Treatment with the vascular disrupting agent combretastatin is associated with impaired AQP2 trafficking and increased urine output

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Submitted 11 October 2011; accepted in final form 27 April 2012

CA4P is believed to mediate its action primarily in tumors because tumor vessels are chaotic and more fragile than normal vessels (46). However, CA4P treatment has been associated with side effects such as anemia, dyspnoea, hypokalemia, headache, and transient sensory neuropathy (6, 40).

We have previously shown that CA4P treatment results in a significant increase in hematocrit, hemoglobin concentration, and in mean arterial blood pressure in nontumor bearing mice (4). On the basis of these results, we hypothesize that water leaves the circulation after CA4P treatment and that this water escape is due either to accumulation in other tissues or excretion through the kidneys.

Using nontumor bearing Munich-Wistar rats, we studied the effect of CA4P on whole body water handling by MRI, as well as renal water and salt handling. Final regulation of the water content in urine takes place in the kidney collecting duct. In the collecting duct, vasopressin in the principal cells binds to the V2 receptor (V2R), and AQP2 is then translocated from intracellular vesicles to the apical plasma membrane, thereby increasing osmotic water permeability (30). Moreover, transcriptional regulation of AQP2 in vivo is thought to be a result of a vasopressin-induced increase in intracellular cAMP levels (19), which is capable of stimulating AQP2 gene transcription in cultured cells by acting through cAMP response element and AP-1 sites in the AQP2 promoter (19, 24, 47). During short-term regulation cAMP activates PKA, which, in turn, phosphorylates intracellularly located AQP2 at serine 256. The phosphorylated AQP2 is then transported to the apical membrane (AQP2 trafficking), probably via microtubules. Incorporation of pS256-AQP2 into the apical membrane facilitates water reabsorption (12, 21, 30). In the present study, the aims were to examine 1) whether CA4P is associated with accumulation of water in the brain, muscle, and kidney tissue using MRI, 2) whether CA4P is associated with changes in the renal water and salt balance, as well as in the expression, localization, and regulation of renal AQP2, and 3) whether CA4P affects microtubule cytoskeleton in collecting duct principal cells.

MATERIALS AND METHODS

Drugs. CA4P was supplied by OXiGENE, (Waltham, MA). The drug was prepared fresh before each experiment, by dissolving in saline, and then kept cold and protected from light until used. In all experiments, CA4P was used in the dose of 30 mg/kg body wt (CA4P groups), and animals in the control group were given saline. Both CA4P and saline were injected intraperitoneally as a bolus injection.

CA4P affects microtubule cytoskeleton in collecting duct principal cells.
The dose of CA4P is considered to be a clinically relevant dose and was chosen according to previous studies (13, 35, 48).

**Animals.** All procedures conformed with the Danish national guidelines for the care and handling of animals and to the published guidelines from the National Institutes of Health. The animal protocols were approved by the board of the Institute of Clinical Medicine, University of Aarhus, according to the licenses for use of experimental animals issued by the Danish Ministry of Justice.

Studies were performed in male Munich-Wistar rats (Møllegaard Breeding Centre, Eiby, Denmark) weighing 250–285 g. The rats were kept in cages with a maximum of three rats in each cage and at a 12:12-h light-dark cycle, a temperature of 21 ± 2°C and a humidity of 55 ± 2%. They had free access to a standard rodent diet (Altromin, Lage, Germany) and tap water during the experiments. The rats were allocated into eight sets of experiments, according to Table 1.

