mTORC1/2 and rapamycin in female Han:SPRD rats with polycystic kidney disease (PKD).


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Running title: Rapamycin in female PKD rats.
ABSTRACT

Rapamycin or everolimus slows disease progression in the male Han:SPRD (Cy/+) rat with PKD. The aim of the study was to determine the effect of rapamycin on PKD and the relative contributions of the pro-proliferative complexes mTORC1 and mTORC2 in female Cy/+ rats. Female Cy/+ rats were treated with rapamycin from 4 to 12 weeks of age. In vehicle-treated Cy/+ rats, the kidney volume increased by 40% and the cyst volume density (CVD) was 19%. Phospho-S6 (p-S6) ribosomal protein, a marker of mTORC1 activity, was increased in Cy/+ rats compared to normal littermate controls (+/+), and decreased by rapamycin. Despite activation of mTORC1 in female Cy/+ rats, rapamycin had no effect on kidney size, CVD, the number of PCNA-positive cystic tubular cells, caspase-3 activity or the number of TUNEL positive apoptotic cells. To determine a reason for the lack of effect of rapamycin, we studied the mTORC2 signaling pathway. On immunoblot of kidney, phospho-Akt (p-Akt) (serine 473), a marker of mTORC2 activity, was increased in female Cy/+ rats treated with rapamycin. PKCα (Ser 657), another marker of mTORC2 activity, was unaffected by rapamycin in females. In contrast, in male rats, where rapamycin significantly decreases PKD, p-Akt (serine 473) was decreased by rapamycin. PKCα (Ser 657) was increased in male Cy/+ rats but was unaffected by rapamycin. In summary, in female Cy/+ rats, rapamycin had no effect on PKD and pro-proliferative p-Akt (serine 473) activity was increased by rapamycin. There were differential effects of rapamycin on mTORC2 signaling in female versus male Cy/+ rats.
INTRODUCTION

We have previously demonstrated in male Han:SPRD rats that rapamycin treatment decreases proliferation in cystic and non-cystic tubules, markedly inhibits renal enlargement and cystogenesis and prevents the loss of kidney function (30). Subsequently two other studies in male Han:SPRD rats have demonstrated that mTOR inhibition reduced cyst formation and renal failure in male Han:SPRD rats (37) (35). Rapamycin also reduces cyst formation in two independent mouse models of PKD, the orpk-rescue and bpk models (28). In this study 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, males have a faster progression of renal failure than females (9). The heterozygous Han:SPRD rat exhibits many of the features of ADPKD in humans including more aggressive disease in males than females (5). In male Han:SPRD rats at 8 weeks of age the kidney volume is more than 2-fold increased compared to wild type controls, the rats have chronic renal failure and the renal enlargement is progressive until death from chronic renal failure at approximately 17 months of age (5) (25). In female Han:SPRD rats at 8 weeks of age, the kidney volume is less than 2-fold increased, the rats do not have chronic renal failure and the kidney enlargement regresses after 12 weeks 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 both cystic and non-cystic 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 compared to males and that the response to rapamycin may be less in females than in 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). The mTORC1 and mTORC2 complexes 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 of Akt at serine 473 (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 versus 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 polycystic kidney disease by 12 weeks of age as evidenced by a 30-40% increase in kidney size compared to +/+ 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 Program 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.

**Experimental Protocol**

Female Cy/+ and +/+ rats were weaned at 3 weeks of age and then treated with rapamycin 0.2 mg/kg/d IP or vehicle (20% ethanol in normal saline) for 9 weeks. 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 week of age, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and kidneys were removed and weighed. The left kidney was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 120 minutes and then put into 70% ethanol and embedded in paraffin for histological examinations.

Male Cy/+ and +/+ rats were weaned at 3 weeks of age and then treated with rapamycin 0.2 mg/kg/d IP or vehicle (20% ethanol in normal saline) for 5 weeks as we have previously reported (30).

**Cyst volume density**

Hematoxylin-eosin stained sections were used to determine the cyst volume density. 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 degrees, 180 degrees and 270 degrees from the hilum of each section were selected to guard against field selection variation.
**Immunohistochemistry**

Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) staining was performed using an anti-PCNA antibody (sc-7907, Santa Cruz, 1:50). The sections were incubated with alkaline-phosphatase labeled polymer (DAKO EnVision System, Cat# K4016, DAKO, Carpinteria, CA) and visualized with the substrate chromogen, fast red. Negative control sections showed no staining.

