Long-term treatment with cyclosporine decreases aquaporins and urea transporters in the rat kidney

Sun-Woo Lim, Can Li, Bo-Kyung Sun, Ki-Hwan Han, Wan-Young Kim, Yoon-Wha Oh, Jong-Un Lee, Peter F. Kador, Mark A. Knepper, Jeff M. Sands, Jin Kim, and Chul-Woo Yang.

Long-term treatment with cyclosporine decreases aquaporins and urea transporters in the rat kidney. Am J Physiol Renal Physiol 287: F139–F151, 2004. First published February 24, 2004; 10.1152/ajprenal.00240.2003.—The aim of this study was to evaluate the long-term effects of cyclosporine (CsA) treatment on urinary concentration ability. Rats were treated daily for 4 wk with vehicle (VH; olive oil, 1 ml/kg sc) or CsA (15 mg/kg sc). The influence of CsA on the kidney’s ability to concentrate urine was evaluated using functional parameters and expression of aquaporins (AQP1–4) and of urea transporters (UT-A1–3, and UT-B). Plasma vasopressin levels and the associated signal pathway were evaluated, and the effect of vasopressin infusion on urine concentration was observed in VH- and CsA-treated rats. Toxic effects of CsA on tubular cells in the medulla as well as the cortex were evaluated with aldose reductase (AR), Na-K-ATPase-α1 expression, and by determining the number of terminal transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells. Long-term CsA treatment increased urine volume and fractional excretion of sodium and decreased urine osmolality and free-water reabsorption compared with VH-treated rats. These functional changes were accompanied by decreases in the expression of AQP (1–4) and UT (UT-A2, A3, and UT-B), although there was no change in AQP2 in the cortex and outer medulla and UT-A1 in the inner medulla (IM). Plasma vasopressin levels were not significantly different between two groups, but infusion of vasopressin restored CsA-induced impairment of urine concentration. cAMP levels and Gso protein expression were significantly reduced in CsA-treated rat kidneys compared with VH-treated rat kidneys. CsA treatment decreased the expression of AR and Na-K-ATPase-α1 and increased the number of TUNEL-positive renal tubular cells in both the cortex and medulla. Moreover, the number of TUNEL-positive cells correlated with AQP2 or UT-A3 expression within the IM. In conclusion, CsA treatment impairs urine-concentrating ability by decreasing AQP and UT expression. Apoptotic cell death within the IM at least partially accounts for the CsA-induced urinary concentration defect.

Cyclosporine A (CsA) is a potent immunosuppressant for organ transplantation and autoimmune disorders, but its use is limited by CsA-induced nephropathy. Long-term treatment of CsA has been associated with characteristic histological lesions (striped interstitial fibrosis, arteriopathy, and tubular atrophy). CsA treatment also causes tubular dysfunction that is characterized by polyuria, magnesium wasting, distal tubular acidosis, and hyperkalemia. Of these, impaired urine concentration is one of the predominant features of chronic CsA nephropathy (5, 16, 27, 32), but the mechanisms that underlie impaired urine concentration have yet to be demonstrated.

Aquaporins (AQPs) are a family of membrane proteins that play an important role in the reabsorption of water in the kidney. To date, at least 11 different types of AQPs have been cloned (12). The expression or physiological regulations of AQPs (1–4) in the kidney are well documented (18). AQP1 aids the rapid reabsorption of large quantities (70–80%) of filtered water, and it is constitutively expressed on both apical and basolateral membranes of epithelial cells in the proximal tubule (PT), the descending thin limbs (DTL) of Henle’s loop, and in endothelial cells of the descending vasa recta (DVR) (17). AQP2, AQP3, and AQP4 are involved in the movement of water across the apical membrane in the collecting duct principal cells, and their expressions are localized to the basolateral membrane of the collecting duct principal cells. This reabsorbed free water (FW) determines the osmolality of the final voided urine, and their expressions are controlled by vasopressin (18).

The urea transporter (UT) family includes renal urea transporters (UT-A) and erythrocyte urea transporter (UT-B), which are encoded by different genes (21). The role of UTs in the urine-concentrating process has been well established (20). In the kidney, UT-A1 and UT-A3 are responsible for accumulation of urea in the inner medulla (IM), and they are expressed in the inner medullary collecting duct (IMCD). UT-A2 is expressed in the DTL of Henle’s loop and UT-B in the DVR, which are important sites in the recycling of urea and thus for generation of the urea concentration gradient in the deep IM (6, 25, 28, 29). These UTs are regulated by vasopressin and all facilitated transporters, allowing a 10- to 1,000-fold faster equilibration of urea across cell membranes compared with passive diffusion in the kidney (2).

