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Calcium channel inhibition accelerates polycystic kidney disease progression in the Cy/+ rat

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In polycystic kidney disease, abnormal epithelial cell proliferation is the main factor leading to cyst formation and kidney enlargement. Cyclic AMP (cAMP) is mitogenic in cystic but antimitogenic in normal human kidney cells, which is due to reduced steady-state intracellular calcium levels in cystic compared to the normal cells. Inhibition of intracellular calcium entry with channel blockers, such as verapamil, induced cAMP-dependent cell proliferation in normal renal cells. To determine if calcium channel blockers have a similar effect on cell proliferation *in vivo*, Cy/+ rats, a model of dominant polycystic kidney disease, were treated with verapamil. Kidney weight and cyst index were elevated in verapamil-treated Cy/+ rats. This was associated with increased cell proliferation and apoptosis, elevated expression, and phosphorylation of B-Raf with stimulation of the mitogen-activated protein kinase MEK/ERK (mitogen-activated protein kinase kinase/extracellular-regulated kinase) pathway. Verapamil had no effect on kidney morphology or B-Raf stimulation in wild-type rats. We conclude that treatment of Cy/+ rats with calcium channel blockers increases activity of the B-Raf/MEK/ERK pathway accelerating cyst growth in the presence of endogenous cAMP, thus exacerbating renal cystic disease.

Kidney International (2008) **73,** 269–277; doi:10.1038/sj.ki.5002629; published online 17 October 2007

KEYWORDS: ADPKD; calcium; cell signaling; cyclic AMP; proliferation

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Received 15 May 2007; revised 16 August 2007; accepted 21 August 2007; published online 17 October 2007

Autosomal-dominant polycystic kidney disease (ADPKD) is a common hereditary renal disorder caused by mutations in *PKD1* or *PKD2*.[1–6] Aberrant growth of renal epithelial cells leads to the formation of fluid-filled cysts that destroy surrounding tissue and disrupt renal function. Polycystin-1 (PC-1) and polycystin-2 (PC-2), the products of *PKD1* and *PKD2*, respectively, are multifunctional proteins implicated in the regulation of cell proliferation via multiple signaling pathways. PC-1 and PC-2 form a Ca^{2+}-permeable cation channel[7–9] and colocalize to primary cilia of cells.[10,11] It remains unclear how functional loss of PC-1 or PC-2 disrupts intracellular Ca^{2+} regulation and gives rise to an abnormal cell proliferation phenotype. Recent evidence has shown that epithelial cells isolated from human ADPKD cysts have an aberrant intracellular Ca^{2+} response to ciliary sensing of fluid flow[12] and reduced intracellular Ca^{2+} levels compared to normal renal cells.[13] Thus, renal cell hyperplasia in ADPKD may be a result of dysfunctional intracellular Ca^{2+} metabolism.

Cyclic AMP (cAMP) agonists, including arginine vasopressin, accelerate the proliferation of cyst-derived cells through activation protein kinase A and subsequent stimulation of the B-Raf/mitogen-activated protein kinase kinase/extracellular regulated kinase (MEK/ERK) pathway.[13–19] By contrast, cAMP does not activate ERK or proliferation of normal renal cells. This phenotypic difference in the proliferative response to cAMP appears to be secondary to differences in basal intracellular Ca^{2+} levels. Sustained reduction of intracellular Ca^{2+} with Ca^{2+} channel blockers (CCBs), including verapamil and nifedipine (L-type CCBs), predisposed cells cultured from normal human kidneys to cAMP-dependent activation of the B-Raf/MEK/ERK pathway and increased cell proliferation, mimicking the ADPKD cell phenotype.[15] Treatment of ADPKD cells with Ca^{2+} entry blockers amplified cAMP-dependent ERK activation and proliferation, suggesting that further reduction in intracellular Ca^{2+} may accelerate cyst growth.[13]

To investigate the effect of CCBs on growth of renal cysts, verapamil was administered twice daily for 7 weeks to Han:SPRD Cy/+ rats. This classical rodent model of

polycystic kidney disease (PKD) transmits the disease in an autosomal-dominant pattern and thus resembles human ADPKD.[20] This model has been used to investigate factors that modulate the progression of renal cystic disease.[21] Previously, Cowley *et al.*[22] showed that cyst progression could be accelerated by growth-promoting stimuli, including NH_4Cl, K^+ restriction, and increased dietary protein. An advantage of this model is the sexual dimorphism in the progression of Cy/+ renal disease. Male rats have a more rapid disease progression, whereas the disease in females is relatively mild. Thus, drug treatment can be investigated in mild and severe forms of PKD using the same animal model.

In this study, we determined if verapamil, a CCB that reduces Ca^{2+} in cultured renal cells, enhances cell proliferation and cyst enlargement in Cy/+ rats. Cy/+ and +/+ female and male rats were treated with verapamil from age 5 to 12 weeks, and renal morphology, cellular proliferation and apoptosis, and renal activity of the B-Raf/MEK/ERK pathway were determined.

RESULTS

Effect of verapamil on kidney weight, SUN, and renal cystic index

Blood pressure (BP) of 12-week-old Cy/+ rats was significantly higher than that of +/+ rats (174±6 vs 152±4 mm Hg, $P<0.001$) (Figure 1). Treatment with 20 mg kg^{-1} verapamil twice daily decreased BP of the Cy/+ rats to 142±9 mm Hg ($P<0.001$, compared to control-treated Cy/+ rats). This demonstrates that verapamil dosing with this concentration was sufficient to normalize BP in Cy/+ rats without inducing hypotension in +/+ animals (144±3 mm Hg).

