mTORC1/2 and rapamycin in female Han:SPRD rats with polycystic kidney disease

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Belibi F, Ravichandran K, Zafar I, He Z, Edelstein CL—mTORC1/2 and rapamycin in female Han:SPRD rats with polycystic kidney disease. Am J Physiol Renal Physiol 300: F236–F244, 2011. First published October 13, 2010; doi:10.1152/ajprenal.00129.2010.—Rapamycin slows disease progression in the male Han:SPRD (Cy/+ ) rat with polycystic kidney disease (PKD). The aim of this study was to determine the effect of rapamycin on PKD and the relative contributions of the profibrotic mammalian target of rapamycin complexes I and 2 (mTORC1 and mTORC2) in female Cy/+ rats. Female Cy/+ rats were treated with rapamycin from 4 to 12 wk of age. In vehicle-treated Cy/+ rats, kidney volume increased by 40% and cyst volume density (CVD) was 19%. Phosphorylated S6 (p-S6) ribosomal protein, a marker of mTORC1 activity, was increased in Cy/+ rats compared with normal littermate controls (+/+ ) and decreased by rapamycin. Despite activation of mTORC1 in female Cy/+ rats, rapamycin had no effect on kidney size, CVD, number of PCNA-positive cystic tubular cells, caspase-3 activity, or the number of terminal deoxynucleotidyl transferase dUTP-mediated nick-end label-positive apoptotic cells. To determine a reason for the lack of effect of rapamycin, we studied the mTORC2 signaling pathway. On immunoblot of kidney, phosphorylated (Ser473) Akt (p-Akt), a marker of mTORC2 activity, was increased in female Cy/+ rats treated with rapamycin. Phosphorylated (Ser657) PKCα, a substrate of mTORC2, was unaffected by rapamycin in females. In contrast, in male rats, where rapamycin significantly decreases PKD, p-Akt (Ser473) was decreased by rapamycin. PKCα (Ser657) was increased in male Cy/+ rats but was unaffected by rapamycin. In summary, in female Cy/+ rats, rapamycin had no effect on PKD and profibrotic p-Akt (Ser473) activity was increased by rapamycin. There were differential effects of rapamycin on mTORC2 signaling in female vs. male Cy/+ rats.

cyst volume; kidney size; autosomal dominant polycystic kidney disease.

WE PREVIOUSLY DEMONSTRATED in male Han:SPRD rats that rapamycin treatment decreases proliferation in cystic and noncystic tubules, markedly inhibits renal enlargement and cystogenesis, and prevents the loss of kidney function (30). Subsequently, two other studies in male Han:SPRD rats demonstrated that mammalian target of rapamycin (mTOR) inhibition reduced cyst formation and renal failure in male Han:SPRD rats (35, 37). Rapamycin also reduces cyst formation in two independent mouse models of polycystic kidney disease (PKD), the Oak Ridge polycystic kidney (orpk)-rescue and BALB/c polycystic kidney (bpk) models (28). In this study (28), it was not specified whether the mice were males or females. The effect of rapamycin in female rats with PKD is not known.

In humans with PKD, progression of renal failure is faster in males than females (9). The heterozygous Han:SPRD rat exhibits many of the features of autosomal dominant PKD in humans, including more aggressive disease in males than females (5). In male Han:SPRD rats at 8 wk of age, the kidney volume is increased more than twofold compared with wild-type controls, the rats have chronic renal failure, and the renal enlargement is progressive until death from chronic renal failure at ~17 mo of age (5, 25). In female Han:SPRD rats at 8 wk of age, the kidney volume is increased less than twofold, the rats do not have chronic renal failure, and the kidney enlargement regresses after 12 wk of age (5, 20). Human and experimental studies provide strong evidence that abnormal proliferation in tubular epithelial cells plays a crucial role in cyst development and/or growth in PKD (36). In the male Han:SPRD rat, there is intense proliferation in cystic and noncystic tubules (30). Proliferation has not been specifically studied in the female Han:SPRD rat kidney. In view of the less aggressive cyst formation and growth in females, we developed the hypothesis that the degree of proliferation would be less in females than males and that the response to rapamycin may be less in females than males.

