Effectiveness of Vasopressin V2 Receptor Antagonists OPC-31260 and OPC-41061 on Polycystic Kidney Disease Development in the PCK Rat

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cAMP plays a major role in cystogenesis. Recent *in vitro* studies suggested that cAMP stimulates B-Raf/ERK activation and proliferation of cyst-derived cells in a Ca^{2+} inhibitable, Ras-dependent manner. OPC-31260, a vasopressin V2 receptor (VPV2) antagonist, was shown to lower renal cAMP and inhibit renal disease development and progression in models orthologous to human cystic diseases. Here it is shown that OPC-41061, an antagonist chosen for its potency and selectivity for human VPV2, is effective in PCK rats. PCK kidneys have increased Ras-GTP and phosphorylated ERK levels and 95-kD/68-kD B-Raf ratios, changes that are corrected by the administration of OPC-31260 or OPC-41061. These results support the importance of cAMP in the pathogenesis of polycystic kidney disease, confirm the effectiveness of a VPV2 antagonist to be used in clinical trials for this disease, and suggest that OPC-31260 and OPC-41061 inhibit Ras/mitogen-activated protein kinase signaling in polycystic kidneys.

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Evidence that cAMP plays a major role in cystogenesis has accumulated in recent years (1). cAMP stimulates cyst formation by promoting chloride-driven fluid secretion. Recent *in vitro* studies suggested that cAMP also stimulates B-Raf and ERK activation and proliferation of cyst-derived cells in a Ca^{2+}-inhibitable and Ras-dependent manner (2). The increased cAMP levels in polycystic kidneys (3–5), the origin of autosomal recessive (ARPKD) and dominant polycystic kidney disease (ADPKD) cysts predominantly from collecting ducts (6), and the V2 receptor-mediated vasopressin effect on adenylyl cyclase in principal cells (7) provided the rationale for treating PKD with vasopressin V2 receptor (VPV2) antagonists (4,5,8). The administration of the VPV2 antagonist OPC-31260 was shown to lower renal cAMP and inhibit disease development and progression in models of ARPKD (PCK rat), ADPKD ($Pkd2^{WS25/-}$ mouse), and adolescent nephronophthisis (pcy mouse). The goals of the present study were to determine whether OPC-41061 (tolvaptan), an OPC-31260–related VPV2 antagonist currently used in clinical trials for water-retaining states (9), also inhibits the development of PKD. In addition, we wanted to determine whether the Ras/mitogen-activated protein kinase pathway that was proposed recently to mediate the proliferative response to cAMP *in vitro* is activated *in vivo* and inhibited by OPC-31260 and OPC-41061.

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Materials and Methods

Experimental Animals and Study Design

The Institutional Animal Care and Utilization Committee approved the use of PCK rats on a Sprague-Dawley strain (maintained at the animal facilities of the Mayo Clinic, Rochester, MN) and experimental protocols. Rats were divided into control and three treatment groups at 3 wk of age ($n = 80$; 10 animals per treatment group and gender). Drugs were added to ground rodent chow (Purina Mills, Richmond, IN) at the specified concentrations. Animals were killed at 10 wk of age.

Experimental Protocol

Twenty-four-hour urine outputs in metabolic cages and tail-cuff BP were obtained before the rats were killed. The animals were weighed and anesthetized with ketamine 60 mg/kg and xylazine 10 mg/kg, intraperitoneally. Blood was obtained by cardiac puncture for determination of plasma electrolytes and blood urea nitrogen (BUN) levels. The right kidney and part of the liver were placed into preweighed vials that contained 10% formaldehyde in phosphate buffer (pH 7.4). The tissues were embedded in paraffin for histologic studies. The left kidneys were frozen immediately in liquid nitrogen for determination of cAMP and protein studies.