At 12 weeks, serum urea nitrogen (SUN) was relatively normal in control-treated female Cy/+ rats compared to control-treated +/+ rats (Figure 2). Verapamil treatment for 7 weeks increased SUN in female Cy/+ rats from 32.4±1.8 to 42.6±3.8 mg per 100 ml ($P<0.01$). This effect of verapamil on SUN was less pronounced in male Cy/+ rats on a percentage basis, which already have elevated SUN levels above 80 mg per 100 ml, indicating an advanced stage of renal insufficiency at 12 weeks. As such, SUN levels were higher in Cy/+ males following verapamil treatment (83.6±5.6 mg per 100 ml for the control-treated vs 98.1±8.2 mg per 100 ml for verapamil-treated male rats). Verapamil had no effect on SUN levels in male or female +/+ rats.

Body weight of sex-matched Cy/+ and +/+ littermates did not differ and was unaffected by verapamil treatment (Figure 3). By contrast, kidney weight (% body weight) was 2- and 2.9-fold greater in the Cy/+ female and male rats, respectively, compared to +/+ rats. Verapamil increased kidney weight 35% in the Cy/+ females and 50% in Cy/+ males compared to control-treated Cy/+ rats. Measurement of the cystic area in representative histological kidney sections revealed that verapamil increased cystic surface area from 13.0±1.0 to 31.7±0.7% of total area ($P<0.001$) in female kidneys and from 29.7±1.9 to 52.0±3.5% ($P<0.001$) in Cy/+ male kidneys (Figure 4). Importantly, verapamil treatment failed to induce renal cyst formation in normal rats

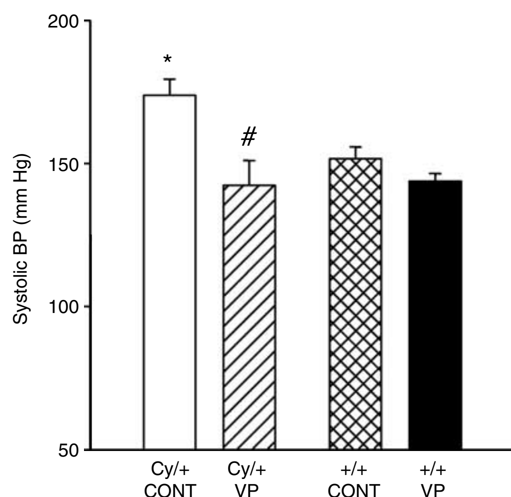


Figure 1 | Effect of verapamil (VP) on systolic BP in Cy/+ and normal +/+ rats. Verapamil (20 mg kg^{-1}) or control solution (CONT) was given by oral gavage every 12 h from age 5 to 12 weeks. Systolic BP was measured at 11.5 weeks by the tail-cuff method. Systolic BP of Cy/+ rats was significantly elevated compared to +/+ rats (*$P<0.01$). Verapamil treatment normalized systolic BP in Cy/+ rats. Comparison between VP vs CONT-treated Cy/+ rats (male and female), #$P<0.001$. There was no effect of VP on the BP of +/+ rats.

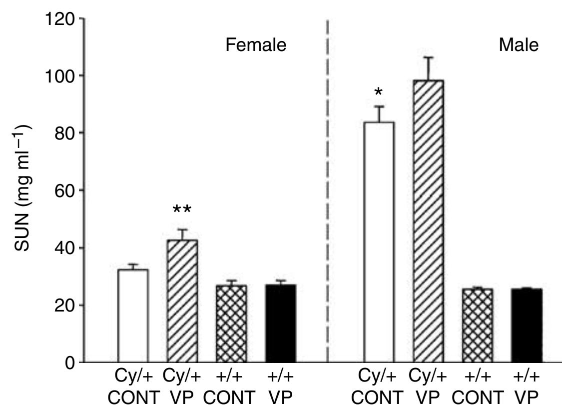


Figure 2 | Effect of verapamil (VP) on serum urea nitrogen (SUN) in Cy/+ and normal +/+ rats. At 12 weeks, control-treated (CONT) female Cy/+ rats ($n=9$) had normal SUN levels compared to CONT female +/+ rats ($n=7$), consistent with a relatively mild stage of renal disease. By contrast, CONT male Cy/+ rats ($n=5$) had SUN values 3.3-fold higher than that in CONT male +/+ rats ($n=9$), indicating advanced stage of renal disease (*$P<0.001$). Treatment with VP caused a significant increase in SUN in female Cy/+ animals ($n=5$), **$P<0.01$. SUN levels in VP-treated Cy/+ males ($n=8$) were not statistically different from CONT-treated Cy/+ males ($P=0.09$). The changes in SUN with verapamil treatment in Cy/+ males ($\Delta=14.5$ mg per 100 ml) were similar (if not greater) than in Cy/+ females ($\Delta=10.24$ mg per 100 ml), indicating that verapamil had an impact on SUN levels in both female and male Cy/+ rats. VP had no effect on SUN levels in +/+ female ($n=10$) or male ($n=7$) rats.