**Tubular cell proliferation**

The number of PCNA positive cells per non-cystic 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. Non-cystic tubules were defined as tubules less than 50 µm diameter. At least 10 non-cystic tubules or cysts in the cortex per sample were randomly selected and counted.

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

**In situ detection of DNA fragmentation.**

The terminal deoxynucleotidyltransferase (TdT) mediated dUTP nick-end labeling (TUNEL) method was used to detect in situ DNA strand breaks. The Deadend™ Colorimetirc TUNEL assay kit (Promega, Madison, WI) was used. Positive and negative
controls for TUNEL stain 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 have previously described (30) (31). Twenty areas per sample were randomly selected at 90 degrees, 180 degrees and 270 degrees from the hilum of each section were selected to guard against field selection variation. To avoid confusion between non-cystic tubules and small cysts as well as potential changes in tubular cells lining massive cysts, TUNEL positive cells were counted in “medium sized cysts” of approximately 250 µm diameter.

Caspase-3 assay

The activity of caspase-3 was determined by use of fluorescent substrates as we have previously described in detail (11). Briefly, whole kidney was mixed with a lysis buffer containing 25 mM Na Hepes, 2 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (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 then centrifuged at 4°C at 100,000 X g in a Beckman Ti70 rotor for 1 hr. The caspase assay was performed on the
resultant supernatants (cytosolic extract). The assay buffer for caspase-3 contained 25 mM K+ Hepes, 1 mM dithiothreitol, 0.1% CHAPS, 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 hr at 30°C using a Cytofluor 4000 series fluorescent plate reader (Perseptive 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 nmol AMC released per minute of incubation time per mg of lysate protein.

**Immunoblotting**

Immunoblot analysis was performed as we have previously described (27). Whole kidney was homogenized in lysis buffer (in mM: 5 Na₂HPO₄, 5 NaH₂PO₄, 150 NaCl, 1 EDTA, 0.1% Triton X-100, 50 NaF, and 0.2 Na₃VO₄, and 0.1% β-mercaptoethanol, pH 7.2) plus proteinase inhibitors: 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 15 μM pepstatin A, 14 μM L-trans-epoxysuccinyl- leucylamide- (4-guanido)- butane (E-64), 40 μM bestatin, 22 μM leupeptin, 0.8 μM aprotonin. 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 minutes) 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% sodium dodecyl sulfate (SDS) and heated at 95°C for 5 minutes. 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 electroblotting. 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 at pH 7.5 overnight at 4°C. Immunoblot analyses was performed with the following antibodies: 1) A p-S6 ribosomal protein (Ser235/236) antibody (Cell Signaling Technology, Beverly, MA. Catalogue number 2211) that detects ribosomal S6 protein only when phosphorylated at serine 235 and 236. The main in vivo S6 ribosomal protein phosphorylation sites for p70S6Kinase are Ser235/236 and Ser240/244 (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 serine 473 (Cell Signaling Technology, Beverly, MA., Catalogue number 9271). p-Akt (serine 473) is recognized as a 60 kDa protein. 3) A rabbit polyclonal antibody raised against a short amino acid sequence containing phosphorylated Ser 657 of PKCα (Santa Cruz Biotechnology, Inc, Catalogue number sc-12356). 4) A rabbit total PKC polyclonal antibody that detects total PKC protein (Cell Signaling Technology, Beverly, MA., Catalogue number 2056). 5) A rabbit monoclonal antibody to β-actin that recognizes a 45 kDa protein (Cell Signaling Technology, Beverly, MA., Catalogue number 4970).

The membranes were incubated with primary antibodies for 1 hour at room temperature, washed in TBST buffer, and further incubated with donkey anti-rabbit IgG coupled to horseradish peroxidase (Amersham) at 1:1000 dilution in TBST buffer for 1 hour at room temperature. Subsequent detection was carried out by enhanced chemiluminescence (Amersham), according to the manufacturer’s instructions. Prestained protein markers (Biorad) were used for molecular mass determination.
Chemiluminescence was recorded with a film, and results were analyzed with the 1D Image Software (Kodak Digital Science).

