

# Increased Water Intake Decreases Progression of Polycystic Kidney Disease in the PCK Rat

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Renal enlargement in polycystic kidney disease (PKD) is caused by the proliferation of mural epithelial cells and transepithelial fluid secretion into the cavities of innumerable cysts. Arginine vasopressin (AVP) stimulates the proliferation of human PKD cells *in vitro* via cAMP-dependent activation of the B-Raf/MEK (MAPK/ERK kinase/extracellular signal-regulated kinase (ERK) pathway. ERK activity is elevated in cells that line the cysts in animals with PKD, and AVP receptor antagonists reduce ERK activity and halt disease progression. For suppression of the effect of AVP physiologically, water intake was increased in PCK rats, a model of PKD, and the effect on renal morphology, cellular mechanism, and function was determined. The addition of 5% glucose in the drinking water increased fluid intake approximately 3.5-fold compared with rats that received tap water. In PCK rats, increased water intake for 10 wk reduced urinary AVP excretion (68.3%), and urine osmolality fell below 290 mOsmol/kg. High water intake was associated with reduced renal expression of AVP V2 receptors (41.0%), B-Raf (15.4%), phosphorylated ERK (38.1%), and proliferating cell nuclear antigen-positive renal cells (61.7%). High water intake reduced the kidney/body weight ratio 28.0% and improved renal function. Taken together, these data demonstrate that water intake that is sufficient to cause persistent water diuresis suppresses B-Raf/MEK/ERK activity and decreases cyst and renal volumes in PCK rats. It is suggested that limiting serum AVP levels by increased water intake may be beneficial to some patients with PKD.

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Mutations in the polycystic kidney disease (PKD) genes (*PKD1*, *PKD2*, and *PKHD1*) (1–4) disrupt intracellular  $\text{Ca}^{2+}$  regulation and, thereby, transform renal epithelial cells into hyperplastic fluid-filled cysts. Epithelial cells that are isolated from cysts of human autosomal dominant (ADPKD) and autosomal recessive (ARPKD) kidneys have a lower basal intracellular  $\text{Ca}^{2+}$  level compared with cells that are derived from normal renal tissue (5). cAMP accelerates the proliferation of ADPKD and ARPKD cells but has no mitogenic effect on normal renal tubule cells (6–8). The proliferative effect of cAMP in PKD cells is mediated by protein kinase A (PKA) stimulation of the MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway through the intermediacy of B-Raf, a kinase that phosphorylates and activates MEK. By contrast, B-Raf is normally repressed in normal human kidney cells, and cAMP inhibits ERK activity and cell proliferation (9). Sustained reduction of intracellular  $\text{Ca}^{2+}$  predisposes normal renal cells to cAMP-dependent stimulation of

the B-Raf/MEK/ERK pathway and cell proliferation (6). Conversely, restoration of intracellular  $\text{Ca}^{2+}$  concentration in cystic cells that are derived from human ADPKD and ARPKD kidneys rescues the normal antimitogenic response of cAMP (5).

Arginine vasopressin (AVP) is an important antidiuretic hormone that mediates its effect through the activation of vasopressin V2 receptors (AVPV2R) and the subsequent stimulation of adenylyl cyclase and synthesis of cAMP (10). Normally, urine is concentrated to an osmolality that is greater than plasma. Day-to-day maintenance of urine output depends on appropriate plasma AVP levels to regulate osmotic water reabsorption by distal tubules and collecting ducts. Relatively normal plasma levels of AVP may be sufficient to stimulate cyst epithelial cell growth and renal enlargement in patients with PKD. Some patients have an intrinsic defect in the capacity to concentrate urine maximally, potentially leading to even greater levels of plasma AVP than normal (11–13).

In this study, we determined whether suppression of plasma AVP levels by increased water intake would be sufficient to slow PKD progression in PCK rats. In this model of PKD, orthologous to human ARPKD, mutations in *Pkhd1* cause cysts to form in the collecting ducts (14). Many features of the disease in PCK rats resemble human ADPKD, such as focal development of cysts, although the pattern of inheritance is autosomal recessive. The results demonstrate that increased water intake

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that was sufficient to suppress the renal effects of AVP decreased MEK/ERK activity in the kidneys and slowed the progression of cystic disease in PCK rats.

## Materials and Methods

### PCK Rat Model

PCK rats that originally were derived from a strain of the Sprague-Dawley rats are maintained at the Education and Research Center of Animal Models for Human Diseases of Fujita Health University. These animals have been characterized previously (15). PCK rats and normal Sprague-Dawley (+/+; Charles River Japan Inc., Kanagawa, Japan) rats were allowed free access to water and food throughout the study. PCK and +/+ rats were randomly assigned to either the control group (normal tap water) or high water intake (HWI) group (water that contained 5% glucose) and treated as such from 4 to 14 wk of age. Twenty-four-hour samples were collected in metabolic cages after 13.5 wk to determine urine volume, water intake volume, and food consumption. At 14 wk of age, rats were anesthetized with pentobarbital sodium (Schering-Plough Corp., Kenilworth, NJ), and both kidneys were removed rapidly, causing exsanguination. Total kidney weight was measured, and then the left kidney was homogenized in lysis buffer to extract proteins and the right kidney was sectioned and immersed in 4% paraformaldehyde.