In vivo and in vitro studies show that CsA-induced nephropathy is closely associated with apoptotic cell death of renal tubular cells (1, 31). In this system, the extent of apoptosis

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cells in the medulla are exposed to a hyperosmotic environment. Tubular cells within the medulla are rarely reported. Tubular injuries have focused on the cortex, and any effects of CsA on renal function have not been well characterized. Our previous studies evaluated the development of tubulointerstitial fibrosis (TIF) in the cortex assessed by trichrome staining but not in the VH-treated rat kidney. Magnification: ×100. 

Fig. 1. Trichrome staining of rat kidneys treated with vehicle (VH; open bar; A) and cyclosporine A (CsA; filled bar; B). CsA treatment for 4 wk resulted in the development of tubulointerstitial fibrosis (TIF) in the cortex assessed by trichrome staining but not in the VH-treated rat kidney. Magnification: ×100. 

Correlates with fibrosis, where cell loss may prevent the kidney’s ability to remodel effectively (31). However, most studies have focused on the cortex, and any effects of CsA on renal tubular cells within the medulla are rarely reported. Tubular cells in the medulla are exposed to a hyperosmotic environment, and enzymes such as aldose reductase (AR), betaine/γ-amino-n-butyric acid transporter-1, and myo-inositol transporter are needed to maintain their integrity by accumulating intracellular osmolytes (22). Therefore, long-term CsA treatment may cause apoptotic cell death by decreasing the levels of these vital enzymes.

Based on the above findings, we hypothesized that CsA treatment impairs urinary concentration ability and that this is associated with apoptotic cell death of renal tubular cells in the medulla. To define our hypothesis, we examined the influence of CsA on mechanisms of urine concentration, apoptotic cell death, and related factors in an animal model of chronic CsA nephropathy.

MATERIALS AND METHODS

Animals and Drugs

The experimental protocol was approved by the Animal Care Committee of The Catholic University. Male Sprague-Dawley rats (Charles River), weighing 220–240 g, were housed in individual cages (Nalge, Rochester, NY) in a temperature- and light-controlled environment. Before the start of the treatment, the rats were placed on a low-salt diet (0.05% sodium, Teklad Premier, Madison, WI) (19, 30). Cyclosporine (Novartis Pharma, Basel, Switzerland) was diluted in olive oil (Sigma Diagnostics, St. Louis, MO) to a final concentration of 15 mg/ml. Exogenous 1-desamino-[8-D-arginine] vasopressin (DDAVP; Sigma) was given using implantable osmotic minipumps.

Experimental Protocol

Two separate experiments were conducted.

Protocol 1. This study was designed to evaluate the effect of long-term treatment of CsA on urine-concentrating ability. Rats were randomized into two groups: 1) the vehicle group (VH; n = 6), in which rats received a daily subcutaneous injection of olive oil at a dose of 1 ml/kg for 4 wk and 2) the CsA group (n = 6), in which rats received a daily subcutaneous injection of CsA at a dose of 15 mg/kg for 4 wk.

Protocol 2. This study was designed to evaluate the effect of exogenous vasopressin administration on urine-concentrating ability in chronic CsA nephropathy. Rats were randomized into four groups: 1) VH, 2) VH+DDAVP, 3) CsA, and 4) CsA+DDAVP. A total of 24 rats was used, and each group comprised 6 rats. Administration of olive oil and CsA was the same as in protocol 1. The dose and infusion route of DDAVP were chosen based on a previous report (13). Briefly, for implantation, minipumps (model 2ML1; Alzet, Palo Alto, CA) were filled with DDAVP in a carrier solution containing a 0.9% NaCl solution. Rats were anesthetized with ketamine, and the minipumps were inserted into the subcutaneous tissue under the skin of the animal’s back. Under these conditions, the pumps delivered 0.1 μg/h of DDAVP to VH- or CsA-treated rats for the last 7 days of the experimental period.

Functional Parameters

After the treatment started, rats were pair-fed and daily body weight was monitored. Before death, animals were individually housed in metabolic cages (Tecniplast, Buguggiate, Italy) for 24-h urine collection. Urine osmolality was measured with a Fiske 2400 Osmometer (Fiske Associates, Norwood, MA). Fractional excretion of sodium (FE_{Na}) and T/W were calculated using standard formulas as previously described by Li et al. (15). Blood samples were obtained to evaluate serum creatinine (S_{Cr}). The creatinine clearance rate (C_{Cr}) was calculated using a standard formula. Whole blood CsA levels were measured by monoclonal radioimmunoassay (Incstar, Stillwater, MN).

