Androgen Receptor Pathway in Rats with Autosomal Dominant Polycystic Kidney Disease

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Androgens have been implicated in mediating disease escalation in autosomal dominant polycystic kidney disease (ADPKD). Dihydrotestosterone (DHT), an agonist, and flutamide (FLT), an antagonist, were administered to Han:SPRD rats with ADPKD, and the role of androgen receptor (AR) abundance and activation on the enlargement and function of cystic kidneys was evaluated. Renal AR abundance determined by immunoblots in 8- to 10-wk-old Cy/+ male rats was naturally increased four-fold above that of littermate +/+ controls. In male Cy/+, castration decreased AR abundance below control +/+ by −89.4%, and AR expression within cyst mural epithelial cells was strikingly decreased. Castration of Cy/+ male rats also reduced the usual increases in kidney weight by −49.7%, kidney cyst area by −34.0%, and serum urea nitrogen by −72.8%; these indices were restored to precastration levels by DHT. In Cy/+ male rats, FLT administration reduced the increase in kidney weight by −27.6% and serum urea nitrogen by −53.7% and decreased the increment in AR expression by −84.2% in comparison with untreated +/+ controls. There was no effect of FLT in female rats. Immunoblot expression of phospho–extracellular signal-regulated kinase 1/2 (P-ERK) and B-Raf, key intermediates in the mitogen-activated protein kinase pathway that are abnormally elevated in Cy/+, was unaffected by castration and/or administration of DHT or FLT. AR was not expressed in renal epithelial cell nuclei of androgen-deficient rats but was displayed in most tubule and mural cyst cell nuclei of androgen-replete rats. In androgen-deficient Cy/+, 80.6% of renal epithelial cells that had entered the cell cycle (proliferating cell nuclear antigen positive) also expressed P-ERK. In androgen-replete rats, proliferating cell nuclear antigen–positive cells co-expressed AR (12.7%), P-ERK (36.4%), and P-ERK + AR (45.0%); 5.9% were probably stimulated by other mitogenic mechanisms. It is concluded that androgens potentiate renal cell proliferation and cyst enlargement through $ERK_{1/2}$-dependent and $ERK_{1/2}$-independent signaling mechanisms in Han:SPRD. It is suggested that the basal rate of cell proliferation is determined by $ERK_{1/2}$ signaling to a major extent and that androgens have additive effects.

J Am Soc Nephrol 16: 2052–2062, 2005. doi: 10.1681/ASN.2004070595

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenetic hereditary renal disorder in adults and is caused by the mutation of *PKD1* or *PKD 2* (1–3). The progressive expansion of kidneys is due to the accelerated proliferation of cells that line cyst cavities and are derived from tubules that creates segmental radial extension of the mural epithelium. Tubule eventration occurs in conjunction with the accumulation of fluid within the expanding cysts in association with basement membrane thickening (4,5). The proliferation of cells within cysts is modulated by external factors, including EGF, together with hormones and autocoids (vasopressin, epinephrine, parathyroid hormone, secretin, vasoactive intestinal polypeptide, adenosine, and prostaglandin-E_2) that activate adenylyl cyclase (6,7). Furthermore, adenosine cAMP, an intracellular mediator of adenylyl cyclase agonists, stimulates the proliferation of cells derived from the cysts of human polycystic kidneys by activating the extracellular signal-regulated kinase (ERK) (6,8,9).

In patients with ADPKD, renal disease progression seems to be gender dependent. Men develop end-stage renal failure about 5 yr earlier than women (10–14). In a rat model of ADPKD (Han:SPRD, Cy/+) the gender effect is seen even more dramatically. Heterozygous male rats (Cy/+) die of uremia at approximately 10 to 12 mo of age, whereas female rats usually live for nearly 2 yr before renal function is seriously compromised. Cystic progression and renal function are strikingly improved by castration of young male rats, whereas female protection can be overridden by the administration of testosterone, suggesting that this steroid hormone influences the progression of renal cystic change in Han:SPRD rats (15). The molecular mechanisms of this gender difference in ADPKD progression have not been elucidated.

In this study, we examined effects of dihydrotestosterone (DHT), an active testosterone analogue, and flutamide (FLT), a testosterone antagonist, on androgen receptor (AR) expression and disease progression in Han:SPRD Cy/+ rats with progres-

Received July 23, 2004. Accepted April 5, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

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ISSN: 1046-6673/1607-2052

sive ADPKD. We also examined the expression of B-Raf and ERK, components of the mitogen-activated protein kinase (MAPK) pathway that are activated in humans and rats with ADPKD (8,16). The results support the view that the gender-dependent effect on disease progression is mediated, in large measure, through mechanisms that are linked to the expression of the androgen receptor.

Materials and Methods

Animals

Han:SPRD Cy rats of the Sprague-Dawley strain were direct descendants of the original colony in Hannover, Germany, maintained for many generations in vivariums at the University of Kansas Medical Center and Fujita Health University. Animals in this study were kept in the vivarium of Fujita Health University, and studies were conducted under Animal Use Committee regulations. Animals were allowed free access to water and food as described previously (16). Heterozygotes were bred to increase the yield of Cy/+ in each litter. Normal animals (+/+) in each litter served as controls.

AR abundance was determined by Western blot and immunohistochemistry in kidneys that were removed from 3-, 8-, and 24-wk-old male and female Han:SPRD rats with (Cy/+) and without (+/+) cystic disease. At 4 wk of age, rats were randomly assigned to one of three groups: Sham operation/vehicle, castration; ovariectomy/vehicle or castration; or ovariectomy/5α-dihydrotestosterone (DHT 50 mg/kg per d; Sigma, St. Louis, MO) or sham operation/FLT (FLT 15 mg/kg per d; Sigma). These protocols were patterned after previously published work using the same reagents (17–19). The gonads were removed at 4 wk of age. Drugs were administered by subcutaneous injection. The solvents (10% ethanol: 90% polyethylene glycol 300; Sigma) for DHT and FLT were administered to control animals until 10 wk of age.

Rats were anesthetized with pentobarbital sodium (Schering-Plough Corporation, Kenilworth, NJ), and both kidneys were removed rapidly, causing fatal exsanguination. Both kidneys were quickly weighed. The left kidney was homogenized in lysis buffer to extract proteins, and the right kidney was sectioned and immersed in 4% paraformaldehyde as described previously (16). The genotype was determined by gross inspection of the kidney sections and confirmed by sections that were stained with hematoxylin-eosin. Kidney cyst area of Cy/+ rats was measured by a naive observer using LUZEX FS software (Kideko Co. Ltd., Tokyo, Japan). Fifteen microscope fields (×4 magnification) were examined per kidney section.