Histomorphometric Analysis and Immunohistology

Four-micron transverse tissue sections, including cortex, medulla, and papilla, were stained with hematoxylin-eosin to measure cyst volumes and with picrosirius red stain for collagen to measure fibrosis volumes (4,5). Image analysis procedures were performed with MetaMorph software (Universal Imaging, West Chester, PA). Immunostaining for proliferating cell nuclear antigen (PCNA), using a monoclonal IgG2a antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and the transferase-mediated dUTP nick-end labeling assay, using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA), were used to measure proliferation and apoptosis. Fields (×400) of renal medulla were randomly selected, and between 500 and 1000 tubular epithelial cell nuclei per tissue section

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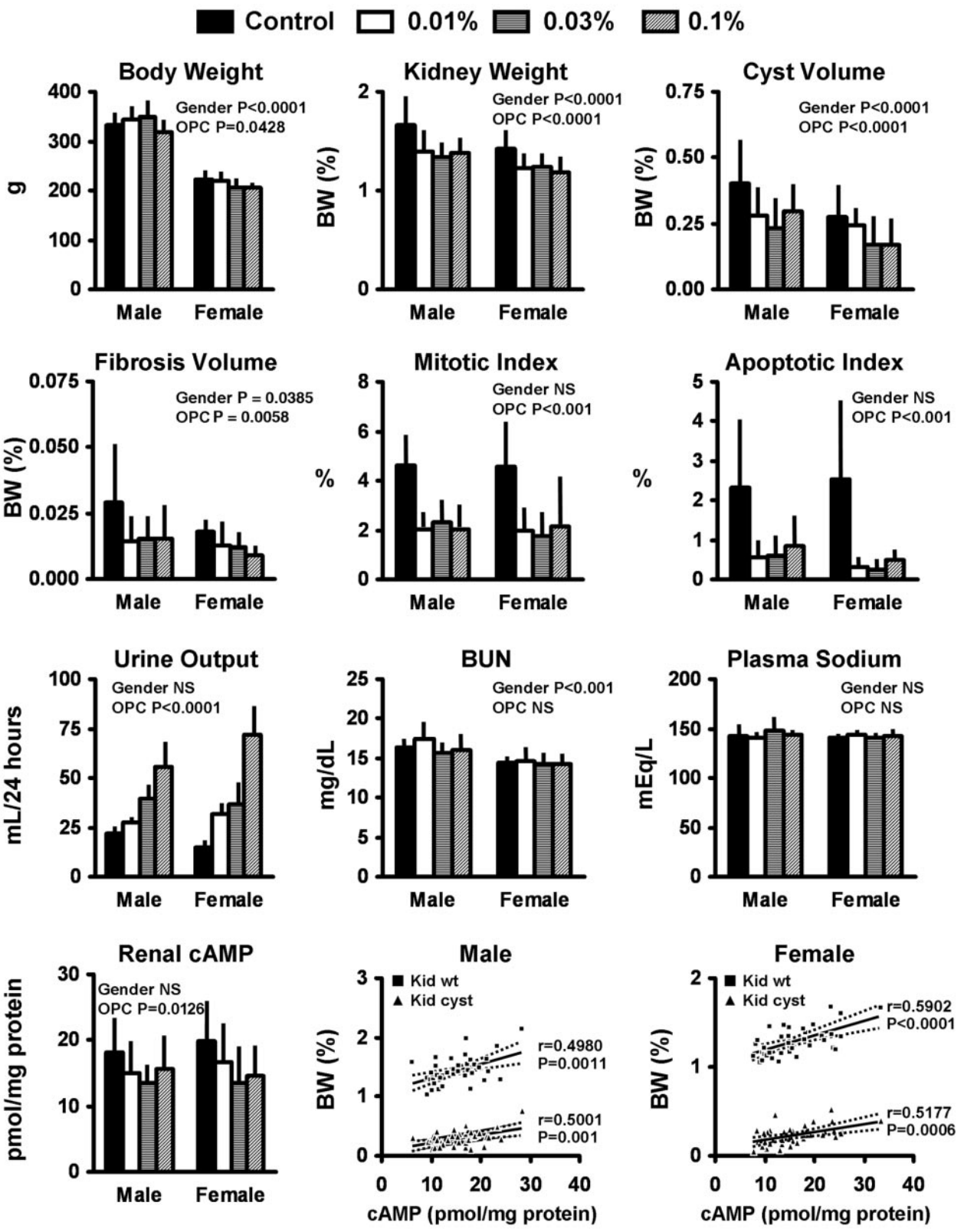