Preservation of Kidneys

The kidneys were preserved by in vivo perfusion through the abdominal aorta. The animals were briefly perfused with 0.01 M PBS to wash out the blood. Then, the left kidney was removed for Western blot analysis and the other kidney was removed after perfusion with a periodate-lysine-paraformaldehyde (PLP) solution. The kidneys were removed and cut into sagittal slices of 1- to 20-mm thickness and postfixed overnight in PLP solution at 4°C. The kidneys were embedded in wax for postimmunostaining for terminal transferase-mediated

Table 1. Functional data in each group

<table>
<thead>
<tr>
<th>Functional Parameter</th>
<th>VH (n = 6)</th>
<th>CsA (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>295 ± 4</td>
<td>255 ± 5*</td>
</tr>
<tr>
<td>Water intake, ml/day</td>
<td>15.8 ± 0.7</td>
<td>28.0 ± 4.0*</td>
</tr>
<tr>
<td>Urine volume, ml/day</td>
<td>9 ± 2</td>
<td>18 ± 1*</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kgH2O</td>
<td>1.539 ± 0.264</td>
<td>665 ± 31*</td>
</tr>
<tr>
<td>FE_{Na}, %</td>
<td>0.04 ± 0.01</td>
<td>0.08 ± 0.01*</td>
</tr>
<tr>
<td>T/W, ml/day</td>
<td>58 ± 3</td>
<td>25 ± 3*</td>
</tr>
<tr>
<td>S_{Cr}, mg/dl</td>
<td>0.64 ± 0.04</td>
<td>1.02 ± 0.09*</td>
</tr>
<tr>
<td>C_{Cr}, ml/min·1·100 g⁻¹</td>
<td>0.35 ± 0.05</td>
<td>0.17 ± 0.03*</td>
</tr>
<tr>
<td>CsA concentration, ng/ml</td>
<td>0.0 ± 0.0</td>
<td>2371 ± 38*</td>
</tr>
</tbody>
</table>

Values are means ± SE. n. No. of rats. VH, vehicle-treated group; CsA, cyclosporine A; FE_{Na}, fractional excretion of sodium; T/W, free-water reabsorption; S_{Cr}, serum creatinine; C_{Cr}, creatinine clearance. *P < 0.05 vs. VH.
dUTP nick end-labeling (TUNEL) assay, AR, and Na-K-ATPase-α1. Vibratome sections for preembedding immunostaining for AQPs and UTs were cut on a Vibratome at a thickness of 50 μm.

**Histopathology**

Trichrome staining was used to assess tubulointerstitial fibrosis (TIF) (15). Under a microscope, 20 nonoverlapping fields from each slide were captured and graded using a color image analyzer (TDI Scope Eye Version 3.0 for Windows, Olympus). A finding of TIF was defined as a matrix-rich expansion of the interstitium with tubular dilatation, tubular atrophy, tubular cast formation, slough of tubular epithelial cells, and thickening of the tubular basement membrane. Data are expressed as relative percentages. Histopathological analysis was performed by a pathologist blinded to the identity of the treatment groups.

**Antibodies**

To determine the expression of AQPs and UTs in rat kidneys, we used specific rabbit polyclonal antibodies against peptides based on the rat renal AQPs (AQP1–3, Chemicon, Temecula, CA; AQP4, Alpha Diagnostic, San Antonio, TX), UT-A (L194 recognizes UT-A1 and UT-A2; Q2 recognizes UT-A3, kindly provided by Dr. M. A. Knepper, National Institutes of Health, Bethesda, MD), and the human erythrocyte UT-B (kindly provided by Dr. J. M. Sands, Emory University of Medicine, Atlanta, GA) (6, 25, 28, 29). Rabbit polyclonal heteromeric G protein subunit Gsα (Calbiochem-Novabiochem, San Diego, CA) and type VI adenylyl cyclase (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used. A goat polyclonal antibody against AR (kindly provided by Dr. P. F. Kador, National Institutes of Health) (26) and a rabbit polyclonal antibody against Na-K-ATPase-α1 (Upstate Biotechnology, Lake Placid, NY) were used.

**Preembedding Method for AQP and UT Immunolabeling**

Vibratome sections were washed with 50 mM NH4Cl in PBS three times for 15 min. Before incubation with the primary antibodies, tissue sections were incubated for 3 h with PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin (solution B). The tissue sections were equilibrated in solution B for 1 h before incubation with the primary antibodies for 3 h at room temperature.