Western Blot Analysis

Kidney lysate samples for immunoblot analysis were obtained as described in a previous study (16). Contents of lysis buffer (TLB buffer) were 20 mmol/L Tris (pH 7.4), 137 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 2 mmol/L dithiothreitol, 1 mmol/L PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin with 1% Triton X-100. Membrane blotted lysates (20 μg protein/lane) 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. Blocked membranes were incubated overnight at 4°C in primary antibody diluted 1:2000 to 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 5000 in 5% milk in TBS-T for 1 h at room temperature. The membranes were washed three times with TBS-T, and reactants were visualized on x-ray film using an enhanced chemiluminescence system (ECL; Amersham Life Sciences, Arlington Heights, IL). Blots were scanned using SCION-modified NIH image software for PC (Scion Corp., Frederick, MD; www.scioncorp.com); images were quantified in arbitrary intensity units. Male +/+ kidneys were used as the point of reference and set to 1.00. Cy/+ kidneys were compared with +/+ male kidneys and expressed as fold changes.

Reverse Transcriptase–PCR Analysis

Total RNA was isolated from whole rat kidney using RNA lysis solution TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA). Contaminating DNA was removed by treating the samples with RNase-free DNAse I (Life Technologies BRL, Gaithersburg, MD). Reverse transcriptase–PCR was performed under linear conditions with respect to RNA input and the number of amplification cycles. PCR were determined as linear for 25 to 30 cycles. Primers for rat ER2 were as follows: upper primer, CTTTCTGTCTCCTTGCCCAC; lower primer, TAGAAGACGCCATCCAAAGG. Primers for G3PDH were as follows: upper primer, ACCACAGTCCATGCCATCAC; lower primer, TCCACCACCCTGTTGCTGTA. These primers were amplified as a control for RNA content and integrity. Amplification products were separated on 2% agarose gel.

Immunohistochemistry

Sections that were obtained from paraffin block were heated to 100°C for 15 min in 10 mM sodium citrate buffer (pH 6.0) to unmask antigens as described previously (16). Sections were incubated with primary antibody at a concentration of 1:3000 for proliferating cell nuclear antigen (PCNA) and 1:100 for AR overnight at 4°C then incubated with 1:100 goat anti-rabbit IgG and goat anti-mouse IgG-conjugated Alexa Fluor, respectively. Photomicrographs of PCNA and AR expression were taken, then sections were heated to 100°C for 15 min in 10 mM sodium citrate buffer (pH 6.0) to remove the primary antibodies. After endogenous peroxidase activity was destroyed, sections were incubated with P-ERK primary antibody at a concentration of 1:3000 overnight at 4°C. After incubation with biotinylated anti-mouse secondary antibody at a concentration of 1:50, peroxidase-conjugated streptavidin (Histofine; Nichirei Biosciences, Tokyo, Japan) immune reaction products were developed using 3,3′-diaminobenzidine as the chromogen, with standardized development times. PCNA-positive cells were counted in four to five fields of three thin sections in each of three groups of Cy/+ cystic animals and 10 fields of three thin sections in each of three groups of +/+ animals.

Protein expression in cystic animals was quantified by co-localization of markers within individual cells. We used PCNA expression as the reference marker and counted 113 to 321 (mean 161) total cells that expressed PCNA in four to five microscope fields prepared from each experimental group. Then, the relative expressions of AR and P-ERK were quantified as a percentage of PCNA-expressing cells. In +/+ rats, PCNA expression was greatly reduced in comparison with Cy/+, and nine fields were examined to obtain an average of 35 PCNA-positive cells per experimental group.

Antibodies

Primary antibodies included $ERK_{1/2}$ (K-23 sc-94), P-ERK (E-4 sc-7383), B-Raf (C-19, sc-166), AR (N-20, sc-816), ERα (H-184, sc-7207) from Santa Cruz Biotechnology (Santa Cruz, CA). AR antibody (ARPG-1, 306-680; Upstate Cell Signaling Solutions, Lake Placid, NY) was used to confirm specificity. PCNA and P-ERK were supplied from Sigma. The AR (N-20) antibody was used for immunohistochemistry. Secondary antibodies conjugated to horseradish peroxidase were goat anti-rabbit IgG (sc-2054), rabbit anti-mouse IgG (sc-2055), and donkey anti-goat IgG (sc-2056) from Santa Cruz Biotechnology. Secondary antibodies

conjugated to biotin for immunohistochemistry were goat anti-rabbit IgG (111-065-003), goat anti-mouse IgG (115-065-003), and rabbit anti-mouse IgG (305-065-003) from Jackson ImmunoResearch (West Grove, PA). Secondary antibodies conjugated to Alexa Fluor for fluorescence staining were goat anti-rabbit IgG (488, Green A11034) and goat anti-mouse IgG (568, Red A11031) from Molecular Probes (Eugene, OR).

Serum Urea Nitrogen

Serum urea nitrogen (SUN) level determinations were performed using a colorimetric assay kit (kit #640; Sigma Chemical Co.).

Statistical Analyses

Mean and SEM were calculated as usual. Levels of significance ($P < 0.05$) were determined by unpaired t test.

Results

AR, B-Raf, and P-ERK Expression in Cystic Kidneys Depends on Age

Rats of the Han:SPRD strain carrying a mutation in only one of the Cy genes develop, within 3 wk after birth, renal cysts that are barely perceptible by gross inspection of cut sections (16). Previous studies have established that by 8 wk, there is significant progression with distinct differences in renal disease severity between male and female rats. Renal function is normal at 8 wk of age in female and slightly decreased in male Cy/+ rats (15,16).

A time-course study was performed to evaluate the renal expression of AR, B-Raf, and P-ERK in Cy/+ and +/+ rats (Figure 1). AR was expressed to an equal extent in male and female +/+ and Cy/+ rats at 3 wk of age. However, by 8 wk of age, AR expression remained prominently elevated only in the kidneys of Cy/+ male rats. AR was weakly expressed in male +/+ rats at 8 and 24 wk of age and undetectable in female +/+ or Cy/+ rats at either age. Examination by immunohistochemistry revealed AR prominently displayed within the nuclei of mural cyst epithelial cells and adjacent normal renal tubule cells (Figure 2A). AR was also expressed in tubule cells nuclei of normal +/+ rats (Figure 2E). There did not seem to be greater expression of AR in cysts than in tubule cells to account for the striking difference between the immunoblots of Cy/+ and +/+ rats at 10 wk of age (Figure 1, Table 1).

The expression of B-Raf and P-ERK was not different between Cy/+ and +/+ male and female rats at 3 wk of age. However, by 8 wk of age AR, B-Raf, and P-ERK expression determined in immunoblots of Cy/+ rats exceeded that of +/+ rats in males (16) (Figure 1, Table 1). This pattern was seen to a lesser extent in female rats (Figure 1, Table 1). At 24 wk, AR, B-Raf, and P-ERK remained greater in male Cy/+ than in +/+ rats; the differences between +/+ and Cy/+ rats were not as great in female rats.