Figure 1. Effect of OPC-41061 treatment at the specified concentrations on the development of polycystic kidney disease, urine output, and renal cAMP concentration in PCK rats (n = 80;10 rats per treatment and gender group). Data are expressed as means ± SD. Two-way ANOVA was used to determine gender and OPC-41061 effects. Correlations between renal cAMP concentrations and kidney weights or cyst volumes at killing in male and female animals.

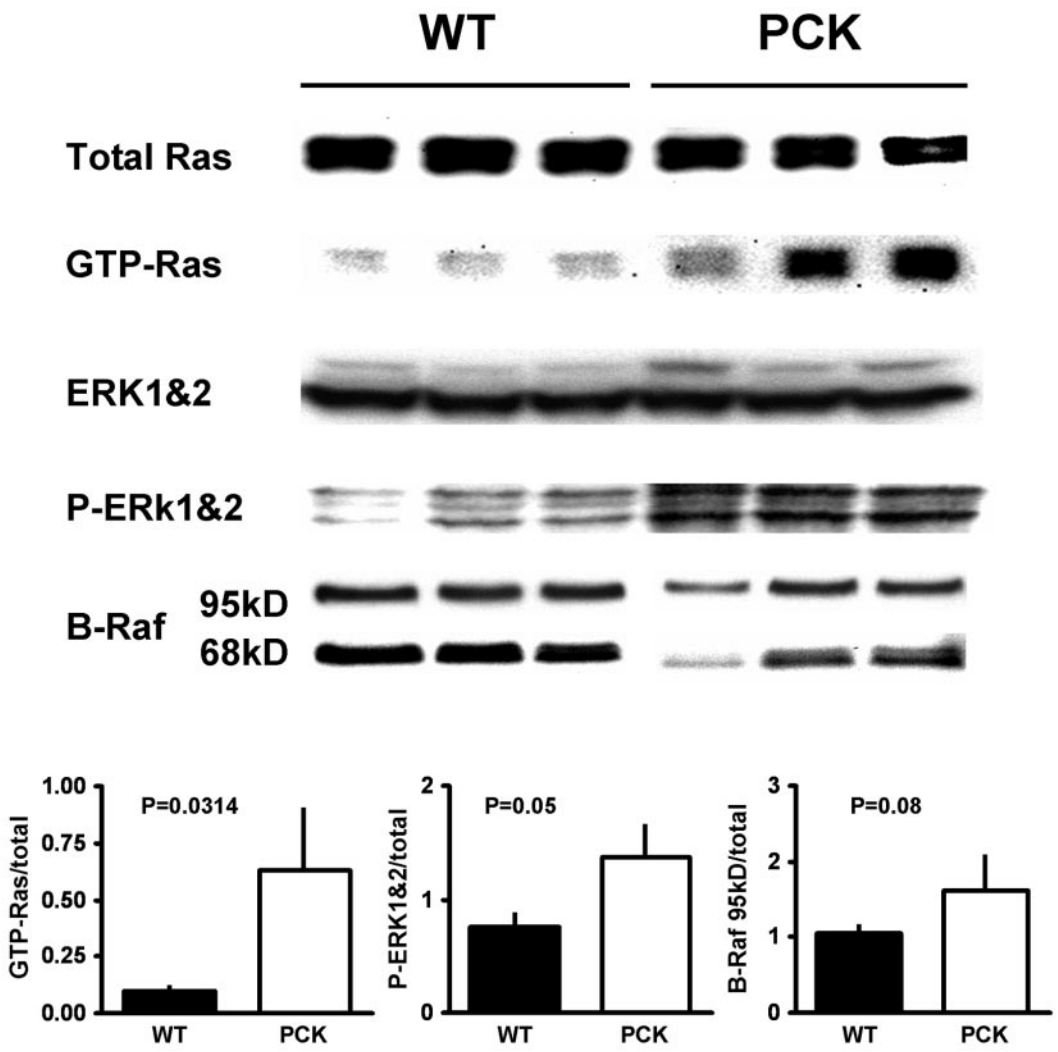

Figure 2. Total and GTP-bound Ras, total and phosphorylated ERK1/2, and 95- and 68-kD B-Raf levels showing activation of Ras and ERK1/2 signaling and an increase in 95-kD B-Raf level relative to 68-kD B-Raf in PCK ($n = 3$) compared with wild-type ($n = 3$) rats.

were counted. The mitotic and apoptotic indices were calculated as the percentage of cells positive for proliferating cell nuclear antigen or the transferase-mediated dUTP nick-end labeling assay.

cAMP Content of Whole Kidneys

The kidneys were ground to fine powder under liquid nitrogen in a stainless steel mortar and homogenized in 10 volumes of cold 5% TCA in a glass-Teflon tissue grinder. After centrifugation at 600 × *g* for 10 min, the supernatants were extracted with 3 volumes of water-saturated ether. After the aqueous extracts were dried, the reconstituted samples were processed without acetylation using an enzyme immunoassay kit (Sigma-Aldrich, St. Louis MO). The results were expressed in pmol/mg of protein (3).

Western Blot Analysis and Affinity Assay for Ras Activation

Total Ras, total ERK1/2, phosphorylated ERK1/2, and B-Raf were measured by Western blot analysis using anti-Ras (F132), -ERK1/2 (C16), -phosphorylated ERK1/2 (E4), and –B-Raf (C19) antibodies (Santa Cruz Biotechnology). The EZ-Detect Ras activation kit (Pierce Biotechnology, Rockford, IL) was used to measure Ras activation. This method uses a GST-fusion protein that contains the Ras-binding domain of Raf1 to specifically pull down active Ras. The pulled-down active Ras was detected by Western blot analysis using an anti-Ras antibody.

Statistical Analyses

Comparisons between groups were made using one-way or two-way ANOVA with least significant difference comparisons of the means or *t* test as appropriate. Data are expressed as means ± SD.

Results and Discussion

The PCK rat is a model of human ARPKD caused by a splicing mutation (IVS35–2A→T) that leads to a frameshift in the ortholog *Pkhd1* as a result of skipping of exon 36 (10). This model is characterized by progressive cystogenesis and impairment of renal function. It exhibits a urine concentration defect, despite increased renal levels of cAMP and expression of aquaporin-2 and the VPV2. We previously showed that OPC-31260

