Demeclocycline attenuates hyponatremia by reducing aquaporin-2 expression in the renal inner medulla

Marleen L. A. Kortenoeven,1,2* Anne P. Sinke,1* Niels Hadrup,1,4 Christiane Trimpert,1 Jack F. M. Wetzels,3 Robert A. Fenton,2 and Peter M. T. Deen1

1Department of Physiology, Radboud University Medical Center, Nijmegen, The Netherlands; 2Department of Biomedicine, Health, Aarhus University, Aarhus, Denmark; 3Department of Nephrology, Radboud University Medical Center, Nijmegen, The Netherlands; and 4Division of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark, Saborg, Denmark

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Demeclocycline attenuates hyponatremia by reducing aquaporin-2 expression in the renal inner medulla. Am J Physiol Renal Physiol 305: F1705–F1718, 2013. First published October 23, 2013; doi:10.1152/ajprenal.00723.2012.—Binding of vasopressin to its type 2 receptor in renal collecting ducts induces cAMP signaling, transcription and translocation of aquaporin (AQP)2 water channels to the plasma membrane, and water reabsorption from the prouine. Demeclocycline is currently used to treat hyponatremia in patients with the syndrome of inappropriate antidiuretic hormone secretion (SIADH). Demeclocycline’s mechanism of action, which is poorly understood, is studied here. In mouse cortical collecting duct (mpkCCD) cells, which exhibit deamino-8-D-arginine vasopressin (dDAVP)-dependent expression of endogenous AQP2, demeclocycline decreased AQP2 abundance and gene transcription but not its protein stability. Demeclocycline did not affect vasopressin type 2 receptor localization but decreased dDAVP-induced cAMP generation and the abundance of adenylate cyclase 3 and 5/6. The addition of exogenous cAMP partially corrected the demeclocycline effect. As in patients, demeclocycline increased urine volume, decreased urine osmolality, and reverted hyponatremia in an SIADH rat model. AQP2 and adenylate cyclase 5/6 abundances were reduced in the inner medulla but increased in the cortex and outer medulla, in the absence of any sign of toxicity. In conclusion, our in vitro and in vivo data indicate that demeclocycline mainly attenuates hyponatremia in SIADH by reducing adenylate cyclase 5/6 expression and, consequently, cAMP generation, AQP2 gene transcription, and AQP2 abundance in the renal inner medulla, coinciding with a reduced vasopressin escape response in other collecting duct segments.

Demeclocycline attenuates hyponatremia by reducing aquaporin-2 expression in the renal inner medulla.

THE KIDNEY is the main organ for the regulation of water homeostasis. In states of hyponatremia or hypovolemia, the hormone arginine vasopressin (AVP) is released from the pituitary and binds to the vasopressin type 2 receptor (V2R), which is expressed in the basolateral membrane of renal collecting duct principal cells (48). Activated V2R increases intracellular cAMP levels via Gs protein and adenylate cyclase (AC), which eventually leads to the activation of PKA and subsequent phosphorylation of aquaporin (AQP)2 water channels (19, 29). This results in the redistribution of AQP2 from intracellular vesicles to the apical membrane (19, 29, 46, 72). Driven by the transcellular osmotic gradient, water enters principal cells through AQP2 and exits cells through AQP3 or AQP4, which are located in the basolateral membrane, resulting in concentrated urine. In addition, AVP also increases the expression of AQP2 via the phosphorylation of cAMP-responsive element-binding protein, which binds and activates transcription via a cAMP-responsive element in the AQP2 promoter, resulting in increased AQP2 transcription (21, 27, 39, 70). Increased AVP levels, leading to hyponatremia with or without hypervolemia, are a hallmark of several diseases, such as the syndrome of inappropriate antidiuretic hormone secretion (SIADH), end-stage cardiac failure, and liver cirrhosis (2, 63, 79). In these diseases, the total and plasma membrane abundance of principal cell AQP2 are increased, illustrating the important role of AQP2 in water retention in these pathological states (1, 18, 47).

Demeclocycline is a bacteriostatic antibiotic of the tetracycline group, which has been shown to cause diuresis and nephrogenic diabetes insipidus (16, 66). Because of the effect on water diuresis, demeclocycline is currently used to treat sustained hyponatremia in patients with SIADH (65, 75, 80). Demeclocycline has been shown to restore the Na+ plasma concentration in SIADH patients to normal levels, permitting unrestricted water intake in these patients (17).

Wilson et al. (77) showed that the aquaretic effect of demeclocycline is exerted by selective inhibition of the water reabsorption in the distal part of the nephron, whereas others (15, 24, 66) have shown that demeclocycline inhibits AVP-induced osmotic water flow in the toad urinary bladder, a model system of the mammalian collecting duct. Just like demeclocycline, the tetracycline antibiotics minocycline, doxycycline, and tetracycline have been shown to reduce water flow in toad bladders (15), and tetracycline has been reported to decrease the urinary concentrating ability in humans (77), indicating that also other tetracycline antibiotics affect collecting duct function and urinary concentrating ability.