mTOR exists in association with two different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is a complex of mTOR and raptor (regulatory associated protein of mTOR), while mTORC2 is a complex of mTOR and rictor (rapamycin-independent companion of mTOR). mTORC1 and mTORC2 regulate proliferation and apoptosis (2). Activation of mTORC1 has been demonstrated in male Han:SPRD rats (35) and in humans (13). The effect of PKD and rapamycin on mTORC1 and mTORC2 in female Cy/+ rats is not known. mTORC2 inhibits apoptosis and increases proliferation by phosphorylation (Ser473) of Akt (2). As proliferation and apoptosis (10, 31) are features of PKD, we developed the hypothesis that activity of mTORC1 and mTORC2 would be different in female vs. male Han:SPRD rats.

MATERIALS AND METHODS

Animals. The study was conducted in heterozygous (Cy/+) and normal littermate control (+/+) Han:SPRD rats. The female Cy/+ Han:SPRD rat develops clinically detectable PKD by 12 wk of age, as evidenced by a 30–40% increase in kidney size compared with +/+ control rats (5, 25). A colony of Han:SPRD rats was established in our animal care facility from a litter that was obtained from the Polycystic Kidney Disease Model Repository at the University of Kansas Medical Center. The study protocol was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee. Rats had free access to tap water and standard rat chow.

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**Experimental protocol.** Female Cy/+ and +/- rats were weaned at 3 wk of age and then treated with rapamycin (0.2 mg·kg·day⁻¹·ip) or vehicle (20% ethanol in normal saline) for 9 wk. Rapamycin was obtained from LC Laboratories (Woburn, MA), and a 1 mg/ml stock solution in 100% ethanol was kept at 4°C. At the end of the 12th wk of age, rats were anesthetized by injection of pentobarbital sodium (50 mg/kg body wt ip), and kidneys were removed and weighed. The left kidney was fixed in 4% paraformaldehyde in PBS for 120 min and then put into 70% ethanol and embedded in paraffin for histological examinations.

Male Cy/+ and +/- rats were weaned at 3 wk of age and then treated with rapamycin (0.2 mg·kg·day⁻¹·ip) or vehicle (20% ethanol in normal saline) for 5 wk, as we previously reported (30).

**Cyst volume density.** Hematoxylin-eosin-stained sections were used to determine the cyst volume density (CVD). This was performed by a reviewer, blinded to the identity of the treatment modality, using point-counting stereology (6). Areas of the cortex at 90°, 180°, and 270° from the hilum of each section were selected to guard against field selection variation.

**Immunohistochemistry.** Immunohistochemical detection of PCNA staining was performed using an anti-PCNA antibody (catalog no. sc-7907, Santa Cruz Biotechnology; 1:50 dilution). The sections were stained using an anti-PCNA antibody (catalog no. K4016, Dako, Carpinteria, CA) and visualized with a rabbit polyclonal antibody that detects total PKC protein (catalog no. 2056, Cell Signaling Technology); and a rabbit monoclonal antibody to the treatment modality, as we previously described (30, 31).

**Tubular cell proliferation.** The number of PCNA-positive cells per noncystic tubule was counted using a Nikon Eclipse E400 microscope equipped with a digital camera connected to Spot Advanced imaging software (version 3.5) by an observer blinded to the treatment modality. Noncystic tubules were defined as <50-μm-diameter tubules. At least 10 noncystic tubules or cysts in the cortex per sample were randomly selected and counted.

To avoid confusion between noncystic tubules and small cysts, as well as potential changes in tubular cells lining massive cysts, PCNA-positive tubular cells were counted in ~250-μm-diameter, “medium-sized,” cysts. At least 10 cysts per sample in the cortex were randomly selected and counted.

**In situ detection of DNA fragmentation.** The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method was used to detect in situ DNA strand breaks. The Deadend colorimetric TUNEL assay kit (Promega, Madison, WI) was used. Positive and negative controls for TUNEL were performed. All cells with apoptotic morphology (cellular rounding and shrinkage, pyknotic nuclei, and formation of apoptotic bodies) that stained positive with the TUNEL assay were counted.