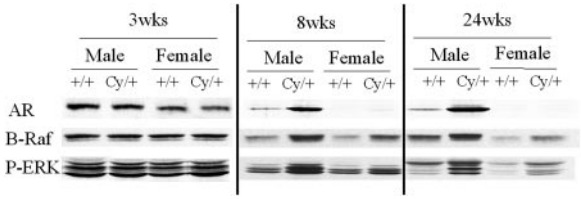

Figure 1. Time course of renal protein expression. Androgen receptor (AR), B-Raf, phospho–extracellular signal-regulated kinase 1/2 (P-ERK), and ERK determined in male and female +/+ and Cy/+ rats at 3, 8, and 24 wk of age.

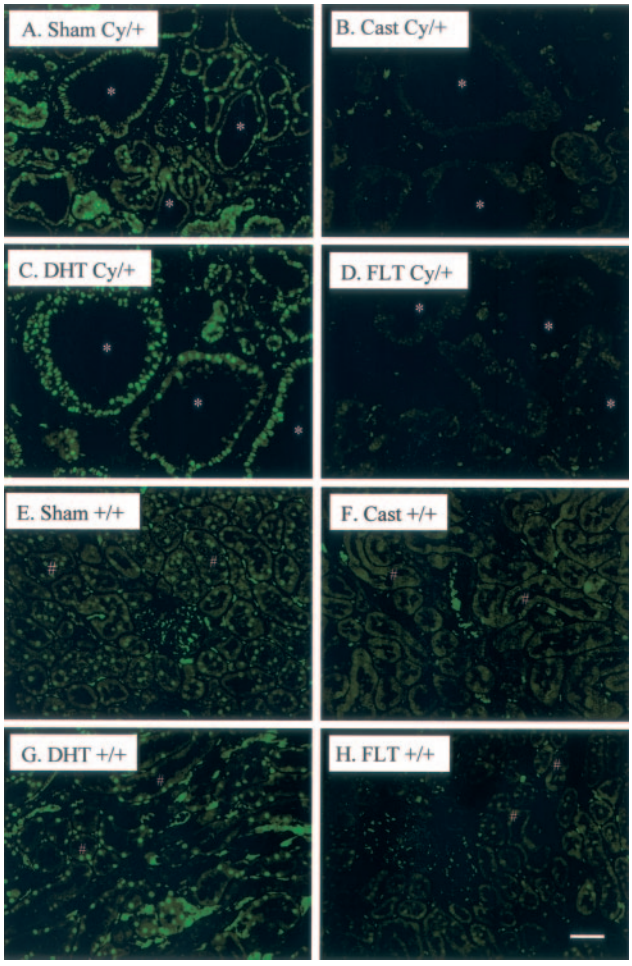

Figure 2. AR cell expression in cross-sections of renal cortex. (A, C, E, and G) High androgen effect. (B, D, F, and H) Low androgen effect. (A through D) Cy/+ male rats. (E through H) +/+ male rats. Sham, control; Cast, castration; DHT, dihydrotestosterone; FLT, flutamide. Animals were 10 wk of age. Marker = 50 μm.

Gonadectomy and Testosterone Alter the Severity of Disease and the Expression of AR but not of B-Raf and P-ERK

To evaluate the relation between AR expression and progression of cystic disease, we administered a testosterone analogue to gonadectomized male and female +/+ and Cy/+ rats. At 10 wk of age, the kidney/body weight ratio (K/B) of sham-operated control Cy/+ male rats was 2.15, compared with 0.70 in +/+ rats, reflecting an increase in renal mass secondary to the appearance of cysts (Table 2). Castration reduced K/B to 1.35 (−49.7%), and DHT restored the ratio to 2.12, indicating that the volume of cysts in the kidneys of male Cy/+ rats is dependent on androgen. Histologic examination of 10-wk-old male rats showed that castration decreased cyst area from 47.6 ± 1.0

Table 1. Age-dependent AR, B-Raf, and P-ERK expression[a]

No.	Genotype	Gender	Weeks		
			3	8	24
AR					
1	+/+	M	1.0 ± 0.1 (4)	0.6 ± 0.0 (3)	0.6 ± 0.0 (3)
2	Cy/+	M	1.1 ± 0.1 (4) NS	2.4 ± 0.7 (3)[b]	2.2 ± 0.2 (4)[c]
3	+/+	F	1.0 ± 0.1 (3)	0.5 ± 0.1 (3)	0.2 ± 0.0 (3)
4	Cy/+	F	0.9 ± 0.0 (3) NS	0.5 ± 0.1 (3) NS	0.2 ± 0.0 (3) NS
B-Raf					
5	+/+	M	1.0 ± 0.0 (4)	0.6 ± 0.1 (3)	0.7 ± 0.1 (3)
6	Cy/+	M	1.4 ± 0.2 (4) NS	1.2 ± 0.1 (3)[c]	2.0 ± 0.2 (4)[c]
7	+/+	F	1.0 ± 0.0 (4)	0.5 ± 0.1 (3)	0.5 ± 0.1 (3)
8	Cy/+	F	1.0 ± 0.0 (3) NS	0.8 ± 0.1 (3) NS	0.8 ± 0.1 (3) NS
P-ERK					
9	+/+	M	1.0 ± 0.0 (4)	0.6 ± 0.1 (3)	0.4 ± 0.0 (3)
10	Cy/+	M	1.0 ± 0.0 (4) NS	1.0 ± 0.1 (3)[c]	1.4 ± 0.1 (4)[c]
11	+/+	F	0.9 ± 0.1 (3)	0.5 ± 0.1 (3)	0.3 ± 0.1 (3)
12	Cy/+	F	1.0 ± 0.0 (3) NS	0.6 ± 0.1 (3) NS	0.6 ± 0.1 (3) NS

Data are means ± SE (*n*).
[a] Age 10 wk. +/+ *versus* Cy/+ in each gender-matched group. AR, androgen receptor; P-ERK, phospho–extracellular signal-regulated kinase 1/2.
[b] $P < 0.05$.
[c] $P < 0.01$.

Table 2. Renal response to gonadectomy[a]

No.	Surgery	Treatment	Genotype	Gender	*N*	K/B (g/100 g)	SUN (mg/dl)
1	Sham	Vehicle	+/+	M	7	0.70 ± 0.01	22.6 ± 0.6
2	Castration	Vehicle	+/+	M	3	0.62 ± 0.01 NS	24.1 ± 0.2 NS
3	Castration	DHT	+/+	M	3	0.81 ± 0.01[b]	22.0 ± 0.5[b]
4	Sham	Vehicle	Cy/+	M	6	2.15 ± 0.04	38.8 ± 1.1
5	Castration	Vehicle	Cy/+	M	5	1.35 ± 0.01[c]	28.5 ± 1.8[c]
6	Castration	DHT	Cy/+	M	6	2.12 ± 0.08 NS	32.2 ± 1.5[b]
7	Sham	Vehicle	+/+	F	3	0.69 ± 0.02	22.0 ± 2.0
8	Ovariectomy	Vehicle	+/+	F	3	0.58 ± 0.01 NS	22.8 ± 0.3 NS
9	Ovariectomy	DHT	+/+	F	3	0.85 ± 0.01 NS	24.1 ± 0.5 NS
10	Sham	Vehicle	Cy/+	F	6	1.08 ± 0.02	24.9 ± 2.3
11	Ovariectomy	Vehicle	Cy/+	F	5	1.21 ± 0.03[c]	26.0 ± 1.1 NS
12	Ovariectomy	DHT	Cy/+	F	5	1.67 ± 0.05[b]	25.1 ± 0.8 NS

[a] Age 10 wk. K/B, kidney/body weight; SUN, serum urea nitrogen; DHT, dihydrotestosterone.
[b] 1 *versus* 3, 4 *versus* 6, 7 *versus* 9, 10 *versus* 12; $P < 0.001$.
[c] 1 *versus* 2, 4 *versus* 5, 7 *versus* 8, 10 *versus* 11; $P < 0.001$.