### Western Blot Analysis

Kidney lysate samples were prepared for immunoblot analysis (16,17). Contents of lysis buffer (TLB buffer) were 20 mmol/L Tris (pH 7.4), 137 mmol/L NaCl, 25 mmol/L  $\beta$ -glycerophosphate, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 2 mmol/L dithiothreitol, 1 mmol/L PMSF, 5  $\mu$ g/ml aprotinin, and 5  $\mu$ g/ml leupeptin with 1% Triton X-100. Membrane-blotted lysates (20  $\mu$ g protein/lane) were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked with 5% milk in TBS-T (20 mmol/L Tris-HCl [pH 8.0], 137 mmol/L NaCl, and 0.05% Tween 20) for 1 h at room temperature and then incubated overnight at 4°C in primary antibody diluted 1:2000 to 1:5000 in 5% milk-TBS-T. Membranes then were

washed three times with TBS-T and incubated with secondary antibody conjugated to horseradish peroxidase diluted 1:2000 to 1:5000 in 5% milk in TBS-T for 1 h at room temperature. The membranes were washed three times with TBS-T, and specific antibody signals were detected using an enhanced chemiluminescence system (ECL or ECL Advance Western Blotting Detection System; Amersham Life Sciences, Arlington Heights, IL). Images of the blots were captured, and the intensity of the protein bands was quantified using a CS Analyzer 2.0 with a CCD camera (ATTO Corp., Tokyo, Japan). Relative band intensity was compared with gender-matched +/+ kidneys from rats that received tap water (set to 1.0).

### Immunohistochemistry

Sections that were obtained from paraffin blocks were heated to 100°C for 15 min in 10 mM sodium citrate buffer (pH 6.0) to unmask antigens. Endogenous peroxidase activity was destroyed by incubating sections in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol for 30 min, and then the sections were incubated with primary antibody (1:3000) for phosphorylated ERK (P-ERK) or proliferating cell nuclear antigen (PCNA) overnight at 4°C. Sections were rinsed with PBS, incubated with biotinylated anti-mouse secondary antibody, and then incubated with streptavidin conjugated to peroxidase (Histofine; Nichirei Biosciences, Tokyo, Japan). Immune reaction products were developed using 3,3'-diaminobenzidine. Cystic surface area was measured from 10 random fields ( $\times 100$  magnification) of hematoxylin-eosin-stained sections of renal cortex ( $n = 3$  PCK rats). Cyst area ( $\mu^2$ /field) was measured by a naive observer using LUZEX FS software (Kideko Co. Ltd., Tokyo, Japan). ApoptTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA) was used to detect apoptotic cells. Apoptotic cells and P-ERK or PCNA-positive nuclei were counted from 500 to 600 cells ( $\times 400$  magnification) per three thin sections from either PCK or +/+ rat kidneys.

### Antibodies

Primary antibodies to B-Raf (F-7, SC-5284), ERK<sub>1,2</sub> (K-23, SC-94), P-ERK (E-4, SC-7383), and Raf-1 (C-12, SC-133) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) for immunoblot analysis.

Table 1. Effect of HWI on urine volume and osmolality and urinary AVP<sup>a</sup>

Genotype	Treatment	<i>n</i>	Water Intake (mL)	Urine Volume (mL)	Urine Osmolality (mOsmol/kg H <sub>2</sub> O)	Urinary AVP (pg/mg creatinine)
Male						
PCK	CONT	8	18 $\pm$ 1	18 $\pm$ 2	1088 $\pm$ 96	257 $\pm$ 22
PCK	HWI	10	68 $\pm$ 5 <sup>b</sup>	58 $\pm$ 5 <sup>b</sup>	232 $\pm$ 47 <sup>b</sup>	114 $\pm$ 46 <sup>b</sup>
+/+	CONT	8	10 $\pm$ 5	9 $\pm$ 1	1498 $\pm$ 130	160 $\pm$ 19
+/+	HWI	8	94 $\pm$ 26 <sup>c</sup>	91 $\pm$ 10 <sup>b</sup>	197 $\pm$ 73 <sup>b</sup>	88 $\pm$ 38
Female						
PCK	CONT	10	18 $\pm$ 3	16 $\pm$ 2	982 $\pm$ 74	435 $\pm$ 83
PCK	HWI	8	63 $\pm$ 9 <sup>b</sup>	49 $\pm$ 8 <sup>b</sup>	284 $\pm$ 52 <sup>b</sup>	111 $\pm$ 59 <sup>b</sup>
+/+	CONT	7	13 $\pm$ 5	12 $\pm$ 1	1559 $\pm$ 211	335 $\pm$ 49
+/+	HWI	10	107 $\pm$ 10 <sup>b</sup>	80 $\pm$ 11 <sup>b</sup>	131 $\pm$ 25 <sup>b</sup>	31 $\pm$ 4 <sup>b</sup>

<sup>a</sup>Addition of 5% glucose to drinking water increased water intake 3.5-fold in PCK rats and 8.2-fold in normal Sprague-Dawley (+/+) rats (composites of male and female rats). Rats drink more water that contained glucose because they have a preference for sweet fluid (33). Urine volume increased accordingly. High water intake (HWI) decreased urine osmolalities below 290 mOsmol/kg H<sub>2</sub>O. Urinary arginine vasopressin (AVP), an indicator of plasma AVP levels, decreased significantly in both PCK and +/+ rats with HWI. Statistical differences between HWI and control (CONT) rats (either +/+ or PCK) were determined by one-way ANOVA.