Using in vitro and in vivo model systems, in the present study, we investigated how tetracycline antibiotics in general and demeclocycline in particular cause diuresis.

METHODS

Cell culture. MpkCCD cells were cultured as previously described (21). Cells were seeded at a density of 1.5 × 104 cells/cm² on semipermeable filters (pore size: 0.4 μm, Transwell, Corning Costar, Cambridge, MA) and cultured for 8 days. Unless stated otherwise, cells were exposed to 1 nM of the stable AVP analog deamino-8-D-arginine vasopressin (dDAVP) at the basolateral side for the last 4 days to induce AQP2 expression (37). Tetracycline, minocycline, and demeclocycl-
caine (Sigma-Aldrich, Steinheim, Germany) were added to both sides of the filters for the last 2–24 h with or without 50 μM cycloheximide, 10 μM forskolin, or 100 μM 8-Br-cAMP. In experiments using 8-Br-cAMP, medium was replaced after 12 h. Unless stated otherwise, demeclocycline was used at a concentration of 50 μM. Transcellular electrical resistance (as a measurement of monolayer integrity) and electrical potential difference were measured using a Millicell-ERS meter at the end of the experiment (Millipore, Bedford, MA). Transfection and generation of a stable mpkCCD cell line with a 3.0 AQP2 promoter-luciferase reporter construct was performed as previously described (34). MpkCCD cells were stably transfected with wild-type V2R COOH-terminally tagged with green fluorescent protein (GFP) (64) using the calcium-phosphate precipitation technique as previously described (8). Transfected cells were selected by supplementation of the medium with 0.25 mg/ml G-418. Individual clones were isolated (9).

Immunocytochemistry. Immunocytochemistry and confocal laser scanning microscopy were performed as previously described (9). To analyze the localization of V2R, cells were fixed in 3% paraformaldehyde in PBS and mounted, and GFP expression was subsequently analyzed by confocal microscopy. For AQ2P detection, fixed cells were incubated with affinity-purified rabbit AQ2P antibodies [1:100 (11)] and goat anti-rabbit antibodies coupled to Alexa 488 (1:100, Molecular Probes, Eugene, OR).

Immunoblot analysis. MpkCCD cells from a 1.13-cm² filter or 5–10 μg of kidney material were lysed in Laemmli buffer, sonicated, and denaturated for 30 min at 37°C. For PNGase-F digestion, cell lysate was incubated with PNGase-F for 1 h at 37°C. Subsequently, Laemmli buffer was added, and samples were immunoblotted. PAGE, blotting, and blockade of polyvinylidene difluoride membranes were done as previously described (28). Membranes were incubated for 16 h at 4°C with either 1:3,000-diluted affinity-purified rabbit R7 AQ2P antibodies (11), 1:2,000-affinity-purified rabbit pre-COOH-tail AQ2P antibody recognizing amino acids 236–255 (71), or 1:200 anti-AC3 or 1:200 anti-AC5/6 (both Santa Cruz Biotechnology), all in Tris-buffered saline and Tween 20 (TBST) supplemented with 1% nonfat dried milk.

Blots were incubated for 1 h with 1:5,000-diluted goat anti-rabbit IgGs (Sigma-Aldrich) as secondary antibodies coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (Pierce, Rockford, IL). Densitrometric analyses were performed using Bio-Rad quantification equipment (Bio-Rad 690c densitometer, Laemmli buffer was added, and samples were immunoblotted. PAGE, blotting, and blockade of polyvinylidene difluoride membranes were done as previously described (28). Membranes were incubated for 16 h at 4°C with either 1:3,000-diluted affinity-purified rabbit R7 AQ2P antibodies (11), 1:2,000-affinity-purified rabbit pre-COOH-tail AQ2P antibody recognizing amino acids 236–255 (71), or 1:200 anti-AC3 or 1:200 anti-AC5/6 (both Santa Cruz Biotechnology), all in Tris-buffered saline and Tween 20 (TBST) supplemented with 1% nonfat dried milk.

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cAMP measurement. MpkCCD cells were grown with or without dDAVP during the last 4 days, with or without 50 μM demeclocycline during the last day, with the last 30 min in combination with 0.5 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich). After lysis, intracellular cAMP levels were measured using the cAMP-Glo assay (Promega, Madison, WI) or cAMP enzyme immunoassay kit (Sigma-Aldrich). After lysis, intracellular cAMP levels were measured using the cAMP-Glo assay (Promega, Madison, WI) or cAMP enzyme immunoassay kit (Sigma-Aldrich) according to manufacturer’s instructions. Results were related to a standard curve based on the measurement of defined cAMP solutions. All measurements were done in triplicate.

Luciferase assay. Luciferase activity was measured using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Luminescence was measured for 10 s using an EG&G Berthold Lumat LB9507 luminometer. To verify that equal amounts of protein per sample were used, protein concentrations were determined using the Bio-Rad protein assay according to manufacturer’s instructions.