### Table 1. Overview of experimental groups used in the study

<table>
<thead>
<tr>
<th>Experimental Subgroup</th>
<th>Treatment</th>
<th>No. of Animals</th>
<th>Procedures Performed</th>
<th>Acquired Parameters</th>
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<tr>
<td><strong>Experiment 1</strong></td>
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<tr>
<td>a</td>
<td>CA4P - 0 h</td>
<td>4</td>
<td>Five control groups and five CA4P groups were included; after administration of CA4P or saline, a blood sample was withdrawn from the femoral artery either at 0, 1, 3, 6, or 8 h under anesthesia with sevoflurane.</td>
<td>Hemoglobin and blood-gas parameters</td>
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<td>b</td>
<td>CA4P - 1 h</td>
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<td>c</td>
<td>CA4P - 3 h</td>
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<td>d</td>
<td>CA4P - 6 h</td>
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<td>e</td>
<td>CA4P - 8 h</td>
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<td>f</td>
<td>Saline - 0 h</td>
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<td>j</td>
<td>Saline - 8 h</td>
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<td><strong>Experiment 2</strong></td>
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<td>Hemoglobin</td>
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<td>a</td>
<td>CA4P</td>
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<td>All rats were bilaterally nephrectomized under sevoflurane anesthesia. After the operation, CA4P or saline was given, and rats were maintained anesthetized for 1 h, after which a blood sample was acquired as in experiment 1.</td>
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<tr>
<td>b</td>
<td>Saline</td>
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<td><strong>Experiment 3</strong></td>
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<td>Percentage water content in muscle, brain, and kidney</td>
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<td>a</td>
<td>CA4P</td>
<td>8</td>
<td>Rats anesthetized using isoflurane were placed in a standard imaging wrist coil of a Horizon Echospeed LX 1.5 Tesla clinical MR scanner. Two scans were performed to determine baseline water content in the tissues of interest. Either CA4P or saline was given, and the animal underwent five additional scans every hour in the following 5 h.</td>
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<td>b</td>
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<td><strong>Experiment 4</strong></td>
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<td>Urinary and plasma sodium, potassium, creatinine, urea, osmolality and urinary NGAL, PGE2, and aldosterone</td>
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<td>a</td>
<td>CA4P</td>
<td>6</td>
<td>Rats were kept in individual metabolic cages. The rats were left in the cage for 24 h to acclimatize. After exactly 24 h, the rats were given CA4P or saline. Urine was collected 1, 2, and 3 h after injection. Three hours after injection, the rats were placed under anesthesia with isoflurane, and a blood sample was withdrawn from the carotid artery.</td>
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<td><strong>Experiment 5</strong></td>
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<td>AQP2, V2R, NHE3, NKCC2, ENaCα, ENaCβ and ENaCγ mRNA level and AQP2, pAQP2, and β-actin protein level</td>
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<tr>
<td>a</td>
<td>CA4P - 1 h</td>
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<td>Either 1 or 3 h after CA4P or saline injection, rats were anesthetized using isoflurane, and kidneys were exsanguinated and divided into cortex, inner stripe of outer medulla, and inner medulla.</td>
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<td>b</td>
<td>CA4P - 3 h</td>
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<td>c</td>
<td>Saline - 1 h</td>
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<td><strong>Experiment 6</strong></td>
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<td>AQP2 and pAQP2 expression in histological slides</td>
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<td>a</td>
<td>CA4P - 1 h</td>
<td>4</td>
<td>Rats were given either CA4P or saline. One or three hours after treatment, animals were sevoflurane-anesthetized, and kidneys were fixed by retrograde perfusion fixation via the abdominal aorta.</td>
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<tr>
<td>b</td>
<td>CA4P - 3 h</td>
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<td>c</td>
<td>Saline - 1 h</td>
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<td>d</td>
<td>Saline - 3 h</td>
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<td><strong>Experiment 7</strong></td>
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<td>Cyclic AMP and medullary osmolality</td>
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<tr>
<td>a</td>
<td>CA4P - 1 h</td>
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<td>One hour after CA4P or saline injection, rats were anesthetized using sevoflurane, kidneys were exanguinated, and inner medulla was used for analysis.</td>
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<td>b</td>
<td>Saline - 1 h</td>
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<td><strong>Experiment 8</strong></td>
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<td>Blood pressure</td>
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<td>a</td>
<td>CA4P - 1 h</td>
<td>6</td>
<td>Two control measurements (baseline corresponding to −2 h and −1 h time-points) were done prior to injection. Then, measurements after injection of either CA4P or saline were made and repeated after 1, 2, 4, 5, and 6 h.</td>
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<tr>
<td>b</td>
<td>Saline - 1 h</td>
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Magnetic resonance imaging. The water content in brain, muscle, and renal tissue was determined by MRI as outlined in Table 1 and as previously described in detail (9). In brief, rats were anesthetized and placed in a standard imaging wrist coil of a Horizon Echospeed LX 1.5 Tesla clinical MR scanner (Signa SR120) with TR/TE of 3000 ms/10ms, TI = 200, 400, 600, 800, 1000, 1200, and 1400 ms. From the mean intensity of these inversion recovery images, a pure T1 image was calculated using a fitting procedure described elsewhere (10). From this T1 map, the total tissue water fraction (fw = g water/g tissue) was calculated as 1/fw = A + B/T1 (9, 10). The values of the constants A and B were determined using gelatin standards of different water contents (68-90) (10). The rats were kept anesthetized during the entire experiment. Two scans were performed to determine baseline water content in the tissues of interest. After these two scans, either CA4P or saline was injected and the animal underwent an additional scan every hour in the following 5 h. All measurements following the injection were normalized to the mean of the two measurements prior to injection.

Urine collection. Rats were kept in individual metabolic cages with free access to a pulverized standard rodent diet and tap water during the experiment. The rats were left in the cage for 24 h to acclimatize. After exactly 24 h, the rats were treated with CA4P or saline intraperitoneally. Urine was collected, 1, 2, and 3 h after injection. Three hours after injection, the rats were anesthetized, and a blood sample was withdrawn from the carotid artery.