($n = 6$) to 31.4 ± 0.4 μ^2/15 fields ($n = 4$) ($P < 0.01$; Figure 3). DHT was administered to castrated animals because it is the activated form of testosterone and does not require metabolic conversion. DHT partially reversed the effect of castration on cyst area. The elevated SUN was decreased from 38.8 to 28.5 mg/dl by castration (increment above control +/+ decreased −72.8%), and administration of DHT to castrated animals elevated the SUN to 32.2 mg/dl.

The renal response to gonadectomy was different in female rats with cystic disease (Table 2). Unlike male rats, the K/B of ovariectomized Cy/+ female rats was significantly greater than in sham-operated controls. As in male rats, DHT administration increased K/B to levels much greater than those observed in normal or ovariectomized Cy/+ female rats. Ovariectomy increased the cyst area from 20.0 ± 1.5 to 30.2 ± 2.4 μ^2/15 fields ($P < 0.05$); administration of DHT to ovariectomized female Cy/+ rats increased cyst area to 32.2 ± 1.7 μ^2/15 fields ($P < 0.01$). Ovariectomy and DHT administration to

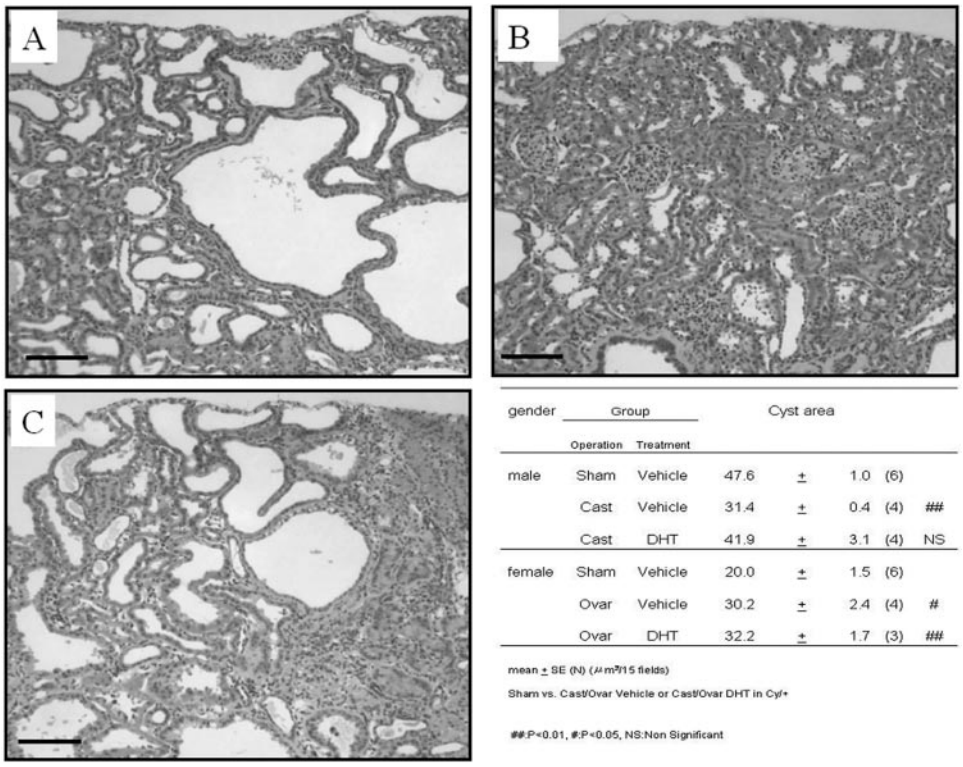

Figure 3. Effects of castration/ovariectomy and DHT on morphology and cyst area of Cy/+ kidney (10 wk old). Cross-section of renal cortex. (A) Untreated male rat showing numerous cysts. (B) Castrated male rat showing diminution in number and size of cysts. (C) Re-expression of the disease in a castrated male rat administered DHT. Bar = 100 μm. (Inset) Effect of FLT on cyst area in male and female Cy/+ rats.

female Cy/+ and +/+ rats had no apparent effects on SUN levels.

As noted in Table 1 and Figure 1, in this group of animals, AR protein expression was greater in Cy/+ male rats than in normal rats (Figure 4, Table 3). Castration, which decreased K/B in male Cy/+ rats (Table 2), also reduced the increase in AR expression by 89.4%, nearly to control levels, whereas administration of DHT increased AR expression 5.3-fold above the control (Table 3). However, castration and the administration of DHT had no apparent effect on the expression of B-Raf, ERK, or P-ERK. AR was expressed weakly in cystic and normal female rats without or with ovariectomy. By contrast, administration of DHT to ovariectomized female rats increased AR expression in both +/+ and Cy/+ rats (Table 3).

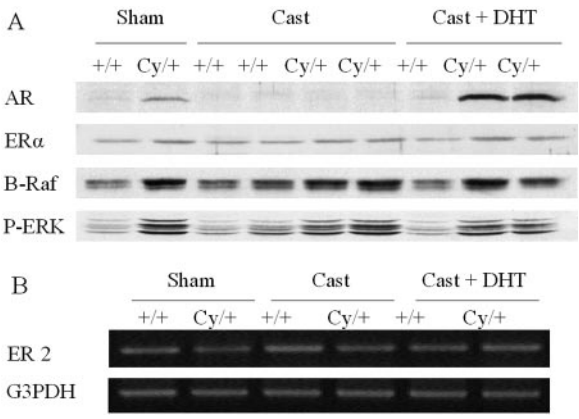

Figure 4. Effects of castration and DHT treatment on renal expression of steroid hormone receptors (AR and ER) and mitogen-activated protein kinase (MAPK) components (B-Raf and P-ERK) in male rats (10 wk of age). (A) Kidney immunoblot. (B) Reverse transcriptase–PCR RNA products of the ERβ subtype of the rat ER2 receptor. G3PDH is a "housekeeping" gene.

In Cy/+ rats, the estrogen receptor (ERα) RNA was normally expressed in both male and female rats. Neither castration of male rats nor ovariectomy of female rats had a detectable effect on ERα RNA expression; neither did administration of DHT (Figure 4). ERβ RNA was not detected in any kidney samples. The ERβ subtype of the rat ER2 receptor was detected by reverse transcriptase–PCR; however, its expression in PKD was not different from normal animals (Figure 4).