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**Fig. 2.** Light micrographs of 50-μm-thick Vibratome sections from VH (A1–A4, B1–B4, and C1–C4) and CsA (A1′–A4′, B1′–B4′, and C1′–C4′)-treated rat kidneys illustrating immunohistochemistry of aquaporin (AQP) 1, 2, 3, and 4. Asterisks, proximal tubule; arrows, brush border of proximal tubule; arrowheads, intercalated cells of collecting duct. DTL, descending thin limb; DVR, descending vasa recta. Magnification: ×200 (B1, B1′, C1, and C1′); ×400, all other panels.
were then incubated overnight at 4°C in rabbit antisera against AQP1 (1:1,000); AQP2 (1:1,000); AQP3 (1:300); AQP4 (1:400); L194 (1:1,000); Q2 (1:800); and UT-B (1:3,000) diluted in PBS containing 1% BSA (solution A). The tissue sections were washed with PBS containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin (solution C) and incubated for 2 h in peroxidase-conjugated donkey anti-rabbit IgG Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in solution A. Tissue sections were rinsed with PBS containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin (solution C) and incubated for 2 h in peroxidase-conjugated donkey anti-rabbit IgG Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in solution A. Tissue sections were rinsed with solution C and 0.05 M Tris buffer (TB), pH 7.6, and incubated in 0.1% 3, 3'-diaminobenzidine (DAB) in TB for 5 min. Tissue sections were then incubated with H2O2 (0.01%) for 10 min. The tissue sections were washed three times with TB, dehydrated in a graded series of ethanol, and embedded in poly/Bed 812 resin (Polysciences, Warrington, CA). The tissue sections were examined with light microscopy and photographed with an Olympus photomicroscope.

Fig. 3. Western blot and densitometric analyses of AQP1, 2, 3, and 4 in the cortex (CO; A), outer medulla (OM; B), and inner medulla (IM; C) of VH (open bars) and CsA (filled bars)-treated rat kidneys. *P < 0.05 vs. VH. **P < 0.001 vs. VH.

Postembedding Method for AR and Na-K-ATPase-α1 Immunolabeling

The tissue sections embedded in wax were cut to 5-μm thickness and mounted on gelatin-coated slides. The tissue sections were de-waxed with xylene and ethanol and were treated with methanolic H2O2 for 30 min after being rinsed under tap water. Before incubation with primary antibodies, the tissue sections were permeabilized by incubation for 15 min in 0.5% Triton X-100 in PBS and then blocked with normal donkey serum diluted 1:10 in PBS for 1 h. Subsequently, the tissue sections were incubated overnight at 4°C with goat polyclonal AR (1:100,000) or rabbit polyclonal Na-K-ATPase-α1 (1:500) antibodies. The tissue sections were rinsed in PBS and incubated for 2 h in peroxidase-conjugated donkey anti-goat or rabbit IgG Fab fragment (Jackson ImmunoResearch Laboratories) diluted 1:100 in solution A.
PBS. After being rinsed with TB, the tissue sections were incubated with a mixture of 0.05% DAB and 0.033% H$_2$O$_2$ for 1–2 min at room temperature. After being rinsed with TB again, the tissue sections were incubated with a mixture of 0.05% DAB and 0.033% H$_2$O$_2$ for 2 min at room temperature. Primary antibodies were diluted in blocking solution as described above and incubated for overnight at 4°C. Blotting was followed by six washes with Tris-buffered saline and incubation with peroxidase-linked anti-rabbit or goat IgG diluted 1:1,000 in blocking solution as secondary antibody (Amersham Biosciences, Buckinghamshire, UK) for 1 h at room temperature. Antibody-reactive protein was detected with enhanced chemiluminescence (Amersham Biosciences). Optical densities were obtained after three determinations for each band. We quantitated the two bands separately, and represented data are averaged values of two band densities.

**Radioimmunoassay of Plasma Vasopressin Levels**

Vasopressin levels in plasma were determined using a vasopressin-directed radioimmunoassay kit (Bühlmann Laboratories) according to the manufacturer’s instructions.