Analysis of urine and blood. Blood and urine were collected as described above and as outlined in Table 1. Blood hemoglobin was determined using an ABL625 (Radiometer, Copenhagen, Denmark). The concentrations of urinary sodium and potassium were determined by standard flame photometry (Eppendorf FC6341, Eppendorf-Netheler-Hinz, Hamburg, Germany). The plasma and urinary concentrations of creatinine and urea and the plasma concentrations of sodium and potassium were determined using a Vitros 950 analyzer (Johnson & Johnson Clinical Diagnostics, Rochester, NY). The urine and plasma osmolality was measured with a vapor pressure osmometer (Osmatot 030, Gonotec, Berlin, Germany). The urinary concentration of neutrophil gelatinase-associated lipocalin (NGAL) was analyzed using a commercially available Rat NGAL ELISA kit (kit 046, BioPorto Diagnostics, Gentofte, Denmark) in accordance with the instructions given by the manufacturer. Urinary PGE2 and aldosterone excretion were measured using commercially available ELISA kits and aldosterone enzyme immunoassay Kit, (both kits from Cayman Chemical, Ann Arbor, MI).

Blood pressure measurements. Systemic blood pressure and heart rate were measured by determining the tail blood volume with a volume pressure recording (VPR) sensor (CODA System, Kent Scientific, Torrington, CT, USA). The VPR sensor has a specially designed differential pressure transducer that measures the systolic and diastolic blood pressure by determining the blood volume in the tail. The measurements were performed automatically without operator intervention. In accordance with manufacturer’s recommendation, rats were trained for the blood pressure measurement procedure during 3 days with 48-h interval prior to the experiment. At the day of the experiment, two control measurements (baseline corresponding to 0 h and −1-h time points) were done prior to injection. Then measurements after injection (time delay ~10 min) were made and repeated after 1, 2, 4, 5, and 6 h.

Cell culture. Mouse collecting duct (mpkCCDc14) cells were essentially grown as described previously (15). Cells were used between passages 22 and 28. During normal growth, cells were incubated at 37°C, in a humidified atmosphere containing 5% CO2. For experiments, mpkCCD cells were grown to confluence and treated with 37°C, in a humidified atmosphere containing 5% CO2. For experiments, mpkCCD cells were grown to confluence and treated with

Preparation of polymer tubulin fractions from intact mpkCCD cells. For polymer fraction preparations, cells were washed twice in PBS and extracted for 3 min with 10 ml lysis buffer: 80 mM Pipes-KOH, pH 6.8, 1 mM MgCl2, 1 mM EGTA, 0.2% Triton X-100, and 10% glycerol-containing protease inhibitor cocktail. After washing briefly with lysis buffer, 2.5 ml of 1% SDS in water was added, cells were scraped, vigorously sonicated for 1 min, and boiled for 3 min in Laemmli sample buffer containing 2% SDS and analyzed by immunoblotting for β-tubulin. Immunoblotting analysis was performed as described below.

Fig. 1. Mean arterial blood pressure and hemoglobin concentration in rats receiving saline (control) or 30 mg/kg CA4P. Mean arterial blood pressure (A) and hemoglobin (B) at various time points after treatment (n = 3–7 per group). C: hemoglobin concentration 1 h after treatment in bilateral nephrectomized rats (n = 4 per group). Results are shown as mean values ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001.
RNA extraction and quantitative PCR. Rat kidneys were collected as outlined in Table 1. Total renal RNA was isolated using the Machery-Nagel’s NucleoSpin RNA II kit. cDNA synthesis was performed with Fermentas RevertAid First-Strand cDNA Synthesis Kit (MBI Fermentas, Burlington, Ontario, Canada), according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed using 100 ng cDNA as a template for PCR amplification. We used Maxima SYBR Green QPCR Master Mix, according to the manufacturer’s instruction (Stratagene, AH Diagnostics, Aarhus, Denmark).

For qPCR experiments, a standard curve was constructed by plotting threshold cycle (Ct values) against serial dilutions of purified PCR product. Specificity of the product was confirmed by postrun melting point analysis and by gel electrophoresis. The following primer sequences were used: AQP2: sense 5′-CTT CCT TCG AGC TGC CTT C-3′ and antisense 5′-CAT TGT TGT GGA GAG CAT TGA C-3′; β-actin: sense 5′-CTG TGG TGG TGA AGC TGT AG-3′ and antisense 5′-TCA TGC CAT CCT GCG TCT-3′; V2R-COOH: sense 5′-CAG CGT GGG ATC CGG AAG CTC TGG AA-3′ and antisense 5′-TCA GGA GGG TGT ATC CTT CAT CAA AGA GGA-3′; NHE3: sense 5′-ACC GAA GCG GAG GAA TAG CA-3′ and antisense 5′-TAT CAA TTC CTG CCC CAG AG-3′; NKCC2: sense 5′-ACC AAG AAC CTC CCT CCT GT-3′ and antisense 5′-TCG GAC ACC AAG GTA CAA AGA C-3′; ENaC/H9251: sense 5′-CGT CAC TGT CTG CAC CCT TA-3′ and antisense 5′-CAC TGT GAC ACC AAG GTA CAA CA-3′; ENaC/H9252: sense 5′-CTG TGT CTT CCA GCC TGA CA-3′ and antisense 5′-GCA GCC TCA GGG AGT CAT AG-3′; and ENaCγ: sense 5′-CTA CCA GCA ACA CCC CAA CT-3′ and antisense 5′-GCT ACA GGA TTG CTT GCA CA-3′.