Experimental animals. As previously reported (73, 74), to induce hyponatremia, male Wistar rats (150–200 g, Harlan Laboratories, Horst, The Netherlands) were infused with dDAVP in isotonic saline at a rate of 5 mg/h via subcutaneously implanted osmotic minipumps (model 1002, Alzet, Cupertino, CA) and were fed a nutritionally balanced rodent liquid formula (ssniff EF R/M AIN 76A, meal water soluble, SSNIFF Spezialdiaten, Soest, Germany) at a caloric density of 1.0 kcal/ml for a total of 8 days. For demeclocycline therapy, rats received daily intraperitoneal injections with 40 mg/kg body wt demeclocycline hydrochloride dissolved in PBS. For the last 48 h, rats were housed in metabolic cages to measure water intake and urine output during the last 24 h. All animal treatments were reviewed and approved by the Animal Experiment Committee of Radboud University Nijmegen Medical Centre.

Tissue preparation. Rats were anesthetized with isoflurane, after which blood was removed by a heart puncture. Rats were killed by cervical dislocation, and the kidneys were rapidly removed. One kidney was prepared for immunohistochemistry by overnight immersion in 4% paraformaldehyde in PBS, whereas of the other kidney, the inner medulla, outer medulla, and cortex were dissected for immunoblot analysis (37). For immunoblot analysis, tissue was homogenized in 1 ml of ice-cold homogenization buffer affer (20 mM Tris, 5 mM MgCl₂, 5 mM Na₂HPO₄, 1 mM EDTA, and 80 mM sucrose and protease inhibitors), cleared of nuclei and unbroken cells by centrifugation at 4,000 g for 15 min, and diluted to 0.5–1 μg protein/μl in Laemmli buffer (36).

Blood and urine analysis. Blood was collected in a BD vacutainer tube SST II Advance (Becton Dickinson, Breda, The Netherlands) for serum collection and centrifuged at 1,500 g for 10 min to sediment red blood cells. Serum and urine samples were analyzed for osmolality, Na⁺, K⁺, urea, phosphate, and creatinine concentrations by standard procedures of the General Clinical Chemical laboratory of Radboud University Nijmegen Medical Centre.

Immunohistochemistry. All procedures as well as affinity-purified antibodies against total AQ2P and pS256 AQ2P have been previously described in detail (42). Additional commercial antibodies used were directed against β-catenin (no. 7199, Santa Cruz Biotechnology) and AQ2P (AQP41-A, Alpha Diagnostics). Primary antibodies were used at final concentrations of 0.05 μg/ml. Labeling was visualized with the use of a peroxidase-conjugated secondary antibody (P448 and

Fig. 1. Structures of demeclocycline (DM), minocycline (Mi), and tetracycline (TC).
Bonferroni correction was applied. Gaussian distribution were compared. For multiple comparisons, the significant. Data are presented as means and camera settings for different groups.

Picrosirius red staining for collagen. Sections were deparaffinized overnight in xylene and rehydrated in graded ethanol solutions. Staining was performed using a commercial picrosirius red staining kit according to the manufacturer’s protocol (Polysciences). Sections were subsequently dehydrated in graded ethanol, cleared in xylene, and mounted. Qualitative assessment of collagen staining was performed using a Leica DMRE microscope with identical microscope and camera settings for different groups.

Statistics. Student’s unpaired t-test was used when two groups with Gaussian distribution were compared. For multiple comparisons, the Bonferroni correction was applied. P values of <0.05 were considered significant. Data are presented as means ± SE unless indicated otherwise.

RESULTS

Effect of tetracycline antibiotics on AQP2 abundance in vitro. The structures of the tetracycline antibiotics demeclocycline, minocycline, and tetracycline are shown in Fig. 1. To study the effect of demeclocycline on AQP2 abundance, mpkCCD cells were grown for 8 days, with the last 4 days in the presence of 1 nM dDAVP to induce maximal endogenous AQP2 expression (37). During the last 24 h, cells were exposed to different concentrations of demeclocycline. Subsequent immunoblot analysis revealed that demeclocycline decreased AQP2 abundance in mpkCCD cells, with significant effects at 50 μM (Fig. 2A). Transepithelial resistance was relatively constant at 2–3 kΩ between 0 and 50 μM demeclocycline but was significantly decreased at 100 μM (Fig. 2B). The electrical potential difference, which is dependent on the epithelial Na+ channel (EnaC) in mpkCCD cells (3, 32), was significantly decreased by 50 and 100 μM demeclocycline, the same concentrations that affect AQP2 expression (Fig. 2C).

To investigate whether the related compounds minocycline and tetracycline also reduce AQP2 abundance, mpkCCD cells were exposed to different concentrations of these compounds during the last 24 h. Minocycline also decreased AQP2, with effects setting in at 25 μM, whereas transcellular resistance was not decreased (Fig. 2D). Tetracycline did not affect transepithelial...
resistance and displayed a nonsignificant \((P = 0.15)\) tendency to decrease AQP2 expression at 100 \(\mu M\) only (Fig. 2E).