AR was found in nuclei of cyst mural epithelial cells by immunohistochemistry in 10-wk-old Cy/+ male rats (Figure 2A) and in renal tubules of male +/+ rats (Figure 2E). AR was not detected in female rats at any age (data not shown). AR protein was not detected in renal epithelial cells of castrated Cy/+ or +/+ rats (Figure 2, B and F) or in rats that received FLT (Figure 4D). By contrast, DHT increased the expression of AR in castrated Cy/+ rats to levels as great as in sham-operated Cy/+ rats (Figure 2, A and C). In female rats, AR expres-

Table 3. Renal protein expression[a]

No.	Surgery	Treatment	Genotype	Gender	AR	B-Raf	P-ERK	ERK
1	Sham	Vehicle	+/+	M	1.0 ± 0.0 (6)	1.0 ± 0.0 (4)	1.0 ± 0.0 (4)	1.0 ± 0.0 (4)
2	Sham	Vehicle	Cy/+	M	2.9 ± 0.3 (6)[b]	1.8 ± 0.0 (4)[b]	2.0 ± 0.1 (5)[c]	1.0 ± 0.0 (5) NS
3	Castration	Vehicle	+/+	M	0.9 ± 0.1 (3)	1.1 ± 0.0 (3)	1.1 ± 0.1 (3)	1.0 ± 0.0 (3)
4	Castration	Vehicle	Cy/+	M	1.1 ± 0.1 (5)[d] NS	1.7 ± 0.1 (3)[e] NS	1.8 ± 0.2 (5)[c] NS	1.0 ± 0.0 (5) NS
5	Castration	DHT	+/+	M	1.3 ± 0.2 (6)	1.3 ± 0.1 (3)	0.9 ± 0.1 (3)	0.9 ± 0.0 (3)
6	Castration	DHT	Cy/+	M	5.3 ± 0.4 (3)[b,d,e]	1.9 ± 0.1 (4)[c] NS	1.8 ± 0.2 (5)[b] NS	0.9 ± 0.0 (5) NS
7	Sham	Vehicle	+/+	F	1.0 ± 0.0 (3)	1.0 ± 0.1 (3)	1.0 ± 0.1 (3)	1.0 ± 0.0 (3)
8	Sham	Vehicle	Cy/+	F	1.1 ± 0.1 (6) NS	1.7 ± 0.1 (6)[b]	2.1 ± 0.3 (5)[b]	1.0 ± 0.0 (5) NS
9	Ovariectomy	Vehicle	+/+	F	1.0 ± 0.1 (3)	0.9 ± 0.1 (3)	1.5 ± 0.2 (3)	0.9 ± 0.0 (3)
10	Ovariectomy	Vehicle	Cy/+	F	1.1 ± 0.1 (5) NS	1.6 ± 0.3 (4)[e] NS	2.0 ± 0.4 (5)[c] NS	0.9 ± 0.0 (5) NS
11	Ovariectomy	DHT	+/+	F	4.7 ± 0.4 (3)	1.1 ± 0.1 (3)	1.0 ± 0.1 (3)	1.0 ± 0.0 (3)
12	Ovariectomy	DHT	Cy/+	F	7.4 ± 1.0 (5)[c,d,e]	1.5 ± 0.1 (5)[c] NS	1.6 ± 0.2 (5)[c] NS	0.9 ± 0.0 (5) NS

[a] Data are means ± SE (*N*). Age 10 wk.
[b,c] +/+ *versus* Cy/+ in each group: [b] $P < 0.01$, [c] $P < 0.05$.
[d] Sham vehicle *versus* castration/ovariectomy vehicle, castration/ovariectomy DHT in Cy/+: $P < 0.01$.
[e] Castration/ovariectomy vehicle *versus* DHT: $P < 0.01$.

sion was detected in nuclei of renal cyst epithelial cells of ovariectomy/DHT-treated Cy/+ rats but not in normal or female Cy/+ rats with or without ovariectomy (data not shown). FLT eliminated AR expression in sham male rats (Figure 2, D and H). These results show that AR expression in renal epithelial cell nuclei is highly dependent on the availability and effectiveness of endogenous and exogenous androgen.

Effect of FLT on Disease Progression and Gene Expression

FLT decreased K/B from 2.15 in vehicle-treated male Cy/+ rats to 1.71 (increment above normal decreased by −27.6%), although there was no effect on K/B in normal rats of either gender (Table 4). FLT administration diminished the SUN level in male Cy/+ rats from 38.8 to 33.8 mg/dl (increment above normal decreased by −72.8%). FLT had no apparent effect on K/B or SUN of female Cy/+ rats. FLT decreased the cyst area of male Cy/+ kidneys (Figure 3) from vehicle control of 47.6 ± 1.0 to 39.3 ± 1.1 μ^2/15 fields ($P < 0.01$) but had no effect in female Cy/+ rats (vehicle control 20.0 ± 1.5 *versus* FLT 20.1 ± 1.4; $P > 0.05$).

FLT strongly suppressed AR expression in Cy/+ male rats; however, the testosterone antagonist had no effect on the increased renal expression of B-Raf and P-ERK in male or female kidneys (Figure 5, Table 5). AR expression in the cyst-lining cells of male Cy/+ kidneys was strikingly decreased by the administration of FLT (Figure 2D).

Co-localization of AR, P-ERK, and PCNA in Renal Epithelial Cells

To explore the extent to which ERK and AR activation may interact in the promotion of cell proliferation, we co-localized these proteins in tissue slices taken from kidneys of Cy/+ and +/+ rats. PCNA-expressing cells were abundant in the mural epithelium of cysts and to a lesser extent in adjacent, apparently noncystic renal tubules (Figure 6, A, C, and E). By contrast, renal PCNA expression in +/+ rats without cysts was much

Table 4. Effect of flutamide on kidney weight and SUN in sham-operated rats[a]

No.	Treatment	Genotype	Gender	*N*	K/B (g/100 g)	SUN (mg/dl)
1	Vehicle	+/+	M	7	0.70 ± 0.01	22.6 ± 0.6
2	FLT	+/+	M	3	0.66 ± 0.02 NS	26.3 ± 2.5 NS
3	Vehicle	Cy/+	M	6	2.15 ± 0.04	38.8 ± 1.1
4	FLT	Cy/+	M	4	1.71 ± 0.04[b]	33.8 ± 1.3[c]
5	Vehicle	+/+	F	3	0.69 ± 0.02	24.9 ± 2.3
6	FLT	+/+	F	4	0.62 ± 0.01 NS	23.0 ± 0.4 NS
7	Vehicle	Cy/+	F	6	1.08 ± 0.02	24.9 ± 2.3
8	FLT	Cy/+	F	3	1.14 ± 0.02 NS	26.0 ± 1.8 NS

[a] Data are mean ± SE. Age 10 wk. FLT, flutamide.
[b] 1 *versus* 2, 3 *versus* 4, 5 *versus* 6, 7 *versus* 8; $P < 0.01$.
[c] 1 *versus* 2, 3 *versus* 4, 5 *versus* 6, 7 *versus* 8; $P < 0.03$.