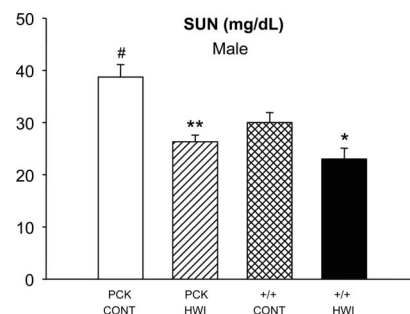
<sup>b</sup> $P < 0.01$ .

<sup>c</sup> $P < 0.05$ .

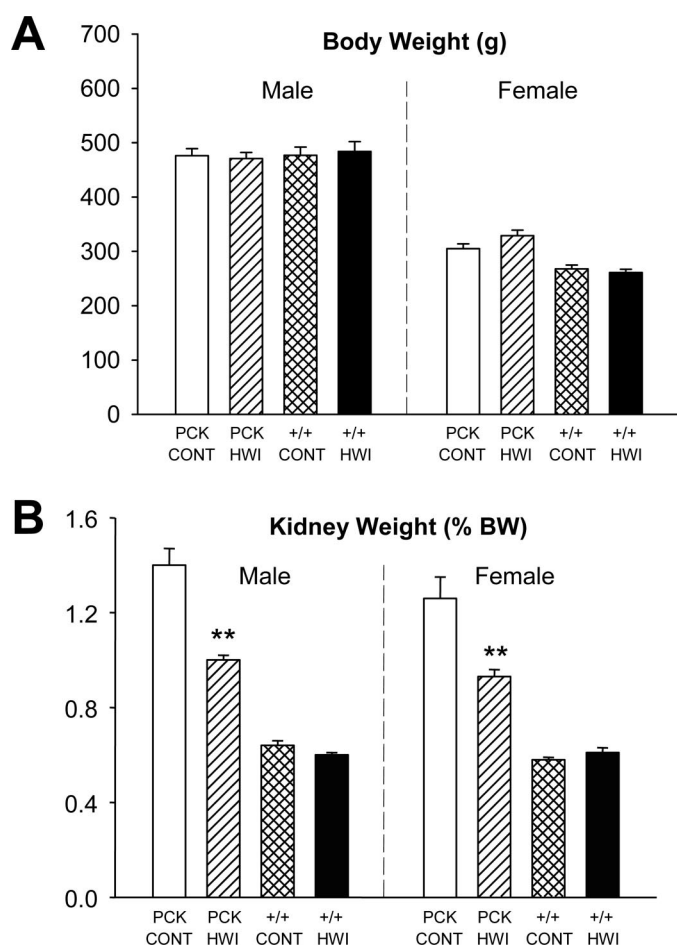
Secondary antibodies conjugated to horseradish peroxidase were goat anti-rabbit IgG (SC-2054) and rabbit anti-mouse IgG (SC-2055) from Santa Cruz Biotechnology. Primary antibodies to AVPV2R (AB1797P; Chemicon International), PCNA (P8825; Sigma Chemical, St. Louis, MO) and P-ERK (M5670; Sigma) were used for immunohistochemistry. Secondary antibody, conjugated to biotin, for immunohistochemistry studies was rabbit anti-mouse IgG+IgA+IgM (HISTOFINE 426032) obtained from Nichirei (Tokyo, Japan).