As demeclocycline is clinically used to treat SIADH, we used 50 \(\mu M\) demeclocycline concentrations in all following experiments. To investigate the timeframe for AQP2 downregulation, cells were incubated for different periods with demeclocycline. Demeclocycline significantly reduced AQP2 abundance after 8 h of incubation. This was not due to loss of cells, as Coomassie brilliant blue staining confirmed equal loading of protein (Fig. 2F).

**Effect of demeclocycline on AQP2 transcription.** The effect of demeclocycline on AQP2 abundance could be caused by a decrease in AQP2 protein/mRNA synthesis and/or an increase in AQP2 degradation. To investigate the involvement of protein degradation, cells were incubated with demeclocycline in the presence or absence of the protein synthesis inhibitor cycloheximide. With cycloheximide, demeclocycline did not affect AQP2 abundance, whereas demeclocycline still decreased AQP2 abundance in control cells (Fig. 3A). These data indicated that demeclocycline does not increase AQP2 degradation but affects AQP2 transcription or RNA stability.

To further investigate whether demeclocycline reduces AQP2 transcription, pooled colonies of mpkCCD cells stably transfected with a construct of 3.0-kb AQP2 promoter-luciferase cDNA (pGL3-AQP2–3.0-luc) were used. As previously reported (34), dDAVP induced a twofold increase in luciferase activity (Fig. 3B). With demeclocycline, however, luciferase activity was reduced, indicating that demeclocycline affects AQP2 transcription.

**Effect of demeclocycline on cAMP levels.** AVP-induced increases in cAMP levels, via V2R, stimulate AQP2 gene transcription. Thus, we investigated whether demeclocycline affects dDAVP-induced cAMP levels. Cells were incubated with dDAVP, the nonselective phosphodiesterase inhibitor IBMX, and with or without demeclocycline, as described above. Whereas dDAVP induced a threefold increase in the amount of cAMP compared with unstimulated control cells, coincubation with demeclocycline led to significantly reduced cAMP levels in both unstimulated cells and dDAVP-stimulated cells (Fig. 4).

**Effect of demeclocycline on ACs.** cAMP is produced by ACs and activated by AVP-coupled V2R. To test whether the...
decrease in cAMP levels with demeclocycline could be due to reduced expression of V2R at the plasma membrane, we analyzed the effect of demeclocycline on the localization of V2R-GFP in stably transfected mpkCCD cells. As previously shown for Madin-Darby canine kidney cells (56), dDAVP caused internalization of V2R-GFP in mpkCCD cells, which was unchanged with the addition of demeclocycline (Fig. 5). To determine whether the decreased cAMP levels are due to effects of demeclocycline on AC, mpkCCD-AQP2–3.0-luc cells were incubated with or without demeclocycline in the presence/absence of dDAVP for 4 days or the AC activator forskolin for 1 day before being harvested. As anticipated, both dDAVP and forskolin significantly increased AQP2 promoter-driven luciferase activity compared with controls (Fig. 6). Cells exposed to demeclocycline still exhibited increased luciferase activity with forskolin or dDAVP compared with unstimulated control cells, but this was significantly less compared with dDAVP/forskolin application in the absence of demeclocycline. These results indicated that demeclocycline directly affects AC activity.

AC3 and AC6 have been shown to be expressed in collecting duct principal cells, and knockdown of AC3 and AC6 has been shown to reduce AVP-stimulated cAMP generation in primary cultured mouse inner medullary collecting duct cells (23, 25, 68). To test whether demeclocycline affects their expression, mpkCCD cells were incubated with dDAVP as described above and treated with demeclocycline for different periods. Subsequent immunoblot analysis revealed a reduction of AC3 abundance to <40% of control levels after 24 h, with reduced abundance initiating at 8 h of demeclocycline incubation (Fig. 7A). Demeclocycline had similar effects on AC5/6, with levels reduced to <25% of control levels after 24 h (Fig. 7B).

ACS/6 expression was already significantly decreased after 8 h, in line with the decrease in AQP2 seen at this time point. Note that the antibody used recognizes both AC5 and AC6 and that, for quantification, samples were treated with PNGase-F to remove glycosylation of AC5/6.

**Effect of demeclocycline on AQP2 localization.** As AVP-induced cAMP levels normally lead to a redistribution of AQP2

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**Fig. 5.** Demeclocycline does not affect vasopressin type 2 receptor (V2R) localization. MpkCCD-V2R-GFP cells were grown to confluence and incubated without (−) or with 1 nM dDAVP for the last 4 days and for the last 24 h in the absence or presence of 50 μM demeclocycline. Cells were fixed in 3% parafomaldehyde (PFA) and mounted, and green fluorescent protein (GFP) expression was subsequently analyzed by confocal microscopy. Cross-sectional confocal images are shown. Three independent experiments were performed, and data from a representative experiment are shown.