**Membrane Preparation and Adenylyl Cyclase Activity**

The membrane preparation was obtained as described previously (10). The IM of the kidney was separated and homogenized in ice-cold homogenizing buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.2 mM PMSF, and 250 mM sucrose) and centrifuged at 40,000 g for 30 min at 4°C. Afterward, each protein concentration of the lysate was determined with a protein microassay using the Bradford method (Bio-Rad). Equal amounts of protein were resolved on 10% SDS-PAGE and then electroblotted onto nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked in Tris-buffered saline, pH 8.0, containing 5% nonfat powdered milk (blocking solution) for 1 h at room temperature. Primary antibodies were diluted in blocking solution as described above and incubated for overnight at 4°C. Blotting was followed by six washes with Tris-buffered saline and incubation with peroxidase-linked anti-rabbit or goat IgG diluted 1:1,000 in blocking solution as secondary antibody (Amersham Biosciences, Buckinghamshire, UK) for 1 h at room temperature. Antibody-reactive protein was detected with enhanced chemiluminescence (Amersham Biosciences). Optical densities were obtained after three determinations for each band. We quantitated the two bands separately, and represented data are averaged values of two band densities.
3,000 and 100,000 g in succession. The resulting pellet was used as the membrane preparation. Protein concentrations were measured with a bicinchonic acid assay kit (Bio-Rad). Adenylyl cyclase activity was assayed by the method of Bar (3), with a slight modification. AVP was used to start the reaction, in which protein contents were 10 μg in 100 μl working solution (50 mM Tris-HCl, pH 7.6, containing 1 mM ATP, 20 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 6.4 mM MgCl₂, 1 mM 3-isobutyl-1-methylxanthine, and 0.02 mM GTP). After 15 min, the reaction was stopped by cold application of a solution consisting of 50 mM sodium acetate, pH 5.0, and centrifuged at 3,000 g for 10 min at 4°C. cAMP was measured in the supernatant by equilibrated radioimmunoassay. Iodinated 2⁻-O-monosuccinyl-adenosine 3',5'-cyclic monophosphate tyrosyl methyl ester was prepared as described by previous investigators (23). Standards or samples were taken up in a final volume of 100 μl of 50 mM sodium acetate buffer (pH 4.8). Dilute cAMP antiserum (Calbiochem-Novabiochem) and iodinated 2⁻-O-monosuccinyl-adenosine 3',5'-cyclic monophosphate tyrosyl methyl ester (10,000 cpm/100 μl), 100 μl each, were added and incubated for 15 h at 4°C. The bound form was separated from the free form by charcoal suspension, and the supernatant was counted in a gamma counter (Packard Instrument, Meriden, CT). All samples were analyzed in a single assay. Nonspecific binding was <2.0%. The 50% intercept was at 16.5 ± 0.8 fmol/tube (n = 10). Intra- and interassay coefficients of variation were 5.0 ± 1.2% (n = 10) and 9.6 ± 1.9% (n = 10), respectively. Results are expressed as moles of cAMP generated per milligram protein per minute.

**Statistical Analysis**

Data are expressed as means ± SE. Comparisons between groups were done by Student’s t-test. Pearson’s single-correlation coefficient (SPSS software, version 11.0, Microsoft) was used to compare TUNEL-positive cells with AQPs and UTs. Statistical significance was assumed as P < 0.05.

### Table 2. Urine volume and urine osmolality

<table>
<thead>
<tr>
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<th>Urine Volume, ml/day</th>
<th>Urine Osmolality, mosmol/kgH₂O</th>
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<tbody>
<tr>
<td></td>
<td>Day 21</td>
<td>Day 28</td>
</tr>
<tr>
<td>VH</td>
<td>12.0 ± 3.0</td>
<td>9.3 ± 1.4</td>
</tr>
<tr>
<td>VH + DDAVP</td>
<td>13.3 ± 1.5</td>
<td>4.6 ± 0.3*</td>
</tr>
<tr>
<td>CsA + DDAVP</td>
<td>15.7 ± 3.2*</td>
<td>9.2 ± 1.5#</td>
</tr>
<tr>
<td></td>
<td>1,224.0 ± 8.0</td>
<td>1,107.0 ± 117.6</td>
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<tr>
<td></td>
<td>1,275.8 ± 7.3</td>
<td>2,971.6 ± 137.9*</td>
</tr>
<tr>
<td></td>
<td>711.8 ± 116.4*</td>
<td>475.5 ± 68.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats/group. DDAVP, 1-desamino-[8-d-arginine] vasopressin. *P < 0.05 vs. VH. #P < 0.05 vs. CsA.

![Fig. 7. Western blot (top) and densitometric (bottom) analyses of Gsα protein and adenylyl cyclase VI in VH (open bars)- and CsA-treated rat kidneys.](http://ajprenal.physiology.org/)

Fig. 8. cAMP production in response to arginine vasopressin in the IM of VH (○) - and CsA (●)-treated rat kidneys. Values are means ± SE of 3 experiments. *P < 0.05 vs. VH.

![Fig. 8. cAMP production in response to arginine vasopressin in the IM of VH (○) - and CsA (●)-treated rat kidneys.](http://ajprenal.physiology.org/)
RESULTS

Induction of Chronic CsA Nephropathy

The concentration of CsA in whole blood was 2,371 ± 38 ng/ml (Table 1). CsA treatment significantly decreased body weight compared with the VH group and produced typical striped TIF, as shown in Fig. 1. Quantitative analysis revealed an increased percentage of TIF in CsA-treated rats compared with VH-treated rats (23 ± 3 vs. 0 ± 0%, P < 0.001).

Functional Parameters

Table 1 summarizes the functional data for each group. CsA treatment significantly decreased urine osmolality (665 ± 31 vs. 1,539 ± 264 mosmol/kgH2O, P < 0.05) and TW (25 ± 3 vs. 38 ± 3 ml/24 h, P < 0.05) compared with VH-treated rats, whereas urine volume (18 ± 1 vs. 9 ± 2 ml/24 h, P < 0.05) and FENa (0.08 ± 0.01 vs. 0.04 ± 0.01%, P < 0.05) increased.