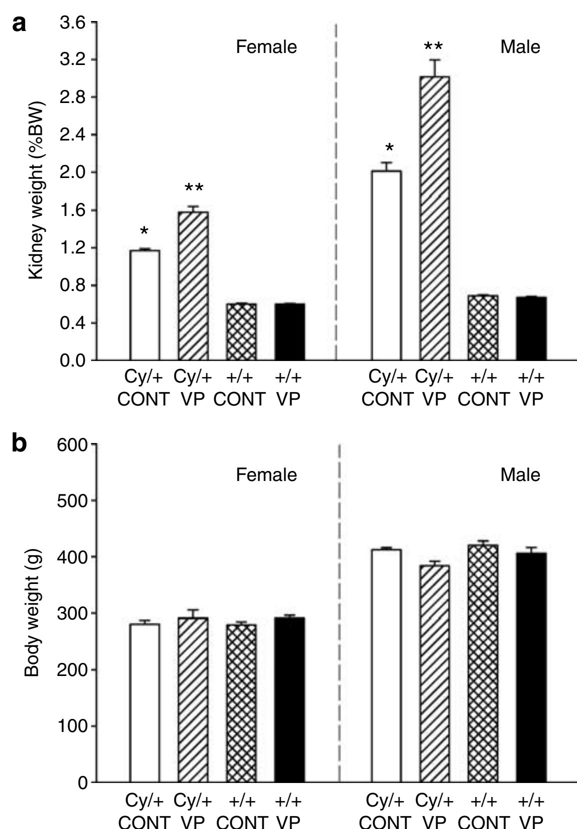


Figure 3 | Effect of verapamil (VP) treatment on kidney weight in Cy/+ and normal rats. Female and male Cy/+ and +/+ rats were treated with VP or control solution by twice daily oral dosing. At 12 weeks of age, total body weight was measured and kidneys were collected to determine total kidney weight, represented as a percentage of body weight (%BW). (**a**) Cy/+ female and male rats had larger kidneys than +/+ littermates (*$P<0.001$). Verapamil treatment caused a significant increase in kidney weight in both genders of Cy/+ rats (**$P<0.001$), but had no effect on total kidney weight of +/+ rats. (**b**) Total body weight did not differ between sex-matched Cy/+ and +/+ rats and was unaffected by verapamil treatment. Statistical differences between VP and control (CONT)-treated rats (either Cy/+ or +/+) were determined by one-way analysis of variance.

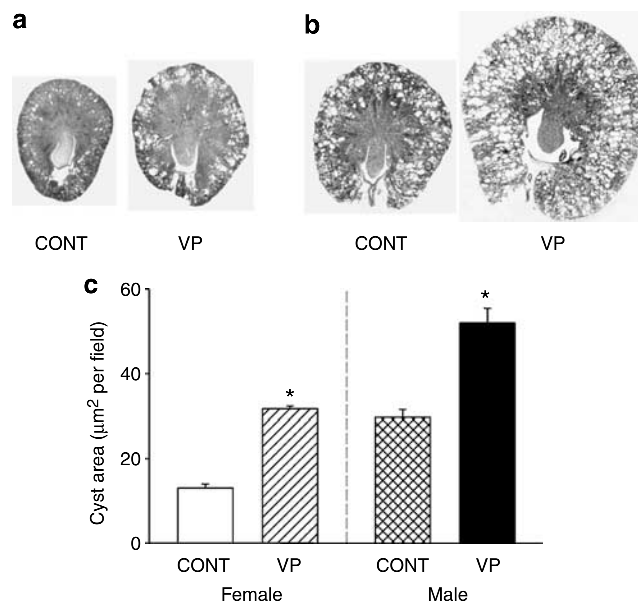


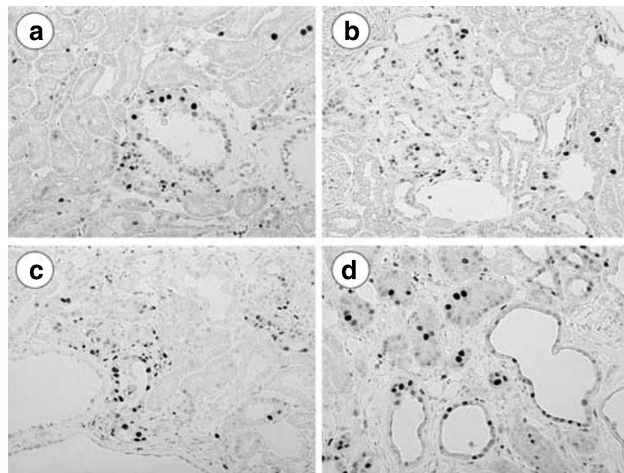
Figure 4 | Effect of verapamil (VP) on renal cyst development in female and male Cy/+ rats. Representative kidney sections obtained from (**a**) female or (**b**) male Cy/+ rats treated with either CONT or VP. (**c**) Cross-sectional surface area of cysts, represented as % of total area (mean ± s.e.), from representative sections of Cy/+ kidneys was measured by morphometric analysis. Comparisons between CONT and VP showed that VP increased cyst area 140% in the female kidneys and 75% in Cy/+ male kidneys, *$P<0.001$.

(data not shown) and had no effect on kidney weight (Figure 3).

Effect of verapamil on cell proliferation and activity of the B-Raf/MEK/ERK pathway in Cy/+ kidneys

Intracellular cAMP is a central mitogenic factor for the progression of renal disease. We found that urinary cAMP levels were fourfold higher in the Cy/+ male rats than in normal rats (1540 vs 6289 pmol per 24 h, $n=2$ per group), suggesting that renal cAMP production was elevated in Cy/+ kidneys consistent with several other rodent models of PKD.[17,18,23] Immunohistochemical analysis revealed that control-treated Cy/+ kidneys had significantly higher levels of proliferating cell nuclear antigen (PCNA)-positive cells than control-treated +/+ kidneys (Figure 5). Verapamil increased the number of PCNA-positive cells in female Cy/+ kidneys from 20.6 ± 4.2 to 39.3 ± 1.2% ($P<0.01$) and in male Cy/+ kidneys from 24.3 ± 1.3 to 30.0 ± 1.0% ($P<0.001$). By contrast, there were few PCNA-positive cells in +/+ kidneys and verapamil had no effect on the number of proliferating cells. In female Cy/+ rats, verapamil treatment increased the number of PCNA-positive epithelial cells in normal and dilated renal tubules, and cysts compared to control-treated animals (Table 1). A similar trend was observed in the male Cy/+ kidneys. Verapamil treatment had no effect on the proliferation of interstitial cells.