**Fig. 6.** Demeclocycline decreases AQP2 transcription independent of V2R signaling in mpkCCD cells. MpkCCD cells containing a 3.0-kb AQP2 promoter-luciferase construct were incubated without (−) or with 1 nM dDAVP for 4 days or 10 μM forskolin (Fors) for 24 h and for the last 24 h in the absence or presence of 50 μM demeclocycline. Cells were lysed, and light emission was measured. Data are given as means ± SE of three samples. *Significant difference (P < 0.05). Three independent experiments were performed, and data from a representative experiment are shown.
from intracellular vesicles to the apical plasma membrane, the effect of demeclocycline on intracellular AQP2 localization was investigated by immunocytochemistry. As shown in Fig. 8, cells treated with dDAVP for 4 days showed predominantly apical AQP2 expression. Cells incubated with demeclocycline during the last 24 h showed a lower AQP2 abundance. The remaining AQP2 was spread throughout the cell and showed clearly less apical expression than cells treated with dDAVP alone. This indicates that demeclocycline also affects AQP2 localization.

**Effect of cAMP addition on demeclocycline-induced AQP2 downregulation.** As our data revealed that demeclocycline reduced AC abundance and cAMP levels, we investigated if the addition of high levels of cell-permeable extracellular cAMP could prevent the effect of demeclocycline on AQP2. For this, cells were incubated during the last 24 h with demeclocycline or 8-Br-cAMP only or together. Cells were analyzed by immunocytochemistry and immunoblot analysis (Fig. 9). As typically found in unstimulated control cells, a patchy distribution of high and low levels of AQP2 expression was observed, with AQP2 predominantly localized inside the cell. The addition of 8-Br-cAMP yielded higher abundance of evenly distributed AQP2, which was located predominantly in the apical membrane. Consistent with a reduced expression with demeclocycline, cells treated with demeclocycline revealed diminished AQP2 levels and a speckled intracellular AQP2 staining pattern. In cells treated with demeclocycline in combination with 8-Br-cAMP, AQP2 showed an apical expression of AQP2 at a clearly higher labeling intensity than demeclocycline alone, although there seemed to be a small decrease in AQP2 abundance compared with 8-Br-cAMP and more cells showing punctuate AQP2 labeling. These data indicated that 8-Br-cAMP can, at least partly, overcome the effects of demeclocycline on AQP2 localization and expression levels.

Immunoblot analysis was used to study the effect on AQP2 abundance semiquantitatively (Fig. 9B). Demeclocycline significantly decreased AQP2 abundance in control cells. The addition of 8-Br-cAMP increased the total AQP2 abundance sixfold compared with control cells. The addition of 8-Br-cAMP to cells treated with demeclocycline yielded an AQP2 abundance that was significantly higher than of cells treated with demeclocycline only, but these levels were not greater than AQP2 levels under control conditions and were significantly lower than in cells treated with 8-Br-cAMP alone.

**Effect of demeclocycline on hyponatremia in rats.** To determine the physiological relevance of our in vitro findings, we investigated the mechanism of action of demeclocycline in vivo. This was done by feeding rats a liquid diet and infusing them with dDAVP for 8 days to induce water retention and dilutional hyponatremia. Rats were split into demeclocycline or saline, respectively. Treatment with demeclocycline resulted in a significant reduction of hyponatremia and a nearly significant correction of the hypoosmolality \( (P = 0.08) \). Demeclocycline significantly increased urine volume, decreased urine osmolality, and caused a significantly increased fractional excretion of water. In addition, demeclocycline treatment resulted in increased fractional Na\(^+\) excretion. Blood K\(^+\) levels were significantly increased, but
Fig. 9. Effect of cAMP and demeclocycline on AQP2 localization and abundance. MpkCCD cells were grown for 8 days and for the last 24 h in the absence or presence of 50 μM demeclocycline or 100 μM 8-Br-cAMP (cAMP) only or together. Next, cells were fixed in 3% PFA and subjected to AQP2 immunocytochemistry (A) or lysed and subjected to AQP2 immunoblot analysis followed by semiquantification. A: top view confocal images and corresponding cross-sections. B: mean values ± SE of three samples of AQP2 abundance relative to control cells. *Significant difference (P < 0.05). Molecular masses (in kDa) are indicated on the left. Three independent experiments were performed, and data from a representative experiment are shown.

