variance with the Bonferroni correction.
P < 0.05 was taken as significant. Data are expressed as means ± SE.
All animal experiments were ethically approved by the University of
Utah Institutional Animal Care and Use Committee.  

RESULTS  

Characterization of CD ET-1 KO mice. CD ET-1 KO mice
developed normally until at least 1 yr of age and had no gross
morphological abnormalities. As previously described (1), CD-
specific knockout of ET-1 was confirmed by in situ hybridiza-
tion of kidney for ET-1 mRNA, genomic PCR of microdis-
sected CD for evidence of ET-1 gene recombination, genomic
PCR for ET-1 gene recombination in an organ panel of 15
different organs, determination of renal cell Cre activity in
mice heterozygous for AQP2-Cre and the ROSA26-YFP re-
porter, and measurement of urinary ET-1. Thus CD ET-1 KO
mice have CD-specific inactivation of the ET-1 gene.  

Renal function during a normal water diet. As previously
described (1), all mice were ration fed so they had exactly
matched food and water intake. This was achieved by using a
gel diet that contained all food and water and that met all
nutritional needs. Mice eat all of the provided gel (9 ml of gel
containing 6.3 ml of water) and do not drop any of it into the
bottom of the cage (it is quite gummy). Under these baseline
conditions, there were no differences in urine volume, urine or
plasma osmolality, or plasma Na concentration (Table 1). Mice
have high insensible water loss, so urine volume is much less
than water intake. As previously reported (1), creatinine
clearance was similar between floxed ET-1 and CD ET-1 KO mice
(data not shown). Notably, plasma AVP concentration was
substantially less in CD ET-1 KO mice, despite all other
aspects of water metabolism being similar (Table 1). These
data indicate that CD ET-1 KO mice excrete similar amounts
of water and osmoles compared with control mice but that
this occurs at lower plasma AVP levels.  

Renal function during high water intake. CD-derived ET-1
may be important in mediating the diuretic response to in-
creased water intake. To assess this, CD ET-1 KO mice were
chronically water loaded (19 ml of gel diet containing 14.1 ml
of water). Mice ate all the gel (they ate more gel than provided
with the control diet due to the lower concentration of nutrients
in the water-loading diet) and had closely matched intakes of
water (14.0 ± 0.2 ml in controls and 14.1 ± 0.2 ml in CD ET-1
KO). As shown in Fig. 1, there was no difference in urine
volume or urine osmolality between CD ET-1 KO and control
mice for up to 7 days of increased water intake. Similarly,
after the 7 days of water loading, there was no difference in plasma
Na concentration or plasma osmolality between the two
groups, whereas plasma AVP levels were suppressed in both
groups to essentially the lower limits of detectability and not
significantly different from zero (Table 2). Thus CD ET-1 KO
mice have no apparent defect in their ability to handle a chronic
water load.  

Renal function during DDAVP administration. The reduced
plasma AVP levels during a normal water diet in CD ET-1 KO
mice suggested that these mice have enhanced responsiveness
to the hydromotic effects of AVP. To test this, mice were

Table 1. Water metabolism in control and CD ET-1 KO
mice during normal water intake