**Quantitation of tubular cell proliferation and apoptosis.** The number of TUNEL-positive cells per tubule was counted using a Nikon Eclipse E400 microscope equipped with a digital camera connected to Spot Advanced imaging software (version 3.5) by an observer blinded to the treatment modality, as we previously described (30, 31). Twenty areas per sample, randomly selected at 90°, 180°, and 270° from the hilum of each section, were selected to guard against field selection variation. To avoid confusion between noncystic tubules and small cysts, as well as potential changes in tubular cells lining massive cysts, TUNEL-positive cells were counted in ~250-μm-diameter, medium-sized, cysts.

**Caspase-3 assay.** The activity of caspase-3 was determined by use of fluorescent substrates, as we previously described in detail (11). Briefly, whole kidney was mixed with a lysis buffer containing 25 mM Na⁺ HEPES, 2 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin A (pH 7.2) and homogenized with 10 strokes in a glass-Teflon homogenizer. The lysate was centrifuged at 4°C at 100,000 g in a Beckman Ti70 rotor for 1 h. The caspase assay was performed on the resultant supernatants (cytosolic extract). The assay buffer for caspase-3 contained 25 mM K⁺ HEPES, 1 mM EDTA, 0.1% CHAPS, and 50 mM KCl (pH 7.4). Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC) in 10% DMSO was used as a susceptible substrate for caspase-3. Peptide cleavage was measured over 1 h at 30°C using a Cytosensor 4000 series fluorescent plate reader (Perceptive Biosystems) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. An AMC standard curve was determined for each experiment. Caspase activity was expressed in nanomoles of AMC released per minute of incubation time per milligram of lystate protein.

**Immunoblotting.** Immunoblot analysis was performed as we previously described (27). Whole kidney was homogenized in lysis buffer [5 mM NaKHPO₄, 5 mM NaH₂PO₄, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 50 mM NaF, 0.2 mM Na₂VO₄, and 0.1% β-mercaptoethanol, pH 7.2] plus proteinase inhibitors: 1 mM 4-2-aminoethoxy)benzenesulfonyl fluoride, 15 μM pepstatin A, 14 μM l-trans-epoxy succinyl-leucyl-leucylamida (4-guanido)-butane (E-64), 40 μM bestatin, 22 μM leupeptin, and 0.8 μM aprotinin. Hearts were powdered in liquid nitrogen and then homogenized in lysis buffer, as described above. The homogenates were centrifuged (14,000 rpm at 4°C for 10 min) to remove unbroken cells and debris. Supernatants were mixed with sample buffer containing 50 mM Tris-base (pH 6.8), 0.5% glycerol, 0.01% bromphenol blue, and 0.75% SDS and heated at 95°C for 5 min. Equal amounts of protein (60 μg/lane) were fractionated by Tris-glycine-SDS-12.5% PAGE. The electrophoretically separated proteins were then transferred to a nitrocellulose membrane (Millipore, Bedford, MA) by wet electrobolting. The membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween buffer) overnight at 4°C. Immunoblot analyses were performed with the following antibodies: 1) a phosphorylated (Ser²³⁵/Ser²³⁶) S6 (p-S6) ribosomal protein antibody (catalog no. 2211, Cell Signaling Technology, Beverly, MA) that detects ribosomal S6 protein only when phosphorylated at Ser²³⁵ and Ser²³⁶ [the main in vivo ribosomal S6 protein phosphorylation sites for p70 S6 kinase are Ser²³⁵/Ser²³⁶ and Ser²⁴⁰/Ser²⁴⁴ (12, 14); p-S6 ribosomal protein is recognized as a 32-kDa protein]; 2) a rabbit monoclonal antibody that detects endogenous levels of Akt only when phosphorylated at Ser⁷³⁷ [p-Akt (Ser⁷³⁷) is recognized as a 60-kDa protein (catalog no. 9271, Cell Signaling Technology)]; 3) a rabbit polyclonal antibody raised against a short amino acid sequence containing phosphorylated (Ser⁶⁵⁷) PKCα (catalog no. sc-12356, Santa Cruz Biotechnology); 4) a rabbit total PKC polyclonal antibody that detects total PKC protein (catalog no. 2056, Cell Signaling Technology); and 5) a rabbit monoclonal antibody to β-actin kinase are Ser²³⁵/²³⁶ and Ser²⁴⁰/²⁴⁴ (12, 14); p-S6 ribosomal protein is recognized as a 32-kDa protein].