Table 1. Summary of metabolic parameters

<table>
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<th>Liquid Diet + dDAVP</th>
<th>Liquid Diet + dDAVP + Demeclocycline</th>
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<tbody>
<tr>
<td>Number of rats</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Drinking water, ml·kg⁻¹·24 h⁻¹</td>
<td>26.9 ± 1.4</td>
<td>57.1 ± 11.9*</td>
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<tr>
<td>Urine volume, ml·kg⁻¹·24 h⁻¹</td>
<td>44.8 ± 9.3</td>
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<td>Urine osmolality, mosM/kg</td>
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<td>820 ± 148*</td>
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<td>Osmolar excretion, mosM/24 h</td>
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<td>Serum osmolality, mosM/kg</td>
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<td>272 ± 15</td>
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<td>Serum Na⁺, mmol/l</td>
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<td>Serum K⁺, mmol/l</td>
<td>3.67 ± 0.29</td>
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<td>Serum creatinine, μmol/l</td>
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<td>Liquid diet consumption, g·kg⁻¹·24 h⁻¹</td>
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<td>199 ± 27</td>
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<td>Urine Na⁺, mmol/l</td>
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<td>Urine K⁺, mmol/l</td>
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<td>Urine creatinine, mmol/l</td>
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<td>Total Na⁺ excretion, mmol</td>
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<td>Total phosphate excretion, mg</td>
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<td>Total urea excretion, mmol</td>
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<td>Fractional excretion of Na⁺</td>
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<td>Fractional excretion of K⁺</td>
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<td>Fractional excretion of water</td>
<td>0.53 ± 0.14</td>
<td>2.07 ± 0.44†</td>
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Values are means ± SE. dDAVP, deamino-8-D-arginine vasopressin. *P < 0.05, demeclocycline-treated rats compared with control rats; †P < 0.01, demeclocycline-treated rats compared with control rats.
fractional K⁺ excretion was not changed. Total osmolar excretion and urinary urea and phosphate excretion were similar between both groups.

Effect of demeclocycline on AQP2 and AC abundance in vivo. Immunoblot analysis revealed a significant 75% reduction in AQP2 abundance in the inner medulla of rats treated with demeclocycline compared with control rats (Fig. 10A). However, demeclocycline treatment led to significant 2.5- and 2.0-fold increases in AQP2 abundance in the outer medulla and cortex, respectively (Fig. 10, B and C). In kidney sections from control rats, immunostaining with a total AQP2 antibody demonstrated clear labeling of the apical and basolateral plasma membrane of inner medullary collecting duct cells (Fig. 11, A and C). In comparison, in kidney sections from demeclocycline-treated rats, AQP2 labeling intensity was clearly reduced (Fig. 11B). In addition, the cellular distribution of AQP2 was different in demeclocycline-treated rats; AQP2 was less prominent at the apical and basolateral plasma membrane, and greater numbers of AQP2-positive intracellular vesicles were observed (Fig. 11D). The effect of demeclocycline was, however, not uniform, and, in some tubules, apical labeling was still visible. Between the groups, no observable differences in total AQP2 labeling intensity or distribution were observed in the cortical and outer medullary collecting ducts (data not shown). Immunohistochemistry using an antibody against pS256 AQP2 showed a large decrease in labeling intensity in demeclocycline-treated rats compared with control rats (Fig. 11, E and F, for the inner medulla).

Immunoblot analysis revealed that AC5/6 abundance was 50% reduced in the inner medulla of demeclocycline-treated rats (Fig. 12A). In the outer medulla, AC5/6 expression was increased (Fig. 12B), whereas it was undetectable in cortex samples (data not shown).

Demeclocycline and nephrotoxicity. As tetracyclines are nephrotoxic at high concentrations and the window at which demeclocycline reduced AQP2 abundance in mpkCCD cells without affecting epithelial integrity was small, we also tested whether demeclocycline showed nephrotoxic effects in vivo. Immunohistochemistry, however, revealed no clear morphological differences between kidneys from demeclocycline-treated and control rats (not shown). Moreover, neither picrosirius red staining (Fig. 13), which labels collagen that is increased in expression with toxicity and fibrosis (52), nor inducible nitric oxide synthase staining (not shown), which is indicative for inflammation (6), differed between kidneys of control and demeclocycline-treated rats.

To analyze possible toxicity effects in more detail and whether the effects of demeclocycline on AQP2 and AC abundance were specific, kidneys were also immunolabeled for AQP4 (Fig. 14, A and B) and β-catenin (Fig. 14, C and D). In both groups, however, a similar protein abundance and distribution of AQP4 and β-catenin were observed. In agreement, immunoblot analysis of the inner (Fig. 14E) and outer medullary (Fig. 14F) segments revealed similar abundances for AQP4 in both groups. Altogether, these data indicate that the effect of demeclocycline in the renal inner medulla is specific for AQP2 and AC5/6 and not secondary to a toxicity effect of demeclocycline.

DISCUSSION

Demeclocycline and minocycline reduce AQP2 abundance in mpkCCD cells. In this study, we show that the tetracycline antibiotic demeclocycline downregulates the AVP-regulated water channel AQP2 in mpkCCD cells. In addition, demeclocycline affects AQP2 localization, reducing its expression at the apical plasma membrane. Besides demeclocycline, minocycline also decreased AQP2 abundance, whereas only a small effect of tetracycline on AQP2 abundance was observed.