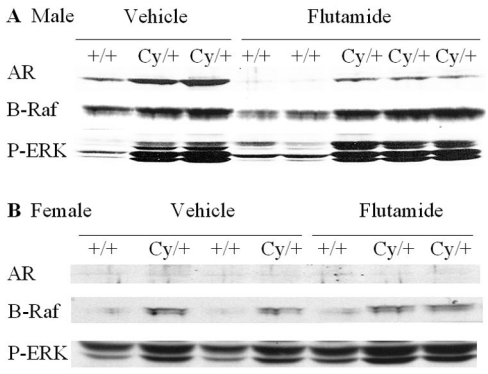

Figure 5. Effect of FLT on AR, B-Raf, and P-ERK expression.

more rare. Accepting that the nuclear expression of PCNA identifies cells that have recently entered the cell cycle and may be expected to divide, we examined tissue sections to which antibodies to PCNA, AR, and P-ERK had been applied. PCNA and AR were assessed independently, and after the images were merged, P-ERK was identified in some cells. PCNA-expressing cells were counted to determine the extent to which AR or P-ERK were co-expressed (Figure 6, Table 6).

PCNA cells stained red, and AR stained green. Expression of both proteins in the same nucleus of merged images yielded a yellow-orange color (Figure 6, A and C). (It is important to note that identical tissue sections are shown in Figures 2A and 6, A and B, and in Figures 2C and 6, C and D.)

AR was expressed in cyst epithelial cells and tubules of Cy/+ male rats (Figures 2A and 6A) without apparent co-expression of PCNA or P-ERK (green arrows). In PCNA-expressing cells, AR was co-expressed with PCNA in approximately 12.7% of cells in androgen-replete Cy/+ rats (Table 6), suggesting a P-ERK–independent link between androgen and cell proliferation. P-ERK was expressed with PCNA in approximately 36.4% of cells (red arrows), suggesting an androgen-independent mitogenic mechanism. P-ERK, AR, and PCNA were co-expressed in 45.4% of cells in Cy/+ rats (yellow-orange arrows), suggesting that both signaling pathways may have been at work. Altogether, 94.1% of the PCNA-positive cells expressed AR or P-ERK, suggesting that these agonist systems account for most of the proliferative activity in cyst epithelial cells.

The respective effects of AR and P-ERK on PCNA expression was much clearer in female Cy/+ rats and castrated or FLT-treated male Cy/+ rats (Table 6; Figures 2, 4, B, D, F, and H, and 6, E and F). AR was undetectable in renal nuclei of androgen-deficient animals; however, the cysts were laden with PCNA-positive mural cells that co-expressed P-ERK. This suggests that the ERK signaling pathway maintains a basal level of aberrant cell proliferation and that androgens have complementary effects.

Discussion

In ADPKD, the kidneys enlarge owing to the expansion of cysts derived from renal tubule epithelium. Increased epithelial proliferation and transepithelial fluid secretion are major determinants of how fast the cysts and the kidneys enlarge (5,12). In the Han:SPRD model of dominantly inherited cystic disease, most of the tubules remain patent and obstruction is not a prerequisite for cyst formation (20). Consequently, transepithelial fluid secretion has a minor role in cyst development and expansion in these animals because the cysts can fill with unreabsorbed glomerular filtrate. Moreover, fluid secreted into the cystic segment would simply drain into the tubule segment downstream. It seems, therefore, that epithelial proliferation is the dominant factor determining the extent to which polycystic kidneys enlarge in these rats (21,22).

Male gender seems to accentuate the progression of ADPKD in humans but to a lesser extent than in Han:SPRD rats (23–28). The availability of an ADPKD animal model with a powerful male gender–positive impact on disease progression offered an opportunity to explore the molecular mechanisms underlying this enhanced acceleration. The cysts in this animal model develop initially in proximal tubules; consequently, the disorder may not mimic human ADPKD in every detail. Nonetheless, this model has served to elucidate important pathogenetic processes that seem to be applicable to most types of renal cystic disease (16,20,21). Previous studies of Han:SPRD rats showed that testosterone accelerated renal enlargement, implicating this hormone in the gender effect on progression (15). Castration of male rats slowed progression to a rate approximately equal to that observed in female Cy/+ littermates, and the administration of testosterone to female rats accelerated progression to a degree approximating that seen in male rats with normal testicles. We hypothesized, therefore, that the major contributor to gender-positive progression in the Han:SPRD rat was an effect of testosterone on the proliferation of cyst epithelial cells.

We found that androgen receptor was abundantly expressed in the kidneys of normal male rats 3 wk of age, but by 8 wk, the intensity of expression was greatly reduced in both male and female rats (Figure 1). AR abundance was greater in the kidneys of rats with cystic disease than in normal littermates at 8, 10, and 24 wk of age.

Immunohistochemical evaluation showed that AR was strongly expressed in the nuclei of cyst mural epithelial cells of male rats, and this expression disappeared after castration or administration of FLT (Figure 2). However, it was not possible to tell by morphologic examination whether there was greater expression of AR in cyst epithelial cells of Cy/+ male rats in comparison with normal tubule cells of +/+ rats.

AR expression in mural cyst cells of castrated male rats with cystic kidneys was restored by the administration of DHT (Figure 2C). In a similar vein, FLT inhibition of AR activation decreased renal AR expression in male rats with cystic disease (Figure 2, Table 5). Conversely, the administration of DHT to female rats with renal cysts increased AR expression and disease severity (Table 2). Thus, the study has demonstrated strong correlation between the availability of testosterone and the abundance and activity of androgen receptors in both male and female rats. Downstream targets of AR were not examined in this study, so we do not know how the increase in AR abundance and activity caused by endogenous androgen and

Table 5. Effect of flutamide on AR, B-Raf, and ERK

No.	Treatment	Genotype	Gender	AR	B-Raf	P-ERK	ERK
1	Vehicle	+/+	M	1.0 ± 0.0 (6)	1.0 ± 0.0 (4)	1.0 ± 0.0 (4)	1.0 ± 0.0 (4)
2	Vehicle	Cy/+	M	2.9 ± 0.3 (6)[b]	1.8 ± 0.0 (4)[b]	2.0 ± 0.1 (5)[b]	1.0 ± 0.0 (5) NS
3	FLT	+/+	M	0.8 ± 0.0 (3)	0.8 ± 0.1 (3)	0.9 ± 0.1 (3)	0.9 ± 0.0 (3)
4	FLT	Cy/+	M	1.1 ± 0.0 (4)[b,c]	1.7 ± 0.1 (4)[b] NS	2.0 ± 0.1 (4)[b] NS	1.0 ± 0.0 (4) NS
5	Vehicle	+/+	F	1.0 ± 0.0 (3)	1.0 ± 0.1 (3)	1.0 ± 0.1 (3)	1.0 ± 0.0 (3)
6	Vehicle	Cy/+	F	1.1 ± 0.1 (6) NS	1.7 ± 0.1 (6)[b]	2.1 ± 0.3 (5)[b]	1.0 ± 0.0 (5) NS
7	FLT	+/+	F	1.0 ± 0.0 (4)	1.4 ± 0.2 (4)	1.4 ± 0.2 (4)	0.9 ± 0.0 (4)
8	FLT	Cy/+	F	1.0 ± 0.0 (3) NS	1.6 ± 0.2 (3) NS	1.7 ± 0.4 (3) NS	1.0 ± 0.0 (3) NS

[a] Data are mean ± SE (N). Age 10 wk.
[b] +/+ *versus* Cy/+ in each group, $P < 0.01$.
[c] Cy/+ vehicle *versus* FLT, $P < 0.01$.