Chemistry

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

Rapamycin levels

Rapamycin was administered daily IP. 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/+ rats just before the next dose at 10 weeks of age. Mean levels of rapamycin (ng/mL) were 5.9 ± 0.9 in female Cy/+ rats (n=5) compared to 6.6 ± 0.1(n=3) that we have previously reported in 8 week old male Cy/+ rats treated with 0.2 mg/kg/d rapamycin (39).

Statistical Analysis

Non-normally distributed data were analyzed by the nonparametric unpaired Mann Whitney test. Multiple group comparisons were performed using analysis of variance (ANOVA) with posttest according to Newman-Keuls. A $P$ value of <0.05 was considered statistically significant. Values are expressed as means ± SE.
RESULTS

Effect of rapamycin on body weight, two kidney/total body weight ratio (2K/TBW), cyst volume density (CVD) and BUN.

Rapamycin significantly reduced the body weight by 15% (Table 1). The weight loss of 15% in the present study in females was less than the 22% 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 2 kidney/total body weight ratio (2K/TBW) was determined to correct for the lower body mass caused by the rapamycin. 2K/TBW (%) was 40% increased in Cy/+ vehicle-treated vs. +/+ vehicle-treated rats. Rapamycin did not reduce the kidney enlargement (Table 1).

Cyst volume density (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 +/+, +/+ treated with rapamycin, Cy/+ and Cy/+ rats treated with rapamycin stained with hematoxylin-eosin, at the same magnification are demonstrated in Figure 1. These representative sections show that the kidney size
is bigger in Cy/+ compared to +/- and that the kidney size and kidney cysts are not
different between female Cy/+ treated with vehicle and Cy/+ treated with rapamycin.

We have 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 non-cystic tubules in the cortex
was not different between vehicle-treated +/- rats, vehicle-treated Cy/+ rats and
rapamycin-treated Cy/+ rats (Figure 2A). Representative pictures are shown in Figure 2
B, C and D. The number of PCNA positive cells per tubule in non-cystic 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/+ rats and rapamycin-treated Cy/+ rats (Figure 3A).
Representative pictures are shown in Figure 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 cyst in the
female vehicle-treated Cy/+ rats was 16% of the number of PCNA positive tubular cells
per cyst 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 Cy/+ mice treated with the vehicle and 0.3 ± 0.05 in Cy/+ mice treated with rapamycin (P=Not Significant vs. vehicle-treated, N=4) (Figure 4A). Representative pictures of TUNEL staining are shown in Figure 4B and C.

Caspase-3 is the major mediator of apoptosis. In support of the data that there is no significant difference in apoptosis with rapamycin treatment, caspase-3 activity was not significantly affected by rapamycin. Caspase-3 activity is demonstrated in Figure 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 to kidneys from normal littermate controls (+/+) (Figure 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 to kidneys from normal littermate controls (+/+) (Figure 5B). The increase in p-S6 ribosomal protein in Cy/+ kidneys was inhibited by rapamycin.

p-Akt (serine 473)

p-Akt (serine 473) is directly phosphorylated by mTORC2 and is a marker of mTORC2 activation (2) (38). p-Akt (serine 473) was increased in female Cy/+ rats
compared to normal littermate control females (+/+), but the increase did not reach statistical significance. However, p-Akt (serine 473) was significantly increased by rapamycin in Cy/+ rats (Figure 6). Total Akt, used as a control, was not different between the groups.

p-Akt (serine 473) was increased in male Cy/+ rats compared to normal littermate control males (+/+) and, in contrast to the female Cy/+ rats, was decreased by rapamycin (Figure 7). Total Akt, used as a control, was not different between the groups.

p-PKCα (serine 657)

In mouse embryonal fibroblasts (MEFs), ablation of rictor decreases phosphorylation of p-PKCα (serine 657) (16). p-PKCα (serine 657) is a substrate for mTORC2 (1). p-PKCα (serine 657) was not inhibited by rapamycin in female Cy/+ rats (Figure 8). Total PKCα, used as a control, followed the same pattern as p-PKCα (serine 657). Equal protein loading was confirmed by Coomassie Blue staining of the membranes.

p-PKCα (serine 657) was increased in male Cy/+ rats compared to normal littermate control males (+/+). The decrease in p-PKCα (serine 657) by rapamycin did not reach statistical significance (Figure 9). Total PKCα, was significantly increased in male Cy/+ rats compared to normal littermate control males (+/+), thus following the same pattern as p-PKCα (serine 657). In this regard it was demonstrated that both p-PKCα (serine 657) and total PKCα were decreased in rictor deficient MEFs confirming previous studies that PKCα phosphorylation is necessary to maintain PKCα stability.
(16). Equal protein loading was confirmed by Coomassie Blue staining of the membranes.