#### Measurement of Urine AVP, Creatinine and Osmolality, and Serum Urea Nitrogen

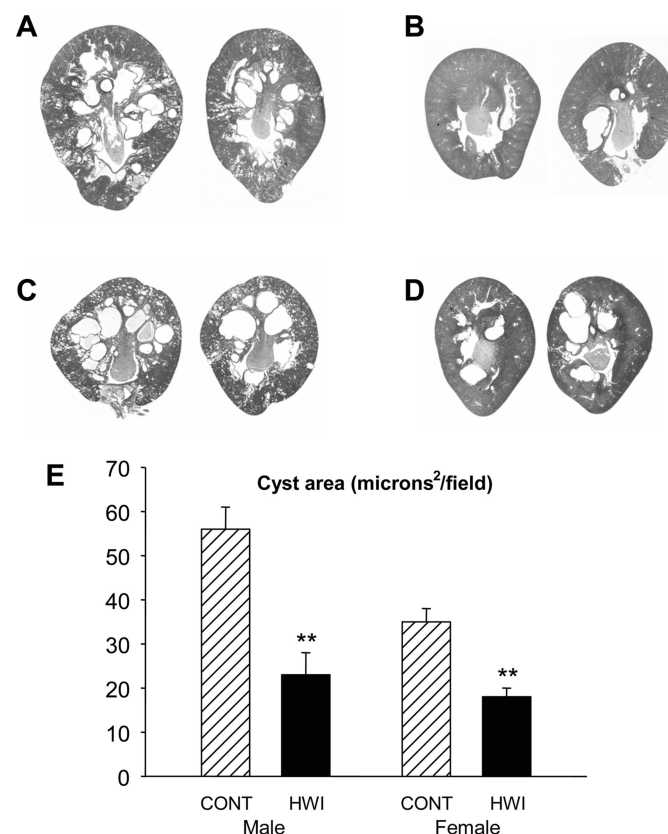
Urine AVP levels were measured with a Correlate-EIATM arg8–Vasopressin Enzyme Immunoassay Kit (cat. no. 900-017; Assay Designs, Inc., Ann Arbor, MI). Urine creatinine measurements were determined by a colorimetric microplate assay kit (Kit CR01; Oxford Biomedical Research, Inc., Oxford, MI). For an AVP concentration below the level of detection ( $<3.39$  pg/ml), a value of 3.38 pg/ml was used for the calculation of AVP per creatinine concentration (pg



**Figure 2.** Effect of HWI on serum urea nitrogen (SUN) in PCK and normal rats. Male PCK rats at 14 wk of age had an elevated SUN compared with age-matched  $+/+$  rats. HWI decreased SUN to control levels in PCK rats. The small but significant decrease in SUN in  $+/+$  rats with HWI is consistent with increased urea excretion as a result of elevated fluid flow in the distal nephron. Statistical differences between HWI and control (CONT) rats (either  $+/+$  or PCK) were determined by one-way ANOVA;  $*P < 0.05$ ,  $**P < 0.01$ .



**Figure 1.** Effect of high water intake (HWI) on kidney weight of PCK and normal rats. (A) The addition of 5% glucose to the drinking water did not affect body weight of either the PCK or normal Sprague-Dawley ( $+/+$ ) rats. (B) HWI caused a significant decrease in total kidney weight, represented as percentage of body weight in both male and female PCK rats. By contrast, HWI had no effect on total kidney weight of  $+/+$  rats. Statistical differences between HWI and control (CONT) rats (either  $+/+$  or PCK) were determined by one-way ANOVA;  $**P < 0.01$ .



**Figure 3.** Effect of HWI on renal cyst development in male and female PCK rats. Micrographs of representative kidney sections, stained with hematoxylin-eosin, were taken at the same magnification from a PCK male rat with normal water intake (A), a PCK male rat with HWI (B), a PCK female rat with normal water intake (C), and a PCK female rat with HWI (D). (E) Surface area of cysts (mean  $\pm$  SE) from representative sections of male and female PCK kidneys was measured by morphometric analysis. Increased water intake decreased cyst area 60 and 50% in male and female PCK kidneys, respectively. Comparison between HWI and CONT rats (male or female),  $**P < 0.01$ .



Table 2. Effect of HWI on male Han SPRD (Cy/+ ) rat<sup>a</sup>

Genotype	Treatment	n	Body Weight (g)	Total Kidney Weight (g)	Kidney Weight/Body Weight (%)
Cy/+	CONT	10	337 ± 17	7.15 ± 0.27 <sup>b</sup>	2.17 ± 0.16 <sup>b</sup>
Cy/+	HWI	11	327 ± 7	7.53 ± 0.40	2.31 ± 0.13 <sup>b</sup>
+/+	CONT	5	370 ± 11	2.88 ± 0.05	0.78 ± 0.03
+/+	HWI	4	339 ± 26	2.44 ± 0.20	0.72 ± 0.03

<sup>a</sup>Total kidney weight and kidney weight/body weight were increased in Cy/+ rats compared with normal Sprague-Dawley rats. Cy/+ male rats that were offered water that contained 5% sucrose increased fluid intake from 64 ± 5 ml (*n* = 6) to 155 ± 8 ml (*n* = 15). There was no effect of HWI on body weight, total kidney weight, or kidney weight/body weight.

<sup>b</sup>Comparison between Cy/+ and control (+/+) rats, *P* < 0.001, determined by unpaired *t* test. The lack of an effect of HWI on kidney weight in Cy/+ suggests that vasopressin may not contribute significantly to the progression of the proximal tubule cysts in kidneys of Cy/+ rats.

AVP/mg creatinine). Osmolality was determined with a freezing-point osmometer (Osmostat OM-6040; Kyoto Diichi Kagaku, Kyoto, Japan). Serum urea nitrogen (SUN) determinations were performed using a colorimetric assay kit using a Urease-Indophenol method (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

### Statistical Analyses

Data represent mean and SE. Statistical significance was determined by one-way ANOVA and Student-Newman-Keuls post test for multiple comparisons or unpaired *t* test for comparison between control and treated animals.