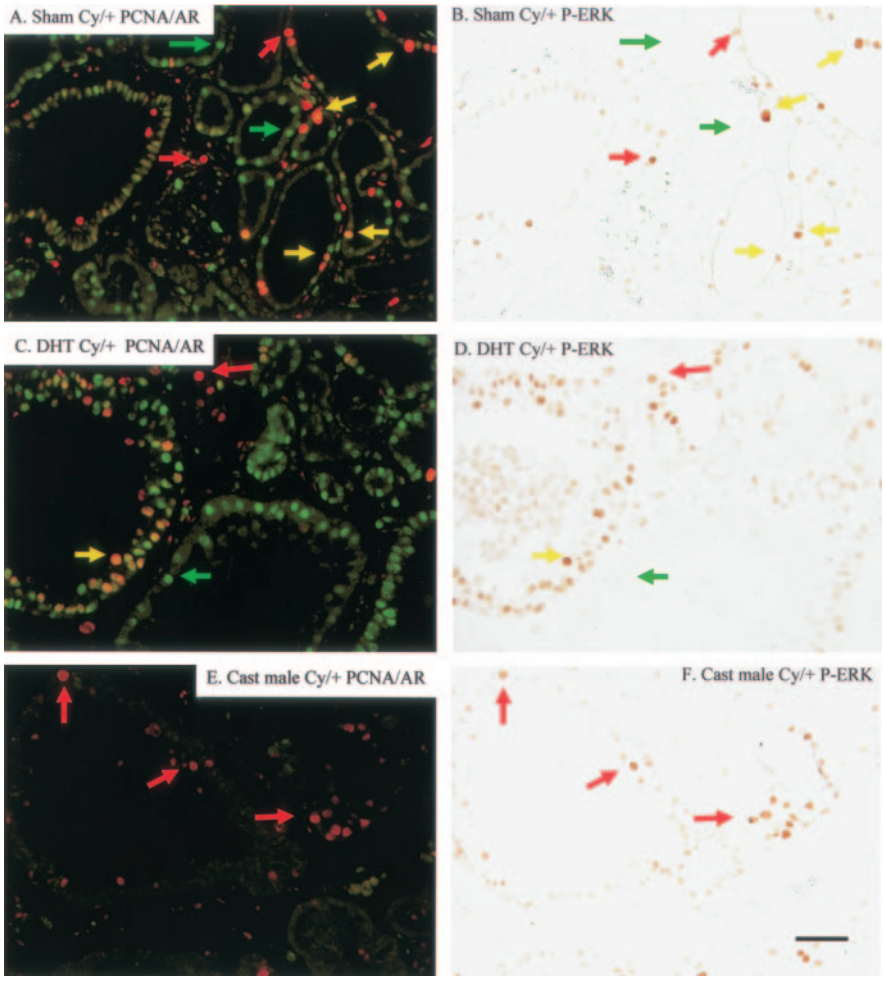

Figure 6. Co-localization of AR, P-ERK, and proliferating cell nuclear antigen (PCNA) in male Cy/+ rats. A and B, C and D, and E and F are adjacent sections. Green arrows, AR alone; red arrows, PCNA and P-ERK; yellow-orange arrows, AR, PCNA, and P-ERK. A is the same section shown in Figure 2A; B is the same section shown in Figure 2B.

exogenous testosterone are specifically linked to cyst expansion.

The intrarenal sites of AR enhancement were explored by immunohistochemistry. AR was clearly expressed in the nuclei and cytoplasm of cells lining cysts of male rats (Figures 2 and 6). The expression of AR in cyst mural cells was strikingly diminished by castration or treatment with FLT. DHT restored AR expression in mural epithelial cells in castrated male rats (Figures 2 and 6). However, AR expression was not detected in cysts of female Cy/+ rats unless DHT was administered. The

Table 6. Co-localization of AR and P-ERK in Cy/+ renal cells (% of PCNA expressing cells)[a]

	Gender	Disease Severity	AR	P-ERK	AR + P-ERK	PCNA Only
Low androgen effect						
sham	F	+	0	90	0	10
castration	F	+	0	73	0	27
FLT	F	+	0	83	0	17
castration	M	+	0	79	0	21
FLT	M	+	0	78	0	22
mean			0	80.6	0	19.4
High androgen effect						
sham	M	+++	16	32	46	5
DHT	M	++++	18	23	51	8
DHT	F	+++	4	54	38	4
mean			12.7	36.4	45	5.7

[a] PCNA, proliferating cell nuclear antigen.

striking difference in renal AR protein expression between rats with high *versus* low androgen effects suggested that androgen receptor activation may have a direct effect on the proliferative elements of cysts to account for their action to increase renal enlargement. Indeed, immunohistochemistry revealed nuclear AR but no P-ERK in approximately 12.7% of PCNA-expressing cells (Figure 6, Table 6), suggesting that androgen may have independently supported a low level of entry into the cell cycle. P-ERK co-localized without AR in 36.4% of the PCNA-expressing cells, suggesting that MAPK may have promoted mitogenesis without androgen assistance. Although this study cannot determine the priority of nuclear binding, AR and P-ERK seemed to have complementary effects because they were co-localized in 45% of the PCNA-positive cells (Table 6). The immunohistochemical analysis indicates that most of the proliferative activity in the Han:SPRD model of ADPKD can be accounted for by mechanisms linked to P-ERK and AR activation. It seems reasonable to suppose that AR and P-ERK have both independent and complementary effects to promote cell proliferation in mural cyst epithelial cells (Figure 7).

Although it is not possible to determine which process may be driving mitogenesis, the observations in female Cy/+ or castrated/FLT-treated male rats indicate that P-ERK is associated with cyst growth and disease progression in the absence of AR (Figures 1, 4, 5, and 6; Tables 1, 3, 5, and 6). Moreover, the administration of androgen to female or castrated male Cy/+ rats strikingly increased the expression of AR, but P-ERK levels were not appreciably changed (Figure 4, Table 3). On the basis of these findings, it seems reasonable to conclude that the MAPK pathway contributes to the basal rate of disease progression and is not regulated by androgen. However, androgens could have a complementary effect with MAPK if they engage the cell cycle at a point beyond the effect of P-ERK (Figure 7).