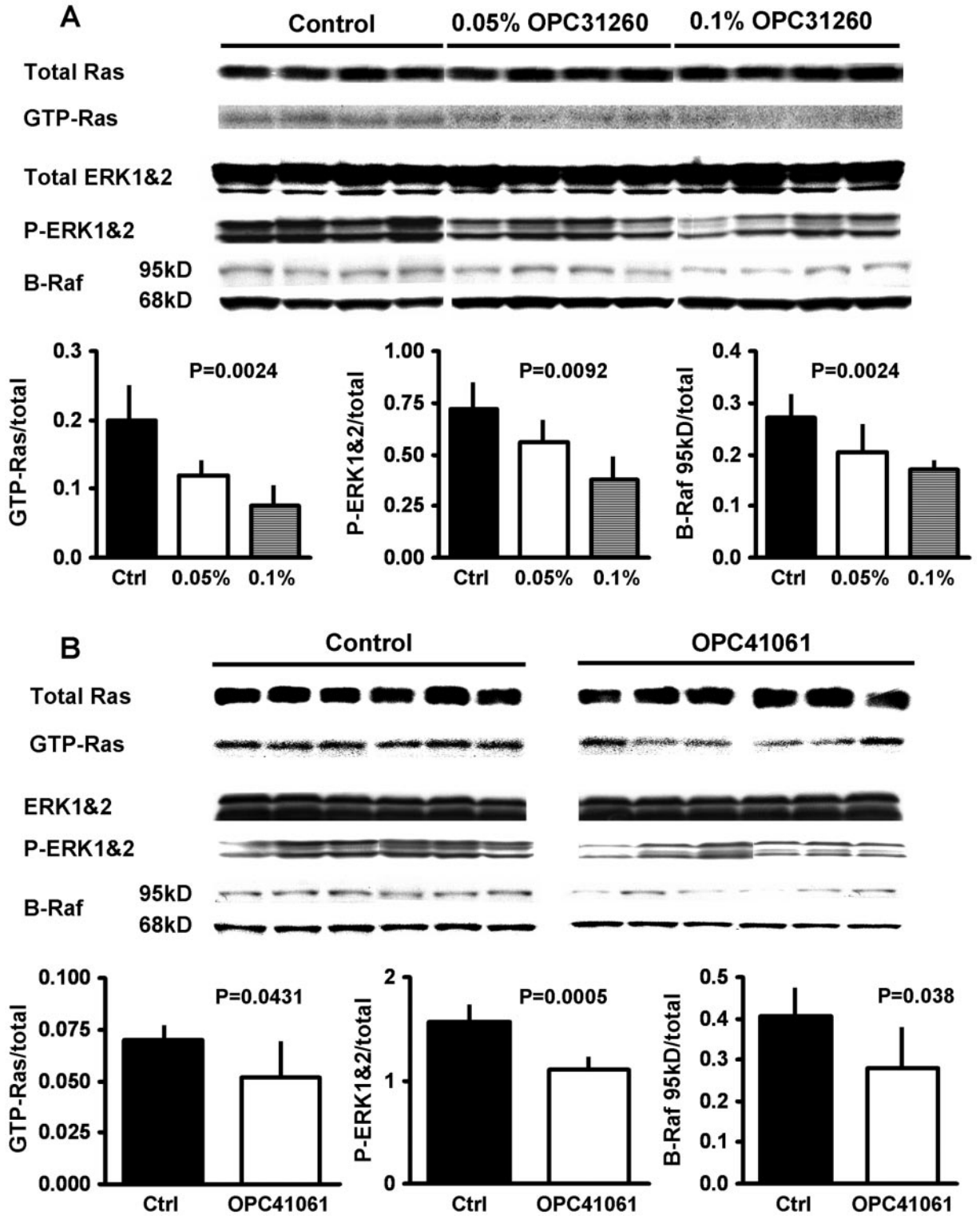

Figure 3. Total and GTP-bound Ras, total and phosphorylated ERK1/2, and 95- and 68-kD B-Raf levels in PCK rats that were treated with 0.05% ($n = 4$) or 0.1% ($n = 4$) OPC-31260 compared with untreated control animals ($n = 4$) (A) or with 0.1% OPC-41061 ($n = 6$) compared with untreated control animals ($n = 6$) (B). OPC-31260 and OPC-41061 caused inhibition of Ras and ERK1/2 signaling and a reduction in 95-kD B-Raf relative to 68-kD B-Raf levels.

administration markedly reduces the renal accumulation of cAMP and inhibits disease development and progression (4). Similar observations have been made in three additional models of PKD, the $Pkd2^{WS25/-}$ mouse (orthologous to human ADPKD), the pcy mouse (orthologous to human adolescent nephronophthisis), and the cpk mouse (a model of rapidly

progressive ARPKD without a known human homologue) (4,5,7).