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<th>Control</th>
<th>ET-1 KO</th>
<th>P&lt;</th>
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<tr>
<td>Water intake, ml/day</td>
<td>6 ±0.1 (9)</td>
<td>6 ±0.1 (9)</td>
<td>NS</td>
</tr>
<tr>
<td>Urine volume, ml/day</td>
<td>2.4 ±0.5 (9)</td>
<td>2.4 ±0.6 (9)</td>
<td>NS</td>
</tr>
<tr>
<td>Urine osmolality,</td>
<td>2613 ±351 (9)</td>
<td>2685 ±402 (9)</td>
<td>NS</td>
</tr>
<tr>
<td>mosmol/kgH₂O</td>
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<tr>
<td>Plasma [Na], meq/l</td>
<td>145 ±3 (9)</td>
<td>147 ±4 (9)</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma osmolality,</td>
<td>309 ±3 (8)</td>
<td>313 ±3 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>mosmol/kgH₂O</td>
<td></td>
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<tr>
<td>Plasma [AVP], pg/ml</td>
<td>30.1 ±7.2 (16)</td>
<td>19.5 ±3.9 (16)</td>
<td>0.01</td>
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Data are expressed as means ± SE. Numbers in parenthesis refer to sample size. CD ET-1 KO, collecting duct endothelin-1 knockout; NS, not significant.
given DDAVP via an osmotic minipump for up to 7 days, while being on a normal water intake (9 ml of the normal water intake gel diet). This procedure was designed to fix plasma AVP at maximal water-retaining levels. Chronic DDAVP administration significantly increased urine osmolality in CD ET-1 KO mice but not in control animals (Fig. 2). The failure to see an increase in urine osmolality in controls was assumed to relate to the relatively hydropenic state under which the mice must normally exist. Notably, urine osmolality did not remain elevated in CD ET-1 KO mice beyond day 3 of DDAVP administration, indicating that compensatory mechanisms came into play. No significant decrease in urine volume could be detected in either group of mice; however, such a decrease would be difficult to detect due to the intrinsic high variability in urine volume within groups. However, CD ET-1 KO mice did have more weight gain, at least on day 2 of DDAVP administration, compared with controls, indicating that they must have retained more fluid than controls (Fig. 3). CD ET-1 KO mice tended to have lower plasma Na concentration (130 ± 3 meq/l in CD ET-1 KO vs. 134.2 ± 3 meq/l in controls) and plasma osmolality (301 ± 4 in CD ET-1 KO vs. 305 ± 5 in controls), but this did not achieve statistical significance. Thus these data suggested that CD ET-1 KO mice do, in fact, have an enhanced antidiuretic response to AVP.

The above studies raised the question as to whether CD ET-1 KO mice have impaired AVP “escape,” i.e., have less decrease in urine osmolality and less increase in urine volume in response to combined DDAVP administration and water loading. As shown in Figs. 3 and 4, there was no difference in urine volume, urine osmolality, or weight gain between control and CD ET-1 KO mice on any day during the 5 days of water loading during DDAVP administration. There was also no significant difference in plasma Na concentration or plasma osmolality (data not shown). Thus CD ET-1 KO does not impair the ability to escape from the hydrosomotic effects of DDAVP.

Effect of CD ET-1 KO on V2 receptor and AQP2 expression. Given that CD ET-1 KO did not change urine volume or urine osmolality under basal or high water intake conditions, it seemed unlikely that either V2 or AQP2 expression would be altered in these mice. However, to confirm this, mem-

<table>
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<th>Control</th>
<th>ET-1 KO</th>
<th>P&lt;</th>
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<tbody>
<tr>
<td>Plasma [Na], meq/l</td>
<td>142±4 (6)</td>
<td>143±4 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma osmolality,</td>
<td>307±3 (5)</td>
<td>305±3 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>mosmol/kg H2O</td>
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<tr>
<td>Plasma [AVP], pg/ml</td>
<td>1.0±0.9 (5)</td>
<td>1.0±0.8 (5)</td>
<td>NS</td>
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Data are expressed as means ± SE. Numbers in parenthesis refer to sample size.
brane-associated AQP2 and phosphorylated AQP2 protein, AQP2 mRNA, and V2 mRNA (there are no reliable antibodies to V2) levels were determined. As expected, total and phosphorylated AQP2 protein levels were not altered in CD ET-1 KO mice, compared with controls, on a normal water diet or after 7 days of water loading (Fig. 5). V2 mRNA levels were also not affected; however, AQP2 mRNA was slightly, albeit significantly, increased in CD ET-1 KO mice, compared with controls, up to 3 h after an acute water load. These data suggest, therefore, that CD-derived ET-1 plays a role in mediating the diuretic response to water loading, at least within the first several hours of increased water intake.

If CD ET-1 were involved in mediating, at least in part, the diuretic response to an acute water load, then it would be reasonable to expect an increase in CD ET-1 production during this acute phase. To first assess this, urinary ET-1 excretion was determined after an acute water load. Because renal ET-1 excretion is relatively low in mice, it was possible to only accurately assess urinary ET-1 excretion for the entire 6-h period after water loading (i.e., not on an hourly basis). ET-1 KO mice excreted markedly less ET-1

Thus these data again suggest enhanced AVP responsiveness in CD ET-1 KO mice on a normal water diet.