Previously, we found that ERK activity generally reflected the mass of cystic epithelia within Cy/+ kidneys.[24] To determine if verapamil treatment increased the activity of the B-Raf/MEK/ERK pathway, we measured levels of B-Raf, ERK, and phosphorylated form of B-Raf (P-BRaf) and ERK (P-ERK) in normal and cystic kidneys by immunoblot analysis (Figure 6, Table 2). There was no consistent difference between normal and Cy/+ female kidneys in the expression of the 95 kDa isoform of B-Raf and ERK; and the level of P-ERK was only slightly increased in female Cy/+ kidneys consistent with the mild cystic phenotype.[24] Treatment with verapamil increased the expression of B-Raf and increased the levels of P-BRaf/B-Raf and P-ERK/ERK in Cy/+ female kidneys. By contrast, renal B-Raf expression and the activity of the B-Raf/MEK/ERK pathway were already elevated in male Cy/+ rats. There were higher levels of P-BRaf and P-ERK in the male Cy/+ kidneys treated with verapamil; however, a statistically significant effect of



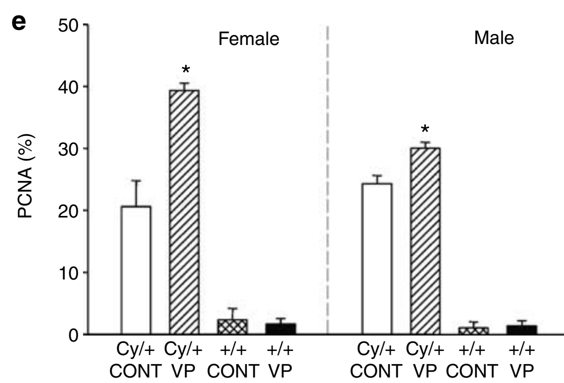


Figure 5 | Effect of verapamil (VP) treatment on cell proliferation in Cy/+ and +/+ kidneys. Representative kidney sections from (**a**) CONT-treated Cy/+ female, (**b**) VP-treated Cy/+ female, (**c**) CONT-treated Cy/+ male, and (**d**) VP-treated Cy/+ male rats stained with an antibody to proliferating cell nuclear antigen (PCNA), a proliferation marker. All images are at the same magnification. (**e**) The number of PCNA-positive cells is represented as % of total cells in the kidney section. Approximately 20–25%' of the cells in the Cy/+ kidneys (female and male) stained positive for PCNA. VP treatment increased the PCNA-positive cells in the Cy/+ kidneys. Comparison between VP vs CONT-treated Cy/+ rats (female or male), *$P<0.01$. By contrast, there were few PCNA-positive cells in +/+ kidney sections and VP had no effect on the proliferative index (representative sections of +/+ kidneys not shown).

verapamil on P-BRaf and P-ERK could not be demonstrated because of the already elevated levels. By contrast, verapamil treatment had no effect on B-Raf, ERK, P-BRaf, or P-ERK levels in +/+ kidneys. We propose that renal cystic disease is related to the activity of the B-Raf/MEK/ERK pathway in the Cy/+ rats, and CCB increases the activity of B-Raf/MEK/ERK pathway and PKD severity. This effect of verapamil was best demonstrated in female Cy/+ rats, which have a milder disease phenotype. Male Cy/+ kidneys already have elevated B-Raf expression and ERK activity and advanced renal cystic disease.

Cellular hyperplasia and apoptosis are both hallmarks of hereditary cystic kidney diseases. To determine if verapamil treatment altered the number of apoptotic cells, we used a

Table 1 | Effect of VP on PCNA-positive cells in Cy/+ kidneys

Treatment	N	Epithelial cells: Normal tubules	Epithelial cells: Dilated tubules	Epithelial cells: Cysts	Interstitial cells
Female Cy/+					
CONT	3	7.8±0.8	8.9±1.1	9.1±2.7	18.2±5.4
VP	3	13.7±1.7*	16.2±2.0*	21.6±1.5*	14.0±3.4
Male Cy/+					
CONT	3	14.2±0.6	15.9±3.6	17.3±0.2	16.1±4.5
VP	3	20.2±4.8	23.8±2.7	23.6±2.0*	10.3±2.3

CONT, control; PCNA, proliferating cell nuclear antigen; VP, verapamil.
Summary of the effect of VP treatment on PCNA-positive epithelial cells of normal tubules (diameter ≤50 μm), dilated tubules (50 μm < diameter ≤ 100 μm), and cysts (diameter > 100 μm); and interstitial cells in Cy/+ male and female kidneys. The data are % PCNA-positive cells (mean±s.e.) counted from 900 to 1400 cells per thin section of cortex. VP (20 mg kg^{-1} given twice daily) caused a significant increase in PCNA-positive cells in normal and dilated tubules, and cysts within the female Cy/+ kidneys (*$P<0.05$). In male Cy/+ kidneys, a significant increase in PCNA-positive cells was demonstrated only in the cysts (*$P<0.05$); however, the number of cells in normal and dilated tubules that stained positive for PCNA was greater in VP-treated compared to CONT-treated kidneys. There was no effect of VP on interstitial cells in female or male Cy/+ kidneys. A comparison of the effect of VP treatment on PCNA-positive epithelial cells (tubules and cysts) of male and female Cy/+ and +/+ kidneys is shown in Figure 5.