The decrease in AQP2 abundance explains the aquaretic effect of tetracycline antibiotics, which have previously been shown to affect water reabsorption in the distal part of the nephron and the toad urinary bladder (15, 24, 66, 77). Demeclocycline downregulated AQP2 in mpkCCD cells at concentrations that were in line with those measured in urine from
patients (14), indicating that the effects observed are at pharmacologically relevant doses. At a demeclocycline concentration of 50 μM, resistance measurements and Coomassie staining indicated that there were no decreases in cell viability but still decreased AQP2 abundance. However, the loss of resistance at 100 μM suggests that the cell monolayer is seriously affected by demeclocycline at higher concentrations, which is in line with reports (40, 59) on the risk of nephrotoxicity of demeclocycline. Minocycline reduced AQP2 abundance at similar concentrations but with a less pronounced effect as demeclocycline, which is in agreement with the effect of minocycline on the water flow seen in toad urinary bladders (15). In contrast, at the concentrations tested, tetracycline was not able to significantly affect AQP2 abundance, which corresponds in line with reports (40, 59) on the risk of nephrotoxicity of demeclocycline. Minocycline reduced AQP2 abundance at similar concentrations but with a less pronounced effect as demeclocycline, which is in agreement with the effect of minocycline on the water flow seen in toad urinary bladders (15). In contrast, at the concentrations tested, tetracycline was not able to significantly affect AQP2 abundance, which corresponds
Reduced AC activity partially explains the diminished AQP2 abundance by demeclocycline in mpkCCD cells. Our results reveal that the reduced AQP2 abundance is independent of any effect on V2R, as demeclocycline did not visibly affect the localization of V2R-GFP in mpkCCD cells in the presence or absence of dDAVP. Moreover, the effect of demeclocycline was also observed when cells were incubated with a direct activator of AC, forskolin, thereby bypassing regulation by V2R (Fig. 6). This AVP-independent effect is in line with the study of Horattas et al. (26), who showed that in patients undergoing surgery, plasma AVP concentrations were increased with demeclocycline treatment, despite the observed decrease in their urinary concentrating ability. The effect of demeclocycline was abolished upon cotreatment with cycloheximide (Fig. 3A), implying that demeclocycline does not decrease existing AQP2. Instead, our data show that demeclocycline directly acts on ACs, as it reduced the abundance of AC3 and AC5/6 (Fig. 7) and fully blocked basal and dDAVP-induced cAMP generation (Fig. 4). As AQP2 transcription is cAMP dependent (39, 78), the observed reduced transcription from the AQP2 promoter-luciferase reporter construct is in line with the affected cAMP levels.

Although the addition of 8-Br-cAMP to demeclocycline-treated cells induced AQP2 translocation to the apical membrane, as anticipated, and increased the AQP2 abundance to control levels, the AQP2 abundance was still significantly lower than in cells treated with 8-Br-cAMP only. These data suggest that demeclocycline may also affect AQP2 abundance at a post-AC level in mpkCCD cells. It remains to be established whether this post-AC effect also exists in vivo. Recently, a collecting duct-specific AC6 knockout mice has been developed, which displayed a urinary concentrating defect and lower AQP2 levels (58). Future studies using these mice might further clarify the role of AC6 and potential post-AC targets in the mechanism of demeclocycline-induced diuresis.

Demeclocycline reduces hyponatremia in vivo. In agreement with results of others (30, 44, 45, 60, 73), our liquid diet/dDAVP-treated control rats developed all the characteristics of SIADH. Due to increased AVP-mediated renal water uptake, they were hyponatremic (99 ± 1 mM) and hypoosmotic (236 ± 10 mosM), as untreated control rats held under similar conditions displayed plasma Na⁺ concentrations and osmolalities of 141 mmol/l and 283 mosM, respectively (32). The severity of hyponatremia observed in our model is similar to that of other hyponatremic rat models, which have reported serum Na⁺ concentrations between 100 and 115 mmol/l (30,
In line with the beneficial effects of demeclocycline on hyponatremic patients (17, 51), demeclocycline increased the fractional excretion of water, resulting in increased urine volume, decreased urine osmolality, and attenuated hyponatremia.

In our rats, we observed a relatively high water intake compared with their urine production. For rats, insensible water losses of ~40–50 ml·kg⁻¹·day⁻¹ have been reported (4, 55), whereas these were 75 ± 14 and 115 ± 22 ml·kg⁻¹·day⁻¹ for control and demeclocycline-treated rats, respectively, in our experiment. We believe that the large discrepancy between consumed liquid and produced urine is likely due to technical difficulties and an overestimation of the consumed volume, because, in the cages, some gelled food was smeared out.

**Attenuated hyponatremia by demeclocycline is associated with decreased AC5/6 and AQP2 expression in the renal inner medulla.** In line with our in vitro data, demeclocycline decreased AC5/6 and, consequently, total and pS256 AQP2 abundance in the inner medulla of our demeclocycline-treated rats. As this segment defines the final urine osmolality, our data indicate that the reduced AC5/6 and AQP2 abundance explains the therapeutic effect of demeclocycline in hyponatremia. Despite clinical reports of nephrotoxicity (49, 51, 76), we did not observe any difference in renal morphology, abundance of non-cAMP-regulated proteins, and markers indicative for fibrosis and inflammation between the two groups, indicating that in our experiments, demeclocycline specifically affected AC5/6 and AQP2 in the inner medulla. As our in vitro data indicated that a doubling of the demeclocycline concentration affected the integrity of the cellular monolayer, longer treatment periods or more variable demeclocycline concentrations due to differences in urine concentrations in humans may explain the nephrotoxicity observed in humans.