**Table 1. Rapamycin in female Han:SPRD rats**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n = 9)</th>
<th>Rapamycin (n = 8)</th>
<th>Vehicle (n = 11)</th>
<th>Rapamycin (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>244 ± 3.0</td>
<td>207 ± 4.0†</td>
<td>251 ± 8.0</td>
<td>212 ± 3‡</td>
</tr>
<tr>
<td>2K/TBW, %</td>
<td>0.6 ± 0.01</td>
<td>0.7 ± 0.03</td>
<td>1.0 ± 0.03*</td>
<td>1.0 ± 0.03*</td>
</tr>
<tr>
<td>CVD, %</td>
<td>1 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>19 ± 1.0*</td>
<td>17 ± 1.0*</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>22 ± 3</td>
<td>23 ± 3</td>
<td>20 ± 1.0</td>
<td>23 ± 2.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. 2K/TBW, ratio of 2-kidney weight to total body weight; CVD, cyst volume density; BUN, blood urea nitrogen. *P < 0.01 vs. +/- vehicle and +/- rapamycin. †P < 0.01 vs. +/- vehicle. □P < 0.01 vs. Cy/+ vehicle.
that recognizes a 45-kDa protein (catalog no. 4970, Cell Signaling Technology).

The membranes were incubated with primary antibodies for 1 h at room temperature, washed in TBST buffer, and further incubated with donkey anti-rabbit IgG coupled to horseradish peroxidase (Amer- sham) at 1:1,000 dilution in TBST buffer for 1 h at room temperature. Subsequent detection was carried out by enhanced chemiluminescence (Amersham), according to the manufacturer’s instructions. Prestained protein markers (Bio-Rad) were used for molecular mass determination. Chemiluminescence was recorded with a film, and results were analyzed with 1D Image Software (Kodak Digital Science).

Chemistry. Blood urea nitrogen (BUN) was measured using quantitative colorimetric urea determination (QuantiChrom urea assay kit DIUR-500, Bioassay Systems, Hayward, CA).

Rapamycin levels. Rapamycin was administered daily intraperitoneally. Rapamycin levels were measured using liquid chromatography-mass spectrometry by the Clinical Laboratory at University Hospital. Trough levels of rapamycin were measured in female Cy/+/H11001 rats just before the next dose at 10 wk of age. Mean levels of rapamycin (ng/ml) were 5.9 ± 0.9 in female Cy/+ rats (n = 5) compared with 6.6 ± 0.1 (n = 3), which we previously reported in 8-wk-old male Cy/+ rats treated with 0.2 mg·kg⁻¹·day⁻¹ rapamycin (39).

**Statistical analysis.** Nonnormally distributed data were analyzed by the nonparametric unpaired Mann-Whitney test. Multiple group comparisons were performed using ANOVA with post test according to Newman-Keuls. P < 0.05 was considered statistically significant. Values are means ± SE.

**RESULTS**

Effect of rapamycin on body weight, two kidney-to-total body weight ratio, CVD, and BUN. Rapamycin significantly reduced body weight by 15% (Table 1). The weight loss of 15% in the present study in females was less than the 22% weight loss we previously reported with short-term treatment in males (30). Food intake was monitored in vehicle- and rapamycin-treated rats. The weight loss occurred without any apparent decrease in food intake. Despite the loss in body weight, all the rats appeared healthy during the study. None of the rats died during the study.

The two kidney-to-total body weight ratio (2K/TBW) was determined to correct for the lower body mass caused by the rapamycin. We observed a 40% increase in 2K/TBW in Cy/+ rats.
vehicle-treated vs. +/- vehicle-treated rats. Rapamycin did not reduce the kidney enlargement (Table 1).

CVD was 19% in Cy/+ vehicle-treated rats. Rapamycin did not reduce the CVD (Table 1).