TdT-mediated dUTP nick end labeling assay to count the number of apoptotic cells in Cy/+ rats treated with either verapamil or vehicle control. Consistent with previous reports,[27] the number of apoptotic cells (% of total cells counted per field) in the kidneys of Cy/+ rats was significantly higher than in +/+ kidneys (Table 3). Verapamil treatment increased the % apoptotic cells in Cy/+ female and male kidneys 70 and 87%, respectively. Apoptotic cells in dilated tubules and cysts were significantly increased in verapamil-treated Cy/+ rats. By contrast, apoptotic levels were unaffected in normal tubule cells or interstitial cells.

DISCUSSION

Intracellular Ca^{2+} signaling in epithelial cells is important for regulation of a variety of cellular functions, including cell volume regulation, ion and fluid transport, differentiation and cell proliferation.[28] Various types of Ca^{2+} channels have been identified in renal tubule cells, including non-selective cation channels,[29,30] store-operated Ca^{2+} channels,[31] and voltage-activated Ca^{2+} channels.[32] There are at least four types of voltage-dependent Ca^{2+} channels (L, N, T, and P) in excitable cells that can be distinguished on the basis of electrophysiological and pharmacological properties. Several studies have demonstrated that L-type Ca^{2+} channels are also present in non-excitable tissues.[33,34] Ca^{2+} channels have been identified in renal tubule cells, which are sensitive to the dihydropyridine Ca^{2+} channel blocker nifedipine and the activator Bay-K8644,[35,36] characteristics of L-type Ca^{2+} channels. L-type Ca^{2+} channels are composed of multiple subunits (α1, α2, β, γ, and δ) with the α1-subunit comprising the channel core, voltage sensor, and drug-binding sites for dihydropyridine, phenylalkylamines (that is, verapamil), and benzothiazepines (that is, diltiazem).[37,38] The α1c-subunit of the cardiac L-type Ca^{2+} channel localizes to both the

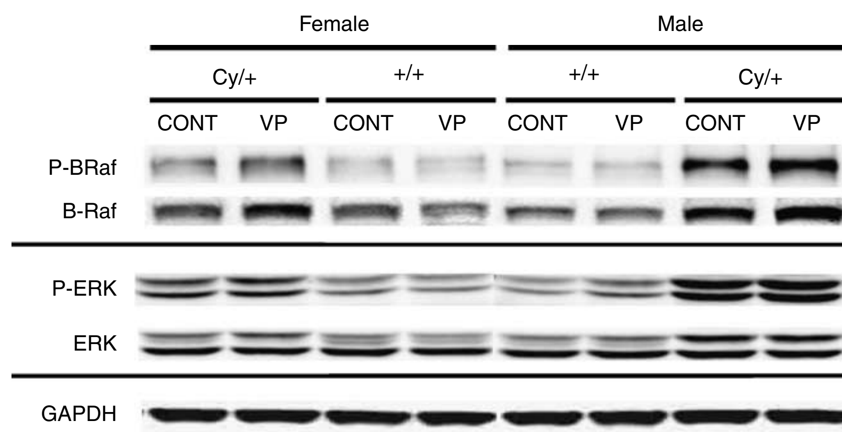


Figure 6 | Effect of verapamil (VP) treatment on the renal activity of the B-Raf/MEK/ERK pathway in Cy/+ and +/+ kidneys. Immunoblot analysis was used to compare expression levels of total B-Raf and ERK, and phosphorylated levels of B-Raf (P-BRaf) and ERK (P-ERK) in kidneys from Cy/+ and +/+ (female and male) rats. In the female Cy/+ kidneys, P-BRaf and P-ERK were slightly elevated above +/+ kidneys, and treatment with VP increased both P-BRaf and P-ERK. B-Raf, ERK, P-BRaf, and P-ERK were elevated in male Cy/+ kidneys compared to +/+ kidneys, and VP treatment did not cause an appreciable increase in these levels. There was no effect of VP on B-Raf and ERK activity in the +/+ kidneys. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. A summary of the effects of verapamil on the expression of B-Raf, ERK, and levels of P-BRaf and P-ERK is presented in Table 2.

Table 2 | Effect of VP on the activity of the B-Raf/MEK/ERK pathway in Cy/+ and +/+ kidneys

Genotype	Treatment	*N*	P-BRaf/B-Raf	B-Raf	P-ERK/ERK	ERK
Female						
+/+	CONT	5	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
+/+	VP	5	1.15 ± 0.11	0.76 ± 0.17	1.04 ± 0.08	0.98 ± 0.11
Cy/+	CONT	5	$1.81 \pm 0.07^{\ddagger}$	1.16 ± 0.05	2.07 ± 0.21	1.16 ± 0.10
Cy/+	VP	5	$2.16 \pm 0.17^{\ddagger,**}$	$1.41 \pm 0.11^{*}$	$3.75 \pm 0.63^{\ddagger,\#}$	1.03 ± 0.05
Male						
+/+	CONT	5	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
+/+	VP	5	1.00 ± 0.08	0.94 ± 0.03	1.62 ± 0.50	0.98 ± 0.08
Cy/+	CONT	5	$1.88 \pm 0.23^{\dagger}$	$1.65 \pm 0.24^{*}$	$4.77 \pm 0.58^{\dagger}$	1.13 ± 0.14
Cy/+	VP	5	$1.96 \pm 0.27^{\dagger}$	$1.86 \pm 0.27^{*}$	$6.50 \pm 0.98^{\ddagger}$	1.09 ± 0.12