DISCUSSION

Rapamycin treatment for 9 weeks had no effect on kidney size and cyst volume density at 12 weeks of age in female Cy/+ rats with PKD. In contrast rapamycin markedly slows disease progression in male Cy/+ rats (30). The lack of effect of rapamycin in females was despite the same dose, similar blood levels and a similar degree of rapamycin-induced weight loss as in males. Also, the females were treated for 4 weeks 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 current 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 that 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.
The mTORC1 and mTORC2 complexes regulate apoptosis (2). The increased p-AKT (ser473) with rapamycin in female animals was not accompanied by an anti-apoptotic (pro-survival) 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, non-cystic tubules from mice with PKD (33) and Han:SPRD rats (23) have higher proliferation rates than 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 non-cystic tubules (30). In the female Han:SPRD rat, the amount of proliferation in non-cystic tubules is not different in rats with PKD compared with normal control age-matched littermates. 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 non-cystic 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/mitogen-activated protein kinase (MAPK) pathway (3), cAMP-induced fluid secretion by activating the cystic fibrosis trans-membrane conductance regulator (CFTR) chloride channel (3), EGF signaling (21), increased expression of proto-oncogenes like
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 (serine 473) in females, but rapamycin had no effect on p-PKCα (serine 657). There are many possible reasons for the different effect of rapamycin on the rictor substrates p-Akt (serine 473) and p-PKCα (serine 657). The selectivity of rictor on p-Akt (serine 473) and p-PKCα (serine 657) has been shown in cultured cells (2;16) (38) but has not been described in the kidney which contains 3 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 (serine 473) and p-PKCα (serine 657) independent of rictor. Recent studies have demonstrated that mTORC1 directly regulates mTORC2 via S6K1 (8) (2). In insulin-treated MEFs, rapamycin inhibited mTORC1, as indicated by decreased S6K1, with a resultant increase in p-Akt (serine 473) (8). Also, p-PKCα (serine 657) can undergo autophosphorylation (18) and ceramide-1-phosphate can phosphorylate p-PKCα (serine 657) (15). The effect of rapamycin on downstream targets of rictor like p-PKCα (serine 657) is not well described and merits further study.

The mTORC2 complex was originally described to be independent of rapamycin. However recent studies have demonstrated that long term or high dose rapamycin therapy is able to inhibit mTORC2 assembly and resultant phosphorylation of phospho- Akt (serine 473) (24) (40). In the male Cy/+ rats there was an increase in p-Akt (serine 473) that was decreased by rapamycin. As rapamycin also inhibited p-Akt (serine 473), it is possible that the therapeutic effect of rapamycin in males is due to
inhibition of both mTORC1 and 2. Our studies clearly demonstrate differential effects of rapamycin on mTORC2 signaling in female versus male Cy/+ rat kidneys.

The differential effects of rapamycin on mTORC2 signaling in female versus male Cy/+ rats may be due to gender hormones. Gender hormones play an important role on the progression of PKD in Cy/+ rats (29). Female gender is protective. However, ovariectomy attenuates the protective effect of female gender in Cy/+ rats (29). In castrated Cy/+ males, it was demonstrated that androgens potentiate renal cyst proliferation and cyst enlargement via extracellular signal-related kinase (ERK) 1 and 2-dependent and independent signaling mechanisms (22). To establish the effect of gender hormones on mTORC1 and 2, the effect of ovariectomy or castration or androgen agonists or antagonists on mTORC1 and 2 signaling merits further study in Cy/+ rats.

What are the implications of our study for human PKD?. There is significant proliferation of noncystic and cystic tubules (19) (17) and increased mTORC1 activity (13) in human PKD suggesting that mTOR inhibition may be a therapeutic option. There are at least 5 interventional clinical studies of mTOR inhibition in PKD (See ClinicalTrials.gov). As males have a faster progression of renal failure than females (9), a different effect of mTOR inhibition in females versus males should be considered when analyzing the data from the human studies.