Primary antibodies. For semiquantitative immunoblotting and immunohistochemistry, we used previously characterized polyclonal antibodies summarized as follows: AQP2 (H7661) (29) and pS256-AQP2 (KO307), a new antibody raised against the sequence of immunizing peptide [GRRQ(pS)VELHSPC] as the previously characterized antibody (5), as well as anti-β-tubulin monoclonal antibody (Abcam, AH Diagnostics, Aarhus, Denmark) and β-actin antibody (BioVision, AH Diagnostics, Aarhus, Denmark).

Semiquantitative immunoblotting. Rat kidneys were collected as outlined in Table 1. The inner medulla was homogenized in dissecting buffer (41) using an Ultra-Turrax T8 homogenizer (IKA Laborteknik, Staufen, Germany), and the homogenate was centrifuged at 1,500 g for 15 min at 4°C. The total protein concentration in the supernatant was measured using a Pierce BCA protein assay kit (Roche, Hvidovre, Denmark), according to the manufacturer’s instructions. Gel samples were prepared from the supernatant in Laemmli sample buffer containing 2% SDS. Samples were run on 12% polyacrylamide gels (Bio-Rad Protean II, Bio-Rad Laboratories, Copenhagen, Denmark), electroeluted to nitrocellulose membranes and subjected to immunolabeling (34).

Immunohistochemistry and immunofluorescence. As outlined in Table 1, either 1 h or 3 h after treatment (CA4P or saline), rats were
The kidneys were fixed by retrograde perfusion via the abdominal aorta with 3% paraformaldehyde in PBS buffer, pH 7.4. Moreover, the kidneys were immersion fixed for 1 h and washed 3 × 10 min with PBS buffer. The kidney blocks were dehydrated, embedded in paraffin, and cut in 2-μm sections on a rotary microtome (Leica Microsystems, Herlev, Denmark). For immunoperoxidase labeling, the sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 5% H2O2 in absolute methanol for 10 min at room temperature. To expose antigens, kidney sections were boiled in a target retrieval solution (1 mM Tris, pH 9.0, with 0.5 mM EGTA) for 10 min. The sections were cooled and nonspecific binding was prevented by incubating in 50 mM NH4Cl in PBS for 30 min, followed by blocking in PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated with primary antibodies diluted in PBS with 0.1% BSA and 0.3% Triton X-100 overnight at 4°C. After being washed 3 × 10 min with PBS wash buffer, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (P448, goat anti-rabbit immunoglobulin; DAKO, Glostrup, Denmark) for 1 h at room temperature. The sections were rinsed with PBS wash buffer, and the sites of antibody-antigen reactions were visualized using 0.05% 3,3′-diaminobenzidine tetrachloride (DAB; Kem-en Tek, Taastrup, Denmark) dissolved in distilled water with 0.1% H2O2. The light microscopy was carried out with Leica DMRE (Leica Microsystems). Cells were cultured in Lab-Tek chamber slides (Life Technologies, Naerum, Denmark). Cells were fixed in 4% formaldehyde in PBS and permeabilized with 0.1% Triton X-100. After blocking 30 min with 10% goat serum in TBS, cells were incubated with primary antibodies and afterward with FITC secondary antibody (goat anti-rabbit or goat anti-mouse immunoglobulin, both from Jackson ImmunoResearch Laboratories, AH Diagnostics, Aarhus, Denmark). Fluorescence images were taken with an Olympus inverted microscope.

Semiquantitative confocal laser scanning microscopy. Labeling was performed as previously described (26). For the semiquantitative analysis, microscope settings (PMT offset and gain, sampling period, and averaging) were identical for all rats (5 control and 4 experimental animals). The dynamic range was set, so that the sections with the most intense fluorescence signal only had a few saturated pixels. From each rat, six images of inner medullary collecting duct (IMCD) tubule segments labeled with pS256-AQP2 were obtained. Digital images were analyzed using ImageJ software to assess the total pixel intensity of each image in relation to the total area of labeled IMCD tubules, thus providing semiquantitative data of the pS256-AQP2-labeling abundance per IMCD tubule. Data were analyzed using GraphPad Prism Software (San Diego, CA). The apical-to-basolateral pixel distribution was assessed similarly. Multiple regions of interest (12 per image), representing single cross sections through labeled IMCD cells, were selected and the distribution of pixel intensities assessed using ImageJ software. For publication (not analysis), image brightness was linearly adjusted using Photoshop.