CONT, control; ERK, extracellular-regulated kinase; MEK, mitogen-activated protein kinase kinase; VP, verapamil.
Summary of the effect of VP treatment on renal expression and activity of B-Raf (95 kDa isoform), and ERK in normal (+/+) and Cy/+ female and male rats. Expression levels for ERK and B-Raf were normalized to glyceraldehyde-3-phosphate dehydrogenase, a loading control. Antibodies were used to detect the phosphorylation of S^{446} of B-Raf (P-BRaf) and Y^{204} of ERK (P-ERK) and relative activity levels were expressed as P-BRaf/B-Raf and P-ERK/ERK, respectively. It has been reported that S^{446} (previously S^{445}) of B-Raf is constitutively phosphorylated in proliferating cells and tumor cell lines.[25] However, it has been shown recently that phosphorylation at this site is negatively regulated by Rheb, and that S^{446} phosphorylation promotes binding of B-Raf to H-Ras.[26] Comparison to +/+ kidneys ($^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$); comparison between CONT and VP treatment in either Cy/+ or +/+ kidneys ($^{**}P<0.05$, $^{\#}P<0.01$).

basolateral and apical membranes of rat medullary collecting ducts, and the mRNA for the α1c-subunit has been demonstrated in rat proximal tubules, and cortical and medullary collecting ducts.[39] Moreover, Ca^{2+} channels with characteristics of L-type Ca^{2+} channels have been identified in apical membranes of rabbit proximal tubules, cortical thick ascending limbs, distal convoluted tubules, and cortical collecting ducts.[35,40,41] Taken together, these localization studies suggest a broad distribution of L-type Ca^{2+} channels throughout the nephron and collecting duct.

Epithelial Ca^{2+} channels share structural similarities to channels found in excitable cells, but may have different channel properties. Currently, it is unclear if these epithelial Ca^{2+} channels are the same voltage-gated channels expressed in excitable tissue. Zhang and O'Neil[42] cloned a Ca^{2+} channel cDNA from renal epithelial cells that had a sequence identical to the α1c-subunit, except for a 11 amino-acid deletion, indicating a splice variant of the cardiac Ca^{2+} channel. In excitable cells, L-type channels can be distinguished from other voltage-gated channels by activation at relatively large membrane depolarization and by slow channel inactivation. In contrast, Ca^{2+} entry in distal convoluted tubule cells was found to be accompanied by membrane hyperpolarization.[43] Thus, Ca^{2+} signaling in renal epithelial cells may be mediated by unique Ca^{2+} channels that are distinct from the classical L-type Ca^{2+} channels. Nevertheless, pharmacological properties of these renal Ca^{2+} channels may be similar to L-type Ca^{2+} channels in

Table 3 | Effect of VP on apoptosis in Cy/+ and +/+ kidneys

Genotype	Treatment	*N*	Apoptotic index (%)
Female			
+/+	CONT	3	0.4 ± 0.2
+/+	VP	3	0.0 ± 0.0
Cy/+	CONT	3	4.0 ± 0.8*
Cy/+	VP	3	6.8 ± 1.2†
Male			
+/+	CONT	3	0.6 ± 1.3
+/+	VP	3	0.6 ± 0.3
Cy/+	CONT	3	3.8 ± 0.2*
Cy/+	VP	3	7.1 ± 0.2**

VP, verapamil.
The number of apoptotic cells was counted from approximately 700 cells per section of Cy/+ or +/+ kidneys. Apoptotic cells were detected by the ApopTag Peroxidase *In situ* Apoptosis Detection Kit (Chemicon International). Female and male Cy/+ kidneys had elevated levels of apoptosis (*$P<0.05$) compared to +/+ rats. Treatment with VP increased the number of apoptotic cells in the Cy/+ female (†$P<0.05$) and male (**$P<0.01$) kidneys, but did not affect the level of apoptosis in +/+ rat kidneys. Apoptotic cells were increased in dilated tubules plus cysts of VP-treated Cy/+ female (2.6 ± 0.4 vs 7.2 ± 0.9, $P<0.02$) and male (3.4 ± 0.4 vs 8.2 ± 1.1, $P<0.02$) kidneys, whereas normal tubule cells and interstitial cells were unaffected by VP treatment.

that they are sensitive to the three classes of CCBs (nifedipine, verapamil, and diltiazem).