The potency and selectivity of vasopressin receptor antagonists is species dependent and varies between humans and rodents. OPC-41061 was developed through a series of structural conversions of OPC-31260 and selected for use in clinical trials because it is a more potent and selective human VPV2 antagonist than OPC-31260 (11). Despite the similarities between OPC-31260 and OPC-41061 in regards to chemical structure and mechanism of action, experiments were needed to confirm that OPC-41061 is also capable of inhibiting the development of PKD. Here we show that the administration of OPC-41061 between 3 and 10 wk of age lowers renal cAMP and exerts a protective effect on the development of PKD in PCK rats. This is reflected by significantly lower kidney weights, cyst and fibrosis volumes, and mitotic and apoptotic indices (Figure 1). Plasma blood urea nitrogen levels were normal and not significantly different between the groups. A statistically significant positive correlation was detected between the tissue levels of cAMP and the severity of PKD (Figure 1), consistent with a causal relationship between the two. Most of the protective effect of OPC-41061 was detectable with the lowest dose used in the study, a dose that caused only modest aquaresis. Limited additional protection was achieved by a 10-fold increase in the dose. Possibly, antagonism for the vasopressin V1a receptor and inhibition of intracellular calcium release at high concentrations of OPC-41061 may limit the potential benefit of higher doses of this compound (12). The administration of OPC-41061 was well tolerated, and no significant effect on plasma sodium was detected. Systolic BP were similar in the control (male, 124 ± 4; female, 125 ± 4 mmHg) and treated (male, 126 ± 3, 125 ± 3, 126 ± 3; female, 122 ± 4, 125 ± 3, 125 ± 4 mmHg) rats. As previously observed with OPC-31260, the administration of OPC-41061 did not inhibit the development of fibropolycystic liver disease, consistent with the absence of VPV2 in the liver.

A recent study showed that prolonged (at least 3 h) incubation of principal cells in a low-calcium medium or in the presence of calcium channel blockers allows cAMP activation of a B-Raf/ERK pathway and stimulation of cell proliferation in a PKA-, Src-, and Ras-dependent manner (2). The effect of calcium restriction was thought to be due to PI3K and Akt inhibition (replicated by PI3K or Akt inhibitors). Prolonged calcium restriction was deemed necessary to increase B-Raf levels (via adjustments in the synthesis and/or turnover rate). Because transfection of principal cells with a dominant negative polycystin-1 C-tail construct induced cAMP-dependent B-Raf and ERK activation, inhibitable by a calcium ionophore, the authors concluded that the proliferative cellular phenotype of PKD is linked directly to abnormalities in intracellular calcium homeostasis.

To determine whether these mechanisms described *in vitro* are operational *in vivo*, we measured Ras and ERK activation and B-Raf levels in kidneys from wild-type Sprague-Dawley and PCK rats. We found increased levels of Ras-GTP and phosphorylated ERK in the polycystic compared with the control kidneys (Figure 2). Total Ras and ERK were similar. Contrary to observations in cultured cells, we did not find increased levels of B-Raf *in vivo*. However, the ratio of the 95- and 62-kD B-Raf isoforms was somewhat higher in the PCK kidneys. This is consistent with recent reports indicating that the effect of cAMP on ERK activation and cell proliferation depends on the relative expression of these two splicing variants (95 and 62 kD) (13,14). cAMP stimulates cell proliferation in cells that express mostly the 95-kD isoform, whereas it is inhibitory in cells that do not express B-Raf or express mostly the 62-kD isoform. The relative expression of these two isoforms depends not only on cell type but also on cellular density (predominantly 95-kD isoform in subconfluent cells and 62-kD isoform in confluent cells) and may work as a molecular switch to activate and inhibit ERK and cell proliferation (15).

To determine whether the protective effects of OPC-31260 and OPC-41061 could be mediated, at least in part, by the inhibition of this Ras/B-Raf/ERK pathway, we measured the expression pattern and activation of Ras, B-Raf, and ERK in control and treated animals. Both compounds caused a significant inhibition of Ras and ERK, without affecting the total protein levels (Figure 3). These changes were accompanied in both cases by a significant reduction in the ratio of the 95- and 62-kD isoforms.

In summary, these results support the importance of cAMP in the pathogenesis of PKD, demonstrate the effectiveness in the PCK rat of a VPV2 antagonist to be used in ADPKD clinical trials (OPC-41061), and suggest that OPC-31260 and OPC-41061 inhibit Ras/MAPK signaling in polycystic kidneys.

Acknowledgments

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See related editorial, "V2 Receptor Antagonists in Cystic Kidney Diseases: An Exciting Step towards a Practical Treatment," on pages 838–839.