In summary, despite evidence of m-TORC1 activation, rapamycin had no effect on kidney size and cyst volume in females. In females, the pro-proliferative p-Akt (serine 473) activity in Cy/+ kidneys was increased by rapamycin. In males, rapamycin has an inhibitory effect on p-Akt (serine 473) that is associated with a therapeutic effect.
Differential effects of rapamycin on mTORC2 signaling in females versus males is demonstrated and may have implications in the use of mTOR inhibition in the treatment of females with ADPKD.

ACKNOWLEDGEMENTS

This work was supported by NIH DK074835, NIH DK07483503S1, a PKD Foundation Bridging Grant to CLE and a NIH minority supplement to FB.
REFERENCES


erratum appears in Proc Natl Acad Sci U S A 1988 Apr;85(8):2578].


Table 1: Rapamycin in female Han:SPRD rats.

<table>
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*P<0.01 vs. +/+ vehicle and +/+ rapamycin, ** P<0.01 vs. +/+ vehicle
*** P<0.01 vs Cy/+ vehicle
**FIGURE 1**

**Effect of rapamycin on PKD in female Cy/+ rats.** Representative kidney sections of +/+, +/- treated with rapamycin (+/+Rapa), Cy/+ and Cy/+ rats treated with rapamycin (Cy/+ Rapa) stained with hematoxylin-eosin, at the same magnification. These representative sections show that the kidney size is bigger in Cy/+ compared to +/+ and that the kidney size and kidney cysts are not different between female Cy/+ treated with vehicle and Cy/+ treated with rapamycin.

**FIGURE 2**

**Tubular cell proliferation in non-cystic tubules.** (A) The number of PCNA positive cells per tubule in non-cystic tubules in the cortex was not different in vehicle (Veh)-treated +/- rats, vehicle-treated Cy/+ rats and rapamycin-treated Cy/+ rats. NS=not significant. (B) (C) (D). Representative pictures of PCNA staining (arrows) in non-cystic tubules in vehicle (Veh)-treated +/- rats (B), vehicle-treated Cy/+ rats (C) and rapamycin-treated Cy/+ rats (D). n=4 per group.

**FIGURE 3**

**Tubular cell proliferation in cysts.** (A) The number PCNA positive cells in tubular epithelial cells lining the cysts was not different in Cy/+ vehicle-treated and Cy/+ rapamycin –treated rats. NS=not significant. (B) (C) Representative pictures of the PCNA staining (arrows) in cysts of vehicle-treated Cy/+ (B) and rapamycin-treated Cy/+ (C). n=4 per group.
FIGURE 4

Apoptosis. A. Quantitation of TUNEL positive cells was not different between Cy/+ treated with vehicle (Cy/+ Veh) and Cy/+ treated with rapamycin (Cy/+ Rapa) (N=4).
B,C. Representative pictures of TUNEL positive cells in Cy/+ Veh and Cy/+ Rapa. D. Caspase-3 activity was not different between the groups (N=4).

FIGURE 5

mTORC1 signaling. A. Females. There was an increase in p-S6 protein, a marker of mTORC1 activation, in 12-week-old female (Cy+) rats compared to normal littermate controls (+/+). The increase in the p-S6 ribosomal protein was inhibited by rapamycin in Cy/+ rats. Total S-6, used as a control, was not different between the groups. B. Males.
There was an increase in p-S6 ribosomal protein in vehicle-treated Cy/+ kidneys at 8 weeks of age compared to kidneys from normal littermate controls (+/+). The increase in p-S6 ribosomal protein in Cy/+ kidneys was inhibited by rapamycin. Total S-6, used as a control, was not different between the groups. Pos=positive control-HEK-292 cells from American Type Culture Collection (ATCC), Manassas, VA. Densitometry of p-S6 reflects three different experiments. *P<0.05 vs. Cy/+ and +/+.
p-Akt (serine 473) in females. Rapamycin increases p-Akt (serine 473). p-Akt (serine 473) is a marker of mTORC2 activity that is directly phosphorylated by the mTOR-Rictor or mTORC2 complex. p-Akt (serine 473) was increased in female Cy/+ rats compared to normal littermate control females (+/+), but the increase did not reach statistical significance (P=0.08). p-Akt (serine 473) was increased with rapamycin treatment in female Cy/+ rats (Cy/+Rapa) compared to vehicle treated (Cy+) and normal controls (+/+). Total Akt, used as a control, was not different between the groups. B-actin used as a loading control was not different between the groups. Densitometry of p-Akt reflects four different experiments. *P<0.01 vs. Cy/+ and +/+.