Polycystins are multifunctional proteins that appear to be involved in Ca^{2+} signaling in renal epithelial cells and may transmit environmental signals into a cellular response that regulates cell differentiation and proliferation. PC-2 is a member of the family of transient receptor potential channels.[4,44,45] PC-1 interacts with PC-2 through a coiled-coil domain and may regulate PC-2 channel activity within the plasma membrane. Recent evidence suggests that functional loss of the polycystins leads to disruption of flow-mediated Ca^{2+} signaling[3] and lower basal Ca^{2+} levels in cystic cells compared to non-cystic cells.[13] Aberrant intracellular Ca^{2+} signaling in renal epithelial cells allows cAMP activation of the MEK/ERK pathway and increased cell proliferation,[15] an important factor in the enlargement of renal cysts.[13–15,18,19,46–48] This phenotypic difference in the cAMP mitogenic response between normal renal cells and PKD cells is linked to cAMP-dependent B-Raf signaling to the MEK/ERK pathway. Normally, B-Raf is repressed by Akt (protein kinase B), which is downstream of the phosphatidylinositol 3-kinase pathway.[15] This mitogenic response to cAMP could be induced experimentally in normal renal cells by reducing intracellular Ca^{2+} with L-type CCBs, verapamil and nifedipine, and the non-selective cation channel blocker gadolinium. Restriction of intracellular Ca^{2+} decreased Akt activity allowing cAMP-dependent activation of B-Raf and cell proliferation, thus mimicking ADPKD cells.[15] Basal activity of Akt is diminished in human ADPKD cells compared to normal renal cells. CCB treatment of ADPKD cells, which already have a reduced Ca^{2+} level, caused a further reduction in Akt activity and amplified the cAMP-dependent ERK activation and cell proliferation.[13] These findings led us to hypothesize that CCB treatment in animals with PKD may have an adverse effect on disease progression by amplifying the activity of the B-Raf/MEK/ERK pathway and, thereby, increasing the proliferation rate of cyst-lining cells.

In the Cy/+ model of dominantly inherited cystic disease, kidneys enlarge owing to aberrant proliferation of tubule epithelial cells mediated by ERK activation.[24] We found that urinary cAMP levels were increased in Cy/+ rats compared to +/+ rats, consistent with an elevation of intracellular cAMP in the kidneys in several rodent models of PKD.[17,18] This increased production of cAMP, either basal or stimulated by activation by G-protein-coupled receptors, could account for this elevation in ERK activity. Cysts develop primarily in the proximal tubule in this model due to mutations in samcystin, which has an undefined function.[49] The reliable progression of kidney disease makes this a particularly useful model for identifying factors that alter disease severity.[50,51]

Treatment of Cy/+ rats with verapamil for 7 weeks returned BP to a normal level (Figure 1). However, this sustained treatment with a CCB caused a significant increase in total kidney weight (Figure 3) and in the size of cysts (Figure 4). Moreover, Cy/+ females, which have a mild disease phenotype at 12 weeks, had significantly increased SUN following CCB treatment, reflecting an adverse effect on renal function. The increase in cystic area was associated with increased proliferation of tubule and cystic epithelial cells (Figure 5 and Table 1) and elevated apoptosis of cells in dilated tubules and cysts (Table 3). Examination of signaling intermediates revealed that verapamil treatment increased the level of B-Raf and the activity of the MEK/ERK pathway (Figure 6 and Table 1). Thus, verapamil treatment, presumably through inhibition of Ca^{2+} entry, increased renal activity of the B-Raf/MEK/ERK pathway and stimulated the proliferation of the epithelial cells, causing acceleration in PKD progression that was significant in the female Cy/+ rats. The effect of verapamil in male Cy/+ kidneys was even more pronounced, but because of the already elevated activity of this pathway, did not show a statistically significant increase. The results also demonstrated that verapamil did not induce cyst formation in normal kidneys. Previously, we showed that restriction of intracellular Ca^{2+} induced a phenotypic switch in the cAMP proliferative response in cultured renal cells. As we suggested previously,[15] it is possible that altered gene expression may be required for the cAMP-dependent proliferative phenotype induced by intracellular Ca^{2+} restriction.

Potential clinical relevance

CCBs are widely used for treatment of hypertension associated with chronic kidney diseases, including PKD. The three chemical types of L-type CCBs (phenylalkylamine, benzothiazepines, and dihydropyridines) are generally believed to have similar actions. However, these compounds have clear differences in their chemical structures, binding sites, tissue selectivity, and clinical activity.[52]

This study suggests that CCBs can have an adverse effect on renal disease in PKD; however, additional studies will be necessary to determine if other L-type CCBs, such as nifedipine and diltiazem, have similar effects as verapamil, and if CCBs have adverse effects on PKD progression in other animal models. Angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and CCBs have similar beneficial effects on BP control;[53] however, there is no consensus on the type of antihypertensive therapy most appropriate for PKD patients. In a preliminary report, Nutahara *et al.*[54] compared the renal-protective effects of amlodipine, a dihydropyridine L-type CCB, and candesartan, an angiotensin II receptor blocker, in 49 ADPKD patients with serum creatinine $\leqslant 2$ mg per 100 ml who were treated for 3 years for hypertension. Patients receiving candesartan (2–8 mg day^{-1}) had less urinary protein and albumin excretion, better renal function, and a greater renal event-free survival rate than patients receiving CCB. The authors concluded that angiotensin II receptor blockers may be more effective than CCBs for renal protection in patients with ADPKD, independent of the capacity to control of hypertension.

In conclusion, treatment with verapamil, a classical phenylalkylamine L-type CCB, increased the renal activity of the B-Raf/MEK/ERK pathway and caused acceleration in growth of renal cysts in Cy/+ rats. Elevation in epithelial cell proliferation in conjunction with increased apoptosis suggests that a reduction in intracellular Ca^{2+} levels with CCB treatment increases the growth and turnover of cyst-lining epithelial cells, leading to accelerated enlargement of cysts and kidneys in this rodent model of PKD. The relevance of these findings to other CCBs and the impact of these agents in the treatment of PKD remain to be determined.