BUN was not different in vehicle-treated +/- rats, rapamycin-treated +/- rats, vehicle-treated Cy/+ rats, and rapamycin-treated Cy/+ rats (Table 1). Thus, despite a 40% increase in 2K/TBW and a CVD of 19%, the female Cy/+ rats do not develop renal impairment as measured by BUN.

Representative kidney sections of +/-, rapamycin-treated +/-, Cy/+, and rapamycin-treated Cy/+ rats stained with hematoxylin-eosin, at the same magnification, are shown in Fig. 1. These representative sections show that the kidney size is larger in Cy/+ than +/- rats and that the kidney size and

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**Fig. 3. Tubular cell proliferation in cysts.**

- **A:** number of PCNA-positive cells in tubular epithelial cells lining the cysts was not different in vehicle-treated Cy/+ and rapamycin-treated Cy/+ rats ($n = 4$ per group).
- **B** and **C:** representative images showing PCNA staining (arrows) in cysts of vehicle-treated Cy/+ (**B**) and rapamycin-treated Cy/+ (**C**) rats.

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**Fig. 4. Apoptosis.**

- **A:** quantitation of terminal deoxynucleotidyl transferase dUTP-mediated nick-end label (TUNEL)-positive cells was not different between vehicle-treated Cy/+ and rapamycin-treated Cy/+ rats ($n = 4$).
- **B** and **C:** representative images showing TUNEL-positive cells in vehicle-treated Cy/+ and rapamycin-treated Cy/+ rats.
- **D:** caspase-3 activity was not different between the groups ($n = 4$).
kidney cysts are not different between female vehicle-treated Cy/+ and rapamycin-treated Cy/+ rats.

We previously reported that rapamycin significantly decreases 2K/TBW and CVD and improves kidney function, as determined by BUN, in male Cy/+ rats (30).

Tubular cell proliferation. The number of PCNA-positive cells per tubule in noncystic tubules in the cortex was not different between vehicle-treated +/+ rats, vehicle-treated Cy/+ rats, and rapamycin-treated Cy/+ rats (Fig. 2A). Representative images are shown in Fig. 2, B–D. The number of PCNA-positive cells per tubule in noncystic tubules in males has previously been described in detail by us (30). The number of PCNA-positive tubular cells per normal tubule in the female vehicle-treated Cy/+ rats was 46% of the number of PCNA-positive tubular cells per normal tubule that we previously reported in male vehicle-treated Cy/+ rats (30).

The number of PCNA-positive cells per cyst in the cortex was not different between vehicle-treated Cy/+ and rapamycin-treated Cy/+ rats (Fig. 3A). Representative images are shown in Fig. 3, B and C.

The number of PCNA-positive cells per cyst in males has previously been described in detail by us (30). The number of PCNA-positive tubular cells per normal tubule in vehicle-treated Cy/+ rats was 46% of the number of PCNA-positive tubular cells per normal tubule that we previously reported in male vehicle-treated Cy/+ rats (30).

Apoptosis and caspase-3. The number of TUNEL-positive apoptotic cells per cyst was 0.3 ± 0.1 in vehicle-treated Cy/+ rats and 0.3 ± 0.05 in rapamycin-treated Cy/+ rats (P = not significant vs. vehicle-treated, n = 4; Fig. 4A). Representative images of TUNEL staining are shown in Fig. 4, B and C.

Caspase-3 is the major mediator of apoptosis. In support of the data that show no significant difference in apoptosis with rapamycin treatment, caspase-3 activity was not significantly affected by rapamycin. Caspase-3 activity is demonstrated in Fig. 4D.

p-S6 ribosomal protein. On immunoblotting of female rats, there was an increase in p-S6 ribosomal protein in vehicle-treated Cy/+ kidneys compared with kidneys from normal littermate controls (+/+; Fig. 5A). The increase in p-S6 ribosomal protein in Cy/+ kidneys was inhibited by rapamycin.