## Results

In PCK rats and normal Sprague-Dawley (+/+) rats that were offered water that contained 5% glucose, fluid intake increased approximately 3.5- and 8.2-fold, respectively, in comparison with rats that were allowed free access to tap water (Table 1). The rate of urine AVP excretion, an indicator of plasma AVP levels (18), was decreased 68.3%. Suppression of the renal effects of AVP decreases intracellular cAMP levels and reduces the water permeability of collecting ducts. We found that HWI increased urine volume and decreased urine osmolality to <290 mOsmol/kg H<sub>2</sub>O in both normal and cystic animals.

Body weight was unaffected by water intake in either the +/+ or the PCK rats (Figure 1A); however, kidney weight (% body weight) decreased 29.8 and 27.0% in PCK male and female rats, respectively (Figure 1B). In normal rats, changes in water intake had no effect on kidney weight. A slight elevation in SUN has been reported in PCK rats at 70 and 128 d of age (14). In this study, we found that the PCK male rats had a significant elevation in SUN at 14 wk (98 d) of age (Figure 2); in contrast, PCK female rats had normal SUN at this age (data not shown). Increased water intake for 10 wk decreased SUN from 38.7 to 26.3 mg/dl in the PCK male rats, a level that was similar to that of normal rats that drank increased water. HWI also caused a small but significant decrease in SUN in +/+ rats. Elevation in tubule fluid flow increases both facilitated and active urea secretion by inner medullary collecting ducts (19), thereby removing urea from the medullary interstitium and ultimately decreasing plasma urea levels. Hence, the reduction in SUN in the PCK rats with HWI was possibly due to a

combination of increased urine flow as well as reduced renal disease (Figure 1B).

Representative histologic sections revealed that HWI treatment diminished cystic area 54% in kidneys of PCK rats (59% in male and 49% in female rats; Figure 3), compared with rats that drank tap water. Therefore, increased fluid intake that was sufficient to cause water diuresis and reduce urinary excretion of AVP, ostensibly as a result of reduced plasma AVP levels, slowed the progression of cystic disease in PCK rats. By contrast, in Hans SPRD (Cy/+) rats, which develop cysts in the proximal tubules (a segment that lacks AVPV2R), HWI caused polyuria and decreased urine osmolality but failed to reduce the severity of cystic disease (Table 2). Therefore, polyuria and decreased urine osmolality *per se* do not account for the striking reduction in kidney size that is caused by HWI.

Previously, Gattone *et al.* (20) reported overexpression of mRNA for AVPV2R in kidneys of PCK rats and C57BL/6J-cpk/

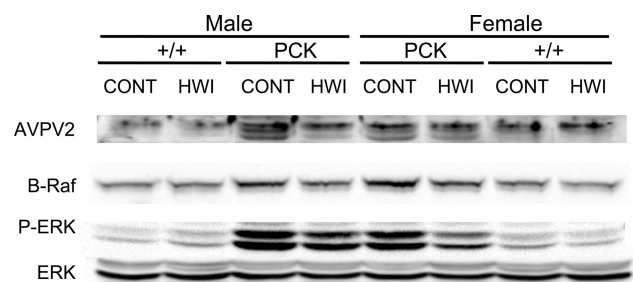


Figure 4. Effects of HWI on the expression of the vasopressin V2 receptor (AVPV2R) and renal activity of the B-Raf/MEK (MAPK/ERK kinase)/extracellular signal-regulated kinase (ERK) pathway in PCK and +/+ rats. Immunoblot analysis was used to compare expression levels of AVPV2R, B-Raf, phosphorylated ERK (P-ERK), and total ERK in kidneys of PCK and +/+ rats that drank normal water (CONT) and 5% glucose (HWI). AVPV2R, B-Raf (95 kD), and P-ERK were elevated in the PCK kidneys compared with control kidneys. HWI decreased levels of AVPV2R, B-Raf, and P-ERK in PCK kidneys. Total ERK levels were similar between +/+ and PCK kidneys and were unaffected by HWI. A summary of the effects of HWI on the relative expression of AVPV2R, B-Raf, P-ERK, and ERK is presented in Table 3.

cpk mice, another model for recessive PKD (12). In this study, we confirmed that protein expression of AVPV2R was elevated in PCK kidneys compared with control kidneys (Figure 4). Therefore, overexpression of AVPV2R in the epithelial cells of collecting duct cysts could contribute to persistently high levels of cAMP (21). It is interesting that HWI normalized AVPV2R expression in the PCK kidneys (Table 3).