Interestingly, demeclocycline treatment led to increased AQP2 and AC5/6 abundance in the outer medulla and cortex.

**Fig. 14.** Demeclocycline does not affect either the expression or localization of AQP4 or β-catenin in hyponatremic rats. Rats were treated as described in Fig. 6 with (B and D) or without (A and C) demeclocycline. One kidney was processed for sectioning and incubated with affinity-purified antibodies against AQP4 (A and B) and β-catenin (C and D). The other kidney was dissected into the inner medulla (E), outer medulla (F), and cortex (no signal observed; data not shown), lysed, and subjected to AQP4 immunoblot analysis (top). Mean values ± SE of AQP4 abundance are given relative to control (bottom). The insets in A and B show higher-magnification images. *Significant differences (P < 0.05) from control.
Although it cannot be excluded that demeclocycline may only be able to enter inner medullary principal cells, the most likely explanation for the reduced AC5/6 and AQP2 abundance in the inner medulla only is that, due to fact that urine becomes more concentrated from the cortex to the inner medulla, demeclocycline concentrations will be the highest in this renal segment. In line with this hypothesis, urinary phosphate excretion, which is induced by increased cAMP levels via ACs in the proximal tubule (43), was not increased with demeclocycline.

Previously, it has been shown that in SIADH and SIADH models, free water excretion is higher and AQP2 abundance is lower than anticipated, considering the high blood AVP levels, a phenomenon called “vasopressin escape” (13). Vasopressin escape indicated that variables other than AVP, such as hypervolemia or hypotonicity, influence AQP2 expression (7, 22, 34, 60). Based on this data, it is to be expected that some vasopressin escape is present in our animals, meaning that AQP2 levels would have likely been higher without this escape. However, these effects are expected to be similar in both groups. As demeclocycline treatment, the only difference between the groups, induced aquaresis by acting on the inner medulla, the elevated AQP2 abundances observed in the cortex and outer medulla can best be interpreted as an attenuation of the vasopressin escape adaptation.

Effects of demeclocycline on Na⁺ excretion. The Na⁺ excretion of 0.23 mmol/day, as observed in our control SIADH group, is low but is similar to that previously reported in a rat model of SIADH (67). The low Na⁺ excretion can be explained by the molecular effects of dDAVP, as vasopressin increases Na⁺ reabsorption via Na⁺-K⁺-Cl⁻ cotransporter 2 (NKCC2), Na⁺-Cl⁻ cotransporter (NCC), and ENaC, either by increasing their abundance, activating phosphorylation, or increasing their membrane expression (5, 12, 20, 31, 50). In addition, it has been shown that in rat models of SIADH, renal ENaC and NCC abundances and plasma and urinary levels of aldosterone levels were increased compared with rats treated with dDAVP only (67). The increased aldosterone synthesis might be a consequence of the hyponatremia in these rats and could lead to the increased NCC and ENaC abundance (38, 53, 62).

In our demeclocycline-treated rats, we observed a doubling of the Na⁺ excretion. Although not delineated in detail, demeclocycline has been reported to increase Na⁺ excretion in some patients (40). At present, the underlying mechanism is unclear. As demeclocycline reduces the renal reabsorption of water and consequently diminishes the hyponatremia in SIADH rats, it is likely to attenuate the aldosterone response and, hence, renal Na⁺ reabsorption. In addition, the observed natriuresis may also be due to a direct effect of demeclocycline on ENaC, as this transporter is regulated by AC6 (57). Indeed, treatment with 50 and 100 μM demeclocycline, which affected AQP2 and AC abundances, also significantly decreased the transcellular potential difference in mpkCCD cells (Fig. 2C), which is an ENaC-dependent process in these cells (3, 32). Demeclocycline might affect other AC-regulated transporters as well, such as NKCC2 and NCC (54); however, as urinary phosphate excretion, which is induced by AC-generated cAMP in the proximal tubule (43), was not increased with demeclocycline, this suggests that demeclocycline does not affect AC-regulated transport in general.

In conclusion, our in vitro and in vivo data show that the aquaretic effect of tetracycline antibiotics such as demeclocycline and minocycline are mediated via downregulation of the AVP-regulated water channel AQP2. Demeclocycline decreases AQP2 gene transcription by decreasing AC5/6 expression and cAMP generation and possibly by affecting a post-AC target, resulting in less AQP2 protein expression in the inner medulla. This results in increased water loss and attenuates the hyponatremia in SIADH.

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AUTHOR CONTRIBUTIONS

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