The levels of Scr in CsA-treated rats were significantly higher than in VH-treated rats (1.02 ± 0.09 vs. 0.64 ± 0.04 mg/dl, P < 0.05), and the Ccr in CsA-treated rats was significantly reduced compared with VH-treated rats (0.17 ± 0.03 vs. 0.35 ± 0.05 ml/min⁻¹·100 g⁻¹, P < 0.05).

Expression of AQPs

AQP1 was expressed in PT, DTL, and DVR. In the cortex (CO), AQP1 was highly expressed along the brush border and

Fig. 9. Differential interference contrast (DIC) micrographs on 5-μm-thick wax sections from the CO (A and D), OM (B and E), and IM (C and F) of VH (A and C)- and CsA (B and D)-treated rat kidneys illustrating terminal transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells. Inset in F: double immunolabeling with AQP2 and TUNEL assay; asterisks, IMCD. G, glomerulus; PT, proximal tubule; TAL, thick ascending limb. Bottom: open bars, VH; filled bars, CsA. Magnification: ×400 (inset in F); ×100, all other panels. #P < 0.05 vs. VH. ##P < 0.001 vs. VH.
was weakly expressed in the basolateral region of the PT in VH-treated rat kidneys (Fig. 2A1), whereas its immunoreactivity was remarkably reduced in the kidneys of CsA-treated rats (Fig. 2A1'). Similar observations were observed in the OM and IM. CsA-treated rat kidneys showed a decrease in AQP1 immunoreactivity in the DTL of Henle’s loop and DVR (Fig. 2B1, B1', C1, and C1'). Densitometric analysis of AQP1 protein demonstrated a marked reduction in AQP1 expression in CsA-treated rat kidneys (CO, 70 ± 4 vs. 100 ± 8%,  P < 0.05; OM, 54 ± 3 vs. 100 ± 6.5%,  P < 0.05; IM, 35 ± 7.5 vs. 100 ± 6.7%,  P < 0.001). AQP2 was strongly expressed throughout the apical region of collecting duct cells in the kidneys of VH-treated rats (Fig. 2, A2–C2), but CsA treatment decreased immunoreactivity for AQP2 only in the IMCD (Fig. 2, C2 and C2'). This was confirmed by Western blot analysis. In the IM, CsA-treated kidneys showed a significant decrease in AQP2 expression (70 ± 7 vs. 100 ± 15%,  P < 0.05). Interestingly, AQP2 expression in the OM was slightly increased in CsA-treated rat kidneys (112 ± 16 vs. 100 ± 12%), although it is not statistically significant. AQP3 and AQP4 were mainly expressed in the basolateral region of the collecting duct cells in VH-treated rat kidneys. However, CsA treatment significantly decreased AQP3 and AQP4 immunoreactivity throughout the collecting duct (cortical collecting duct, OMCD, and IMCD) relative to VH treatment (Fig. 2). Results obtained from Western blot analysis were consistent with those from the immunohistochemical study. CsA treatment decreased the abundance of AQP3 (CO, 62 ± 7 vs. 100 ± 14%; OM, 80 ± 2 vs. 100 ± 8%; IM, 71 ± 10 vs. 100 ± 7%,  P < 0.05, respectively) and AQP4 (CO, 62 ± 10 vs. 100 ± 7%; OM, 85 ± 5 vs. 100 ± 3%; IM, 54 ± 5 vs. 100 ± 5%,  P < 0.05, respectively) compared with VH treatment (Fig. 3).

Expression of UTs

UT-A1 and UT-A3 were normally expressed in the IMCD in the VH-treated rat kidneys. Long-term CsA treatment markedly decreased UT-A3 immunoreactivity, (Fig. 4, E and F) but did not affect UT-A1 (Fig. 4, A and B). UT-A2 was chiefly located in the DTL of Henle’s loop in the OM and in the initial portion of IM in VH-treated rat kidneys, but its expression was evidently reduced by CsA treatment (Fig. 4, C and D). CsA treatment also reduced the expression of UT-B compared with VH-treated rat kidneys in the endothelial cells of the DVR (Fig. 4, G and H). High magnifications of UT-A2, -A3, and -B (Fig. 4, C'-H') revealed decreased transporter expression in each tubule in CsA-treated rat kidneys. Similarly, Western blot analysis revealed decreased expression of UT-A2 (24 ± 7 vs. 100 ± 17%,  P < 0.05), UT-A3 (42 ± 8 vs. 100 ± 6%,  P < 0.05), and UT-B (59 ± 9 vs. 100 ± 11%,  P < 0.05) in CsA-treated rat kidneys relative to VH-treated rat kidneys. However, there was no difference in UT-A1 between the two groups (111 ± 13 vs. 102 ± 5%; Fig. 5).

Plasma Vasopression Levels

Figure 6 shows plasma vasopressin concentration in VH- and CsA-treated rats. The results showed no significant difference between VH- and CsA-treated rats (47.1 ± 6.7 vs. 37.6 ± 6.3 pg/ml).