The levels of a 95-kD isoform of B-Raf and P-ERK were elevated in kidneys of male and female PCK rats compared with gender-matched +/+ rats (Figure 4, Table 3), consistent with previous findings (22). HWI decreased the level of P-ERK 33% in male and 41% in female rats (composite average 38.1%) but had no effect on P-ERK levels in +/+ rats. Furthermore, increased water intake reduced B-Raf abundance 19.7% in PCK male kidneys. There also was a small reduction (11.1%) in B-Raf abundance in PCK female kidneys; however, this difference was not statistically significant. Immunohistochemical analysis revealed that HWI decreased the number of cells that stained positive for PCNA, a proliferation marker, by approximately 60% (male and female; Figure 5). The number of cells (% of cells per section) that stained positive for P-ERK was reduced 52% in PCK male rats and 44% in PCK female rats (Figure 6), confirming the observations made by immunoblot analysis. Moreover, the number of apoptotic cells (%) in the kidneys of PCK rats was elevated compared with +/+ rats (Figure 7). HWI reduced the number of apoptotic cells in both male and female PCK kidneys. Taken together, these data show that the renal protective effects of increased water intake in PKD likely were mediated by reductions in AVPV2R expression, B-Raf abundance, and activity of the B-Raf/MEK/ERK pathway.

### Discussion

We determined whether reduction of plasma AVP levels by increased water intake slows the progression of renal cystic disease in PCK rats. The central observations are that increased water intake (1) decreased urine osmolality; (2) reduced renal

expression of AVPV2 receptors, B-Raf, P-ERK, and PCNA-positive renal cells; (3) decreased the size of renal cysts and total kidneys; and (4) improved renal function. Therefore, physiologic inhibition of AVP by simply increasing fluid intake was sufficient to suppress cAMP-dependent B-Raf/MEK/ERK activity and proliferation of the cyst-lining cells to slow renal enlargement in the PCK rat.

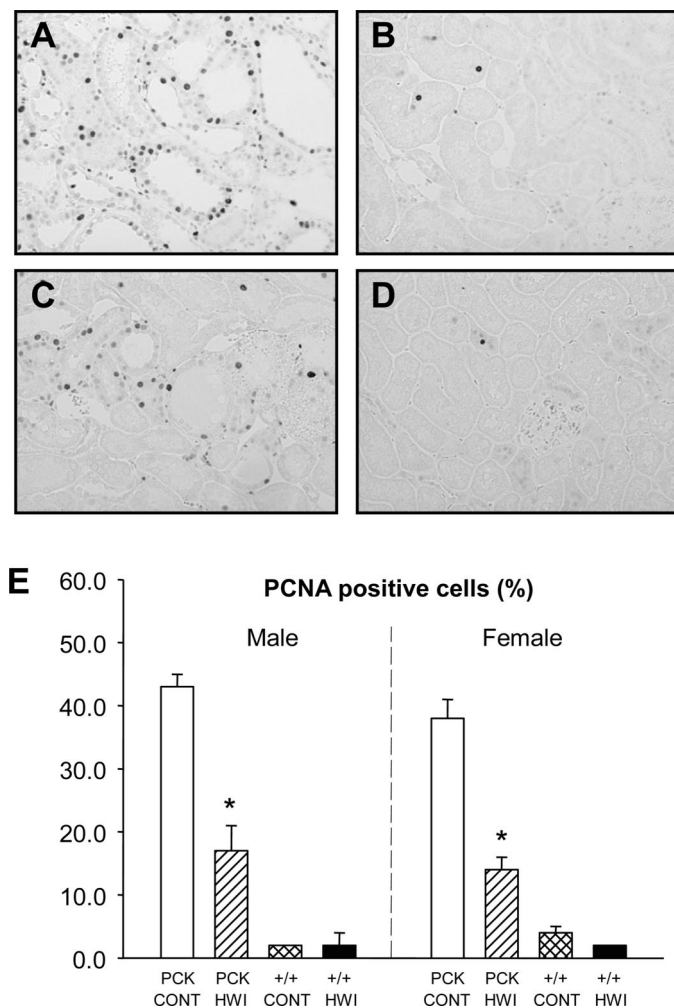
ARPKD is characterized as a disease that affects predominantly the collecting ducts. In ADPKD, cysts can arise from glomeruli and most segments of the nephron; however, several studies have demonstrated that the majority of ADPKD cysts are derived from collecting ducts and distal tubules. Verani and Silva (23) found that 69% of human ADPKD cysts (ranging in size from 0.1 to 6 cm in diameter) stained *Arachis hypogaea*, a lectin that is specific for collecting ducts. By contrast, cysts of proximal tubule origin could not be identified using the lectin *Lotus tetragonolobus*. Previously, we found that the majority of cells that were cultured from a mixed population of surface cysts from an ADPKD kidney stained *Arachis hypogaea* and *Dolichos biflorus* (another marker for collecting duct and distal nephron) (9). Relatively few cells stained *Lotus tetragonolobus*. Immunocytochemical studies for the localization of aquaporin-1 and gp330, a proximal tubule marker, in human ADPKD kidneys suggest that only 30 to 44% of >1000 cysts examined were derived from proximal tubules (24,25). More recently, 70 to 80% of the cysts in the renal cortex of PKD2<sup>WS25/-</sup> mice, a disease homologous to human ADPKD, were identified as being of either distal tubule or collecting duct origin (26). The morphology of these cysts was indistinguishable from that of human ADPKD cysts. Therefore, both ADPKD and ARPKD cysts seem to be derived largely from collecting ducts and distal tubules.