On immunoblotting of male rats, the findings were similar to females. There was an increase in p-S6 ribosomal protein in vehicle-treated Cy/+ kidneys compared with kidneys from normal littermate controls (+/+; Fig. 5B). The increase in p-S6 ribosomal protein in Cy/+ kidneys was inhibited by rapamycin.

p-Akt (Ser473). p-Akt (Ser473) is directly phosphorylated by mTORC2 and is a marker of mTORC2 activation (2, 38). p-Akt (Ser473) was increased in female Cy/+ rats compared with normal littermate control females (+/+), but the increase did not reach statistical significance. However, p-Akt (Ser473) was significantly increased by rapamycin in Cy/+ rats (Fig. 6). Total Akt, used as a control, was not different between the groups.
p-Akt (Ser473) was increased in male Cy/+ rats compared with normal littermate control males (+/+), and, in contrast to the female Cy/+ rats, was decreased by rapamycin (Fig. 7). Total Akt, used as a control, was not different between the groups.

p-PKCα (Ser657). In mouse embryonal fibroblasts (MEFs), ablation of rictor decreases phosphorylation of p-PKCα (Ser657) (16). p-PKCα (Ser657) is a substrate for mTORC2 (1). p-PKCα (Ser657) was not inhibited by rapamycin in female Cy/+ rats (Fig. 8). Total PKCα, used as a control, followed the same pattern as p-PKCα (Ser657). Equal protein loading was confirmed by Coomassie blue staining of the membranes.

Fig. 7. p-Akt (Ser473) in males. Rapamycin decreases p-Akt (Ser473). p-Akt (Ser473) was increased in male Cy/+ rats compared with normal littermate control male rats. p-Akt (Ser473) was decreased in rapamycin-treated compared with vehicle-treated rats. Total Akt, used as a control, was not different between groups. β-Actin, used as a loading control, was not different between groups. Densitometry of p-Akt reflects 4 different experiments. *P < 0.01 vs. Cy/+ and +/+.

DISCUSSION

Rapamycin treatment for 9 wk had no effect on kidney size and CVD at 12 wk of age in female Cy/+ rats with PKD. In contrast, rapamycin markedly slows disease progression in male Cy/+ rats (30). There was no effect of rapamycin in females, despite use of the same dose and blood levels as in males and the degree of rapamycin-induced weight loss was similar to males. Also, the females were treated for 4 wk longer than the males. However, it is possible that rapamycin may have had a therapeutic effect in females, if the time course of treatment was extended, such that the female rats developed renal functional impairment similar to male animals. The present study investigates possible mechanisms for the lack of an effect of rapamycin in female Cy/+ rats.

Human and experimental data provide strong evidence that abnormal proliferation in tubular epithelial cells plays a crucial role in cyst development and/or growth in PKD (36). In the female Cy/+ rat, the number of PCNA-positive tubular cells per cyst was 15% of the number we previously reported in male Cy/+ rats (30). Despite the fact that mTORC1 signaling was increased in female Cy/+ kidneys and reduced by rapamycin, the number of PCNA-positive cells was not different in normal and Cy/+ rat kidneys, and rapamycin had no effect on the proliferation in cystic tubules. It is possible that the lack of...
an effect of rapamycin in female Han:SPRD rats may be related to the low degree of proliferation in the cystic epithelium.

mTORC1 and mTORC2 regulate apoptosis (2). The increased p-Akt (Ser\(^{473}\)) with rapamycin in female animals was not accompanied by an antiapoptotic (prosurvival) effect. This likely rules out inhibition of apoptosis as a possible explanation for the lack of a therapeutic effect of rapamycin in the female Cy/+ animals. Next, we investigated other factors that may play a role in cyst formation or the lack of effect of rapamycin in female Cy/+ rats.

It is known that proliferation in noncystic tubules is a precursor of cyst formation (23). Genetic manipulations that induce the proliferation of tubular epithelial cells in mice cause cysts to form in the kidney (26, 34). While the proliferation index is consistently highest in cystic tubular epithelium, proliferation rates are higher in noncystic tubules from mice with PKD (33) and Han:SPRD rats (23) than in tubules from age-matched controls. We have demonstrated that the decrease in cyst formation in male Cy/+ rats with rapamycin is associated with a decrease in tubular cell proliferation in noncystic tubules (30). In the female Han:SPRD rat, the amount of proliferation in noncystic tubules is not different in rats with PKD compared with normal control age-matched littersmates. It is possible that the lack of an effect of rapamycin in female Han:SPRD rats may be related to the low degree of proliferation in the noncystic epithelium.