MATERIALS AND METHODS

Han:SPRD Cy rat model

Han:SPRD Cy rats were originally derived from a strain of Sprague–Dawley rats in Hanover (Germany),[55] and descendants of this colony have been maintained at Fujita Health University. Male and female heterozygote (Cy/+) rats produced litters containing Cy/+, Cy/Cy, and +/+ littermates. Rats were allowed free access to water and food throughout the study. Cy/Cy rats die around 3 weeks of age.

There is early onset of renal cyst development in both male and female Cy/+ rats, but by 8 weeks, there is an obvious difference in the severity of the disease. Male Cy/+ rats have a more rapid disease progression and earlier loss of renal function compared to female Cy/+ rats. Male Cy/+ rats often die of uremia at approximately 1 year, whereas female rats often live to 2 years of age before significant loss of renal function. This sexual dimorphism in the progression of cystic disease is related, in part, to the renal androgen receptor pathway.[24] In this study, we examined the effect of a Ca^{2+} entry blocker on mild and rapid disease progression by treating female and male Cy/+ rats with verapamil.

At 5 weeks of age, female and male Cy/+ and +/+ rats were randomly assigned to one of the two groups: treatment with 20 mg kg^{-1} verapamil (VP; Sigma Chemical, St Louis, MO) or vehicle control (water) by gavage every 12 h from 5 to 12 weeks of age. Systolic BP was determined at 11.5 weeks by tail-cuff using a sphygmomanometer (BP-98A; Softron INC., Tokyo, Japan). Rats were anesthetized at 12 weeks with sodium pentobarbital and both kidneys were removed causing fatal exsanguination. Total body weight and kidney weight were measured; the left kidney was homogenized in lysis buffer for protein extraction, and the right kidney was immersed in 4% paraformaldehyde, embedded in paraffin, and sectioned for immunohistochemistry. The protocol for the use of these animals was approved by the Animal Care and Use Committee at Fujita Health University.

Western blot analysis

Kidney lysates were prepared for immunoblot as described previously.[24,46] Contents of lysis buffer were 20 mM Tris (pH 7.4), 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg ml^{-1} aprotinin, and 5 μg ml^{-1} leupeptin with 1% Triton X-100. Proteins (20 μg protein per lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in TBS-T (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature and then incubated overnight at 4°C in primary antibody diluted at 1:2000–5000 in 5% milk–TBS-T. Membranes were then washed three times with TBS-T and incubated with secondary antibody conjugated to horseradish peroxidase diluted 1:2000–5000 in 5% milk in TBS-T. Specific antibody signals were detected using an enhanced chemiluminescence system (ECL Advance Western Blotting Detection System; Amersham Life Sciences, Arlington Heights, IL, USA). 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 Corporation, Tokyo, Japan). Relative band intensity was compared to gender-matched +/+ kidneys gavaged with control solution (set to 1.0).

Immunohistochemistry

Paraffin sections were heated to 100°C for 15 min in 10 mM sodium citrate buffer (pH 6.0) to unmask antigens. Endogenous peroxidase was destroyed by incubating sections in 0.3% H_2O_2/methanol, and the sections were incubated with primary antibody (1:3000) for PCNA overnight at 4°C. Sections were incubated with biotinylated anti-mouse secondary antibody and then 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 (×100 magnification) of hematoxylin and eosin-stained sections of renal cortex ($N=3$). Cyst area (% of total field) was measured by a naive observer using LUZEX FS software (Kideko CO. LTD, Tokyo, Japan). ApopTag Peroxidase *In situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) was used to detect apoptotic cells. PCNA-positive cells were counted from 900 to 1400 cells per thin section and apoptotic cells from approximately 700 cells per section from cortex of Cy/+ or +/+ rat kidneys (×400 magnification).

Antibodies

Primary antibodies to $ERK_{1,2}$ (K-23, SC-94), P-ERK (E-4, SC-7383), and B-Raf (F-7, SC-5284) were obtained from Santa Cruz Technology (Santa Cruz, CA, USA), and P-BRaf (no. 2696, Ser[445]) was purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies conjugated to horseradish peroxidase were

goat anti-rabbit IgG (SC-2054) and rabbit anti-mouse IgG (SC-2055) from Santa Cruz Technology. Primary antibodies to PCNA (P8825; Sigma Chemical) were used for immunohistochemistry. Secondary antibodies, conjugated to biotin, were goat rabbit anti-mouse IgG + IgA + IgM (HISTOFINE 426032) obtained from Biosciences.

Measurement of SUN and urinary cAMP levels

SUN determinations were performed using a colorimetric assay using a urease–indophenol method (Wako Pure Chemical Industries Ltd, Osaka, Japan).[46] To measure urinary cAMP levels, male Cy/+ and Sprague–Dawley rats ($n = 2$ per group) were given free access to food and water in metabolic cages, and urine was collected for a 24 h period. cAMP levels were analyzed using a cAMP radioimmunoassay kit (Yamasa, Chiba, Japan).

Statistics

Data are represented as mean ± s.e. Statistical significance was determined by one-way analysis of variance and Student–Newman–Keuls post-test for multiple comparisons or unpaired *t*-test for comparison between control and treated animals (Instat; Graphpad Software Inc., San Diego, CA, USA).

ACKNOWLEDGMENTS

This work was supported by grants from the Japanese Ministry of Education Culture, Sports, Science and Technology ((High-Tech Research Center Project (2002), Open Research Center Project (2003), and 21st Century COE Program (2003)), the National Institutes of Health (P50 DK074043 (DPW and JPC)) and the PKD Foundation (DPW). We are grateful to Dr JJ Grantham for reading this paper and Mrs Ayumi Ohono for technical assistance.

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