Medullary osmolality. Renal inner medulla was collected as outlined in Table 1, and tissue was processed according to a method developed by Schmidt-Nielsen et al. (37) and modified by Fenton et al. (11). Briefly, kidneys were removed, and the inner medulla was frozen in liquid nitrogen, and stored at −80°C. During the analysis, the inner medulla was placed in preweighed tubes. Samples were weighed immediately and subsequently dried over a desiccant at 60°C for 3.5 h (after which the weight remained constant). After reweighing, 200 μl of distilled water was added to each tube, and tubes were capped, boiled in water bath for 5 min, and, after brief centrifugation, they were stored at 4°C for 24 h for diffusion. After centrifugation for 1 min at 8,000 g, supernatant osmolality was determined using a vapor pressure osmometer.

cAMP. One hour after CA4P or saline injection, rats were anesthetized, kidneys were excised, and the renal inner medulla was frozen in liquid nitrogen immediately after collection. The cyclic AMP concen-
Effect of CA4P on mean arterial blood pressure and hemoglobin concentration. The changes in mean arterial blood pressure and hemoglobin concentration in intact rats treated with CA4P or saline (control rats) are shown in Fig. 1, A and B. Mean arterial blood pressure was significantly increased between a half an hour and 4 h after CA4P administration compared with control rats (Fig. 1A). Hemoglobin increased significantly 1–6 h after CA4P treatment compared with control rats. After 8 h of treatment, hemoglobin levels did not differ between CA4P-treated and control rats (Fig. 1A). To examine whether the kidneys play a role, directly or indirectly, in the CA4P-mediated hemoglobin increase, anesthetized rats were subjected to bilateral nephrectomy. Nephrectomized rats were treated with either CA4P or saline and kept anesthetized for 1 h before measurement of the hemoglobin concentration. CA4P treatment was not associated with increased hemoglobin concentration 1 h after treatment in these rats (Fig. 1C).

Tissue water accumulation. Using MRI, we detected no significant changes in the water content in either brain (data not shown), muscle (data not shown), or kidney (Fig. 2) following CA4P treatment. However, MRI showed that there was a tendency to changes in water content in renal inner and outer medulla, where tissue water content changed in inner medulla (Fig. 2C) and to a lesser extent in outer medulla (Fig. 2B) in CA4P-treated rats compared with control rats.

Effect of CA4P on renal water and salt balance in rats. Rats were placed in metabolic cages and were treated with either CA4P or saline. Water intake did not differ between the two groups 3 h after treatment (Fig. 3A). However, 1 h after CA4P treatment, urine output increased significantly (Fig. 3B). The urine-excretion:water-intake ratio after 3 h CA4P treatment is 4.5 for the control group and 4.0 for the CA4P group. We were not able to calculate the ratio after 1 h CA4P treatment because water intake was not detected after such a short time period.

Urinary osmolality was significantly lower in the CA4P-treated rats compared with control rats 2 h after treatment (Fig. 3C), whereas medullary osmolality was unchanged between CA4P-treated and control rats (Fig. 3D).

Urinary sodium excretion increased 1 h after CA4P treatment compared with control rats but did not differ 2 h after CA4P treatment (Table 2). Urinary PGE2 excretion showed a tendency to increase in the CA4P-treated rats compared with control after 1 h, although it was not significant (Table 2). The plasma sodium and potassium concentration was not affected by CA4P treatment (Table 2). Urine potassium, creatinine, and NGAL and aldosterone concentration did not differ 1 h after CA4P treatment compared with controls (Table 2). Furthermore, creatinine clearance was unchanged in response to 3 h CA4P treatment (Table 2).

Effect of CA4P on AQP2 and pS256-AQP2 expression and trafficking in inner medulla. To study the molecular mechanisms involved in the increased urine output in response to CA4P treatment, renal expression of AQP2 and pS256-AQP2 was examined. One hour after CA4P treatment, AQP2 mRNA and protein levels did not change (Fig. 4, A and B). However, immunohistochemistry showed a diffuse distribution of pS256-AQP2 within the principal cells in the inner medulla in the CA4P-treated rats compared with control rats (Fig. 4, C–F).

Expression of AQP2 mRNA levels and protein levels of AQP2 and pS256-AQP2 in kidney inner medulla 3 h after CA4P administration demonstrated significantly increased AQP2 mRNA levels compared with control rats (Fig. 5A). AQP2 protein levels did not change after treatment (Fig. 5B), and no change in distribution of AQP2 was observed (Fig. 5, C–F). However, there was a significant increase in pS256-AQP2 protein levels in inner medulla after CA4P treatment (Fig. 6A). However, no clear change was observed in labeling and distribution of pS256-AQP2 in inner medullary collecting duct principal cells of the CA4P-treated rats compared with control rats after 3 h (Fig. 6, B–E).

To further clarify the changes in pS256-AQP2 distribution 1 and 3 h after CA4P treatment, we did confocal microscopy analysis and quantification of pS256-AQP2 (Fig. 7). These experiments demonstrated a mostly apical distribution of pS256-AQP2 in control conditions and 3 h after CA4P treatment.
AQP2 mRNA and pS256 AQP2 (pAQP2) protein level and immunohistochemical localization in rat renal inner medulla 1 h after receiving saline (control rats) or 30 mg/kg CA4P. A: AQP2 mRNA level relative to β-actin mRNA level 1 h after treatment. B: immunoblotting and band intensity of AQP2 protein relative to β-actin in renal inner medulla 1 h after treatment (n = 6 per group). The bars represent the mean value (n = 6 per group). C–F: representative histological pictures of pS256-AQP2 localization in inner medullary collecting ducts (×25 and ×63 magnification) 1 h after treatment.