p-Akt (serine 473) in males. Rapamycin decreases p-Akt (serine 473). p-Akt (serine 473) was increased in male Cy/+ rats compared to normal littermate control males (+/+). p-Akt (serine 473) was decreased in rats treated with rapamycin (Cy/+Rapa) compared to vehicle treated rats (Cy/+). Total Akt, used as a control, was not different between the groups. B-actin used as a loading control was not different between the groups. Densitometry of p-Akt reflects four different experiments. *P<0.01 vs +/+ and Cy/+Rapa.
FIGURE 8

**p-PKCα (serine 657) in females.** p-PKCα (serine 657) is a substrate for mTORC2. p-PKCα (serine 657) was unaffected in Cy/+ rats or by rapamycin. Specifically, p-PKCα (serine 657) was not inhibited by rapamycin in female Cy/+ rats. Total PKCα, used as a control, followed the same pattern as p-PKCα (serine 657). Equal protein loading was confirmed by Coomassie Blue staining of the membranes. Densitometry of p-PKCα (serine 657) reflects six different experiments. NS=not significant.

FIGURE 9

**p-PKCα (serine 657) in males.** p-PKCα (serine 657) is a substrate for mTORC2. p-PKCα (serine 657) was increased in male Cy/+ rats compared to normal littermate control males (+/+). The decrease in p-PKCα (serine 657) by rapamycin did not reach statistical significance. Total PKCα followed the same pattern as p-PKCα (serine 657) confirming previous studies that PKCα phosphorylation is necessary to maintain PKCα stability. Equal protein loading was confirmed by Coomassie Blue staining of the membranes. Densitometry of p-PKCα (serine 657) reflects six different experiments. *P<0.05 vs. +/-
FIGURE 2

A

PCNA (+) cells per normal tubule

+/-
Veh

+/
Rapa

Cy/+ Veh

Cy/+ Rapa

B

C

D

Cyst

Cyst

Cyst
FIGURE 4

A. Apoptotic cells per cyst

B. Cy/+ Veh

C. Cy/+ Rapa

D. Caspase-3 activity (nmol/min/mg)
FIGURE 5B

p-S6 32 kDa

Total-S6 32 kDa

<table>
<thead>
<tr>
<th>Pos</th>
<th>++</th>
<th>Cy/+</th>
<th>Cy/+ Rapa</th>
</tr>
</thead>
</table>

Arbitrary units $\times 10^3$

- ++: 0.0
- Cy/+: 200.0
- Cy/+ Rapa: 100.0
FIGURE 6

![Image of Western blot results showing protein bands for p-AKT, Total-AKT, and β-actin with corresponding molecular weights and quantitative analysis for different genotypes and treatments.](image_url)

- p-AKT: 60 kDa
- Total-AKT: 60 kDa
- β-actin: 45 kDa

![Bar chart showing quantitative analysis of protein expression levels for different genotypes and treatments.](chart_url)

- Arbitrary units x 10^3
- (+/+), (+/+ Rapa), (Cy/+), (Cy/+ Rapa)
FIGURE 8

p-PKCα
80 kDa

Total PKCα
80 kDa

Arbitrary units X 10^3

<table>
<thead>
<tr>
<th>Pos</th>
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<th>Cy/+ Rapa</th>
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NS

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<th>50.0</th>
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</thead>
<tbody>
<tr>
<td>++</td>
<td>Cy/+</td>
<td>Cy/+ Rapa</td>
</tr>
</tbody>
</table>
FIGURE 9

Legend:
- **p-PKCα 80 kDa**
- **Total PKCα 80 kDa**

Bar chart:
- ++
- Cy/+ (with * symbol)
- Cy/+ Rapa

Arbitrary units X 10^3

Graph shows the comparison of p-PKCα and Total PKCα levels across different genotypes with and without Rapa treatment.