**Effect of acute water loading.** Although CD ET-1 KO mice exhibited increased AVP responsiveness, no data thus far indicated that this was of particular physiological relevance. Based on previous studies discussed earlier, it seemed most likely that CD-derived ET-1 would likely help mediate the diuretic response to water loading. A significant problem with our water-loading studies was that they measured renal function only at 24-h intervals and not until 24 h after water loading. It was possible, therefore, that differences in renal water handling might be observed more acutely. Indeed, as illustrated in Fig. 7, CD ET-1 KO mice had an impaired response to acute water loading (2 ml ip). In particular, ET-1 KO mice had reduced water excretion, compared with controls, up to 3 h after an acute water load. These data suggest, therefore, that CD-derived ET-1 plays a role in mediating the diuretic response to water loading, at least within the first several hours of increased water intake.

If CD ET-1 were involved in mediating, at least in part, the diuretic response to an acute water load, then it would be reasonable to expect an increase in CD ET-1 production during this acute phase. To first assess this, urinary ET-1 excretion was determined after an acute water load. Because renal ET-1 excretion is relatively low in mice, it was possible to only accurately assess urinary ET-1 excretion for the entire 6-h period after water loading (i.e., not on an hourly basis). ET-1 KO mice excreted markedly less ET-1

Fig. 4. Effect of ET-1 KO on urine volume (A) and urine osmolality (B) in mice given DDAVP via osmotic minipump together with a high-water diet; n = 6 each data point.

Fig. 3. Effect of ET-1 KO on daily weight change in mice given DDAVP via osmotic minipump for 7 days, either with a normal water diet (A) or a high-water diet (B); n = 6 each data point. *P < 0.05 vs. control.
than did controls over the 6-h period (2.46 ± 0.45 pg ET-1 for CD ET-1 KO and 4.85 ± 0.93 pg ET-1 for controls). To more directly assess CD ET-1 production, IMCDs were acutely isolated from control kidneys after acute water loading [CD ET-1 KO IMCD do not have ET-1 mRNA (1)]. As shown in Fig. 8, there was a qualitative increase in IMCD ET-1 mRNA levels that were first evident 2 h after water loading and persisted up to at least 6 h. Thus these data provide further evidence that CD-derived ET-1 plays a role in the diuretic response to an acute water load.

**Agonist-stimulated cAMP accumulation.** To more directly assess increased CD sensitivity to AVP in CD ET-1 KO mice, IMCDs were acutely isolated and stimulated with AVP. Note that outer MCDs or cortical CDs were not studied due to inability to acutely isolate sufficient quantities of these nephron segments. IMCD from CD ET-1 KO kidneys had significantly greater AVP-stimulated cAMP accumulation, and this occurred at AVP concentrations as low as 100 pM (Fig. 9). To determine whether this augmented AVP response was due, at least in part, to postreceptor mechanisms, IMCDs were stimulated with forskolin. Similar to AVP, forskolin increased cAMP levels much more in IMCD from CD ET-1 KO mice compared with controls (Fig. 9). Thus at least part of the enhanced AVP responsiveness observed in vivo can be related to increased AVP-induced CD cAMP accumulation via postreceptor mechanisms.

**DISCUSSION**

The present study demonstrates that CD-derived ET-1 regulates renal water excretion. CD-specific knockout of ET-1 reduces plasma AVP levels yet does not alter renal water excretion under baseline conditions or in response to a chronic water load. Thus, while the relationship between plasma AVP levels and urine osmolality was reset, other mechanisms were able to fully compensate. However, when plasma AVP levels are not permitted to reset by exogenous AVP administration, CD ET-1 KO mice exhibit enhanced...
urine osmolality. The physiological relevance of this ET-1-mediated downregulation of AVP responsiveness was apparent during acute water loading, wherein CD ET-1 KO mice had impaired ability to mount a diuresis. Increased AVP responsiveness was further confirmed by demonstrating enhanced AVP-induced cAMP accumulation in acutely isolated CD ET-1 KO IMCD. Taken together, these studies provide the first demonstration of altered AVP responsiveness due to deficiency of a peptide not directly involved in water transport.