Fig. 4. AQP2 mRNA level and pS256 AQP2 (pAQP2) protein level and immunohistochemical localization in rat renal inner medulla 1 h after receiving saline (control rats) or 30 mg/kg CA4P. A: AQP2 mRNA level relative to β-actin mRNA level 1 h after treatment. B: immunoblotting and band intensity of AQP2 protein relative to β-actin in renal inner medulla 1 h after treatment (n = 6 per group). The bars represent the mean value (n = 6 per group). C–F: representative histological pictures of pS256-AQP2 localization in inner medullary collecting ducts (×25 and ×63 magnification) 1 h after treatment.

ment (Fig. 7, A, C, and D), whereas a more diffuse distribution was seen 1 h after CA4P treatment (Fig. 7B). Quantification of the apical-basal fluorescent intensity showed a highly significant decrease in pS256-AQP2 apical distribution 1 h after

Fig. 5. AQP2 mRNA and protein level and immunohistochemical localization of AQP2 in rat renal inner medulla 3 h after receiving saline (control rats) or 30 mg/kg CA4P. A: AQP2 mRNA level relative to β-actin mRNA level in renal inner medulla 3 h after treatment. B: immunoblotting and band intensity of AQP2 protein relative to β-actin in renal inner medulla 3 h after treatment. The bars represent the mean value (n = 6 per group). C–F: representative histological pictures of AQP2 localization in inner medullary collecting ducts (×25 and ×63 magnification) 3 h after treatment. *P < 0.05.
CA4P treatment compared with control rats (Fig. 7E). This decrease was also observed 3 h after CA4P treatment, though not as pronounced (Fig. 7, E and F). Both changes were highly significant (P < 0.0001).

**Effect of CA4P on sodium transport protein expression in cortex and inner medulla.** To study the mechanism behind the increased sodium excretion after 1 h CA4P treatment, we measured the expression of major sodium transporters, like NHE3, NKCC2, and ENAC. NHE3 mRNA level was significantly decreased after 1 h in CA4P-treated rats compared with control rats (Table 3). However, cortical and outer medullary NKCC2, as well as cortical and medullary ENaC expression, were not changed after 1 h CA4P administration (Table 3). Furthermore, there was no change in the expression of NHE3 and NKCC2 after 3 h CA4P treatment (Table 3).

**CA4P disrupts the microtubule cytoskeleton and mediates changes in pS256-AQP2 localization.** To study whether CA4P affects the collecting ducts principal cell (mpkCCD) microtubules, we performed cell studies to see whether CA4P blocks tubulin polymerization. Cells were treated with CA4P and cytoskeletal detergent-insoluble polymerized fraction was extracted and analyzed by Western blot analysis. As shown in Fig. 9A, 10 μM of CA4P-mediated complete disruption of microtubules evidenced by the absence of polymerized tubulin. Immunofluorescence tubulin staining of mpkCCD cells showed that 1 μM CA4P caused the disruption of microtubules within 1 h (Fig. 9, B and C). Apical staining of pS256AQP2 was weaker in CA4P-treated mpkCCD cells compared with control cells (Fig. 9, D and E). The anti-pS256-AQP2 antibody produced signals in the nucleus that might be caused by the antibody binding to histone H2A1, as has previously been shown to occur with the anti-AQP2 antibody (16).

**DISCUSSION**

The main results of the present study were that CA4P treatment increased hemoglobin concentration after 1 h. In parallel, urine production transiently increased after CA4P treatment, which could be associated with impaired trafficking of pS256-AQP2 to the apical plasma membrane of collecting duct principal cells.

It has previously been shown that the vascular disrupting agent CA4P mediates its effect primarily in tumors, where vessels are fragile (46). In a previous study (4), we have shown that CA4P increases hematocrit, hemoglobin concentration, and mean arterial blood pressure in nontumor-bearing mice. In this study, we show that CA4P significantly increases mean arterial blood pressure and hemoglobin concentration in non-tumor-bearing rats. The rapid increase in hemoglobin could be caused by water leaving the circulatory system. In this study, we show that CA4P causes a reduction in AQP2 trafficking, and, thus, a decrease in water reabsorption by the kidneys. Furthermore, CA4P disrupts the microtubule cytoskeleton and reduces apical labeling of pS256-AQP2 in collecting duct principal cells.