The initiation of renal cyst formation is caused by mutations in PKD genes (*PKD1*, *PKD2*, and *PKDH1*), whereas AVP mediated by intracellular cAMP seems to be central for cyst progression in both ADPKD and ARPKD (20,27,28). The underlying

Table 3. Effects of HWI on AVPV2 expression and B-Raf/MEK/ERK activity in PCK and +/+ kidneys<sup>a</sup>

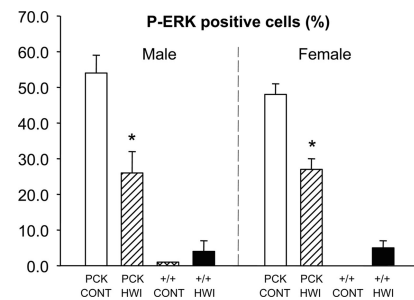
Genotype	Treatment	AVPV2R	B-Raf	P-ERK	ERK
Male					
PCK	CONT	1.48 ± 0.14 <sup>b</sup>	1.47 ± 0.1 <sup>c</sup>	5.70 ± 0.06 <sup>c</sup>	1.13 ± 0.03
PCK	HWI	0.79 ± 0.09	1.18 ± 0.1 <sup>d</sup>	3.80 ± 0.42 <sup>e</sup>	1.07 ± 0.04
+/+	CONT	1.00 ± 0.00	1.00 ± 0.0	1.00 ± 0.00	1.00 ± 0.00
+/+	HWI	0.65 ± 0.07 <sup>d</sup>	1.00 ± 0.0	1.14 ± 0.11	1.09 ± 0.03
Female					
PCK	CONT	2.11 ± 0.25	1.44 ± 0.0 <sup>c</sup>	4.47 ± 0.53 <sup>b</sup>	0.99 ± 0.07
PCK	HWI	1.33 ± 0.04 <sup>e</sup>	1.28 ± 0.1	2.65 ± 0.17 <sup>e</sup>	0.99 ± 0.05
+/+	CONT	1.00 ± 0.00	1.00 ± 0.0	1.20 ± 0.05	1.00 ± 0.00
+/+	HWI	0.90 ± 0.20	1.01 ± 0.1	0.94 ± 0.01	0.99 ± 0.03

<sup>a</sup>Summary of the fold changes in renal expression of AVPV2R, 95-kD isoform of B-Raf, P-ERK, and total ERK in PCK and +/+ rats (male and female) treated with normal water intake (CONT) or HWI. These data were obtained from animals listed in Table 1. MEK, MAPK/ERK kinase; AVPV2R, vasopressin V2 receptor; P-ERK, phosphorylated extracellular signal-regulated kinase.  
 Comparisons between PCK and +/+ rats, <sup>b</sup>*P* < 0.05, <sup>c</sup>*P* < 0.01; comparison between HWI and control (CONT) rats (either +/+ or PCK), <sup>d</sup>*P* < 0.01, <sup>e</sup>*P* < 0.05.

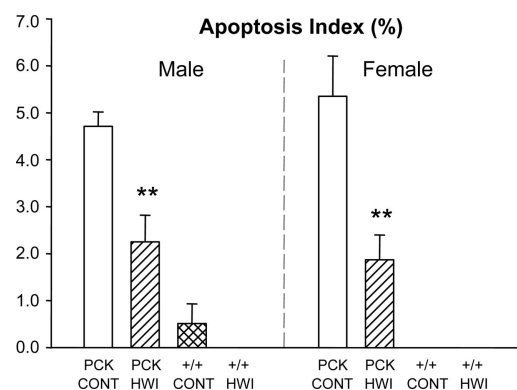


**Figure 5.** Effect of HWI on cell proliferation in PCK and +/+ kidneys. Representative kidney sections from PCK and +/+ rats (male and female) that were treated with normal water intake (CONT) or HWI. Sections were stained with an antibody to proliferating cell nuclear antigen (PCNA), a proliferation marker. Micrographs show PCNA-positive nuclei in cells of kidney sections from PCK-CONT (A), PCK-HWI (B), +/+CONT (C), and +/+HWI rats (D). (E) Number of cells (percentage of total) that were positive for PCNA. Kidney sections of +/+ rats had very few PCNA-positive cells. By contrast, approximately 40% of the cells in the PCK kidney stained positive for PCNA. Increased water intake decreased the percentage of proliferating cells in the kidneys of PCK rats. Comparison between HWI- and CONT-treated PCK rats (male or female), \* $P < 0.05$ , \*\* $P < 0.01$ .

basis for the cAMP-mitogenic phenotype in PKD seems to be due to dysregulation of intracellular  $\text{Ca}^{2+}$  secondary to mutations in the PKD genes (5,6). ADPKD and ADPKD cyst-derived cells have  $[\text{Ca}^{2+}]_i$  that is lower than tubule cells derived from noncystic regions of ADPKD kidneys or normal human kidneys. Therefore, cyst-lining cells are associated with lower basal  $[\text{Ca}^{2+}]_i$  and cAMP-dependent activation of B-Raf/MEK/ERK and cell proliferation. The cellular pathway for  $\text{Ca}^{2+}$  regulation of the cAMP-mitogenic phenotype remains to be established,