How absence of CD-derived ET-1 leads to enhanced AVP responsiveness and a reduced acute diuretic response is not yet fully understood. Previous in vitro studies suggested that CD-derived ET-1 could act in an autocrine manner by activating CD ETRB with resultant decreased AVP-stimulated cAMP accumulation (10, 21, 39). Our finding that CD ET-1 KO mice also have increased forskolin-stimulated cAMP accumulation in acutely isolated IMCD is in agreement with previous in vitro studies (21) and suggests that enhanced AVP responsiveness in CD ET-1 KO IMCD is due, at least partially, to postreceptor mechanisms. Which mechanisms are involved in such altered signaling remains to be determined, although potential candidates have been identified. For example, ET-1 may increase CD nitric oxide (NO) production (45), whereas NO reduces AVP-stimulated water permeability in isolated cortical CD (12). The increased AVP responsiveness is associated with augmented AQP2 phosphorylation and elevated mRNA levels for the V2 receptor and AQP2 (as seen in kidneys from CD ET-1 KO mice given DDAVP and a normal water diet). These changes may well be related to increased AVP-induced cAMP accumulation; however, this was not directly assessed. It is possible that CD ET-1 KO leads to increased AVP binding, through altered V2 receptor expression or affinity, which could partially account for increased AVP responsiveness. This could be tested using radiolabeled AVP analog binding to acutely isolated IMCD; such studies are planned as part of follow-up investigations on the mechanism of CD ET-1 KO-mediated alterations in AVP responsiveness.

CD-derived ET-1 could also modulate water reabsorption through paracrine effects. Adjacent medullary interstitial cells express ET receptors (9) but do not synthesize ET-1 (43). ET-1 elicits cultured medullary interstitial cell NO production (47), which could conceivably act on neighboring CD to inhibit water reabsorption. In addition, medullary CD increases in ET-1 release might lead to augmented medullary blood flow (4, 11), thereby washing out the medullary gradient and facilitating water excretion.

The factors responsible for augmenting CD ET-1 production within a few hours of water loading are unknown. Hypertonicity has been found to reduce cultured CD ET-1 production (20, 33, 38), although this has not been consistently noted (26, 44). Volume depletion, which increases medullary tonicity, decreases renal medullary ET-1 content, whereas volume expansion increases medullary ET-1 levels (20). Another interesting possibility is that increased tubule fluid flow rate may regulate CD ET-1 production. Cai et al. (5) reported that shear stress increases all three NO synthase isoforms in cultured IMCD; the possibility is raised that ET-1 could also be increased and may, at least in part, lead to increased NO production. Given that the IMCD is the major nephron source of ET-1 and that the IMCD is a major site of AVP-stimulated water reabsorption, clarification of this regulatory system is of substantial importance.

In summary, the present studies demonstrate that CD-derived ET-1 is involved in the acute diuretic response to water loading and chronically modulates CD AVP responsiveness. In contrast, CD-derived ET-1 is not necessary for a maximal diuretic response to water loading nor does it play a significant role in mediating AVP escape. Further studies are needed on the mechanisms by which CD-derived ET-1 is regulated and the mechanisms by which it regulates renal water excretion.

**GRANTS**

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![Fig. 8. Effect of acute water loading (2 ml ip) on inner medullary CD ET-1 mRNA levels in control mice. A representative blot showing the PCR products is shown. SNRP mRNA levels were similar for all mice (data not shown). Numbers indicate hours after water loading.](image-url)

![Fig. 9. Effect of 1 μM forskolin (A) or varying concentrations of AVP (B) on cAMP accumulation in inner medullary CDs acutely isolated from control or ET-1 KO mice; n = 12 each data point. *P < 0.001 vs. control.](image-url)
COLLECTING DUCT ET-1 KNOCKOUT ALTERS WATER METABOLISM

DISCLOSURES

There is no conflict of interest for these studies.

REFERENCES