Androgens also enhance the production of cAMP in epithelial cells derived from the kidney (29). Although we did not measure cAMP in this study, we previously found increased expression of phosphorylated protein kinase A (PKA) in cyst

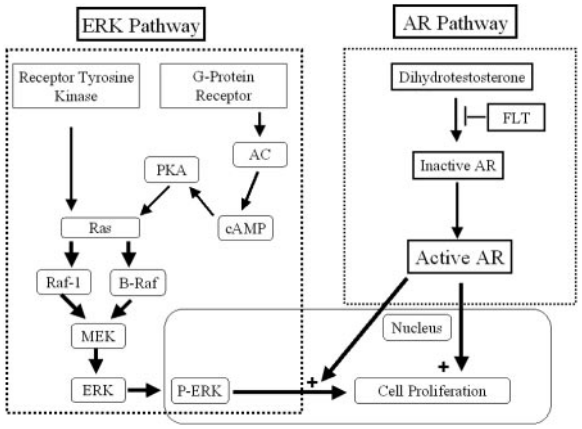

Figure 7. Hypothetical scheme by which MAPK (ERK) and AR agonist pathways may interact in mural cyst epithelial cells. ERK is activated by extracellular agonist's effect on receptor tyrosine kinase or adenylyl cyclase (AC). P-ERK and activated AR migrate to the nucleus. Nuclear binding of AR or P-ERK seems to directly augment proliferation, but in addition, AR may potentiate the MAPK pathway beyond the activation of ERK. See text for details.

mural epithelial cells of male Cy/+ rat, implicating a role for cAMP in this model of ADPKD (16). We did not determine whether androgens increased the expression of phosphorylated PKA because reagents are not available to perform quantitative Western blot analysis. The possibility remains that by augmenting production of PKA, androgens may stimulate ERK activation in cells that also express AR.

Enhanced AR expression was strongly associated with increases in kidney weight, cyst volume, and SUN in male and female cystic rats (Tables 1 through 5). Taken together, the data are consistent with the interpretation that testosterone-mediated AR activation in Han:SPRD rats promotes a significant portion of the increase in renal size and dysfunction, hallmarks of PKD afflicting the Han:SPRD rat and humans with ADPKD.

This study also holds some clues to the role that AR-medi-

ated proliferation may play relative to other pathways that are believed to regulate cyst epithelial cell proliferation. The extracellular MAPK mechanism has been studied extensively in renal cystic disease models and in human cells (30). Evidence suggests that in renal cystic disease, the MAPK pathway may be activated by either of two major agonist systems: (*1*) Receptor tyrosine kinase (RTK) activation by EGF (7,31–34) and (*2*) adenylyl cyclase with activation by G-protein–mediated hormones and autocoids such as vasopressin, epinephrine, and prostaglandin (Figure 7) (35–37).

EGF has been shown to activate ERK 1/2 in Han:SPRD rats with cystic disease; moreover, administration of a small molecule inhibitor of RTK ameliorates renal enlargement and improves renal function (7). Increased renal cAMP production has been associated with cyst growth in four different hereditary types of PKD (37–39).

Nagao *et al.* (16) demonstrated increased expression of P-ERK and B-Raf in cyst mural epithelial cells in the same strain of animals used in this study; the endogenous agonists have not been specifically determined in this model of cystic disease. As demonstrated in this study, the robust proliferative stimulation caused by testosterone was eliminated by castration or administration of FLT to male rats. It is interesting that removing the effects of testosterone suppressed AR abundance, renal enlargement, and azotemia without causing a detectable change in renal ERK and B-Raf expression. To obtain a clearer view of the role that the MAPK-mediated pathway might have on disease progression, we compared ERK 1/2 and B-Raf expression between female Cy/+ and castrated male rats or male rats that were treated with FLT (Figures 3 and 6, Tables 2 through 5). Under conditions in which the androgen-mediated stimulation of disease progression was minimized (castration and FLT administration), the kidneys of male rats expressed ERK 1/2 and B-Raf at modestly higher levels than in female rats. Thus, there seems to be a small male gender–positive effect on the basal levels of MAPK pathway components that are associated with mural hyperplasia within cysts (16,30). We adduced the extent to which cyst and kidney enlargement may be dependent on AR, as opposed to other pathways that signal to proliferation, by analyzing data in Table 2. In Cy/+ male rats, relative renal weight after castration (K/B 1.35) minus relative renal weight of castrated +/+ rats (K/B 0.62) provides an indication of testosterone-independent size (K/B 0.73). Cy/+ relative kidney weight minus +/+ kidney weight (K/B 2.15 to 0.70 = 1.45) reflects the combined contributions of the testosterone-dependent and testosterone-independent increase in renal kidney size to the total weight of Cy/+ kidneys. The difference between the total increase in relative K/B in Cy/+ male rats and the testosterone-independent K/B is equal to the testosterone-independent K/B. Thus, approximately 49.7% of the increase in renal size caused by PKD in male rats seems to be dependent on androgen stimulation. More than likely, the remaining 50.3% is due to other factors, including the MAPK and adenylyl cyclase signaling pathways.

As reported previously, the MAPK and adenylyl cyclase pathways seem to interact in a cell-specific way in the mural epithelium of polycystic kidneys (30). In normal cells, cAMP, the product of adenylyl cyclase activation, does not increase the rate of epithelial cell proliferation. Rather, the nucleotide inhibits at Raf-1 growth factor signals activating receptor tyrosine kinase (Figure 7). By contrast, in polycystic kidney cells, cAMP stimulates cell proliferation by activating ERK 1/2 through proximal activation of B-Raf, a kinase that activates MEK. In ADPKD cyst epithelial cells, Raf-1 inhibition by PKA is relieved, and the RTK and adenylyl cyclase systems have complementary effects to activate MEK and ERK (6,8). A recent study of mouse cortical collecting duct cells (M1) (40) suggested that Ras may be the preferred small G-protein that carries the activation signal from PKA to B-Raf. It seems reasonable to suppose that ERK could be activated by RTK or G-protein–coupled receptors and work in parallel with AR receptor stimulation by androgens to accelerate cell proliferation and ultimately the course of ADPKD (Figure 7).

This study highlights an important practical consideration in patient care. Some athletes illicitly use androgenic substances to enhance performance. To the extent that the findings in rats hold true for humans, androgens could be extremely harmful to young athletes who have ADPKD and use such agents.

Acknowledgments

This work was supported by grants from the National Institutes of Health and by grants from the Japanese Ministry of Education Culture, Sports, Science and Technology and the National Institutes of Health Polycystic Kidney Center Grant P50 DK57301 (University of Kansas).

We thank Ayumi Ohono for technical assistance.

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