**Figure 6.** Effect of HWI on P-ERK-positive cells in PCK and +/+ kidneys. Number of cells (percentage of total) positive for P-ERK was measured from micrographs of kidney sections of PCK and +/+ rats (male and female) that were treated with CONT or HWI. Male and female PCK rats on HWI had a significant reduction in the number of cells that stained positive for P-ERK, indicating that the renal MEK/ERK activity was reduced by increased water intake. Comparison between HWI- and CONT-treated PCK rats (male or female), \* $P < 0.05$ .



**Figure 7.** Effect of HWI on cellular apoptosis in PCK and +/+ kidneys. The number of apoptotic cells (percentage of total) in kidney sections of CONT- and HWI-treated PCK and +/+ rats was determined using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method. There was a striking elevation in the percentage of apoptotic cells in the PCK kidneys compared with +/+ kidneys. Increased water intake (HWI) decreased the percentage of apoptotic cells 52 and 65% in the male and female PCK rats, respectively. Comparison between HWI- and CONT-treated PCK rats (male or female), \*\* $P < 0.01$ .

but the phosphatidylinositol 3-kinase/Akt pathway seems to be involved (5,6). Basal Akt activity was found to be reduced in ADPKD cells compared with normal human kidney cells, and agents that increased  $[\text{Ca}^{2+}]_i$  also stimulated Akt and blocked cAMP-dependent B-Raf activation of the MEK/ERK pathway. Recently, Boca *et al.* (29) showed that the expression of full-length polycystin-1 in renal cells increased the activity of phosphatidylinositol 3-kinase/Akt signaling pathway, supporting a role for this pathway in cystogenesis.

Normal plasma AVP levels which respond to fluctuations in extracellular fluid osmolality are likely to maintain renal intracellular cAMP at levels that activate the MEK/ERK pathway and proliferation of PKD cystic epithelial cells, promoting cyst



and kidney enlargement. Several rodent models of PKD, including the PCK rat, have been reported to have elevated renal cAMP levels (12,21). Gattone and Torres (reviewed by Torres [13]) found that novel AVPV2R antagonists OPC-31261 and OPC-41061 (Tolvaptan; Otsuka Pharmaceutical, Tokyo, Japan) reduced renal cAMP levels and halted PKD progression in four different animal models, including PKD2<sup>WS25/-</sup> mice, as well as PCK rats (13,20,28). Treatment with OPC-41061 caused a concomitant reduction in renal levels of B-Raf and P-ERK (22). On the basis of these studies, the capacity for OPC-41061 to reduce PKD progression is being examined in clinical trials (30).

As with other models of PKD, PCK rats are characterized by tubular epithelial cell hyperplasia and increased rates of apoptosis, hallmarks of human PKD (31). We used immunohistochemistry to evaluate the effect of HWI on the proliferation and apoptosis of cyst-lining cells in PCK rat kidneys. The number of cells that stained positive for PCNA, a mitotic indicator, was measured in thin kidney sections from PCK rats. Consistent with a reduction in cyst area and kidney weight, HWI decreased cell proliferation of cyst epithelial cells (PCNA-positive cells decreased approximately 60%; Figure 5E). Inhibition of cell proliferation was associated with decreased levels of B-Raf and P-ERK, suggesting that increased water intake diminished the renal activity of the MEK/ERK pathway. Moreover, the number of apoptotic cells in the cyst epithelium of water-loaded PCK rats was significantly reduced compared with control-treated PCK rats (Figure 7). Male PCK rats at 14 wk of age were found to have increased SUN. Elevated SUN was corrected by increased water intake, demonstrating that suppression of plasma AVP decreased renal cyst formation and improved renal function.

In a retrospective analysis of patients with chronic renal insufficiency, Hebert *et al.* (32) found that high urine volume was associated with a faster decline of GFR. The authors suggested that high urine volume and low urine osmolality may be risk factors for the progression of renal disease, including PKD. Conversely, an inability to concentrate urine as kidney function declines could account for the high urine volume in their study. Therefore, it is impossible to establish a cause–effect relationship between water intake and the decline in GFR on the basis of their results. In this study, we found that high fluid intake slowed the progression of PKD and significantly improved renal function in the PCK rats. On the basis of these results, we suggest that increased water intake may be beneficial to patients with PKD.

## Conclusion

This study provides evidence for a central role of AVP in cAMP-dependent activation of the B-Raf/MEK/ERK pathway in cyst-lining cells and renal cyst enlargement. Increased water intake that was sufficient to cause a sustained reduction in plasma AVP levels decreased AVPV2R expression, reduced ERK activity and renal cell proliferation, and slowed PKD progression in PCK rats. We propose that sustained hydration by increased water intake may be beneficial to some patients with PKD by limiting the detrimental effects of AVP on renal cyst growth.

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