Effect of DDAVP Infusion on Urine-Concentrating Ability in Chronic CsA Nephropathy

Table 2 shows the effects of DDAVP infusion on urine concentration in VH and CsA groups. Treatment of rats in the VH group with DDAVP for 7 days significantly decreased urine production and increased urine osmolality compared with groups not given vasopressin treatment. Similar effects were observed in CsA-treated rat kidneys. Rats treated with DDAVP for 7 days significantly decreased urine volume and increased urine osmolality compared with rats not given vasopressin treatment.

Expression of Gsα and Adenylyl Cyclase VI

Figure 7 presents the results of Western blot analysis of Gsα and adenylyl cyclase VI protein in the CO, OM, and IM. CsA treatment significantly decreased the expression of Gsα protein in all regions of the kidney compared with VH-treated rat kidneys (CO, 31 ± 9 vs. 100 ± 16,  P < 0.05; OM, 14 ± 7 vs. 100 ± 15,  P < 0.05; IM, 24 ± 5 vs. 100 ± 8,  P < 0.05).
However, there were no significant differences in the levels of adenylyl cyclase VI expression in CO, OM, and IM compared with VH-treated rat kidneys (CO, 112 ± 3 vs. 100 ± 6; OM, 103 ± 3 vs. 100 ± 24; IM, 96 ± 17 vs. 100 ± 21).

Adenylyl Cyclase Activity

Figure 8 shows the results of adenylyl cyclase activity stimulated by AVP in the IM. The generation of cAMP was significantly blunted after treatment with CsA compared with those treated with VH.

Apoptotic Cell Death

CsA treatment increased the number of TUNEL-positive cells in the CO (69 ± 3 vs. 11 ± 1, P < 0.001), OM (55 ± 6 vs. 10 ± 1, P < 0.001), and IM (10 ± 2 vs. 3 ± 1, P < 0.05) compared with the group given VH treatment (Fig. 9). TUNEL-positive cells in the kidneys of CsA-treated rats were mainly seen among PT, thick ascending limb (TAL), and IMCD cells, and the presence of TUNEL-positive cells in IMCD cells was confirmed by double immunolabeling with AQP2 and TUNEL assay (Fig. 9F, inset). Furthermore, there was good correlation between the number of TUNEL-positive cells and AQP2 (r = −0.671, P < 0.05) or UT-A3 (r = −0.827, P < 0.05) expression in IM of CsA-treated rat kidneys (Fig. 10).

Expression of AR and Na-K-ATPase-α1

AR was mainly expressed in the ascending thin limb of Henle’s loop (ATL) in the midportion of the IM and IMCD and interstitial cells of VH-treated kidneys. After CsA treatment, AR immunoreactivity only decreased dramatically in the ATL relative to VH-treated rat kidneys. Western blot analysis showed the level of AR expression was significantly decreased from 86 ± 9% in the VH to 54 ± 7% in the CsA-treated rat kidneys (P < 0.05; Fig. 11). Expression of Na-K-ATPase-α1 was constitutive in the distal convoluted tubule and TAL in VH-treated rat kidneys. However, CsA treatment markedly reduced Na-K-ATPase-α1 immunoreactivity in both distal convoluted tubule and TAL compared with VH-treated rat kidneys (Fig. 12).

DISCUSSION

The present study clearly demonstrates that long-term CsA treatment significantly decreases the expression of AQPs (AQP1–4) and UTs (UT-A2, UT-A3, and UT-B) in different regions of the kidney. These phenotypic changes were associated with an increase in urine volume and fractional excretion of sodium and a decrease in urine osmolality and TcW. Our observations suggest that the decrease in the expression of AQPs and UTs in chronic CsA nephropathy may be one of the mechanisms for the impairment of urine-concentrating capacity.

Expression of AQPs and UTs Is Decreased in Rats with Chronic CsA Nephropathy

In the present study, long-term CsA treatment significantly decreased the expression of AQPs and UTs. CsA treatment decreased expression of AQP1 by 65% in the IM and by 30% in the CO; AQP2 in the IM by 30%; AQP3 in the CO by 28%, OM by 20%, and IM by 29%; and AQP4 in the CO by 38%, OM by 15%, and IM by 46%. CsA treatment also decreased expression of UT-A2, UT-A3, and UT-B proteins by ~76, 58, and 41%, respectively. Our findings suggest that CsA inhibits water reabsorption by downregulating AQPs and decreases delivery of concentrated urea to the IM interstitium and its recycling by inhibiting UT expression. These molecular changes account, at least in part, for the CsA-induced urinary concentration defect.