It is possible that factors other than mTORC1-induced proliferation play a role in the cyst formation in female Han:SPRD rats. These factors include abnormalities of the primary cilia and Wnt signaling (32), cAMP-induced abnormal cell proliferation via the Ras/MAPK pathway (3), cAMP-induced fluid secretion by activation of the CF\(_{trans}\)-membrane conductance regulator (CFTR) chloride channel (3), EGF signaling (21), increased expression of protooncogenes (e.g., c-myc, c-fos, c-jun, and c-ki-ras) in the polycystic kidney (4, 7), and alterations in the extracellular matrix (21).

Rapamycin resulted in an increase in p-Akt (Ser\(^{473}\)) in females, but rapamycin had no effect on p-PKC\(_{\alpha}\) (Ser\(^{657}\)). There are many possible reasons for the different effect of rapamycin on the rictor substrates p-Akt (Ser\(^{473}\)) and p-PKC\(_{\alpha}\) (Ser\(^{657}\)). The selectivity of rictor on p-Akt (Ser\(^{473}\)) and p-PKC\(_{\alpha}\) (Ser\(^{657}\)) has been shown in cultured cells (2, 16, 38) but has not been described in the kidney, which contains three different Akt isoforms. The potency of rictor to phosphorylate downstream targets may differ in different cell types. Also, there may be other pathways that affect p-Akt (Ser\(^{473}\)) and p-PKC\(_{\alpha}\) (Ser\(^{657}\)) independent of rictor. Recent studies have demonstrated that mTORC1 directly regulates mTORC2 via S6 kinase (S6K1) (2, 8). In insulin-treated MEFs, rapamycin inhibited mTORC1, as indicated by decreased S6K1, with a resultant increase in p-Akt (Ser\(^{473}\)) (8). Also, p-PKC\(_{\alpha}\) (Ser\(^{657}\)) can undergo autophosphorylation (18), and ceramide-1-phosphate can phosphorylate p-PKC\(_{\alpha}\) (Ser\(^{657}\)) (15). The effect of rapamycin on downstream targets of rictor, such as p-PKC\(_{\alpha}\) (Ser\(^{657}\)), is not well described and merits further study.

mTORC2 was originally described to be independent of rapamycin. However, recent studies demonstrated that long-term or high-dose rapamycin therapy is able to inhibit mTORC2 assembly and resultant phosphorylation of p-Akt.
mTORC2 signaling merits further study in Cy/H11001 or androgen agonists or antagonists on mTORC1 and mechanisms (22). To establish the effect of sex hormones on gens potentiate renal cyst proliferation and cyst enlargement (29). In castrated Cy/H11001 kidneys was increased by rapamycin. In males, rapamycin has mTORC2 signaling in females vs. males are demonstrated and mycin had no effect on kidney size and cyst volume in females. analyzed.

What are the implications of our study for human PKD? There is significant proliferation of noncystic and cystic tu-
bules (17,19) and increased mTORC1 activity (13) in human PKD, suggesting that mTOR inhibition may be a therapeutic option. There are at least five interventional clinical studies of mTOR inhibition in PKD (see ClinicalTrials.gov). As progressi-
on of renal failure is faster in males than females (9), a different effect of mTOR inhibition in females vs. males should be considered when the data from the human studies are analyzed.

In summary, despite evidence of mTORC1 activation, rapamycin had no effect on kidney size and cyst volume in females. In females, the proliferative p-Akt (Ser\(^{73}\)) activity in Cy/+ rats was increased by rapamycin. In males, rapamycin has an inhibitory effect on p-Akt (Ser\(^{73}\)) that is associated with a therapeutic effect. Differential effects of rapamycin on mTORC2 signaling in females vs. males are demonstrated and may have implications in the use of mTOR inhibition in the treatment of females with autosomal dominant PKD.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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