**CA4P increases hemoglobin levels and urine output in rats.** Previously, it was demonstrated that treatment with the vascular disrupting agent CA4P increases hematocrit, hemoglobin concentration, and mean arterial blood pressure in nontumor-bearing mice (4). This finding was confirmed in the present study. We hypothesize that the increased hemoglobin concentration is due to escape of water from the circulation. To examine how water is removed from the circulation, we measured the effect of CA4P treatment on MRI-determined tissue water content and on renal water excretion. CA4P did not...
significantly change the water content in muscle, brain, or renal tissue. We can, however, not exclude the possibility that changes in water content in soft tissue could lead to swelling, which would not be detectable by MRI. Our results demonstrated a transient decrease of water content in the control, but not in the CA4P-treated rats. This might be explained by the use of isoflurane, which inhibits vasoconstriction and thus diuresis through vasopressin (2, 38). We suggest that this inhibition causes the observed decrease in renal water content in the kidneys shown in Fig. 2, B and C. In the CA4P-treated rats, we did not observe any decrease, which is consistent with our data demonstrating an increased diuresis independent of vasopressin. To examine whether the kidneys are directly or indirectly involved in the CA4P-induced hemoglobin increase, a subset of rats was subjected to bilateral nephrectomy before CA4P administration. After the nephrectomy, CA4P treatment did not cause hemoglobin changes, indicating that the kidneys could be involved in the CA4P-mediated increase in hemoglobin concentration.

Urine output increased significantly 1 h after CA4P treatment and then normalized after 2 and 3 h of treatment. Despite the increased water intake in CA4P-treated rats, the enhanced hemoglobin concentration indicated that the increased urine output was not solely a result of the augmented water intake.
COMBRETASTATIN TREATMENT AFFECTS RENAL WATER RETENTION

Fig. 8. Vasopressin V2 receptor (V2R) mRNA expression and cAMP tissue concentration in rat renal inner medulla 1 h after receiving saline (control rats) or 30 mg/kg CA4P. A: V2R mRNA level relative to β-actin mRNA level in renal inner medulla 1 h after treatment. B: cAMP concentration in inner medullary tissue 1 h after treatment. Results are shown as mean values (n = 6 per group).

Furthermore, the CA4P-mediated increase in urinary output cannot be explained by an increase in renal glomerular filtration, as the creatinine clearance remains unchanged between CA4P-treated and control rats. The increased urinary output is instead suggested to be a result of a concentrating defect further down the nephron. The generation of a hypertonic medullary interstitium is essential for the ability to concentrate urine (14); a defect would, thus, be associated with urinary concentration problems. However, in our study, we see no change in the renal medullary interstitial osmolality. Therefore, hypertonicity does not explain the increased urinary output. Moreover, urinary osmolality was not significantly changed 1 h after CA4P treatment. We observed no significant changes in the urinary potassium and creatinine concentrations after 1 h of CA4P treatment. Consequently, we believe that the increased urinary output is associated with an increase in the absolute number of excreted electrolytes, leading to an unchanged osmolality. In contrast, we observed a clearly significant increase in urinary sodium concentration 1 h after CA4P treatment, indicating alterations in the renal sodium transport. We observed a decrease in cortical NHE3 expression after 1 h CA4P treatment. Furthermore, urinary PGE2 excretion showed a tendency to increase after 1 h in CA4P rats compared with control rats. This could explain the natriuresis observed after 1 h of CA4P treatment.

CA4P affects AQP2 and pS256-AQP2 expression and trafficking in the inner medulla. Because of the major change in urine output, we investigated the effect of CA4P on AQP2 and the active pS256-AQP2 expression and trafficking 1 and 3 h after treatment. AQP2 plays an important role in the regulation of water transport in the kidney collecting duct (23, 31, 33). The results demonstrated that V2R mRNA expression and inner medullary cAMP concentration, as well as AQP2 mRNA levels, did not change 1 h after CA4P treatment. However, immunohistochemistry and semiquantitative confocal laser-scanning microscopy analysis of pS256-AQP2 showed changes in trafficking from a clearly apical localization in inner medullary collecting duct principal cells in control rats to a more diffuse or basolateral distribution in the CA4P-treated animals. Short-term regulation of AQP2 leads to activation of the V2R, which increases cAMP levels. cAMP then activates PKA, which phosphorylates AQP2 at serine 256 in intracellular vesicles. pS256-AQP2 is transported to the apical membrane. Incorporation of pS256-AQP2 into the apical membrane facilitates water reabsorption (32). Our observations imply that vesicular trafficking is decreased 1 h after CA4P treatment and that the defect in urinary concentrating capacity is likely to be caused by decreased membrane association of pS256-AQP2.

It has previously been shown that the diuretic response to vasopressin is partially dependent on the existence of a sustained microtubular network (42). Klussmann et al. (22) have demonstrated that inactivation of Rho, a GTP-binding protein involved in organization of the actin cytoskeleton (36), results in depolymerization of F-actin-containing stress fibers and in translocation of AQP2 to the cell membrane and, conversely, that the F-actin cytoskeleton prevents translocation of AQP2-bearing vesicles to the cell membrane. Likewise, microtubule-disrupting agents have been shown to slow down the membrane shuttle mechanism of the vesicles that contain the water channels (42, 45). CA4P is a microtubule-disrupting agent and Kanthou and Tozer (20) have shown that CA4P treatment of various cell types in vitro either lead to Rho activation-dependent formation of actin stress fibers or F-actin reorganization and accumulation in dense spherical bands. Consistent with this, we observed that CA4P disrupts the microtubule cytoskeleton and reduces apical labeling of pS256-AQP2 in collecting duct principal cells. This suggests that CA4P can influence trafficking of AQP2 in collecting duct principal cell, and thus renal water reabsorption, by affecting the microtubular network directly instead of indirectly through AVP signaling.