Results obtained during our study show that expression of AQP2 and UT-A1 was slightly increased in the IM of CsA-
treated rat kidneys compared with the kidneys of VH-treated rats. The reason for upregulation of these transporters is unclear, but it may represent a compensatory mechanism for the loss of collecting duct cells during conditions of increased urine volume and decreased urine osmolality. Similar results have been observed in rats with uncontrolled diabetes that have shown increases in UT-A1 and AQP2 expression despite continuing osmotic diuresis (4, 9).

Chronic CsA nephropathy is associated with striped interstitial fibrosis (30, 32). Therefore, it is reasonable to assume that reduced expression of AQPs and UTs is related to the tubular loss associated with chronic CsA nephropathy. However, interstitial fibrosis is rarely observed in the medulla in this model. Moreover, each tubule examined under higher magnification showed decreased immunoreactivity with AQPs and UTs, as shown in Figs. 2 and 4. Therefore, reduced expression of these transporters caused by CsA can be attributed to decreased tubular expression in this model.

Impaired renal function is an important factor of decreased urine concentration. Similar observations have been observed in an experimental model of 5/6 nephrectomy (13). Therefore, we conclude that renal dysfunction caused by CsA is partly responsible for the decreased urine concentration reported in this study. In addition to renal dysfunction, disturbances in
food intake associated with CsA administration per se can affect urinary concentration ability (8). In this study, VH- and CsA-treated rats were pair-fed to avoid the influence of food intake on urine concentration. Thus we can exclude the possibility that disturbances in food intake associated with CsA treatment may affect our results.

A low-salt diet is essential to induce an animal model of chronic CsA nephropathy. Using the observation that sodium depletion exacerbates CsA nephropathy, Rosen et al. (19), Elzinga et al. (7), and others (32) developed a reproducible animal model of chronic CsA nephropathy. In this model, a low-salt diet induced a histological feature of chronic CsA nephropathy mimicking those described in patients on long-term CsA therapy. As a result, there is a possibility that a low-salt diet itself may influence the expression of AQPs and UTs between two groups. However, a recent publication demonstrated that a low-salt diet did not affect AQP expression (e.g., AQP1 and AQP2) compared with a high-salt diet (14). Furthermore, all animals were placed on same diet (low salt) in this study. On the basis of our study and previous outlined reports, we suggest that changes in the expression of AQPs and UTs in this model may be a consequence of long-term CsA treatment.

Signal Mechanisms of Decreased AQPs and UTs Associated with Chronic CsA Nephropathy

To evaluate the role of vasopressin in CsA-induced impairment of urine-concentrating ability, we measured plasma vasopressin levels and observed the effect of exogenous vasopressin treatment on urine concentration in VH and CsA groups. In this study, there were no significant differences in plasma vasopressin levels between VH- and CsA-treated rats. However, exogenous vasopressin administration increased urine osmolality compared with the CsA group that was not given exogenous vasopressin. This finding suggests that CsA impairs urine concentration by a vasopressin-dependent pathway.

Our study was extended to evaluate the vasopressin/cAMP pathway. The results obtained showed that CsA treatment reduced the expression of Gsα proteins without significantly altering adenyl cyclase VI expression. cAMP generation in response to arginine vasopressin was blunted in CsA-treated rat kidneys. These findings suggest that primary impairment in the pathway leading to the downregulation of AQPs lies at the level of Gsα proteins, and decreases in the abundance of Gsα proteins may result in a diminished generation of cAMP and hence AQPs and UTs (11).

Impaired Urine-Concentrating Ability in Chronic CsA Nephropathy Is Associated with Apoptotic Cell Death

In this study, we confirmed that CsA caused apoptotic cell death in tubular cells of the medulla as well as the CO. CsA treatment increased the number of TUNEL-positive cells in the IM, mainly localized to the collecting duct. Furthermore, decreases in AQP2 or UT-A3 expression in CsA-treated rat kidneys correlated well with the increased number of TUNEL-positive cells. These findings suggest that apoptotic cell death by CsA is responsible for the decreased levels of AQPs and UTs expression in rat kidneys.

To support our hypothesis, we also evaluated AR expression based on previous reports indicating that inhibiting the adaptive responses to hypertonicity by downregulating proteins (AR, betaine/y-amino-n-butyric acid transporter, and heat shock protein 70) and CsA caused apoptosis in the IM (22). Na-K-ATPase-α1 protein also plays an important role in the maintenance of cellular viability. In this study, the expression of AR and Na-K-ATPase-α1 in CsA-treated rat kidneys was markedly reduced compared with VH-treated rat kidneys. Therefore, we propose that CsA causes apoptotic cell death by inhibiting these vital enzymes, and this is functionally associated with impaired urine concentration in chronic CsA nephropathy.

In conclusion, we have demonstrated that long-term CsA treatment affects urine concentration by decreasing AQP and UTs, and induction of apoptosis in renal cells accounts, in part, for the CsA-induced urinary concentration defect. Our findings provide substantial evidence of the mechanism for CsA-induced impaired urine concentration.

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