Three hours after treatment, CA4P mediated a significant increase in AQP2 mRNA level, but no significant changes in the urinary potassium and creatinine concentrations after 3 h of CA4P treatment. As shown in Table 3, QPCR analysis of sodium transporters revealed that CA4P significantly decreased the expression of NHE3 in the kidney cortex and inner medulla, and of ENaCα in the inner medulla.

Table 3. QPCR data from sodium transporters

<table>
<thead>
<tr>
<th>Sodium Transporter</th>
<th>Cortex</th>
<th>CA4P</th>
<th>Inner Medulla</th>
<th>Control</th>
<th>CA4P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHE3</td>
<td>0.76 ± 0.01</td>
<td>0.65 ± 0.05*</td>
<td>0.76 ± 0.11</td>
<td>0.65 ± 0.05*</td>
<td></td>
</tr>
<tr>
<td>NKCC2</td>
<td>0.53 ± 0.05</td>
<td>0.47 ± 0.04</td>
<td>0.53 ± 0.05</td>
<td>0.47 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>ENaCa</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.00</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>ENaCβ</td>
<td>0.10 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>ENaCγ</td>
<td>0.24 ± 0.03</td>
<td>0.22 ± 0.03</td>
<td>0.24 ± 0.03</td>
<td>0.22 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>3 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHE3</td>
<td>1.37 ± 0.01</td>
<td>1.39 ± 0.01</td>
<td>1.37 ± 0.01</td>
<td>1.39 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>NKCC2</td>
<td>1.05 ± 0.01</td>
<td>1.05 ± 0.00</td>
<td>1.05 ± 0.01</td>
<td>1.05 ± 0.00</td>
<td></td>
</tr>
</tbody>
</table>

Results are presented as means ± SE. NHE3, sodium/hydrogen exchanger; NKCC2, sodium-potassium-chloride cotransporter; ENaC, epithelial sodium channel. *Statistically different from control, P < 0.05.

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AQP2 protein level and distribution were found. However, we observed a significant increase in pS256-AQP2 protein level, and furthermore, immunohistochemical analysis showed more apical distribution of pS256-AQP2 in the apical membrane of the collecting duct principal cell. On the contrary, confocal microscopy showed a clear reduction in total pS256-AQP2 abundance 1 h after CA4P treatment and only a small reduction 3 h after treatment compared with control rats. This corresponds well to the urinary output data in which the urinary output is highest 1 h after treatment. The difference between the results obtained with confocal and IHC in pS256-AQP2 abundance 3 h after treatment may be due to the difference in sensitivity between the two methods and to the relatively low sample size used for blotting.

Importantly, quantification of the apical/basal fluorescent intensity 3 h after treatment showed a predominantly apical expression of pS256-AQP2 compared with control and to 1 h after treatment confirming the results from the immunohistochemical analysis. This is consistent with the fact that the increase in urine output is less pronounced at 2 and 3 h after CA4P treatment. This observation might indicate a compensatory mechanism to reabsorb water and, thus, stop the rats from dehydrating and eventually normalizing their hemoglobin levels.

CA4P does not induce acute kidney injury. Our analysis of urinary NGAL excretion within the first hour after CA4P administration did not show any significant changes. NGAL is a small protein found not only in neutrophils but also in certain epithelia, such as renal tubules, where its expression is dramatically increased in ischemic or nephrotoxic injury. NGAL levels rise in urine and blood within 1–2 h of renal insult, making NGAL a biomarker for acute renal injury (25). Our result indicates that CA4P does not induce acute kidney injury, at least not 1 h after treatment. We can, however, not exclude the possibility that CA4P mediates changes in urinary NGAL excretion at later time points.

**Perspectives and Significance**

The present study is an effort to elucidate whether CA4P affects the renal water and salt balance. Our data indicate that CA4P increases the urine output through a local renal effect on collecting duct water permeability due to a transient impairment of AQP2 trafficking. Elucidation of the renal effect of CA4P at the molecular level may provide novel targets to improve treatment strategies to prevent the dysregulation of renal tubular function.

**ACKNOWLEDGMENT**

We thank Line V. Nielsen, Gitte Kall, Gitte Skou, Inger Merete S. Paulsen, and Dorthe Grand for excellent technical assistance. We also thank Jeppe Praetorius for helpful suggestions on image quantification.

**GRANTS**

Support for this study was provided by The Danish Cancer Society, Radiumstationen, Helga and Peter Kornings Foundation, The Lundbeck Foun-
 tion. The Novo Nordisk Foundation, The Commission of the European Union (EU Action Programs), The Danish Medical Research Council, and The University of Aarhus Research Foundation.

DISCLOSURES

Michael R. Horsman is a consultant for OXiGENE. No other conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


